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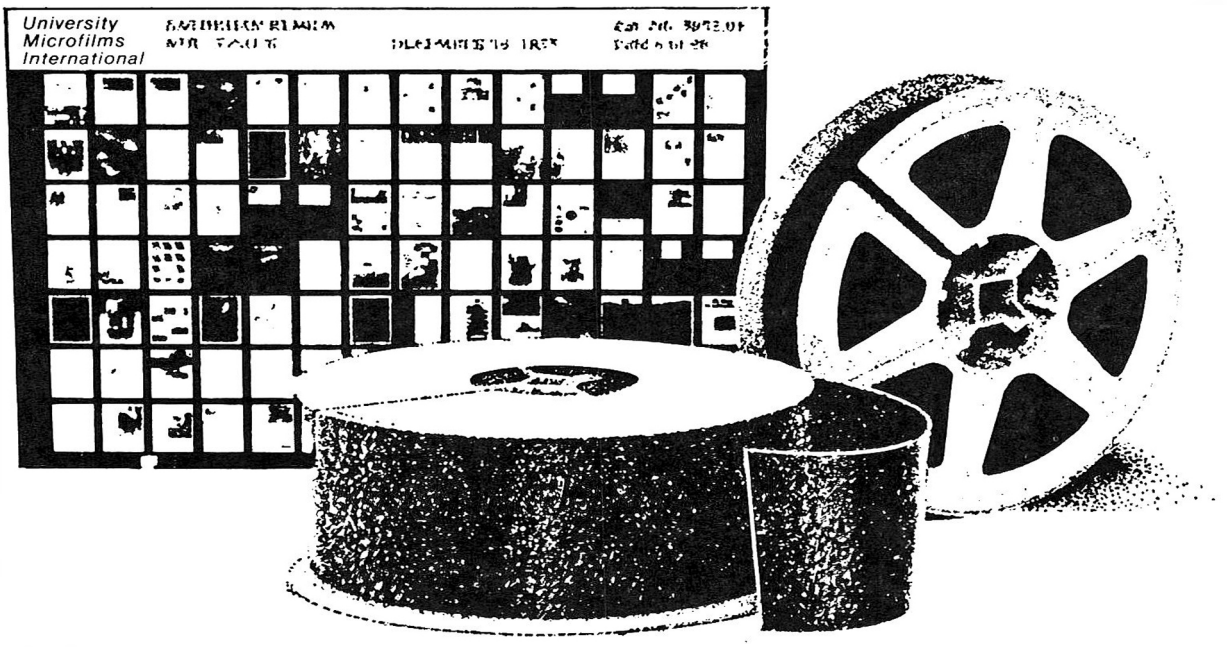
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Influence of Postmortem Changes in Bovine Muscle on the Water-Holding Capacity of Beef. Postmortem Storage of Muscle at 20°C

K. O. HONIKEL, C. FISCHER, A. HAMID, and R. HAMM

ABSTRACT

Rigor mortis occurs in bovine neck muscles as soon as pH 5.9 and an ATP level of about 1 μMol/g are reached. At 20°C muscle contraction does not occur before the onset of rigor. Postmortem changes in water-holding capacity (WHC) were followed by measuring the cooking loss of unsalted and salted (2% NaCl) muscle homogenates prepared after storage of the intact muscle tissue at 20°C for different periods postmortem. At tissue pH above 5.9 addition of salt causes a strong increase of WHC of muscle homogenates. There is a small decrease of WHC of both unsalted and salted homogenates during the prerigor phase which is apparently caused by the postmortem fall of pH. Rigor mortis does not influence the WHC of unsalted muscle homogenates but causes a strong decrease of WHC of salted homogenates. The reason for this difference is discussed. No more than one-third of the total postmortem decrease of WHC and protein solubility in salted muscle homogenates was attributed to the fall of pH, so at least two-thirds were due to the development of rigor.

INTRODUCTION

HOT-BONING of beef carcasses and processing of prerigor meat is attracting increasing interest because of economic reasons (Bendall, 1979; Gunner, 1979; Cross et al., 1979; Taylor, 1979). Furthermore processing of beef immediately after slaughter provides products of excellent water-holding and fat emulsification capacity (Hamm, 1960, 1970, 1972, 1973, 1978) by the combined effects of high levels of adenosine triphosphate (ATP) and high pH values in the prerigor muscle. During 12-24 hr postmortem, a decrease in water-holding capacity (WHC) occurs which is accompanied by the enzymatic breakdown of ATP by a drop of pH cuased by the glycolytic formation of lactic acid, and by the onset of rigor mortis. Consequently, the quality of sausages made by rigor or postrigor beef is much lower compared to that from prerigor beef.

The high WHC of prerigor beef can be retained for several days by salting the minced prerigor meat (Hamm, 1957) despite accelerated ATP breakdown and glycolysis by mincing and salting (Hamm, 1977; Honikel and Hamm, 1978a, b). The high WHC of the prerigor material can also be preserved for months by rapid freezing of bovine muscle and processing without prior thawing. The same result can be achieved by using minced beef salted and freeze-dried in the prerigor state and then rehydrated (Hamm and Potthast, 1975; Hamm, 1978).

In all these types of prerigor-processing of beef a high level of ATP and a high pH of the tissue at the time of mincing and salting seem to be of paramount importance. Detailed studies, however, of the dependence of WHC of beef on ATP concentration and tissue pH have not been reported. In recent studies beef in the early prerigor state, i.e., as soon after slaughter as possible, was compared with tissue in the rigor or postrigor state, i.e. about 24 hr postmortem or later (Bendall, 1976; Hamm, 1972, 1977). From these investigations no information could be obtained on how the changes in ATP and pH and the development of rigor mortis are related to the postmortem changes of WHC. It was also not known if the influence of these postmortem changes on the WHC of meat, measured by the extent of cooking loss, is different for the intact tissue and for unsalted and salted muscle homogenates, the latter presenting the conditions which exist in sausage mixtures. It was the purpose of the experiments presented in this paper to answer these questions.

Authors Honikel, Fischer, and Hamm are with the Institute of Chemistry & Physics at the Bundesanstalt für Fleischforschung, D-8650 Kulmbach, Germany. Author Hamid was visiting scientist in this Institute. He is now with the Pakistan Council of Scientific & Industrial Research, Lahore-16 (Pakistan).

MATERIALS & METHODS

Material

Beef neck muscles (M. sternomandibularis and M. mastoideus) were obtained from the local abattoir within 30-40 min after slaughter and trimmed of visible fat and connective tissue. The lean muscle was portioned in 200-300g pieces of about 1 cm thickness, sealed in polypropylene pouches and stored at about 20°C. At different times of postmortem storage, one of the bags was selected at random; a part of this sample was ground through an electrical mincer using a plate with 4.5 mm holes. About 50-60g were left as intact muscle. Unsalted and salted (2% NaCl) muscle homogenates were prepared in a Moulinette mixer (Moulinex, France) using 66g of ground muscle, 33g of icewater or 33g of an ice-cold % NaCl solution, respectively, and homogenized four times for 3 sec.

pH of tissue

About 5g of the intact muscle was cut into small pieces and homogenized with 20 ml of distilled water for 10-15 sec in the 30 ml flask of a Buhler Homogenizer (Bühler, Tübingen, Germany) equipped with an ice jacket. This was done in triplicate. The pH of the homogenate was measured with the pH-Meter pH-410 (WTW, Weilheim, Germany) using a combined glass electrode.

R-value and ATP determination

The determination of R-value is a fast spectrophotometric method for the estimation of the inosine/adenosine ratio in muscle. In the case of postmortem changes in muscle it represents the degree of transformation of ATP to IMP. The change in the R-value parallels the decrease in the ATP level postmortem (Jolley et al., 1980). The R-value according to the method of Honikel and Fischer (1977) allows an estimation of the ATP content in muscle tissue and was used with some modification. After thorough homogenization of 2-3g muscle with 15 ml of 0.85M perchloric acid for 30 sec the homogenate was spun at about 20,000 x G in a Beckman Model J-21B centrifuge. The supernatant was neutralized with 2M KOH and made up to a final volume of 25 ml. Then 0.1 ml of this solution was mixed with 1.9 ml phosphate buffer, pH 6.5, and the optical density was measured at 250 and 260 nm with phosphate buffer as reference. The R-value is the 250/260 nm absorption ratio. ATP was determined by the method of Jaworek et al. (1970).

Water-holding capacity

Cooking loss was used as a measure of WHC. Immediately after measuring the pH of a muscle sample, unsalted and salted homogenates were prepared. The unsalted samples were used at once, the salted homogenates after an incubation time of 30 min in order to allow the effect of NaCl to reach an equilibrium. This incubation period improved the reproducibility of the results obtained without a significant effect on the values of cooking loss. The unsalted

homogenates, however, had to be measured immediately as grinding and homogenization accelerated the postmortem changes. About 5g of the intact muscle or of the unsalted or salted muscle homogenate was weighed into a preweighed corex centrifuge tube. After covering the tube with a glass marble it was placed in a boiling water bath for 20 min. The tube with contents was then allowed to cool and the juice released by heating was drained off. The cooked meat sample was put on a filter paper, blotted with filter paper, then placed back into the tube which was reweighed for determining the percent moisture loss during cooking. This method is essentially the same as reported by Yu Bang Lee et al. (1978). In the present study duplicate samples were used.

Sarcomere length

Neck muscle slices of about 50g and about 1 cm thickness were taken from four beef carcasses approximately 30 min postmortem and incubated in a water bath of 30°C. After different times of incubation, samples for the determination of pH and R-value were taken. At the same time, about 200 mg of muscle fibers were fixed in a solution of 2% glutardialdehyde and 0.2M sucrose in 0.2M phosphate buffer (pH 7.1) at room temperature. After 30 min the sarcomere length was measured using a laser technique as described by Voyle (1971).

Extensibility of muscle

Bovine neck muscles were taken from the carcasses within 30 min postmortem, cut longitudinally into sections of the same length and similar diameter (3-4 cm) packed in alumina foil and incubated at 20°C. At different times postmortem small samples from these muscles were cut off and their pH and R-value were determined. Immediately after removing these samples the muscles were stretched by loading with 2.3 kg, and the increase in length was measured. After release of the load the storage of the muscles at 20°C was continued.

Protein solubility

Changes in solubility of myofibrillar proteins postmortem are described by Grabowska and Hamm (1979).

SDS polyacrylamide-gel electrophoresis

Preparation of protein samples. 7g of the unsalted or salted muscle homogenate were homogenized for 30 sec with 15 ml distilled water or 2% NaCl-solution, repsectively, in a 30 ml flask of a Bühler homogenizer equipped with an ice-jacket. 3.0 ml of the unsalted or salted homogenate were layered on top of a sucrose solution (0.05M β-glycerophosphate buffer, pH 7.0, containing 0.8M sucrose and 0 or 2% NaCl, respectively) in 50 ml polycarbonate tubes. These were then centrifuged at 10,000 x G in an IS-13 swinging-out rotor (Beckman Instruments) for 30 min. The soluble proteins remained in the top layer above the sucrose solution. The pellet at the bottom of the tube contained insoluble proteins, mainly myofibrillar proteins. 0.2 ml of the layer of soluble proteins was pipetted and 10 mg of the pellet was weighed into 15 ml tubes. These samples were treated with 2.8 ml and 2.0 ml, respectively, of a mixture of 4% sodium dodecyl sulfate (SDS) and an equal volume of 0.5M boric acid/0.25M Tris buffer (pH 8.2). The tubes were placed in a boiling water bath for 20 min to prevent further proteolysis. After cold storage overnight and reheating the solutions were used for electrophoresis.

Gel electrophoresis. The polyacrylamide-gel electrophoresis of these protein fractions was carried out using the method of Hofmann and Penny (1973). Identification of the protein fractions was made by running a mixture of four standard proteins (pig hemoglobin, horse myoglobin, bovine serum albumin, chicken serum albumin) at the same time in each gel. The polyacrylamide concentration was 8%. 30 µl of the SDS digest prepared as described above was applied onto the gel slots. After the electrophoresis, the separated protein bands were strained by Brilliant Coomassie Blue, and the density of the bands was measured using a recording densitometer (Multronics, Heidelberg, Germany).

DSC studies

Differential scanning calorimetry was performed using a Perkin-Elmer DSC-II (Uberlingen, Germany). The instrument was calibrated for temperature using samples of benzoic acid and indium. The quantitative equilibration for the determination of enthalpies was obtained with weighed samples of indium using a value of 6.8 cal/g for its melting enthalpy. Weighed samples of muscle (38-42 mg) were sealed in large-volume capsules. As a reference a large-volume capsule containing 36 mg of water was used. The experiments were conducted with a heating rate of 10°K/min over the temperature range 275-370°K.

RESULTS & DISCUSSION

Dependence of sarcomere length and rigor mortis on pH and ATP concentration

The rigor mortis of normal bovine neck muscles, as defined by the decrease of extensibility (Bendall, 1973) occurs when the postmortem pH decreased to values around 5.9 and the R-value has reached a level around 1.10 (Fig. 1) which corresponds to an ATP concentration of about 1 µMol ATP/g tissue (Honikel and Fischer, 1977).

At 20°C neither the loaded nor the unloaded muscle shortened before these values were reached (Fig. 1). These observations are supported by the measurements of sarcomere length (Fig. 2). Until the onset of rigor mortis, the sarcomere length remained unchanged; with onset of rigor, however, a marked decrease in sarcomere length occurred. The experiments in Figure 2 were carried out at 30°C, because around 15-20°C the small changes due to rigor shortening could not be clearly demonstrated by the method used. The pH postmortem before onset of rigor mortis does not have any influence on extensibility of muscle or on sarcomere length and the development of rigor mortis under the conditions studied is accompanied by shortening of muscle and sarcomeres.

The lack of muscle shortening pre-rigor at temperatures of 20°C and 30°C (Fig. 1 and 2) can be explained by the fact that the activity of the calcium pump within the sarcoplasmic reticulum (SR) membranes, which is relatively high at these temperatures (Whiting, 1980), keeps the Ca^{++}

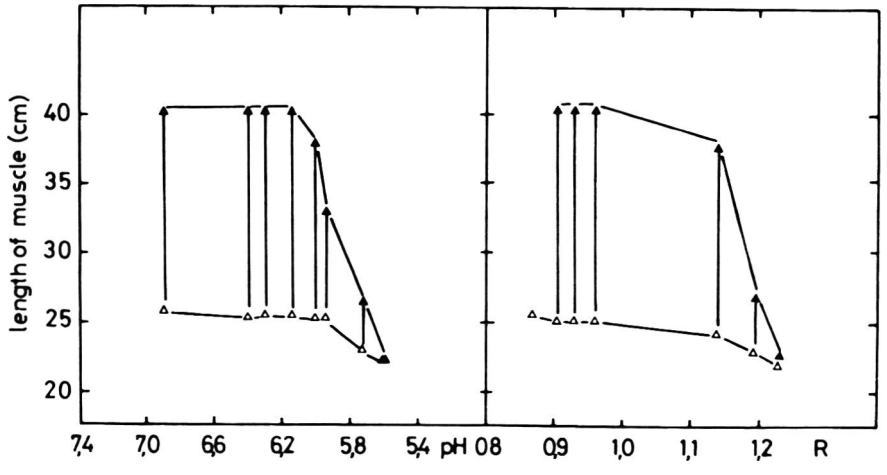


Fig. 1—Extensibility of M. mastoideus of beef in relation to postmortem changes of pH and R-value. Temperature was 20°C. Experiments were repeated three times with similar results. (△) length of muscle without load; (▲) length of muscle under load; the vertical lines indicate the extensibility.

concentration around the myofilaments low enough to maintain the blocking effect of the tropomyosin-troponin system on the myosin-actin interaction and consequently prevents muscular contraction. However, if the ATP level falls below about 1 μMol/g as a result of the postmortem breakdown of ATP, not enough ATP might be available for the operation of the ion pump in the SR; also the postmortem fall of pH has an inhibiting effect on calcium accumulating systems in the muscle cell as shown by Whiting (1980). Therefore, the Ca^{++} concentration around the myofibrils will increase and initiate contraction as well as rigor mortis.

Influence of tissue pH, ATP concentration, and salt on the WHC

Muscles from different animals were incubated at 20°C immediately after slaughter. After different times postmortem the pH was measured in the intact tissue from which unsalted and salted (2% NaCl) homogenates were prepared. In Figure 3 the cooking losses of these homogenates are plotted against the pH of the intact muscle. The relatively low WHC (high cooking losses) of the unsalted muscle homogenates increased slowly and linearly with decreasing pH until the final pH of about 5.4 was reached. With the salted homogenates, however, slow increase in cooking loss occurred only down to about pH 5.9 where the onset of rigor occurs. Then, with further falling pH, a strong linear rise in cooking loss could be observed. In the pH range between 7.0 and 5.9 the cooking loss of the salted homogenates were much smaller than the cooking loss of the unsalted homogenates. Around pH 5.4, however, the differences were virtually none. Furthermore between pH 7 and 5.9 the increase in cooking loss ran parallel in both homogenates indicating a similar pH-dependence of the WHC.

The change in cooking loss with falling pH was studied for each single muscle. In all cases the curves obtained for the unsalted homogenates as well as both parts of the curve for the salted homogenates were similar to those shown for the 21 muscles presented in Figure 3. The fact that neutral salts increase the WHC of meat at high levels of ATP and/or at high pH values much more than after depletion of ATP or at pH values around the isoelectric point (IP) of myofibrillar proteins (pH 5), is well known and can be explained by the electrostatic theory of swelling (Hamm, 1972).

In Figure 4 the cooking losses of salted muscle homogenates are plotted vs R-values. Apparently no linear relationship exists between these parameters. In the prerigor state, i.e. below R = 1.05 almost all values for cooking loss were below 31% whereas in the rigor and postrigor state, i.e. above R = 1.05 cooking losses were between 35 and 60%. Decrease in ATP concentration down to an R value of 1.05, equivalent to about 1 μMol ATP/g, apparently has no significant influence on the WHC of salted muscle homogenates, but further decrease in ATP concentration results in a drop of WHC, probably by the development of rigor.

Protein solubility and WHC in salted muscle homogenates at different muscle pH postmortem

The relationship between muscle pH, protein solubility and WHC is presented in Figure 5. Figure 5A shows that between pH 6.6 and 6.1, i.e. in the prerigor state, a relatively small and linear decrease in the solubility of myofibrillar proteins occurred. Below pH 6.1, however, i.e. during rigor mortis, a strong fall of protein solubility was observed. Readjusting of the pH of rigor muscle homogenates up to the pH values of prerigor homogenates caused an increase in protein solubility, but even so protein solubility was still far lower than that found for the prerigor muscle homogenates.

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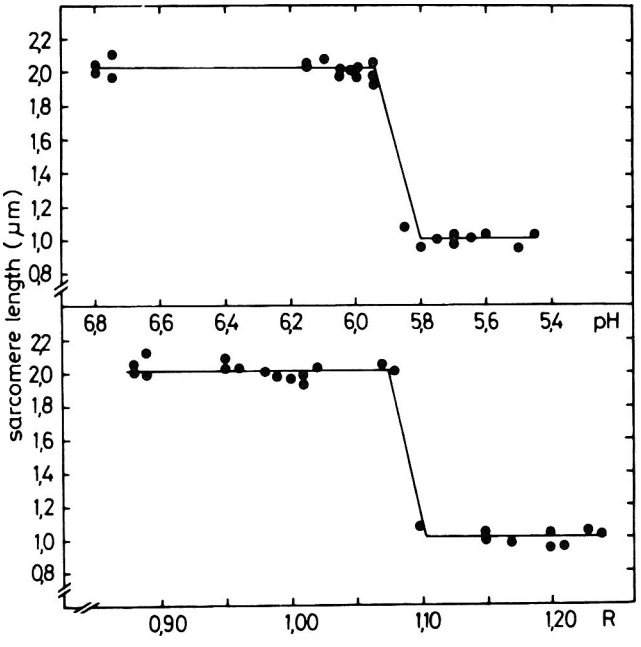


Fig. 2—Relation between rigor shortening and postmortem pH and R-value in bovine neck muscles at 30°C. pH, R-value and sarcomere length measured as described in Methods.

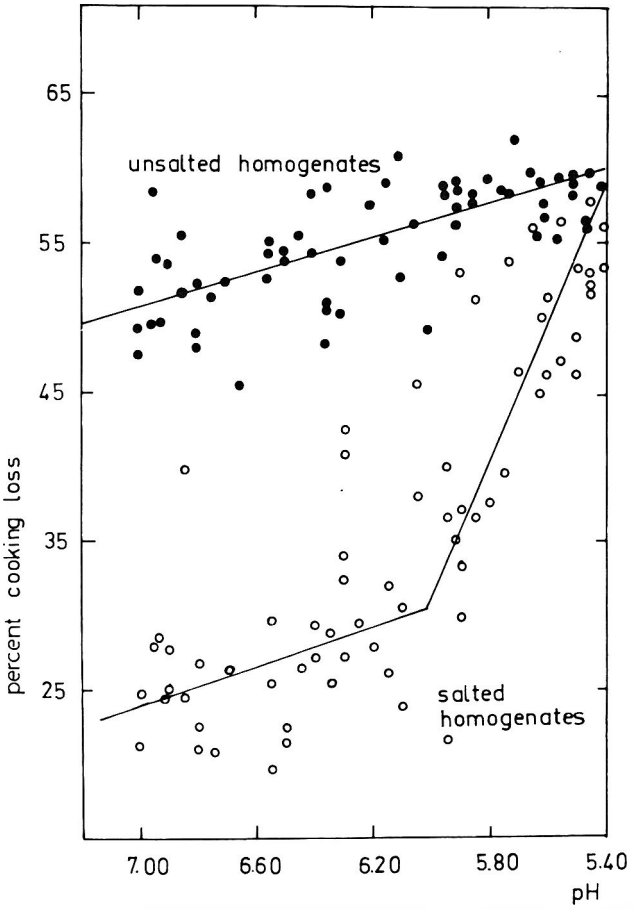


Fig. 3—Relationship of percent cooking loss of unsalted and salted muscle homogenates and pH of the corresponding bovine neck muscles. The pH of the intact muscles were measured and homogenates were prepared and cooking loss measured. The samples were taken from 21 different animals. The points of the salted homogenates were calculated below and above pH 6.0 in two groups by linear regression analysis.

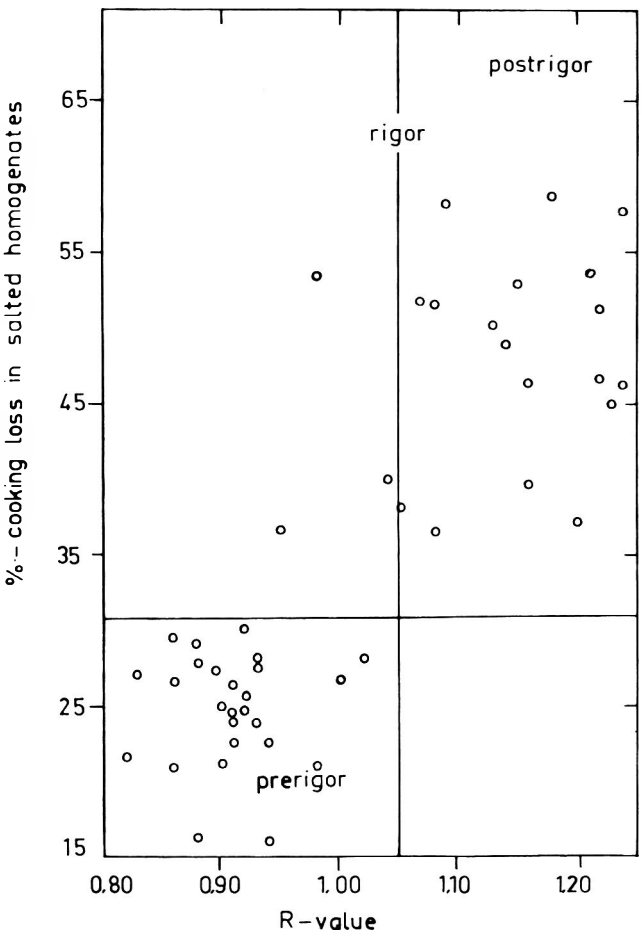


Fig. 4—Cooking loss in salted homogenates and R-value in the corresponding muscle pieces.

The decrease in the solubility of myofibrillar proteins during rigor mortis was also shown using SDS-polyacrylamide gel electrophoresis (Table 1). In unsalted muscle homogenates only small amounts of myosin are soluble, even in the prerigor state. In the supernatant of salted prerigor muscle homogenates (pH 6.1), however, relatively high amounts of myosin could be detected which became insoluble during development of rigor mortis (Table 1).

With regard to WHC, measured by cooking loss, similar effects were obtained (Fig. 5B). With decreasing pH of the intact tissue the WHC of the salted homogenates decreased slowly postmortem until about pH 6.2. During the development of rigor mortis, a large decrease in WHC with decreasing pH occurred. It should be mentioned that in both experiments (Fig. 5A and B) the salted homogenates were prepared at different times postmortem. By readjustment of the pH of rigor muscle homogenates, WHC increased again but it did not approach the original high WHC of the prerigor homogenates. As the insert in Figure 5A shows, there was a continuous decrease in ATP concentration between pH 6.9 and 5.8, with no sign of an increased rate of ATP breakdown during the postmortem fall in pH from 6.0 to 5.8, i.e. during the development of rigor mortis.

The development of rigor mortis, defined by the loss of extensibility and accompanied by a decrease of WHC of salted homogenates is apparently also associated with a remarkable decrease in the solubility of myofibrillar proteins (Fig. 5A, Table 1). This postmortem change in protein solubility has been observed by many authors (Grabowska and Hamm, 1979) and is probably due to the strong association of myosin and actin after depletion of ATP during the onset of rigor. It is noteworthy that before the onset of rigor a small loss of solubility of myofibrillar proteins during postmortem fall of pH could be observed, increasing during development of rigor (Fig. 5A). The decrease in protein solubility with decreasing pH in the prerigor phase (above pH 6.1) parallels the pH dependence of protein

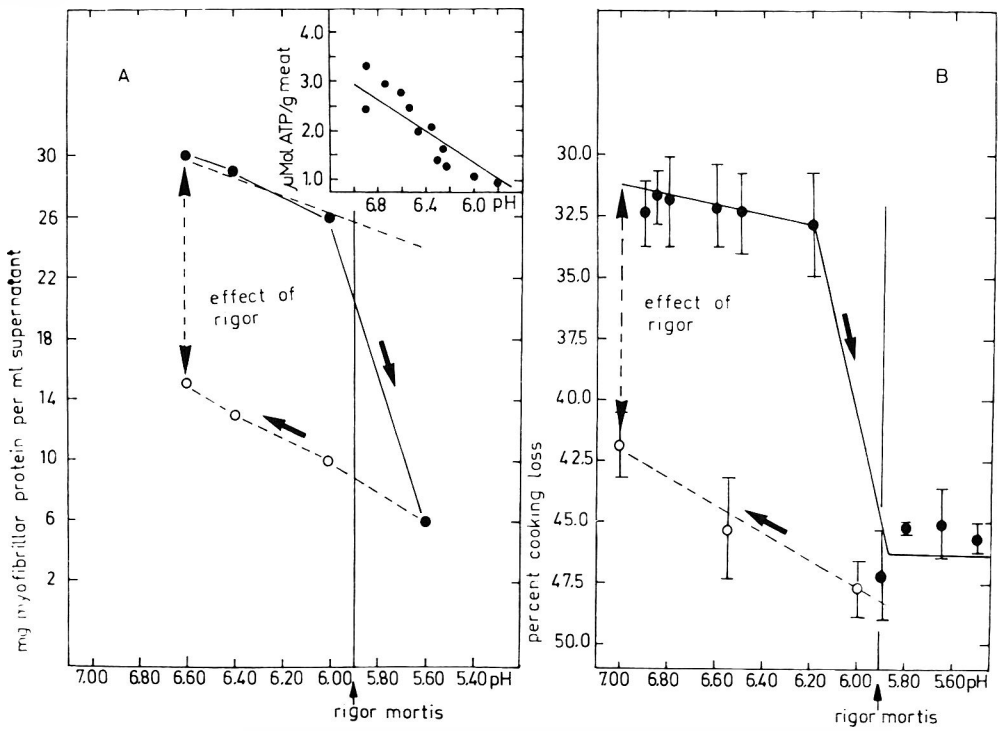


Fig. 5—Changes of soluble myofibrillar protein and cooking loss of salted homogenates vs pH. (A) Bovine neck muscles incubated at 20°C were ground at certain pH values, homogenates with 2% salt were prepared and the amount of soluble myofibrillar proteins determined (as described by Grabowska and Hamm, 1979). After the final pH in the intact muscle was reached the pH was readjusted with 1M NaOH and the amount of soluble myofibrillar protein was determined (o) as described above. (B) The homogenates were prepared as in (A) and the percent cooking loss was determined. Bars indicate standard deviation of 4 experiments. The insert in (A) shows the relationship between ATP concentration and pH postmortem in bovine neck muscles incubated at 20°C.

solubility in the rigor muscle homogenates (Fig. 5A). From this result it can be concluded that the decrease in protein solubility in the prerigor phase is caused by the decrease in pH only and not by the breakdown of ATP. However, at least two-thirds of the strong fall of solubility below pH 6.1 must be attributed to the development of rigor mortis. Hamm (1956) came to a similar conclusion by measuring the WHC of unheated muscle homogenates (of bovine M. longissimus dorsi) using a filter-paper press-method; he found that one-third of the postmortem loss of WHC is due to the fall of pH whereas two-thirds are caused by the disappearance of ATP.

The similar pattern of the curves in Figure 5A and 5B suggests a close relationship between the change of solubility of myofibrillar proteins, induced by postmortem metabolism, and the WHC of salted tissue homogenate. Such a relationship was extensively discussed by Hamm (1972) and more recently by Grabowska and Hamm (1979). Hamm (1960) suggested that ATP increases the WHC of meat at prerigor pH values by sequestering muscular alkaline earth metal-ions (Ca^{++}, Mg^{++}) which are responsible for lowering the WHC of meat. However, in our experiments with salted muscle homogenates no direct correlation between the change in ATP concentration postmortem and WHC was found (see Fig. 4 and insert in Fig. 5A). The strong fall of WHC (increase of cooking loss) starting at about 5.9 and R = 1.05, shows that the development of rigor mortis, initiated by the depletion of ATP, and not the postmortem ATP hydrolysis itself is the main reason for postmortem alteration of WHC of salted muscle homogenates.

Postmortem changes in the conformation of myofibrillar proteins as measured by DSC

Changes in the conformational state of proteins are accompanied by changes in enthalpy (Atkinson, 1977) as also revealed by calorimetric studies. Recently several authors (Martens and Vold, 1976; Wright et al., 1977) have reported that muscles of different kinds of animals show in the prerigor state an exothermal peak with a maximum at 53-55°C in DSC measurements. Postrigor muscles lack this peak. Wright et al. (1977) suggested that this peak is a direct or indirect result of muscle contraction and would be due to ATP turnover during heating. We calculated the energy output during splitting of ATP to ADP and compared it with the measured enthalpy change of muscle. Table 2 shows that the exothermal peak of the intact muscle tissue represents 30-240 times the energy output that could be expected from the effect of ATP hydrolysis and is probably due to conformational changes of myosin. During these studies we also found that the postmortem change in the exothermal peak during the prerigor phase (pH above 5.9, R-value below 1.05) is fairly independent of pH and ATP concentration (Fig. 6). Only small changes were observed before the onset of rigor, followed by a strong decrease during rigor. The exothermal heat output did not decrease until the ATP concentration had dropped down to about 20% of its original level and pH 5.9 where rigor mortis occurs. So, the change in heat output must be due to heat-induced changes in proteins, most probably myofibrillar proteins. These changes in Figure 6 at pH 5.9 and R = 1.10 coincide with the measurements of muscle extensibility and muscle shortening, WHC, and protein solubility of salted homogenates (Fig. 1-5). Aggregation of proteins which form quartenary structures often show a concerted action as has been expressed in the allosteric model of protein function and control (Monod et al., 1965; Atkinson, 1977). Thick and thin filaments of myofibrils are aggregates of proteins. Changes in a filament may occur only after the protein molecules have lost ATP (onset of rigor mortis) which functions in the myofibrillar structure as an allosteric agent. Denaturation processes, however, have been shown to be endothermal reactions (Hägerdahl and Martens, 1976; Martens and Vold, 1976; Ledward, 1978; Wright et al., 1977). Therefore, protein denaturation

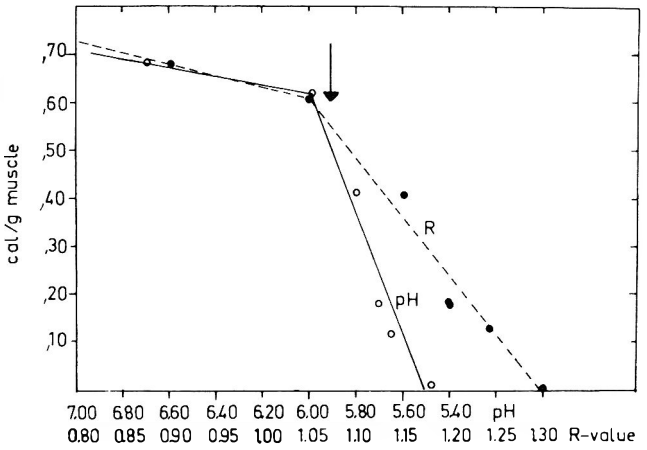


Fig. 6—Area of the exothermal heat output in the DSC thermogram with the peak at about 327 of bovine neck muscles vs pH and R-value. Arrow indicates the onset of rigor mortis (pH 5.9 and R around 1.08).

Table 1—SDS polyacrylamide gel electrophoresis of unsalted and salted (2% NaCl) homogenates of bovine sternomandibularis muscle

Hr postmortem	pH of intact tissue	Salt added to homogenate	% Myosin in electropherogram: Sol. fraction of homogenate	% Myosin in electropherogram: Insol. fraction of homogenate
1	6.9	–	1.0	27.0
6	6.5	–	0.5	26.5
14	6.1	–	3.5	27.0
25	5.6	–	1.0	26.5
1	6.9	+	19.0	12.5
6	6.5	+	18.0	12.5
14	6.1	+	6.0	26.0
25	5.6	+	2.5	34.0

Table 2—Relation of output of exothermal energy and the theoretical exothermal energy set free on ATP hydrolysis

pH of muscle	ATP in muscle (nMol)	A corresponds to free energy output on splitting to ADP (mcal)	Output of heat calc from exothermal peak (mcal)	C/B
	A[a]	B[b]	C[c]	
6.9	135	0.99	29	29
6.7	110	0.84	30	36
6.4	68	0.51	28.5	55
6.2	66	0.50	20	40
6.1	53	0.41	17	41
5.85	35	0.27	16	59
5.8	24	0.18	17	91
5.7	3	0.02	5.5	240

[a] A: 38-42 mg of muscle fibers were weighed into the capsules. Parallel to it the concentration of ATP in the muscle was determined and the amount in the capsules calculated.
[b] B: 1 nMol ATP = 7.6 µcal on hydrolysis of ATP to ADP. This value is valud for standard conditions. The authors are aware that this value depends on pH, temperature, and concentration of ATP, ADP and phosphate. Calculations revealed that this value may be up to four times higher under the conditions in the muscle and hydrolysis to AMP. Even under these conditions the output measured with DSC (C) is 8-60 times higher than by the hydrolysis of ATP (B).
[c] C: Calculated from the area of the peak between 320 and 330°K.

due to low pH and high tissue temperature can be excluded.

As the shape of the curves in Figure 6 agree also very well with the dependence of the postmortem changes in WHC (cooking loss) of salted muscle homogenates on tissue pH (Fig. 3) and R-value (Fig. 4), the conformational changes in myofibrillar proteins as induced by rigor mortis seem to be closely related to the WHC of meat after mincing and salting.

CONCLUSIONS

WHY HAS THE development of rigor mortis an effect on the WHC of salted muscle homogenates rather than on the WHC of unsalted muscle homogenates (Fig. 3) or of the unsalted intact muscle (Jolley et al., 1980)? It is possible that this is due to different types of intermolecular or interfilamental cross-linking occurring postmortem during pH decrease from 7 to 5.9 at which stage the onset of rigor occurs and the myofibrillar protein is approaching the IP. As the IP is approached, there is an increase of oppositely charged groups and, therefore, an increase of intermolecular ionic cross-linkages. In the network of myofibrillar proteins, tightened in this way, less water can be immobilized than in the loose network existing at higher pH values; the result is a decrease of WHC according to the general concept of the influence of protein charges on WHC and swelling of muscle (Hamm, 1972, 1975). This pH-dependent type of intermolecular cross-linking must be so strong that an additional cross-linking between thick and thin filaments (and other conformational changes) caused by rigor development cannot exert an additional effect on WHC in the absence of salt. Addition of salt at a pH higher than the IP causes a strong increase in WHC and swelling of muscle because of a shift of the IP of myofibrillar protein to lower pH values (Hamm, 1960, 1972, 1975). The formation of interfilamental cross-linkages during rigor mortis will hinder this effect. Therefore, the WHC-raising effect of NaCl in muscle homogenates is continuously diminished with decreasing pH of the prerigor muscle. Before onset of rigor (pH 5.9), pH dependent changes of protein charges determine postmortem alteration of unsalted and of salted muscle homogenates to a similar extent but at a different level (Fig. 3).

Hamm (1972, 1977) has suggested that the high WHC of presalted meat, i.e. meat salted in the prerigor state and then stored might be due to a strong electrostatic repulsion between the dissociated myofibrillar proteins myosin and actin caused by the combined influence of ATP, high pH, and increased ionic strength resulting from the salt addition. Conformational changes in protein structure caused by this effect are apparently irreversible because the WHC capacity of this sytem remains high irrespective of the postmortem breakdown of ATP and fall of pH ("presalting"-effect) (Hamm, 1972, 1977). It is conceivable that as long as sufficient ATP is present in the muscle in order to prevent appreciable cross-linking between thick and thin filaments (prerigor state), the electrostatic repulsion between proteins or filaments caused by salting is strong enough to guarantee the presalting effect. Consequently, for the preparation of sausages from hot-deboned beef the meat should be minced and presalted before the onset of rigor, i.e. before pH 5.9 is reached. The actual time postmortem before pH 5.9 is reached depends upon the temperature of the meat as shown in another paper (Honikel et al., 1981).

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Ascorbic Acid Effects on Bovine Muscle Pigments in the Presence of Radiant Energy

CAROLE A. Z. HARBERS, DOROTHY L. HARRISON, and DONALD H. KROPF

ABSTRACT

Untreated and treated (dipped in ascorbic acid solutions of 0, 0.5, 1.0, or 5.0%) raw psoas major muscle samples were exposed to radiant energy in a model system (500W Hg lamp, 577 nm, 2° ± 1°C, 20% O_2) with spectral reflectance measured every 30 min. Results varied with reflectance ratios or differences at 572/525, 630-582, 630/582, or 630-614 nm. Differences between 0 and 4 hr exposure indicated that ascorbic acid retarded pigment oxidation. The K/S ratio at 572/525 nm indicated that 5% ascorbic acid solution retarded pigment oxidation and protected muscle color most. Ascorbic acid retarded lipid oxidation (TBA values) in muscle stored at −26°C from 3-10 wk; a 5% solution gave the least protection of the solutions tested.

INTRODUCTION

THE APPEARANCE of a cut of meat is a primary factor in consumer choice. Although color of the lean does not predict meat quality, color of lean muscle contributes much to appearance (Romans and Ziegler, 1977). Deterioration of the bright red color in 1-3 days reduces red meat salability (Solberg, 1970).

Setser et al. (1973) suggested that lipids in muscle could be involved in the oxidation of heme pigments (color deterioration). Ledward and MacFarlane (1971) stated that lipid oxidation was not related directly to pigment oxidation or to metmyoglobin (MetMb) concentration, and suggested that both lipid oxidation and myoglobin (Mb) oxidation might be controlled by antioxidants.

Many researchers have studied the effects of antioxidants on lipid oxidation or Mb oxidation in ground meat (Watts and Lehman, 1952; Rikert et al., 1957; Caldwell et al., 1960; Greene, 1969; Liu and Watts, 1970; Green et al., 1971; Benedict et al., 1975; Govindarajan et al., 1977; Brown et al., 1978). We found little work on applying antioxidants to intact meat cuts and their effects on color. Hood (1975) found less accumulation of MetMb in steaks from beef animals given intravenous injections of ascorbate just before slaughter than in steaks from control animals. In preliminary studies he found that MetMb formation was inhibited in steaks sprayed with 2.5 or 5.0% ascorbate solutions.

Muscle pigment color has been measured objectively by reflectance spectrophotometry. Methods for recording data at various wavelengths include percentage reflectance, reflectance on absorbance scale, and calculated K/S values. Little work was found that compared the various methods of treating reflectance data to indicate beef color. Kropf et al. (1976) compared reflectance measurements of frozen beef T-bone steaks with visual color scores. Based on correlation coefficients, they found that visual redness correlated highest with R 685 nm and 582/525 nm reflectance ratio.

Attrey and Sharma (1979) analyzed reflectance data at various wavelengths to compared R_A values with K/S ratios as estimates of fresh mutton color. They found that estimates of color from K/S 572/525 nm ratio were more consistent than those from K/S values at 474/525 nm, 507/573 nm, or 473/579 nm, R_A 632 nm, and absorption (A 580 − A 630 nm).

We investigated effects of four concentrations of ascorbic acid on the color of steaks cut from four bovine psoas major (PM) muscles and exposed to radiant energy in a model system. The wavelength of light (577 nm), temperature (2° ± 1°C), and level of atmospheric oxygen (20%) were held constant. Reflectance spectrophotometry was used to study changes in color of the steaks during exposure to radiant energy. Four reflectance ratios or differences were calculated and compared.

MATERIALS & METHODS

Source of materials

Four bovine PM muscles from two steers and two heifers 18-19 months of age with USDA yield grades ranging from 2.1-4.2 and USDA quality grades of Low Choice to Average Choice were obtained from the Kansas State University Dept. of Animal Sciences & Industry. Each muscle was obtained when available for the experiment and held in the dark (24-48 hr) at 3.5°C before being cut into 11 or 12 steaks, each 3 cm thick. Each muscle represented one replication in the experiment. Immediately after being cut, steaks were vacuum packaged (Multi-Vac), frozen at −26°C, and held 3-7 wk in the dark at −26°C.

Treatments

Before application of the treatment combinations, each steak was thawed in the dark at 3.5°C for 16-18 hr, removed from its vacuum package, placed on a styrofoam tray, covered with polyvinyl chloride (PVC) film (oxygen permeable, moisture proof), and "bloomed" in the dark at 3.5°C for 30 min. A 4-cm diameter sample was removed from each steak with a copper sample cutter. Sterile disposable plastic gloves were worn when samples were handled.

Ascorbic acid crystals (Mallinckrodt, Inc.) were dissolved in distilled, deionized water at four concentrations, 0.5, 1.0, 2.0, or 5.0%. Muscle samples were dipped in distilled, deionized water or one of the ascorbic acid solutions for 10 or 20 sec, then drained for 10 sec. One sample from each muscle was untreated.

Exposure and spectral reflectance

Immediately after treatment, a single sample was placed in a sample holder, wrapped with PVC film, and exposed to radiant energy 4 hr. Conditions of exposure consisted of a cooling system where compressed air (20% oxygen) was passed through a copper coil submerged in liquid nitrogen to cool the air to approximately 2°C ± 1°C. The cooled air entered the sample chamber where samples were exposed to radiant energy from a 500-watt medium pressure mercury lamp fitted with a standard mercury line interference filter of 577 nm (Fig. 1). Light intensity was 0.95×10^{-3} w/cm^2 as measured by a IL 500 radiometer (International Light, Newburyport, Mass.) with detector SEE 010 # 127. Before exposure to radiant energy and at 30-min intervals during the exposure period, spectral reflectance of each sample was measured at light wavelengths of 474, 572, 582, 600, 614, and 630 nm with a Bausch and Lomb 600 spectrophotometer equipped with a reflectance attachment. The instrument was standardized by adjusting to 100% reflectance with a reference standard of magnesium carbonate.

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Authors Harbers and Harrison are with the Dept. of Foods & Nutrition, and Author Kropf is with the Dept. of Animal Sciences & Industry, Kansas Agricultural Experiment Station, Kansas State University, Manhattan, KS 66506.

Untreated steaks were handled and exposed to radiant energy in the same manner as those treated with antioxidant or distilled, deionized water. Data from percentage reflectance ratios or differences at 572/525, 630-582, 630/582, and 630-614 nm were converted to K/S values (Judd and Wyszecki, 1963).

Visual scoring

Each time a spectrophotmetric measurement was made, a visual score was given to the sample. Samples were compared to color photographs in "A Guide to Lamb Color" (American Lamb Council, Denver, CO, undated). Scoring was done on a scale of 1-5 with 1R indicating the bright red of oxymyoglobin and 5R, dark red (unacceptable). Scores also were given for browning, indicated by 3B (light brown) to 5B (brown–metmyoglobin), or for bleaching indicated by 3B1 (slight bleach) to 5B1 (much bleach–surface whitening).

pH and ether extract

A second sample was removed from each steak, treated, and held for pH measurement. Each sample was placed in a Whirl-Pak freezer bag, frozen, stored in the dark at −26°C for 1-10 days. Duplicate pH measurements were made on a slurry of 10g of ground muscle and distilled water (Rogers et al., 1967). Untreated samples from each muscle were ground three times in an electric food grinder, frozen, and analyzed for ether extract by the Soxhlet method (AOAC, 1975).

TBA analysis

After final reflectance measurements were made, each sample was placed in a Whirl-Pak freezer bag, frozen at −26°C and held in the dark at −26°C for 3-10 wk. Those samples were analyzed for lipid oxidation according to the 2-thiobarbituric acid (TBA) test (Tarladgis et al., 1960) modified according to Witte et al. (1970) and Kuntapanit (1978).

Preliminary studies

Preliminary work included experiments with PM, semitendinosus, and gluteus medius muscles. The last two muscles were discontinued in favor of the PM muscle, because it was most susceptible to Mb oxidation.

During preliminary trials, exposed and unexposed samples were tested for microbial growth by incubating plates at 21°C for total aerobes or at 7°C for psychotrophs. No indication of a microbial problem was observed. In addition to ascorbic acid, the antioxidants tertiary butylhydroquinone (TBHQ) and propyl gallate (PG) were used and eliminated, because of their extremely low solubility in distilled, deionized water. Because color changes were expected to be accelerated by the light source in the model system, exposure times from 1-6 hr were studied. Additional changes in reflectance measurements were minimal after 4 hr.

Statistical analyses

Reflectance data (differences between 0 and 4 hr exposure to radiant energy) for ratios or differences at 572/525, 630-582, 630/582, and 630-614 nm; calculated K/S ratios; pH values; and TBA values were subjected to analysis of variance (AOV) for a randomized complete block design. Duncan's Multiple-Range Test (Snedecor and Cochran, 1967) was used to determine differences ($P<0.05$) among specific treatment means.

RESULTS & DISCUSSION

Reflectance

F-values for percentage reflectance (R) and K/S values (a ratio of absorption to light scattering calculated from percentage reflectance) for wavelengths studied are in Table 1. When data for ratios 572/525 or 630/582 nm were analyzed, both percentage reflectance and K/S values indicated differences ($P<0.001$ or 0.01) among treatment combinations. With data for 630-582 nm, percentage reflectance, but not K/S values, indicated differences ($P<0.001$) among treatments; whereas, with data for 630-614 nm, neither percentage reflectance nor K/S values indicated significant differences among treatment combinations.

When F-values (Table 1) indicated significant differences among treatment means for the wavelengths studied, Duncan's Multiple-Range Test was used to determine differences between specific means (Table 2). Percentage reflectance at 572/525, 630-582, or 630/582 nm for untreated samples did not differ significantly from those dipped in distilled, deionized water (Table 2). When treatment means

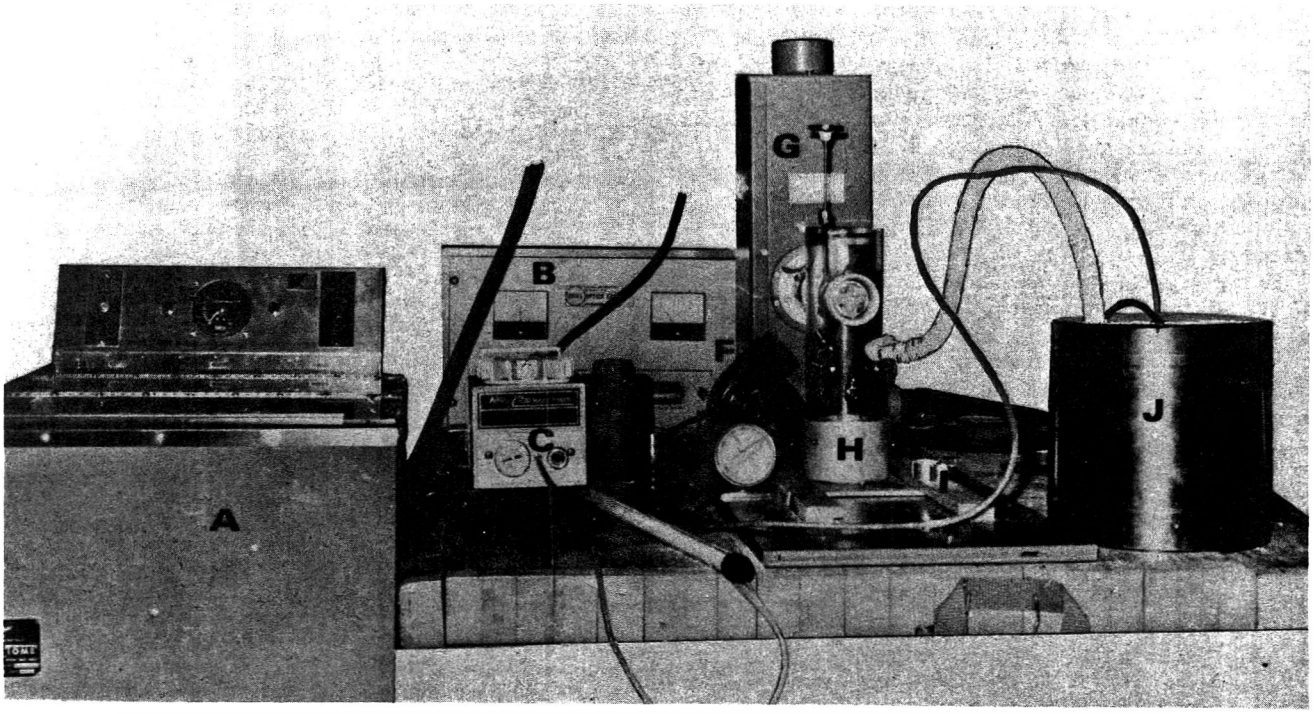


Fig. 1—Light exposure and cooling system: A, dry air source—Cryostat microtome cavity; B, power supply; C, air compressor; D, variable power regulator; E, sample cutter; F, blower to cool filter; G, housing for mercury lamp; H, sample chamber; I, sample holder; J, cooling system.

for R 572/525 nm were compared (Table 2), untreated and ascorbic acid treated samples differed ($P<0.05$), but none of the concentrations of ascorbic acid used differed ($P<0.05$) from one another. Percentage reflectance for dip time (10 vs 20 sec) did not differ significantly.

The K/S ratio at 572/525 nm has been used to estimate the amount of MetMb on the surface of a meat sample (Stewart et al., 1965; Snyder and Armstrong, 1967; Zimmerman and Snyder, 1969; Liu and Watts, 1970; Kropf et al., 1976; Attrey and Sharma, 1979). When K/S ratio treatment means for 572/525 nm for the difference between 0- and 4-hr exposure were compared (Table 2), in addition to differences ($P<0.05$) between untreated and ascorbic acid treated samples, samples dipped 10 sec in a 5.0% solution of ascorbic acid had lower ($P<0.05$) K/S values than those dipped 10 sec in an 0.5% ascorbic acid solution. Reflectance data for K/S 572/525 for incremental 30-min exposure times (Fig. 2 and 3) show that samples treated with ascorbic acid changed little from 0-4 hr (periods 1-8). Color of samples not treated with ascorbic acid deteriorated progressively during exposure. Differences in initial reflectance values (Fig. 2 and 3), were minor.

Table 1—F-values for reflectance data for differences between 0- and 4-hr exposure to radiant energy, pH, and TBA values[c]

Measurement	Source of variation: Treatment df=9	Source of variation: Replication df=3
Wavelength, nm		
572/525		
R (%)[a]	7.28***	3.43*
K/S[b]	6.17***	4.08*
630-582		
R (%)	7.11***	5.17**
K/S	1.80	0.30
630/582		
R (%)	3.74**	1.51
K/S	6.89***	1.60
630-614		
R (%)	1.74	4.89**
K/S	1.61	1.90
pH	7.51***	2.53
TBA	16.30***	0.83

[a] R (%), percentage reflectance
[b] K/S, ratio of absorption to scattering of light
[c] TBA, mg malonaldehyde/1000g sample
*P<0.05
**P<0.01
***P<0.001

Dean and Ball (1960) observed some differences in sample redness on freshly "bloomed" beef surfaces and concluded that initial "bloom" varied in samples from the same animal.

Reflectance of our samples dipped in the 5.0% ascorbic acid solution (10 or 20 sec dip) decreased rapidly during the first 30-min interval, then slowly increased in brightness (Fig. 2 and 3). That was supported by visual observation of an initial slight bleaching followed by a slow return to redness. That phenomenon could be related to pH. The pH of samples dipped in 5.0% ascorbic acid solution was lower ($P<0.05$) than that of the other samples (Table 2). At a lower pH, meat fibrils shrink and scatter more light than fibrils in muscle of higher pH, which likely gives a bleached appearance (Stewart et al., 1965).

Stewart et al. (1965) assumed a linear relationship between percentage MetMb and K/S ratio based on their values for the ratio of 0.56 for 100% MetMb and 1.40 for 0% MetMb. Therefore, decreasing K/S ratios at 572/525 nm should indicate an increase in MetMb formation. Using K/S 572/525 nm with values of 0.56 for 100% MetMb and 1.40 for 0% MetMb, we calculated the percentage MetMb in the PM muscles from mean K/S values at 572/525 nm after both 0- and 4-hr exposure to radiant energy (Table 3). Samples not receiving an antioxidant contained more MetMb after 4-hr exposure than treated samples, and the difference in percentage MetMb between 0 and 4 hr was larger for untreated samples than for any treated sample. Samples dipped in 5.0% ascorbic acid solution for 10 or 20 sec accumulated little MetMb; samples treated with 5.0% ascorbic acid solution, 10 sec dip, appeared to decrease in MetMb content, indicating protection of color. That tends to agree with findings of Watts and Lehmann (1952), who reported that the surface of ground pork with added ascorbic acid brightened after refrigerator storage. Greene et al. (1971) found that a consumer panel rejected samples of raw ground beef with a percentage MetMb greater than 40. After 4-hr exposure to radiant energy, untreated samples in our study contained more than 40% MetMb (Table 3).

R 630 nm is the predominant R_A peak for MetMb, and R 582 nm is the predominant peak for MbO_2. When Van den Oord and Wesdorp (1971) used R_A 630 nm – R_A 580 nm to determine relative proportions of MbO_2 and MetMb, they found a linear relationship between that value and percentage MetMb or percentage MbO_2. However, that relationship depended on the amount of Mb in the sample. Percentage reflectance at 630-582 nm differed ($P<0.001$) among treatments (Table 1). Based on Duncan's Multiple-Range Test that difference was attributable to differences

Table 2—Mean reflectance data for the difference between 0- and 4-hr exposure of bovine PM muscle to radiant energy, mean pH, and TBA values for four muscles

Treatment: Ascorbic Acid (%)	Treatment: Dip time (sec)	572/525: R (%)[g]	572/525: K/S[h]	630-582: R (%)	630/582: R (%)	630/582: K/S	pH	TBA[a]
0.0, untreated	0	−0.140c	0.214c	8.625c	1.103c	−0.189c	5.53c	0.263c
0.0, DDW[b]	10	−0.149c	0.256c	9.500c	0.959c	−0.177c	5.40cd	0.213c
0.5	10	−0.051d	0.109d	4.625d	0.344d	−0.057d	5.42cd	0.118e
1.0	10	−0.002d	0.013de	1.750d	0.130d	−0.016d	5.47cd	0.119e
2.0	10	−0.033d	0.077de	2.125d	0.152d	−0.019d	5.25de	0.117e
5.0	10	+0.006d	−0.016e	1.500d	0.308d	−0.034d	4.97f	0.165de
0.5	20	−0.054d	0.097de	4.000d	0.594cd	−0.075d	5.41cd	0.119e
1.0	20	−0.046d	0.092de	3.750d	0.351d	−0.050d	5.37cde	0.162de
2.0	20	−0.032d	0.069de	3.625d	0.118d	−0.035d	5.15ef	0.130e
5.0	20	−0.014d	0.027de	2.375d	0.195d	−0.034d	4.99f	0.200d

[a] TBA, mg malonaldehyde/1000 g sample
[b] DDW, distilled, deionized water
[cdef] Means bearing different letters within the same column differ significantly ($P<0.05$) as determined by Duncan's Multiple-Range Test
[g] R (%), percentage reflectance
[h] K/S, ratio of absorption to scattering of light

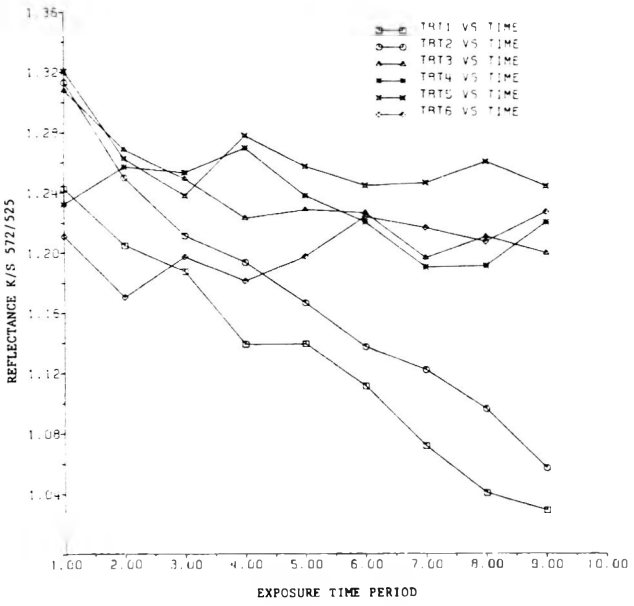


Fig. 2—Changes in K/S 572/525 nm of ascorbic acid treated and untreated PM samples (Treatments 1-6) as a function of exposure time (1 period = 0.5 hr) to radiant energy. Trt 1, 0.0% ascorbic acid (untreated); Trt 2, 0.0% ascorbic acid (distilled, deionized water); Trt 3, 0.5% ascorbic acid, 10-sec dip; Trt 4, 1.0% ascorbic acid, 10-sec dip; Trt 5, 2.0% ascorbic acid, 10-sec dip; Trt 6, 5.0% ascorbic acid, 10-sec dip.

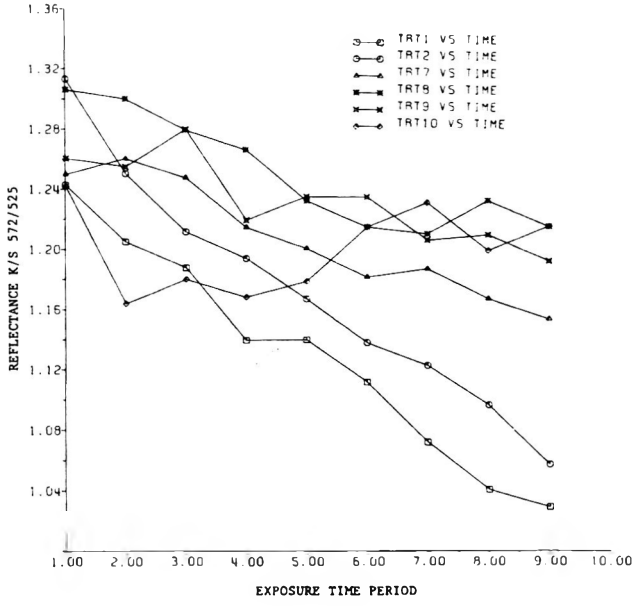


Fig. 3—Changes in K/S 572/525 nm of ascorbic acid treated and untreated PM samples (Treatments 1, 2, 7-10) as a function of exposure time (1 period = 0.5 hr) to radiant energy. Trt 1, 0.0% ascorbic acid (untreated); Trt 2, 0.0% ascorbic acid (distilled, deionized water); Trt 7, 0.5% ascorbic acid, 20-sec dip; Trt 8, 1.0% ascorbic acid, 20-sec dip; Trt 9, 2.0% ascorbic acid, 20-sec dip; Trt 10, 5.0% ascorbic acid, 20-sec dip.

between untreated samples and those treated with ascrobic acid (Table 2), with no significant differences among samples dipped in the four concentrations of ascorbic acid. Strange et al. (1974) reported a linear correlation coefficient of −0.86 between % R 630 − % R 580 nm and consumer acceptability. When we analyzed our R 630-582 nm data according to the K/S ratio (Table 1), we found no differences among treatments. That may be attributable to variability when the values were converted to K/S ratios.

Plots of the data for % R 630-582 nm with various exposure times (Fig. 4 and 5) show steadily decreasing reflectance values for samples receiving no antioxidant. According to Van den Oord and Wesdorp (1971), the almost linear decrease in reflectance indicates increased pigment oxidation. Samples dipped in 0.5, 1.0, or 2.0% ascorbic acid solution declined slowly in reflectance. The initial drop in reflectance for K/S at 572/525 nm (Fig. 2 and 3) of samples dipped in 5.0% ascorbic acid also is evident for R 630-582 nm (Fig. 4 and 5).

Significant differences among treatments were found with data for R 630/582 nm (Table 1), which is a ratio reported to parallel consumer acceptability (Strange et al., 1974) and to visual observations (Leising, 1976). Except for samples dipped in 0.5% ascorbic acid, our untreated and ascorbic acid treated samples differed ($P<0.05$), Table 2. Samples receiving ascorbic acid were brighter (lower K/S values) than untreated samples or samples treated only with distilled, deionized water (Table 2). As shown in Figures 2, 3, 4, and 5, samples dipped for either 10 or 20 sec in a 5% solution of ascorbic acid showed an initial drop in reflectance, then a slow increase to an end point similar to that for the other treated samples.

Values for R 630-614 nm or for K/S 630-614 nm indicated no significant differences among treatments (Table 1). Eagerman et al. (1978), who followed Mb oxidation in intact beef muscle samples with R 630-614, found R values less positive as the samples became browner. In their study, a small negative value indicated a brown, unacceptable sam-

Table 3—Mean reflectance data and percentage MetMb in PM muscle by treatment as calculated from K/S ratio at 572/525 nm after 0- and 4-hr exposure to radiant energy

Treatment combination		0 hr		4 hr		
Ascorbic acid (%)	Dip time (sec)	Mean	MetMb (%)	Mean	MetMb (%)	MetMb (%) Diff. (4-0)
0.0, untreated	0	1.243	18.69	1.029	44.05	25.36
0.0, DDW[a]	10	1.312	10.48	1.057	40.48	30.00
0.5	10	1.308	10.95	1.199	23.81	12.86
1.0	10	1.232	20.00	1.220	21.43	1.43
2.0	10	1.320	9.52	1.244	19.05	9.53
5.0	10	1.211	22.50	1.227	20.24	−2.26
0.5	20	1.250	17.86	1.153	30.00	12.14
1.0	20	1.306	11.19	1.214	22.26	11.43
2.0	20	1.260	16.67	1.191	25.00	8.33
5.0	20	1.241	18.93	1.214	22.62	3.69

[a] DDW, distilled, deionized water.

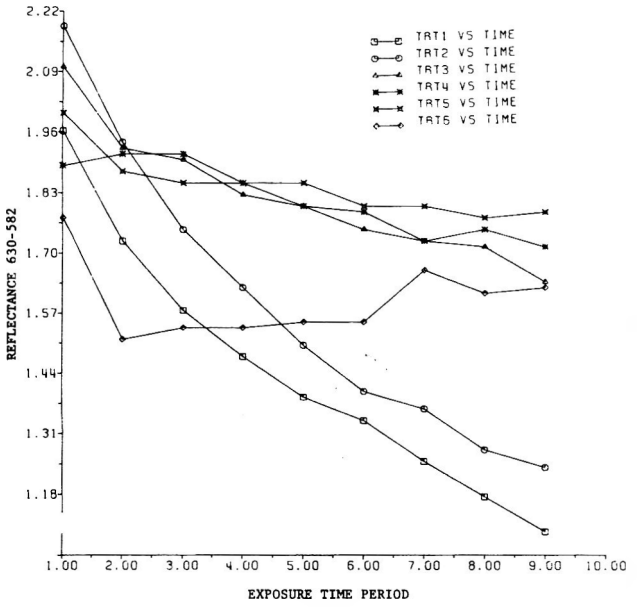


Fig. 4—Changes in R 630-582 nm of ascorbic acid treated and untreated PM samples (Treatments 1-6) as a function of exposure time (1 period = 0.5 hr) to radiant energy. Trt 1, 0.0% ascorbic acid (untreated); Trt 2, 0.0% ascorbic acid (distilled, deionized water); Trt 3, 0.5% ascrobic acid, 10-sec dip; Trt 4, 1.0% ascorbic acid, 10-sec dip; Trt 5, 2.0% ascorbic acid, 10-sec dip; Trt 6, 5.0% ascrobic acid, 10-sec dip.

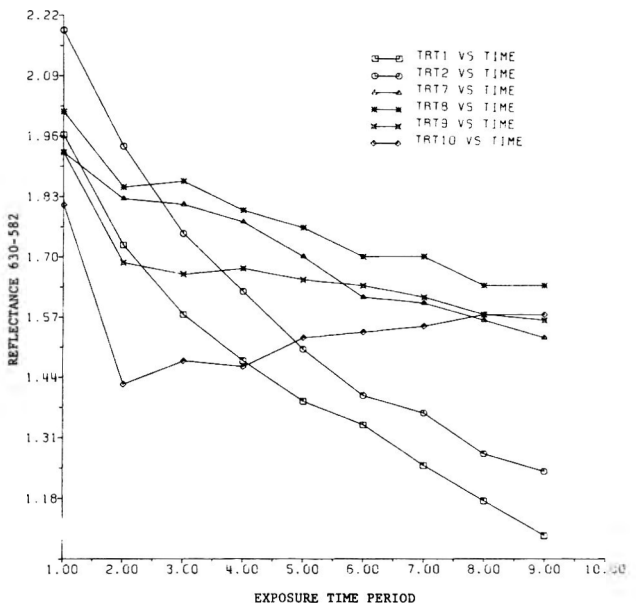


Fig. 5—Changes in R 630-582 nm of ascorbic acid treated and untreated PM samples (Treatments 1, 2, 7-10) as a function of exposure time (1 period = 0.5 hr) to radiant energy. Trt 1, 0.0% ascorbic acid (untreated); Trt 2, 0.0% ascorbic acid (distilled, deionized water); Trt 7, 0.5% ascorbic acid, 20-sec dip; Trt 8, 1.0% ascrobic acid, 20-sec dip; Trt 9, 2.0% ascorbic acid, 20-sec dip; Trt 10, 5.0% ascorbic acid, 20-sec dip.

ple. Although in our study, none of the values for R 630-614 was negative, values for samples receiving no antioxidant were smaller than values for samples dipped in an ascorbic acid solution.

Visual scores

Mean visual scores were not analyzed statistically because of "red," "brown," or "bleached" labels; but visual observation of samples each 0.5-hr interval supported the trends shown in Figures 2 through 5. At the end of 4 hr all samples not treated with ascorbic acid and those treated with a 0.5% ascorbic acid solution were brown. At least one sample remained red or returned to red when 1.0, 2.0, or 5.0% ascorbic acid solution was the dipping medium, which agrees with Rikert et al. (1957), who reported that color of beef cuts improved with the use of ascorbic acid.

The protection of meat color by ascorbic acid may be attributable to its function as a reducing agent. However, at certain concentrations ascorbic acid can act as a prooxidant and accelerate oxidation of Mb and MbO_2 to MetMb (Watts, 1954). We observed no prooxidant effects with the concentrations of ascorbic acid used.

Percentage ether extract

Triplicate measurements for ether extract (EE) were made on one sample of each muscle from which samples for exposure to radiant energy were taken. PM muscles from the two steers contained a medium amount of fat (8.12 and 8.61% EE); whereas, muscles from the heifers were either high (10.93% EE) or low (6.0% EE) in fat.

Values for R 572/525 nm and for K/S ratios at 572/525 nm differed ($P<0.05$) among replications, and data for R 630-582 nm and for R 630-614 nm differed ($P<0.01$) among replications (Table 1). Those differences could be attributed partially to differences in percentage fat among the four muscles (replications). Snyder and Armstrong (1967) stated that fat content could cause differences in reflectance values in intact meat samples, and Brown et al. (1978) reported that reflectance data differed ($P<0.05$) among samples of ground beef with three different fat levels.

pH

The F-value (Table 1) for differences in pH among treatments was significant ($P<0.001$). Samples dipped in a 5.0% solution of ascorbic acid had lower ($P<0.05$) mean pH values than untreated samples or samples treated with distilled, deionized water or with 1.0 or 2.0% ascorbic acid solutions (Table 2). pH values did not differ significantly among replications.

TBA values

F-values for the AOV of TBA values for the exposed samples (Table 1) indicated differences ($P<0.001$) among treatments, but no significant differences among replications. The TBA values (Table 2) for all ascorbic acid treated samples were lower ($P<0.05$) than those for the untreated samples or for samples dipped in distilled, deionized water. Oxidation was greater ($P<0.05$) for samples dipped 20 sec in a 5.0% solution of ascorbic acid than for other ascorbic acid treated samples (Table 2). All TBA values were low, indicating little or no rancidity, which agrees with findings of Chang et al. (1961), Liu and Watts (1970), and Greene et al. (1971).

If pigment oxidation and lipid oxidation are two separate reactions as Ledward and MacFarlane (1971) proposed, two different antioxidants may be required to retard both reactions in bovine muscle. Ascrobic acid is water soluble; a fat-soluble antioxidant could be used in combination with ascorbic acid. Greene et al. (1971), who added ascorbic acid and a lipid-soluble antioxidant to ground beef samples, reported that PG or butylated hydroxyanisole (BHA) (fat-soluble antioxidants) plus ascorbic acid protected the samples from lipid and pigment oxidation more than did either antioxidant alone.

—Continued on next page

CONCLUSIONS

UNDER the conditions of our study:

1. Ascorbic acid retards pigment oxidation in intact bovine PM muscle. The K/S ratio for 572/525 nm indicates that a 5.0% solution of ascorbic acid protects muscle color better than do 0.5, 1.0, or 2.0% solutions.

2. Ascorbic acid retards lipid oxidation in intact bovine PM muscle. TBA values indicate that a 5.0% solution of ascorbic acid protects against lipid oxidation less than do concentrations of 0.5, 1.0, or 2.0% solutions.

3. Effects of ascorbic acid on pigment oxidation in bovine PM muscle exposed to radiant energy, as measured by reflectance data, vary according to reflectance ratios or differences at 572/525, 630-582, 630/582, or 630-614 nm. With both 572/525 nm and 630/582 nm, %R and K/S values indicate that ascorbic acid protects the color of bovine PM muscle exposed to radiant energy. With 630-582 nm, only %R indicates that ascorbic acid protects the color of bovine PM muscle; and with 630-614 nm, both %R and K/S values indicate that ascorbic acid does not protect the color of bovine PM muscle exposed to radiant energy, or measures at those wavelengths are not sensitive to changes that occur.

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Effects of Certain Electrical Stimulation Parameters on Quality and Palatability of Beef

F. K. McKEITH, G. C. SMITH, J. W. SAVELL, T. R. DUTSON, Z. L. CARPENTER and D. R. HAMMONS

ABSTRACT

Grain-finished steers and heifers (n=390) were slaughtered in a commercial plant and assigned to one of five groups: (a) electrical stimulation (ES) immediately after exsanguination, (b) ES immediately before evisceration, (c) ES immediately after evisceration, (d) ES immediately after splitting, and (e) untreated (not ES). ES consisted of either 150 or 550 volts for either 1 or 2 min. Electrically stimulated carcasses had more youthful, brighter colored lean and less "heat-ring" than untreated (not ES) carcasses. Steaks from electrically stimulated carcasses had ($P<0.05$) lower shear force values, were more tender, had less panel-detectable connective tissue and higher overall palatability ratings than did steaks from untreated (not ES) carcasses. Steaks from carcasses receiving 550-volt ES usually had more youthful, brighter colored lean and lower shear force values, were more tender, had less panel-detectable connective tissue and higher overall palatability ratings than steaks from carcasses that received 150-volt electrical stimulation. Quality and palatability traits of carcasses electrically stimulated for 1 min were not different from those of carcasses receiving 2 min of electrical stimulation. Electrical stimulation at different stages in the slaughter-dressing sequence did not evoke differential responses in quality or palatability factors.

INTRODUCTION

ELECTRICAL STIMULATION (ES) of beef improves flavor, tenderness, lean color, lean maturity score and marbling, and reduces "heat-ring" and aging time necessary for tenderness assurance (Walker et al., 1977; Davey et al., 1976; Savell et al., 1977, 1978a, b, c, 1979; Gilbert and Davey, 1976; Smith et al., 1977). These results have created considerable interest in the ES process among industry personnel but some consternation regarding the details of its application under commercial conditions.

Some research has been conducted to identify conditions necessary to maximize benefits from electrical stimulation in a commercial slaughter plant. Savell et al. (1978b) compared differing numbers of electrical impulses using a constant voltage (440 volts) and found no apparent advantage for using 75, rather than 25, impulses. Chrystall and Devine (1978) compared the effects of using 320, 150 and 50 volts for electrical stimulation and reported only slight increases in the rate of pH decline with higher voltages. However, Bendall et al. (1976) reported a more rapid pH decline with high voltages and found no difference in the rate of pH decline when carcasses that were electrically stimulated as intact carcasses were compared to carcasses electrically stimulated as split sides. McKeith et al. (1979) reported that electrical stimulation of goats could be performed on sides or unsplit carcasses at any of several stages during the slaughter-dressing sequence. McKeith et al. (1980) determined that electrical stimulation of mature cow carcasses could be accomplished best by use of 16 impulses of 550 volts during 1 min.

Authors McKeith, Smith, Savell, Dutson and Carpenter are with the Meats & Muscle Biology Section, Dept. of Animal Science, Texas A&M Univ., Texas Agri. Exp. Stat., College Station, TX 77843. Author Hammons, now deceased, was with Agricultural Research, U.S. Dept. of Agriculture, College Station, TX 77843.

The present study was conducted to determine the effects of two voltages (150 vs 550 volts), two impulse durations (1 vs 2 min) and ES at each of four stages in the slaughter-dressing sequence of a commercial slaughter plant on the quality-determining characteristics and palatability attributes of beef. Results of this study were used by the LeFiell Company, Inc. to establish parameters for commercial application of the electrical stimulation process.

EXPERIMENTAL

GRAIN-FINISHED STEERS and heifers (n=390) were slaughtered in a commercial plant and assigned to one of five groups: (a) electrical stimulation (ES) immediately after exsanguination–approximately 5 min postmortem; (b) ES immediately before evisceration–approximately 20 min postmortem; (c) ES immediately after evisceration–approximately 30 min postmortem; (d) ES immediately after splitting–approximately 40 min postmortem; and (e) untreated (not ES). Cattle used were from commercial feedlots and had generally similar genetic, nutritional and management backgrounds. Electrical stimulation was performed with an experimental "Lectro-Tender™" unit which was manufactured by the LeFiell Company, San Francisco, Calif. One probe (approx 0.6 x 20 cm) was inserted in the muscles between the scapula and the thoracic vertebrae and the rail served as the ground for the system. Electrical stimulation involved the use of either 150 volts or 550 volts (AC) for either 1 min (16 impulses) or 2 min (32 impulses); impulses were of 1.8 sec duration with a 1.8 sec pause between impulses.

Quality characteristics

Carcasses were ribbed at 24 hr postmortem and USDA (1975) quality grade factors, lean maturity, lean color and incidence of "heat-ring" (Savell et al., 1978b) were determined by Texas Agricultural Experiment Station personnel.

Palatability tests

Sections of the longissimus muscle (10th to 11th ribs) from some of the carcasses were removed and frozen (–34°C) at 24 hr postmortem for sensory panel evaluation. Two steaks (2.5 cm in thickness) were cut from each longissimus muscle section, wrapped in polyethylene-coated paper and maintained at –34°C until cooked. Each steak was removed from the freezer, thawed (2°C) for 18 hr and cooked to an internal temperature of 70°C on Farberware Open Hearth broilers (monitored by use of copper-constantan thermocouples and a recording thermometer). One cooked steak from each carcass was evaluated by an 8-member experienced sensory panel; the second steak was used for Warner-Bratzler shear force determinations.

Data analysis

These data were analyzed by use of analysis of variance and mean separation analysis (Barr et al., 1976) to determine the significance of difference among treatments. Because this study was conducted in a commercial slaughter plant it was extremely difficult to maintain a balanced experimental design; as a result, numbers of observations in certain treatments and comparisons were not always consistent with good experimental design.

RESULTS

PAIRED COMPARISONS of carcass characteristics from all of the beef carcasses in this study (Table 1) and for beef carcasses that were electrically stimulated after exsanguina-

tion (Table 2), before evisceration (Table 3), after evisceration (Table 4) and as sides, after splitting (Table 5) provided data useful for identifying a system that could be used for electrically stimulating beef in commercial slaughter plants. When treated (ES) carcasses were compared to untreated (not ES) carcasses, electrical stimulation improved ($P<0.05$) lean maturity score (5 of 5 comparisons), improved ($P<0.05$) marbling (3 of 5 comparisons) and decreased ($P<0.05$) "heat-ring" incidence (5 of 5 comparisons) in Tables 1, 2, 3, 4, and 5. Use of 550 volts, rather than 150 volts, for electrical stimulation improved ($P<0.05$) lean maturity score (9 of 14 comparisons), improved ($P<0.05$) lean color (9 of 14 comparisons) and decreased ($P<0.05$) "heat-ring" incidence (3 of 14 comparisons) in Tables 1, 2, 3, 4, and 5. Stimulation for 2 min, rather than for 1 min, did not affect ($P>0.05$) lean maturity score in 6 of 9 comparisons, lean color and marbling in 9 of 9 comparisons or "heat-ring" incidence in 8 of 9 comparisons in Tables 1, 2, 3, 4, and 5.

Paired comparisons of palatability characteristics of cooked longissimus muscle for all of the beef carcasses from which wholesale ribs were obtained (Table 6) and for beef from carcasses that were electrically stimulated before evisceration (Table 7), after evisceration (Table 8), and as sides (Table 9) provided data useful for identifying a system that could be used for electrical stimulation in commercial slaughter plants. When steaks from treated (ES) carcasses were compared to steaks from untreated (not ES) carcasses (Tables 6, 7, 8, and 9), electrical stimulation decreased ($P<0.05$) shear force (4 of 4 comparisons), increased ($P<0.05$) muscle fiber tenderness (4 of 4 comparisons), increased ($P<0.05$) flavor desirability (3 of 4 comparisons) and improved ($P<0.05$) overall palatability (4 of 4 comparisons).

Use of 550 volts, rather than 150 volts, for electrical stimulation (Table 6, 7, 8, and 9) decreased ($P<0.05$) shear force (5 of 10 comparisons), increased ($P< 0.05$) muscle fiber tenderness (5 of 10 comparisons), decreased ($P<0.05$) amount of panel-detectable connective tissue (6 of 10 comparisons), increased ($P<0.05$) overall tenderness (5 of 10 comparisons), increased ($P<0.05$) flavor desirability (2 of 10 comparisons), increased ($P<0.05$) juiciness (2 of 10 comparisons) and improved ($P<0.05$) overall palatability (6 of 10 comparisons). Stimulation for 2 min, rather than for 1 min, did not affect ($P>0.05$) shear force in 4 of 4 comparisons, muscle fiber tenderness in 4 of 4 comparisons, amount of panel-detectable connective tissue in 4 of 4 comparisons, overall tenderness in 4 of 4 comparisons or overall palatability in 4 of 4 comparisons in Tables 6 and 9.

Comparisons of characteristics for beef carcasses that were electrically stimulated at different stages during the slaughter-dressing sequence are presented in Table 10. There was no clear indication of superiority in favor of any of the four stages of application of ES with regard to im-

Table 1—Paired comparisons of carcass characteristics for all of the beef carcasses in the study

Paired comparison	N	Lean maturity[c]	Lean color[d]	Marbling[e]	"Heat-ring" incidence[f]
All ES in the study	200[g]	A^{32a}	7.2^{a}	Sm^{08a}	4.3^{a}
All Not ES	190	A^{79b}	5.8^{b}	Sl^{96b}	3.2^{b}
All ES, 550v	118	A^{23a}	7.5^{a}	Sm^{07a}	4.4^{a}
All ES, 150v	82	A^{47b}	6.6^{b}	Sm^{11a}	4.1^{a}
ES, 550v, 1 min	90	A^{27a}	7.4^{a}	Sl^{99a}	4.3^{a}
ES, 150v, 1 min	50	A^{48b}	6.7^{b}	Sm^{17a}	4.3^{a}
ES, 550v, 2 min	28	A^{15a}	7.6^{a}	Sm^{24a}	4.5^{a}
ES, 150v, 2 min	32	A^{45b}	6.6^{b}	Sl^{98a}	4.0^{b}
ES, 150v, 1 min	50	A^{48a}	6.7^{a}	Sm^{17a}	4.3^{a}
ES, 150v, 2 min	32	A^{45a}	6.6^{a}	Sl^{98a}	4.0^{a}
ES, 550v, 1 min	90	A^{27b}	7.4^{a}	Sl^{99a}	4.3^{a}
ES, 550v, 2 min	28	A^{15a}	7.6^{a}	Sm^{24a}	4.5^{a}

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different ($P>0.05$).
[c] Cattle slaughtered at approximate chronological ages of 9-30 months normally produce carcasses of A^{00} to A^{100} maturity (USDA, 1975).
[d] 8 = light grayish-red; 1 = very dark purple.
[e] Sm = " small;" Sl = "slight" (USDA, 1975).
[f] 5 = no "heat-ring;" 1 = extremely severe "heat-ring" (Saveli et al., 1978 b).
[g] Includes 30 carcasses with ES after exsanguination, 72 carcasses with ES before evisceration, 48 carcasses with ES after evisceration, and 50 carcasses with ES after splitting.

Table 2—Paired comparisons of carcass characteristics for beef carcasses that were electrically stimulated after exsanguination

Paired comparison	N	Lean maturity[c]	Lean color[d]	Marbling[e]	"Heat-ring" incidence[f]
All ES after exsanguination	30	A^{27a}	7.2^{a}	Sm^{11a}	3.9^{a}
All Not ES	190	A^{79b}	5.8^{b}	Sl^{96b}	3.2^{b}
All ES, 550v	15	A^{18a}	7.5^{a}	Sm^{31a}	4.1^{a}
All ES, 150v	15	A^{37b}	6.9^{b}	Sl^{90a}	3.7^{a}
ES, 550v, 1 min	10	A^{26a}	7.4^{a}	Sm^{39a}	4.2^{a}
ES, 150v, 1 min	10	A^{42b}	6.6^{b}	Sl^{91a}	3.7^{a}
ES, 550v, 2 min	5	A^{02a}	7.8^{a}	Sm^{16a}	3.8^{a}
ES, 150v, 2 min	5	A^{27b}	7.4^{a}	Sl^{87a}	3.6^{a}
ES, 150v, 1 min	10	A^{42a}	6.6^{a}	Sl^{91a}	3.7^{a}
ES, 150v, 2 min	5	A^{27a}	7.4^{a}	Sl^{87a}	3.6^{a}
ES, 550v, 1 min	10	A^{26b}	7.4^{a}	Sm^{39a}	4.2^{a}
ES, 550v, 2 min	5	A^{02a}	7.8^{a}	Sm^{16a}	3.8^{a}

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different ($P>0.05$).
[c] Cattle slaughtered at approximate chronological ages of 9-30 months normally produce carcasses of A^{00} to A^{100} maturity (USDA, 1975).
[d] 8 = light grayish-red; 1 = very dark purple.
[e] Sm = " small;" Sl = "slight" (USDA, 1975).
[f] 5 = no "heat-ring;" 1 = extremely severe "heat-ring" (Saveli et al., 1978 b).

Table 3—Paired comparisons of carcass characteristics for beef carcasses that were electrically stimulated before evisceration

Paired comparison	N	Lean maturity[c]	Lean color[d]	Marbling[e]	"Heat-ring" incidence[f]
All ES before evisceration	72	A^{39a}	6.9^{a}	Sl^{97a}	4.3^{a}
All Not ES	190	A^{79b}	5.8^{b}	Sl^{96a}	3.2^{b}
All ES, 550v	44	A^{24a}	7.4^{a}	Sl^{87a}	4.5^{a}
All ES, 150v	28	A^{61b}	6.1^{b}	Sm^{11a}	4.1^{b}
ES, 550v, 1 min	32	A^{32a}	7.3^{a}	Sl^{91a}	4.5^{a}
ES, 150v, 1 min	18	A^{63b}	6.2^{b}	Sm^{21a}	4.3^{a}
ES, 550v, 2 min	12	A^{04a}	7.8^{a}	Sl^{79a}	4.4^{a}
ES, 150v, 2 min	10	A^{58b}	6.0^{b}	Sl^{93a}	3.6^{b}
ES, 150v, 1 min	18	A^{63a}	6.2^{a}	Sm^{21a}	4.3^{a}
ES, 150v, 2 min	10	A^{58a}	6.0^{a}	Sl^{93a}	3.6^{b}
ES, 550v, 1 min	32	A^{32b}	7.3^{a}	Sl^{91a}	4.5^{a}
ES, 550v, 2 min	12	A^{04a}	7.8^{a}	Sl^{79a}	4.4^{a}

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different ($P>0.05$).
[c] Cattle slaughtered at approximate chronological ages of 9-30 months normally produce carcasses of A^{00} to A^{100} maturity (USDA, 1975).
[d] 8 = light grayish-red; 1 = very dark purple.
[e] Sm = " small;" Sl = "slight" (USDA, 1975).
[f] 5 = no "heat-ring;" 1 = extremely severe "heat-ring" (Saveli et al., 1978 b).

proving lean maturity, lean color, marbling or "heat-ring" incidence in this population of carcasses. Although differences were not usually consistent enough for statistical significance, numerical values favored ES after splitting to ES after exsanguination (6 of 16 comparisons), ES before evisceration (10 of 16 comparisons) and ES after evisceration (5 of 12 comparisons). ES after evisceration was numerically superior to ES after exsanguination (8 of 12 comparisons) or before evisceration (9 of 12 comparisons).

Comparisons of palatability traits for longissimus muscles from beef carcasses that were electrically stimulated at different stages during the slaughter-dressing sequence are presented in Table 11. Electrical stimulation of carcasses after evisceration produced numerical advantages in palatability traits in 10 of 14 (2 of 14 were statistically significant) comparisons with ES before evisceration. Comparison of ES after splitting with ES before evisceration or ES after evisceration revealed numerical superiority in palatability for ES after splitting in 12 of 14 and 6 of 14 comparisons, respectively.

Based on statistical significance of comparisons, electrical stimulation at different stages during the slaughter-dressing sequence did not evoke differential responses in either carcass characteristics or cooked beef palatability traits. Data further suggest that it is not necessary to electrically stimulate carcasses as individual sides to achieve the desired response in quality and palatability.

Table 4—Paired comparisons of carcass characteristics for beef carcasses that were electrically stimulated after evisceration

Paired comparison	N	Lean maturity[c]	Lean color[d]	Marbling[e]	"Heat-ring" incidence[f]
All ES after evisceration	48	A^{24a}	7.5^{a}	Sl^{87a}	4.5^{a}
All Not ES	190	A^{79b}	5.8^{b}	Sl^{96a}	3.2^{b}
All ES, 550v	33	A^{22a}	7.6^{a}	Sl^{85a}	4.5^{a}
All ES, 150v	15	A^{27a}	7.2^{a}	Sl^{92a}	4.4^{a}
ES, 550v, 1 min	33	A^{22a}	7.6^{a}	Sl^{85a}	4.5^{a}
ES, 150v, 1 min	10	A^{22a}	7.5^{a}	Sl^{91a}	4.6^{a}
ES, 150v, 1 min	10	A^{22a}	7.5^{a}	Sl^{91a}	4.6^{a}
ES, 150v, 2 min	5	A^{38a}	6.6^{a}	Sl^{94a}	4.0^{a}

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different (P>0.05).
[c] Cattle slaughtered at approximate chronological ages of 9-30 months normally produce carcasses of A^{00} to A^{100} maturity (USDA, 1975).
[d] 8 = light grayish-red; 1 = very dark purple.
[e] Sm = "small;" Sl = "slight" (USDA, 1975).
[f] 5 = no "heat-ring;" 1 = extremely severe "heat-ring" (Savell et al., 1978 b).

Table 5—Paired comparisons of carcass characteristics for beef carcasses that were electrically stimulated, as sides, after splitting

Paired comparison	N	Lean maturity[c]	Lean color[d]	Marbling[e]	"Heat-ring" incidence[f]
All ES after splitting	50	A^{39a}	7.0^{a}	Sm^{14a}	4.3^{a}
All Not ES	190	A^{79b}	5.8^{b}	Sl^{96b}	3.2^{b}
All ES, 550v	26	A^{31a}	7.3^{a}	Sm^{09a}	4.3^{a}
All ES, 150v	24	A^{47a}	6.7^{b}	Sm^{18a}	4.3^{a}
ES, 550v, 1 min	15	A^{32a}	7.3^{a}	Sm^{00a}	4.1^{a}
ES, 150v, 1 min	12	A^{50a}	6.5^{a}	Sm^{28a}	4.1^{a}
ES, 550v, 2 min	11	A^{30a}	7.4^{a}	Sm^{22a}	4.5^{a}
ES, 150v, 2 min	12	A^{45a}	6.8^{a}	Sm^{09a}	4.6^{a}
ES, 150v, 1 min	12	A^{50a}	6.5^{a}	Sm^{28a}	4.1^{a}
ES, 150v, 2 min	12	A^{45a}	6.8^{a}	Sm^{09a}	4.6^{a}
ES, 550v, 1 min	15	A^{32a}	7.3^{a}	Sm^{00a}	4.1^{a}
ES, 550v, 2 min	11	A^{30a}	7.4^{a}	Sm^{22a}	4.5^{a}

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different (P>0.05).
[c] Cattle slaughtered at approximate chronological ages of 9-30 months normally produce carcasses of A^{00} to A^{100} maturity (USDA, 1975).
[d] 8 = light grayish-red; 1 = very dark purple.
[e] Sm = "small;" Sl = "slight" (USDA, 1975).
[f] 5 = no "heat-ring;" 1 = extremely severe "heat-ring" (Savell et al., 1978 b).

DISCUSSION

RECENT RESEARCH on electrical stimulation has created impetus for incorporation of the necessary ES equipment into the slaughter-dressing sequence of commercial beef slaughter plants. Because space and time are important factors limiting options of particular beef slaughterers with regard to places on the kill-floor at which ES equipment can be installed, it is essential that research should identify the effects of electrically stimulating carcasses (a) at different stages in the slaughter-dressing sequence, (b) for different periods of time, and (c) with different voltages, on quality-indicating and palatability factors.

Improvements in tenderness have been observed when electrical stimulation was performed on sides (Savell et al., 1977, 1978a, b, c, 1979; Davey et al., 1976; Gilbert and Davey, 1976; Walker et al., 1977; McKeith et al., 1979) and on intact carcasses (Walker et al., 1977; McKeith et al., 1979, 1980). Observation of the ES process reveals that carcass response to ES is much more violent when applied to sides (after splitting) than when applied to intact (unsplit) carcasses. Since Savell et al. (1978a) had proposed that these violent contractions might cause increased tenderness via structural damage, it was essential that we determine whether tenderness enhancement could be achieved without having to split carcasses prior to ES treatment. Savell et al. (1978b; 1979) and Calkins et al. (1980) had also observed improvements in quality-indicating characteristics (color, marbling, etc.) but all of their studies dealt with results from ES of sides. In the present study, electrical stimulation improved palatability and improved quality-indicating characteristics for beef irrespective of the form (sides or intact carcasses) or of the stage in the slaughter-dressing sequence at which it was performed.

Savell et al. (1978b) found no apparent advantages in quality or palatability in response to use of many impulses as opposed to few impulses for electrical stimulation. McKeith et al. (1980) reported that duration of ES (1 min vs 2 min) was inconsequential in assuring that beef from mature cow carcasses had been tenderized. Since a part of the tenderization effect of ES had been attributed to rate of pH decline (in preventing "cold shortening," Bendall et al., 1976; in rupturing lysosomal membranes and freeing lysosomal enzymes, Smith et al., 1977; Dutson et al., 1980) and since rate of pH decline may be associated with the increased firmness and brighter color of ES meat, it could be assumed that 2 min of ES might be more effective than 1 min in achieving pH-related palatability or quality enhancement. In the present study, lean maturity scores were improved slightly when electrical stimulation was performed using 550 volts for 2 min; however, palatability characteristics were not affected by duration (1 min vs 2 min) of stimulation. The pH decline was checked in longissimus muscles of some carcasses (data not reported in tabular form) and no consistent difference was found in pH among carcasses stimulated for 1 vs 2 min.

—Text continued on page 17

Table 6—Paired comparisons of palatability characteristics for longissimus muscles for all of the beef carcasses from which wholesale ribs were obtained

Paired comparison	N	Shear force (kg)	Tenderness: Muscle fiber[c]	Tenderness: Amt. of conn. tissue[d]	Tenderness: Overall[c]	Flavor[e]	Juiciness[f]	Overall palatability[g]
All ES in the study	83[h]	6.8[a]	5.0[a]	6.0[a]	5.0[a]	5.3[a]	4.8[a]	4.9[a]
All Not ES	73	8.9[b]	3.9[b]	5.2[b]	3.9[b]	4.9[b]	4.9[a]	4.0[b]
All ES, 550 volts	48	6.3[a]	5.4[a]	6.3[a]	5.3[a]	5.4[a]	4.9[a]	5.2[a]
All ES, 150 volts	35	7.5[b]	4.6[b]	5.7[b]	4.5[b]	5.1[b]	4.6[a]	4.5[b]
ES, 550 volts, 1 min	37	6.3[a]	5.4[a]	6.3[a]	5.3[a]	5.5[a]	4.9[a]	5.2[a]
ES, 150 volts, 1 min	30	7.5[b]	4.6[b]	5.7[b]	4.6[b]	5.1[b]	4.6[a]	4.5[b]
ES, 550 volts, 2 min	11	6.4[a]	5.4[a]	6.4[a]	5.3[a]	5.4[a]	4.8[a]	5.3[a]
ES, 150 volts, 2 min	5	7.3[b]	4.4[a]	5.4[b]	4.2[b]	5.4[a]	4.5[a]	4.4[b]
ES, 150 volts, 1 min	30	7.5[a]	4.6[a]	5.7[a]	4.6[a]	5.1[a]	4.6[a]	4.5[a]
ES, 150 volts, 2 min	5	7.3[a]	4.4[a]	5.4[a]	4.2[a]	5.4[a]	4.5[a]	4.4[a]
ES, 550 volts, 1 min	37	6.3[a]	5.4[a]	6.3[a]	5.3[a]	5.5[a]	4.9[a]	5.2[a]
ES, 550 volts, 2 min	11	6.4[a]	5.4[a]	6.4[a]	5.3[a]	5.4[a]	4.8[a]	5.3[a]

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different (P>0.05).
[c] 8 = extremely tender; 1 = extremely tough.
[d] 8 = no panel-detectable connective tissue; 1 = abundant panel-detectable connective tissue.
[e] 8 = extremely desirable flavor; 1 = extremely undesirable flavor.
[f] 8 = extremely juicy; 1 = extremely dry.
[g] 8 = extremely desirable in palatability; 1 = extremely undesirable in palatability.
[h] Includes 6 carcasses with ES after exsanguination, 22 carcasses with ES before evisceration, 20 carcasses with ES after evisceration, and 35 carcasses with ES after splitting.

Table 7—Paired comparisons of palatability characteristics of beef from carcasses that were electrically stimulated before evisceration

Paired comparison	N	Shear force (kg)	Tenderness: Muscle fiber[c]	Tenderness: Amt. of conn. tissue[d]	Tenderness: Overall[c]	Flavor[e]	Juiciness[f]	Overall palatability[g]
All ES before evisceration	22	7.6[a]	4.8[a]	5.9[a]	4.8[a]	5.1[a]	4.8[a]	4.6[a]
All Not ES	73	8.9[b]	3.9[b]	5.2[b]	3.9[b]	4.9[a]	4.9[a]	4.0[b]
All ES, 550 volts	11	6.3[a]	5.3[a]	6.2[a]	5.3[a]	5.4[a]	5.2[a]	5.1[a]
All ES, 150 volts	11	9.0[b]	4.2[b]	5.5[b]	4.2[b]	4.8[a]	4.3[b]	4.1[b]
ES, 550 volts, 1 min	11	6.3[a]	5.3[a]	6.2[a]	5.3[a]	5.4[a]	5.2[a]	5.1[a]
ES, 150 volts, 1 min	11	9.0[b]	4.2[b]	5.5[b]	4.2[b]	4.8[a]	4.3[b]	4.1[b]

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different (P>0.05).
[c] 8 = extremely tender; 1 = extremely tough.
[d] 8 = no panel-detectable connective tissue; 1 = abundant panel-detectable connective tissue.
[e] 8 = extremely desirable flavor; 1 = extremely undesirable flavor.
[f] 8 = extremely juicy; 1 = extremely dry.
[g] 8 = extremely desirable in palatability; 1 = extremely undesirable in palatability.

Table 8—Paired comparisons of palatability characteristics of beef from carcasses that were electrically stimulated after evisceration

Paired comparison	N	Shear force (kg)	Tenderness: Muscle fiber[c]	Tenderness: Amt. of conn. tissue[d]	Tenderness: Overall[c]	Flavor[e]	Juiciness[f]	Overall palatability[g]
All ES after evisceration	20	6.7[a]	5.2[a]	6.1[a]	5.2[a]	5.2[a]	4.9[a]	5.0[a]
All Not ES	73	8.9[b]	3.9[b]	5.2[b]	3.9[b]	4.9[b]	4.9[a]	4.0[b]
All ES, 550 volts	11	6.5[a]	5.5[a]	6.3[a]	5.4[a]	5.4[a]	4.9[a]	5.1[a]
All ES, 150 volts	9	6.8[a]	4.9[a]	5.7[a]	4.8[a]	5.1[a]	5.0[a]	4.8[a]
ES, 550 volts, 1 min	11	6.5[a]	5.5[a]	6.3[a]	5.4[a]	5.4[a]	4.9[a]	5.1[a]
ES, 150 volts, 1 min	9	6.8[a]	4.9[a]	5.7[a]	4.8[a]	5.1[a]	5.0[a]	4.8[a]

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different (P>0.05).
[c] 8 = extremely tender; 1 = extremely tough.
[d] 8 = no panel-detectable connective tissue; 1 = abundant panel-detectable connective tissue.
[e] 8 = extremely desirable flavor; 1 = extremely undesirable flavor.
[f] 8 = extremely juicy; 1 = extremely dry.
[g] 8 = extremely desirable in palatability; 1 = extremely undesirable in palatability.

Table 9—Paired comparisons of palatability characteristics of beef from carcasses that were electrically stimulated, as sides, after splitting

Paired comparison	N	Shear force (kg)	Tenderness			Flavor[e]	Juiciness[f]	Overall palatability[g]
			Muscle fiber[c]	Amt. of conn. tissue[d]	Overall[c]			
All ES after splitting	35	6.4[a]	5.1[a]	6.1[a]	5.0[a]	5.5[a]	4.7[a]	5.0[a]
All Not ES	73	8.9[b]	3.9[b]	5.2[b]	3.9[b]	4.9[b]	4.9[a]	4.0[b]
All ES, 550 volts	20	6.3[a]	5.4[a]	6.4[a]	5.4[a]	5.5[a]	4.8[a]	5.3[a]
All ES, 150 volts	15	6.7[a]	4.6[b]	5.8[b]	4.5[b]	5.3[a]	4.5[a]	4.6[b]
ES, 550 volts, 1 min	15	6.1[a]	5.4[a]	6.3[a]	5.3[a]	5.6[a]	4.8[a]	5.3[a]
ES, 150 volts, 1 min	10	6.4[a]	4.7[a]	6.0[a]	4.7[a]	5.3[a]	4.5[a]	4.8[a]
ES, 550 volts, 2 min	5	6.8[a]	5.5[a]	6.6[a]	5.5[a]	5.5[a]	4.9[a]	5.5[a]
ES, 150 volts, 2 min	5	7.3[a]	4.4[a]	5.4[a]	4.2[a]	5.4[a]	4.5[a]	4.3[a]
ES, 150 volts, 1 min	10	6.4[a]	4.7[a]	6.0[a]	4.7[a]	5.3[a]	4.5[a]	4.8[a]
ES, 150 volts, 2 min	5	7.3[a]	4.4[a]	5.4[a]	4.2[a]	5.4[a]	4.5[a]	4.3[a]
ES, 550 volts, 1 min	15	6.1[a]	5.4[a]	6.3[a]	5.3[a]	5.6[a]	4.8[a]	5.3[a]
ES, 550 volts, 2 min	5	6.8[a]	5.5[a]	6.6[a]	5.5[a]	5.5[a]	4.9[a]	5.5[a]

[ab] Means in the same column and in the same paired comparison bearing a common superscript letter are not different (P>0.05).
[c] 8 = extremely tender; 1 = extremely tough.
[d] 8 = no panel-detectable connective tissue; 1 = abundant panel-detectable connective tissue.
[e] 8 = extremely desirable flavor; 1 = extremely undesirable flavor.
[f] 8 = extremely juicy; 1 = extremely dry.
[g] 8 = extremely desirable in palatability; 1 = extremely undesirable in palatability.

Table 10—Comparisons of carcass characteristics for beef carcasses that were electrically stimulated at different stages during the slaughter-dressing sequence

Comparison	Trait	Stages of electrical stimulation[c]			
		After exsanguination	Before evisceration	After evisceration	After splitting
ES, 150 volts, 1 min	Lean maturity[d]	A^{42ab}	A^{63b}	A^{22a}	A^{50ab}
ES, 150 volts, 1 min	Lean color[e]	6.6^{ab}	6.2^{b}	7.5^{a}	6.5^{ab}
ES, 150 volts, 1 min	Marbling[f]	Sl^{91a}	Sm^{21a}	Sl^{91a}	Sm^{28a}
ES, 150 volts, 1 min	"Heat-ring"[g]	3.7^{a}	4.3^{a}	4.6^{a}	4.1^{a}
ES, 150 volts, 2 min	Lean maturity[d]	A^{27a}	A^{58a}	A^{38a}	A^{45a}
ES, 150 volts, 2 min	Lean color[e]	7.4^{a}	6.0^{b}	6.6^{ab}	6.8^{ab}
ES, 150 volts, 2 min	Marbling[f]	Sl^{87a}	Sl^{93a}	Sl^{94a}	Sm^{09a}
ES, 150 volts, 2 min	"Heat-ring"[g]	3.6^{a}	3.6^{a}	4.0^{a}	4.6^{a}
ES, 550 volts, 1 min	Lean maturity[d]	A^{26a}	A^{32a}	A^{22a}	A^{32a}
ES, 550 volts, 1 min	Lean color[e]	7.4^{a}	7.3^{a}	7.6^{a}	7.3^{a}
ES, 550 volts, 1 min	Marbling[f]	Sm^{39a}	Sl^{91a}	Sl^{85a}	Sm^{00a}
ES, 550 volts, 1 min	"Heat-ring"[g]	4.2^{a}	4.5^{a}	4.5^{a}	4.1^{a}
ES, 550 volts, 2 min	Lean maturity[d]	A^{02a}	A^{04a}	–	A^{30b}
ES, 550 volts, 2 min	Lean color[e]	7.8^{a}	7.8^{a}	–	7.4^{a}
ES, 550 volts, 2 min	Marbling[f]	Sm^{16a}	Sl^{79a}	–	Sm^{22a}
ES, 550 volts, 2 min	"Heat-ring"[g]	3.8^{a}	4.4^{a}	–	4.5^{a}

[ab] Means in the same row and for the same comparison bearing a common superscript letter are not different (P>0.05).
[c] Stages were: after exsanguination, before evisceration, after evisceration and after splitting.
[d] Cattle slaughtered at approximate chronological ages of 9-30 months normally produce carcasses of A^{00} to A^{100} maturity (USDA, 1975).
[e] 8 = light grayish-red; 1 = very dark purple.
[f] Sm = "small;" Sl = "slight" (USDA, 1975).
[g] 5 = no "heat-ring;" 1 = extremely severe "heat-ring" (Saveli et al., 1978b).

Because of the comparatively greater danger to workmen on the slaughter-floor of 550 volts as compared to 150 volts, it was essential to compare effects of these two voltages on quality-indicating and palatability characteristics of beef. Most of our ES studies had been conducted with 440-550 volts, with the desired results, yet Chrystall and Devine (1978) found little difference in rate of pH decline between 320, 150 and 50 volts for electrical stimulation and Taylor and Marshall (1980) accomplished tenderization of beef carcasses with ES of 32 volts. Since the objective of this study was twofold—improvement of palatability and improvement in appearance of the longissimus muscle cross-sectional surface at the 12-13th rib—the effectiveness of a high (550 volts) and a low (150 volts) voltage for electrical stimulation was compared. Results of the present study suggested sufficient advantage for high (550 volts) vs low (150 volts) voltage in improving lean maturity, lean color, flavor, tenderness and overall palatability to warrant its use for commercial implementation of electrical stimulation.

Data of the present study indicate that electrical stimulation will improve palatability of beef muscles and that it can be performed at any of the four stages in the slaughter-dressing sequence that we investigated. Electrical stimulation for 2 min rather than for 1 min had no apparent advantage in improving palatability or appearance of beef. However, electrical stimulation with 550 volts rather than with 150 volts improved lean maturity, lean color, flavor,

Table 11—Comparisons of palatability traits of beef from carcasses that were electrically stimulated at different stages during the slaughter-dressing sequence

Comparison	Trait	Stages of electrical stimulation[c] Before evisceration	After evisceration	After splitting
ES, 150 volts, 1 min	Shear force, kg	9.0[a]	6.8[b]	6.4[b]
ES, 150 volts, 1 min	Mus. Fib. tenderness[d]	4.2[a]	4.9[a]	4.7[a]
ES, 150 volts, 1 min	Amt. of conn. tissue[e]	5.5[a]	5.7[a]	6.0[a]
ES, 150 volts, 1 min	Overall tenderness[d]	4.2[a]	4.8[a]	4.7[a]
ES, 150 volts, 1 min	Flavor[f]	4.8[a]	5.1[a]	5.3[a]
ES, 150 volts, 1 min	Juiciness[g]	4.1[a]	4.8[b]	4.5[b]
ES, 150 volts, 1 min	Overall palatability[h]	4.3[a]	5.0[a]	4.8[a]
ES, 550 volts, 1 min	Shear force, kg	6.3[a]	6.5[a]	6.1[a]
ES, 550 volts, 1 min	Mus. Fib. Tenderness[d]	5.3[a]	5.5[a]	5.4[a]
ES, 550 volts, 1 min	Amt. of conn. tissue[e]	6.2[a]	6.3[a]	6.3[a]
ES, 550 volts, 1 min	Overall tenderness[d]	5.3[a]	5.4[a]	5.3[a]
ES, 550 volts, 1 min	Flavor[f]	5.4[a]	5.4[a]	5.6[a]
ES, 550 volts, 1 min	Juiciness[g]	5.2[a]	4.9[a]	4.8[a]
ES, 550 volts, 1 min	Overall palatability[h]	5.1[a]	5.1[a]	5.3[a]

[ab] Means in the same row and for the same comparison bearing a common superscript letter are not different ($P>0.05$).
[c] Stages were: before evisceration, after evisceration and after splitting.
[d] 8 = extremely tender; 1 = extremely tough.
[e] 8 = no panel-detectable connective tissue; 1 = abundant panel-detectable connective tissue.
[f] 8 = extremely desirable flavor; 1 = extremely undesirable flavor.
[g] 8 = extremely juicy; 1 = extremely dry.
[h] 8 = extremely desirable in palatability; 1 = extremely undesirable in palatability.

tenderness and overall palatability. In conclusion, electrical stimulation can be performed at any of several stages during the slaughter-dressing sequence of commercial plants using short duration (1 min) and high voltage (550 volts) stimulation to improve quality-indicating characteristics and palatability of beef.

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Qualitative Alteration of Phospholipids in Beef Patties Cooked After Vacuum and Nonpackaged, Frozen Storage

H. YUSUF GOKALP, HERBERT W. OCKERMAN, RODNEY F. PLIMPTON, and ANDREW C. PENG

ABSTRACT

Alterations during frozen storage of the phospholipids in high quality and very lean (7.5% fat) beef patties as influenced by vacuum packaging were studied. Meat was obtained from a closely trimmed semimembranosus muscle from a U.S. Choice Grade steer carcass. Ground patties were stored 3 days, 1.5, 3, and 4.5 months at −23 ± 1°C. Lipids were extracted after the patties were thawed and cooked. After the 3 days of frozen storage, the vacuum packaged samples indicated the presence of eight different phospholipid classes in decreasing concentration: phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol, lysophosphatidylethanolamine, and diphosphatidylglycerol, in the nonpackaged samples; diphosphatidylglycerol and lysophosphatidylethanolamine were absent. As storage progressed, the amount of certain phospholipid classes, particularly in the nonpackaged samples, declined. After 3 months of storage in both packaged and nonpackaged samples, trace amount of lysodiphosphatidylglycerol became evident.

INTRODUCTION

LIPIDS found in meat may be classified according to location as: (1) depot or subcutaneous; (2) intermuscular; and (3) intramuscular or tissue lipids (Love and Pearson, 1971). The intermuscular lipids are generally stored in specialized connective tissues in relatively large deposits, whereas tissue lipids are integrated into and widely distributed throughout the muscles (Kono and Colowick, 1961). The intracellular lipids exist in close association with proteins and contain a large percentage of total phospholipids (PL) which are the main contributors to oxidations and subsequent rancidity (Watts, 1962).

The phospholipid content of various meats and muscle tissue has been determined (Hornstein et al., 1967; Turkki and Campbell, 1967; O'Keefe et al., 1968). The phospholipids contribute about 0.43-1.00% of the tissue weight in beef (Hornstein et al., 1961). Keller and Kinsella (1973) have reported 5.2-5.5% phospholipids in the total lipids of the round and 2.50-2.75% in the lipids of the chuck.

This very important class of lipids, phospholipids, has not been thoroughly studied, especially during frozen storage of muscle tissue from large meat animals. Surprisingly, complete distribution of phospholipid classes of beef muscle on thin-layer chromatography (TLC) plates are not available in the literature. Keller and Kinsella (1973) indicated the presence of seven different phospholipid classes (lysophosphatidylcholine, sphingomyelin, phosphatidylcholine, phosphatidylinositol, phosphatidylserine, phosphatidylethanolamine and cardiolipin) in fresh hamburger tissue, ground round, ground chuck, and ground beef. The procedures used in this TLC separation of the phospholipid classes were not reported.

Authors Ockerman and Plimpton are with the Dept. of Animal Science, The Ohio State Univ., Columbus, OH 43210, and the Ohio Agricultural Research & Development Center, Wooster, OH 44691. Author Peng is with the Dept. of Horticulture, Ohio State Univ., Columbus, OH 43210. Author Gokalp, formerly with Ohio State Univ., is now with Ataturk Universitesi, Ziraat Fak, Erzurum, Turkey.

Although various workers have noted that tissue lipids were stable during frozen storage (Caldwell et al., 1960; Keskinel et al., 1964; Witte et al., 1970), breakdown of triglycerides and phospholipids by lipases and autoxidation has been observed in frozen muscle tissue held at various temperatures and for different storage times (Awad et al., 1968; Bosund and Ganrot, 1969; Keller and Kinsella, 1973; Younathan and Watts, 1960; Hornstein et al., 1961). The lability of phospholipids to oxidation may be a result of their high unsaturated fatty acid content and the proximity to proteins with their more active structure.

Even though the phospholipid content of meat is relatively small, the susceptibility of phospholipids to oxidation and degradation makes this class of compounds important in the study of postmortem shelf life of muscle tissue. It is postulated that phospholipids play an important role in governing the quality, i.e., off-flavor, off-odors and discoloration of ground beef patties (Lea, 1957; Younathan and Watts, 1959; Watts, 1962; Chipault and Hawkins, 1971.

In the present investigation, the objective was to study the alteration of the phospholipid fraction in USDA Choice grade muscle tissue converted into very lean cooked beef patties, following frozen storage under vacuum packaged (VP) and under nonpackaged (NONP) conditions.

MATERIALS & METHODS

Materials

A semimembranosus muscle was removed from the right round of a USDA Choice grade steer carcass which was obtained from The Ohio State University Meat Laboratory at 1 day postmortem. The internal temperature of the round had reached 5 ± 1°C. After removal of the external fat the muscle was ground through a 1/8 in. (0.3175 cm) plate. The fat percentage of this ground product was found to be 7.5% as determined by ether extraction (Ockerman, 1976). Removal of the outside fat layer increased the relative percentage of the intramuscular lipid fraction since this tissue is richer in phospholipids than ground meat which is not closely trimmed. Ground beef patties of approximately 200g were made with a thickness of 1.6-2.0 cm.

Twenty-four ground beef patties were randomly assigned to one of eight treatments. The treatments included two groups of patties (vacuum packaged–VP and nonpackaged–NONP or unwrapped) and each group was stored at −23 ± 1°C for 3 days, 1.5 months, 3 months, and 4.5 months. Samples were not ananlyzed in the unfrozen or stored uncooked state since the phospholipid analyses would have overlapped the corresponding frozen and stored cooked analysis.

Patties to be vacuum packaged (VP) were immediately placed into 17.78 x 55.88 cm Barrier Bags (Cry-O-Vac B-620 Barrier Bag, WVT gram at 37.78°C-100% RH 0.5-0.6/254 cm^2, 24 hr and oxygen transmission cc at 22.73°C 30-50/m^2, 24 hr 1 atm., thickness 2.12 mils). A vacuum of 560-610 mm was used to gently draw the air from the bags. The bags were sealed with clips. The nonpackaged (NONP) samples were stored unwrapped. All samples, vacuum packaged (VP) and nonpackaged (NONP) were placed in a single layer in cardboard boxes with the bottom lined with freezer paper (Marlon, American Can Company). Boxes were placed in a −23 ± 1°C laboratory-type upright freezer.

At the end of the storage time intervals, patties from both treatments (vacuum and nonpackaged) were removed from the freezer and thawed in a 3 ± 1°C household type refrigerator. The nonpackaged (NONP) samples were thawed in unsealed barrier bags. Com-

plete thawing of samples took from 24-28 hr.

Thawed samples were taken from the bags, exposed to air under laboratory conditions to "bloom" 30 min, and then placed on aluminum trays for cooking in a 165-170°C electric oven. Juice was retained in the trays during cooking. The patties were cooked to an internal temperature of 68°C which resulted in an interior color of light brown and an exterior showing some browning. When the cooking was completed, a spatula was used to homogenize the juice and meat together and the mixture was wrapped very tightly with aluminum foil and stored in a small laboratory refrigerator (3 ± 1°C). A composite sample was used since the objectives of this research were to follow alteration during frozen storage.

Analytical methods

Lipid extraction was started approximately 6 hr after the patties were cooked and continued for 10 hr. The total lipids were extracted from 5g of homogenized patties by the method of Folch et al. (1957). Nonlipid contaminants from the chloroform-methanol ($CHCl_3$-CH_3OH) extract were removed with a Sephadex G-25 column (Wuthier, 1966).

After the solvent was evaporated on a rotary evaporator, the phospholipids were separated from the crude lipid by the method of Rouser et al. (1966), using a 2.5 x 40 cm glass column containing SilicAR cc-7, with special 100-200 mesh (Mallinckrodt Chemical Works, St. Louis, Mo.). In the following sequence, neutral lipids with $CHCl_3$, glycolipids with acetone and phospholipids with CH_3OH were eluted from the column.

The phospholipids were separated into their classes by two-dimensional thin-layer chromatography (Parson and Patton, 1967), using 20 x 20 cm precoated Silica Gel G (250 m thickness) glass plates (Applied Sci. Lab., Inc., State College, Pa.). Each time a standard amount of $CHCl_3$ containing the phospholipids (500 μg, based on the original amount of phospholipids present) was applied at the origin. The separation was accomplished by using a solvent system of $CHCl_3$:CH_3OH:Water:28% aqueous ammonia (130:70:8:0.5, v/v) in the first dimension followed by $CHCl_3$:acetone:CH_3OH:acetic acid:water (100:40:20:20:10, v/v) in the second dimension. The different phospholipid classes were made visible as blue spots by spraying with Molybdenum Blue Reagent (Dittmer and Lester, 1964). The spots were marked with a continuous (solid) or discontinuous (dotted) line according to the color intensity which is an indicator of the amount of phosphorus ester present. The spots were identified by comparison with R_f values of known phospholipid standards (Applied Sci. Lab., Inc., State College, Pa.).

Since the blue color of the phospholipid spots would fade within a day, plates were charged in a 100°C drying oven to produce yellow colored lipid spots that could be retained for a longer period of time. The charging of the plates also helped to visualize the neutral lipids and free fatty acids (FFA) if there were any in the phospholipid fraction. These samples were also visually and sensory evaluated and chemically analyzed for TBA, fatty acids and carbonyls; portions of which will be reported in subsequent papers.

RESULTS & DISCUSSION

AS SEEN FROM FIGURE 1, eight different classes of phospholipid–diphosphatidylglycerol (DPG), phosphatidylethanolamine (PE), phosphatidylcholine (PC), sphingomyelin (Sph), lysophosphatidylethanolamine (LPE), lysophosphatidylcholine (LPC), phosphatidylinositol (PI), and phosphatidylserine (PS)–were found to be present in the vacuum packaged samples after 3 days of frozen storage and cooking. Phosphatidylcholine was the predominant phospholipid class. The phosphatidylethanolamine was the second in area and intensity of color which are the indications of the total amount of phosphatidylethanolamine in the phospholipid fraction. Keller and Kinsella (1973) had observed the presence of seven phospholipid classes–phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, phosphatidylserine, sphingomyelin, lysophosphatidylcholine and cardiolipin (DPG)–in ground round of beef carcass. Their work indicated a very small percent (0.60%) of lysophosphatidylcholine and a large percentage of phosphatidylcholine (57.22%) and phosphatidylethanolamine (23.86%).

A small amount of lysophosphatidylethanolamine which was present in the vacuum packaged samples at 3 days of frozen storage, was not found by Keller and Kinsella (1973). Simon and Rouser (1969) have noted a small percent of lysophosphatidylethanolamine in the ground mixture of the skeletal muscle obtained from the legs of mouse, rat, frog and human and from bovine neck.

Keller and Kinsella (1973) reported 5.72% of diphosphatidylglycerol in the ground round compared to 5.32% of sphingomyelin, 1.92% phosphatidylserine, and 5.41% phosphatidylinositol. In the present work, the close examination of the fresh formed phospholipid spots suggested that the quantity of diphosphatidylglycerol was very small, followed by increasing quantities of phosphatidylinositol, lysophosphatidylethanolamine, phosphatidylserine, lysophosphatidylcholine, and sphingomyelin. The small amount of diphosphatidylglycerol compared to the work of Keller and Kinsella (1973) could be explained by the instability of this phospholipid class during the frozen storage and subsequent thawing and cooking. As the Figures (1, 2, 3 and 4) indicated, no diphosphatidylglycerol was found in the nonpackaged (NONP) beef patties starting at 3 days of frozen storage. However, very small amounts of diphosphatidylglycerol were always noted in the vacuum packaged (VP) samples. A spot corresponding to the lysophosphatidylethanolamine was only present in the vacuum packaged (VP) samples at 3 days of storage.

Only one spot which is marked phosphatidylserine and phosphatidylinositol was present in the nonpackaged (NONP) samples compared to the distinctive spots for phosphatidylserine and phosphatidylinositol in the vacuum packaged (VP) samples at 3 days of storage time. The quantity of the phosphatidylserine and phosphatidylinositol declined very rapidly in both packaging treatments after 3 days of storage. No traces of these compounds were found at the 3 and 4.5 months of storage.

A visually noticeable decline in the quantity of sphingo-

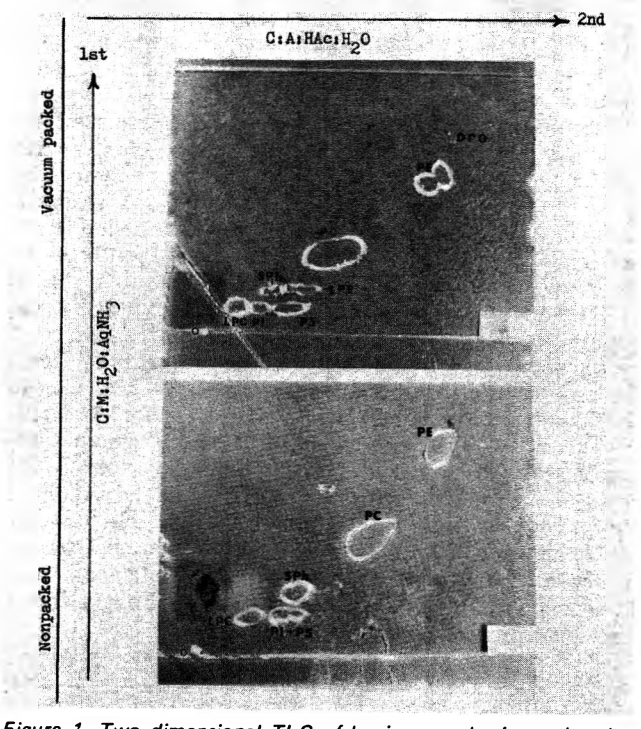


Figure 1–Two dimensional TLC of bovine muscle tissue phospholipids, after 3 days of frozen storage and cooking. Development time 2 hr in each direction. DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PC, phosphatidylcholine; Sph, sphingomyelin; LPE, lysophosphatidylethanolamine; LPC, lysophosphatidylcholine; PI, phosphatidylinositol; PS, phosphatidylserine; O, sample origin.

myelin and lysophosphatidylcholine was noted after the 1.5 months of storage in the nonpackaged (NONP) samples and after 3 months in the vacuum packaged samples. Also a decline in the amount of phosphatidylethanolamine, especially in the nonpackaged (NONP) samples, after 3 months of storage was clearly seen in the figures.

The limited changes in the area and color of phosphatidylcholine spots could be an indication of the stability of this compound under these experimental conditions. As seen in Figure 2 the spot of phosphatidylcholine sometimes could be seen as almost two spots joined together very closely, PC_1 and PC_2. The appearance of this phosphatidylcholine divided spot is in agreement with the work of Lyman et al. (1967) which showed the presence of three closely associated phosphatidylcholine (PC_1, PC_2 and PC_3) and two phosphatidylethanolamine classes (PE_1 and PE_2) in the phospholipids of rat livers.

After 3 and 4.5 months of frozen storage in both treatments (vacuum and nonpackaged) a trace amount of lysodiphosphatidylglycerol started to appear. The presence of the lysodiphosphatidylglycerol in fresh beef muscle tissue has not been reported (Keller and Kinsella, 1973). However, the lysodiphosphatidylglycerol has been seen in extracts of organs allowed to stand for several hours before the extraction of the phospholipid fraction (Rouser et al., 1969). A small percent of lysodiphosphatidylglycerol was also found in ground squirrel heart extract (Aloia et al., 1974) and mitochondrial inner membrane lipids of beef kidney (Rouser et al., 1969).

Oxidation of meat lipids during cooking has been reported (Younathan and Watts, 1960; Chang et al., 1961; Jakobsson and Berngtsson, 1972; Nakanishi and Suzama, 1967; Keller and Kinsella, 1973). Keller and Kinsella (1973) indicated a small increase in the amount of lysophosphatidylcholine and lysophosphatidylethanolamine upon the cooking of beef hamburgers. They concluded that formation of these lysophosphatides may have been caused by phospholipases and/or selective thermal hydrolysis. The present study does not confirm the above conclusion since a very small amount of lysophosphatidylethanolamine was only present in the vacuum packaged samples at 3 days of frozen storage and it was absent in the unprotected samples stored for a longer period of time. The lysophosphatidylcholine was present in all samples but the amount declined as the storage time progressed.

At 1.5 months of frozen storage some of the nonpackaged (NONP) samples indicated a trace quantity of a phosphorus compound (X_1) which might be an intermediate degradation product of a phospholipid or a trace of diphosphatidylglycerol still present. A spot which is marked X_2 in Figure 3 was not identified clearly. It could be a part of phosphatidylcholine (like PC_1 in the vacuum packaged samples in Fig 2) or it could be phosphatidylglycerol (PG) formed as an intermediate product of phospholipid hydrolysis and/or oxidation.

A very small amount of phosphatidic acid (PA) was present in the vacuum packaged samples at 1.5 months of storage. This phosphatidic acid could be an artifact due to the enzymatic hydrolysis and breakdown of phospholipids. The other samples did not yield any traces of phosphatidic acid in their phospholipid fraction.

As frozen storage time progressed, the color intensity and the area of phosphatidylcholine, phosphatidylethanolamine, sphingomyelin, lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol, lysophosphatidylethanolamine, and diphosphatidylglycerol declined. This reduction was more obvious in the nonpackaged (NONP) samples.

Lipolysis of phospholipids during frozen storage has been observed in bovine, fish and chicken muscle (Awad et al., 1968; Bosund and Ganrot, 1969; Davidkova and Khan, 1968). Conceivably the phospholipids decreases noted in this study may have been partly caused by enzymatic hydrolysis. Mammalian tissues contain phospholipases which continuously release fatty acids from the phosphoglycerides (McMurray and Magee, 1972).

In this research work the grinding may have enhanced

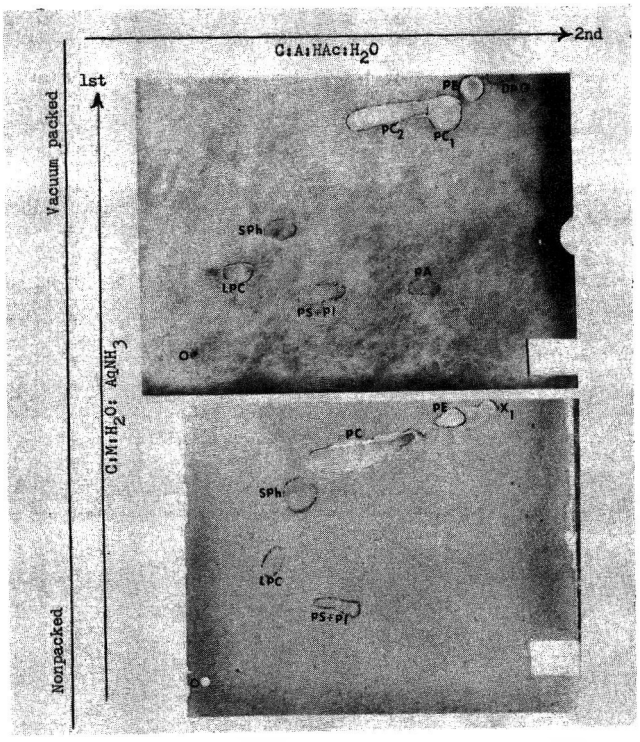


Fig. 2—Two dimensional TLC of bovine muscle tissue phospholipids, after 1.5 months of frozen storage and cooking. Development time 2 hr in each direction. PA, phosphatidic acid; X_1, unknown.

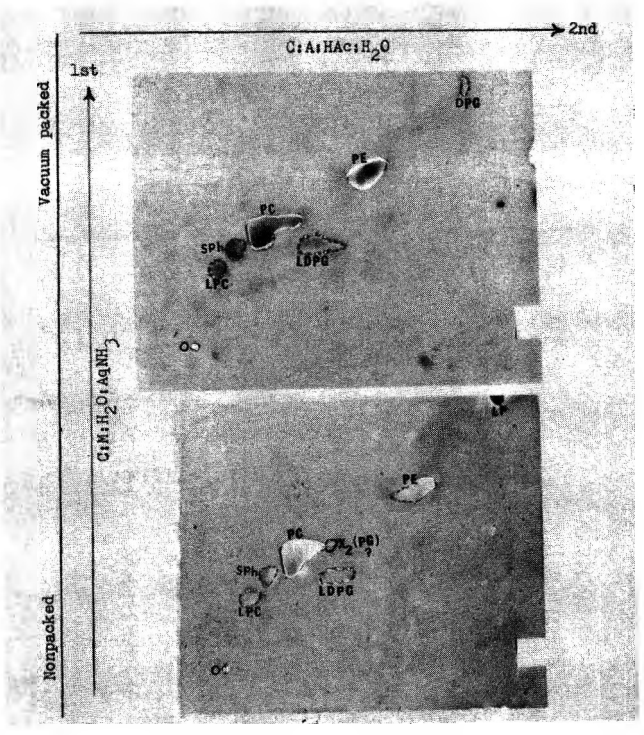


Fig. 3—Two dimensional TLC of bovine muscle tissue phospholipids, after 3 months of frozen storage and cooking. Development time 2 hr in each direction. LDPG, lysodiphosphatidylglycerol; PG, phosphatidylglycerol; X_2, unknown; LP, less polar lipids.

the phospholipid oxidation. The grinding increased the surface area and liberated bound phospholipids which were then easily oxidized (Castell, 1971; Lea, 1957).

The observed loss of different phospholipid classes was not a result of drip loss upon cooking, since drip and meat were homogenized and then samples taken for the lipid extraction. Negligible amounts of phospholipid phosphorus have been observed in the drip of cooked beef patties (Keller and Kinsella, 1973).

In Figures 3 and 4, the spots marked LP (less polar lipids), FFA (free fatty acid) and NL (neutral lipids) were seen. These spots were not apparent when TLC plates were sprayed with Molybdenum Blue Reagent. This points out that they did not contain any phosphorus ester in their chemical structure. They became visible after the plates were heated to 100°C. These are the impurities which were not removed on the SilicAR cc-7 column during the fractionation of the crude lipids.

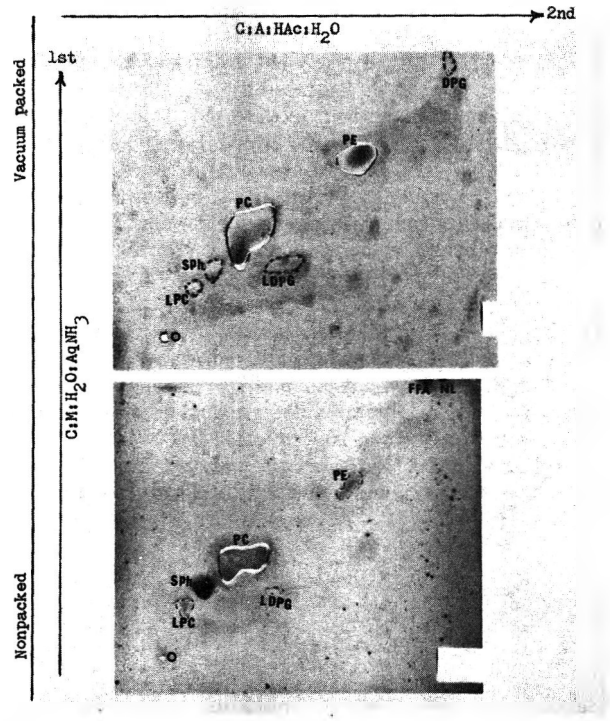


Fig. 4—Two dimensional TLC of bovine muscle tissue phospholipids, after 4.5 months of frozen storage and cooking. Development time 2 hr in each direction. FFA, free fatty acids; NL neutral lipids.

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Influence of Postmortem Changes in Bovine Muscle on the Water-Holding Capacity of Beef. Postmortem Storage of Muscle at Various Temperatures Between 0 and 30°C

K. O. HONIKEL, A. HAMID, C. FISCHER, and R. HAMM

ABSTRACT

Bovine neck muscles were incubated between 0° and 30°C soon after slaughter. At different times postmortem biochemical and physical parameters and cooking loss in homogenates with or without salt were measured. The rate of pH fall in muscle depends on the incubation temperature. At 0.5°C the pH drops fast at first, leveling off afterwards but between 7° and 14°C there is an initial lag period of 2-3 hr. At 30°C no lag phase occurs. The increased rate of postmortem metabolism at 0.5°C is accompanied by cold shortening which occurs before onset of rigor and is followed by little rigor shortening. Above 16°C rigor shortening increases with rising temperature without prerigor contraction. Neither shortening nor development of rigor have an immediate influence of WHC of muscle and unsalted muscle homogenates; the small decrease of WHC postmortem is due to pH fall only and independent of temperature. Salted homogenates prepared from muscles at different time postmortem show above pH 6.0 a similar relationship between pH and WHC. At the onset of rigor mortis (pH 5.9), however, the WHC of the salted homogenate decreases strongly. The practical consequences of these results with regard to processing of beef are discussed.

INTRODUCTION

IN THE PRECEEDING paper of Honikel et al. (1981) the influence of postmortem changes occurring in intact bovine neck muscle on the water-holding capacity (WHC) i.e. the amount of juice released by cooking of unsalted and salted muscle homogenates was investigated. The effect of postmortem fall of muscle pH and of the development of rigor mortis on WHC were particularly elucidated. These studies at 20°C were carried out with special regard to the use of hot-deboned beef for sausage production. However, the influence of postmortem storage of beef at various temperatures on WHC is also of practical interest, particularly the effect of storage at low temperatures because under such conditions not only rigor mortis but also cold-shortening could influence the WHC of meat.

From earlier work it is known that lowering the temperature from about 37°C (immediately after slaughter) to 6-8°C results in a continuous decrease in the rates of ATP turnover and glycolysis; a further decrease of tissue temperature down to the freezing point (about −1°C) causes an acceleration of postmortem metabolism (Honikel and Hamm, 1978; Jeacocke, 1977). These increased rates of metabolic processes coincide with "cold-shortening" which results in a toughening of the cooked meat (Marsh and Leet, 1966).

Cold-shortening occurs before the onset of rigor mortis. Clear information on the influence of cold-shortening on the WHC of beef is not yet available. It would be of particular interest to know if there are differences due to prerigor shortening, rigor shortening and rigor mortis itself with regard to the effect on WHC of meat in terms of cooking loss of salted muscle homogenates. Powell (1978) found that the drip loss of beef stored at temperatures where cold-shortening occurs increased compared with that of beef kept at 10-15°C. Locker and Daines (1974) observed no influence of cold-shortening of muscle on cooking loss. However, in a later paper Locker and Daines (1976) reported a decrease in cooking loss of cold-shortened muscle after the onset of rigor mortis. Contrary to these results, Davey and Gilbert (1975) found an increase in cooking loss as a result of cold-shortening. This obscure situation needed clarification. For this purpose it was necessary to distinguish in our experiments between shortening, measured by decrease in sarcomere length, and rigor mortis, measured by decrease in extensibility of muscle and to find the optimum conditions for conditioning prerigor beef and to determine the length of time the hot-boned meat may be left at a given temperature and still obtain the benefit of a high WHC compared with conventionally chilled rigor or postrigor beef.

Authors Honikel, Fischer, and Hamm are with the Institute of Chemistry & Physics at the Bundesanstalt für Fleischforschung, D-8650 Kulmbach, Germany. Author Hamid was visiting scientist in this institute. He is now with the Pakistan Council of Scientific & Industrial Research, Lahore-16 (Pakistan).

MATERIAL & METHODS

BEEF NECK MUSCLES (M. sternomandibularis and M. sternomastoideus) were obtained from the local abattoir within 30-40 min after slaughter and trimmed of visible fat and connective tissue. For studying the influence of conditioning temperature, the lean neck muscles were divided longitudinally into samples of about 100-200g and about 1 cm thickness. One of the strips was analyzed before incubation (30-40 min postmortem); the other strips were sealed in polypropylene pouches and placed in a cryostat bath of the desired temperature between 0 and 30°C. After 10-15 min incubation, the temperature at the center of the samples had reached the bath temperature. At selected periods of incubation up to 24 hr postmortem a sample was taken for further investigations. The neck muscles from one animal were not sufficient to carry out measurements over the whole range of temperature between 0° and 30°C; therefore, muscles from different animals had to be used and several replications had to be made with regard to temperature and time of postmortem storage and tissue pH, respectively.

For studying the effect of muscle shortening on sarcomere length, neck muscle samples (about 50g, 1 cm in thickness) were sealed 30-60 min postmortem in polypropylene pouches and incubated in a cryostat bath at the desired temperature. After 3 and 24 hr incubation about 200 mg of the sample were used for the determination of sarcomere length as described by Honikel et al. (1981) and Voyle (1971).

The unsalted and salted muscle homogenates were prepared according to the method of Honikel et al. (1981). Methods for the determination of pH value, cooking loss as a measure for WHC, and extensibility of muscle were carried out as described in the preceeding paper by Honikel et al. (1981).

RESULTS & DISCUSSION

Influence of incubation temperature on postmortem changes in the pH of intact muscle tissue

The postmortem changes in pH were measured in the intact muscle samples which were obtained from the carcass immediately after slaughter and were incubated at different temperatures between 0.5° and 30°C. During the first hours postmortem the rate of pH-fall was much faster at 0.5° and 30°C than at 7.5° or 14°C (Fig. 1). Within the first 4 hr postmortem the drop of pH was even faster at

0.5°C than at 30°C. After about 4 hr postmortem at 0.5°C the rate of pH fall in the tissue slowed down whereas at higher temperatures the rate of glycolysis as indicated by the slope pf pH fall remained either constant or increased slightly until about 12 hr postmortem. This change in rate is certainly due to an accelerated turnover of ATP (Newbold and Scopes, 1967; Honikel and Hamm, 1978; Jolley et al., 1980). This phenomenon can be explained by changes in the lipoprotein system of membranes at low temperature which inactivate the ATP-driven calcium pump of the sarcoplasmic reticulum (SR) and/or increase the permeability of membranes of SR or mitochondria for Ca^{++}. This release of calcium ions (Honikel and Hamm, 1978) at still sufficient levels of ATP initiates muscular contraction before onset of rigor mortis (Newbold and Tume, 1977). The period necessary for reaching pH 5.9 (onset of rigor mortis) increased with falling temperature, being about 10 hr at 30°C and more than 24 hr at 0.5°C (Fig. 1). This latter fact, i.e. the influence of temperature on the time necessary for reaching the "final" pH postmortem (around pH 5.5) is well known.

Influence of incubation temperature on postmortem shortening and rigor development in muscles

As the rapid biochemical alterations, which cause the rapid decline in pH during the first hours postmortem at 0.5°C, result in cold shortening of muscle (Marsh and Leet, 1966) we studied the influence of incubation temperature postmortem on the sarcomere length in the intact muscle

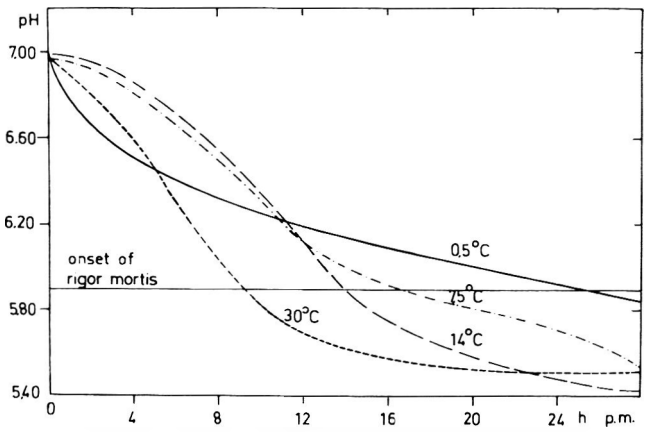


Fig. 1—Relation between the pH fall and the time postmortem in bovine neck muscles at different temperatures.

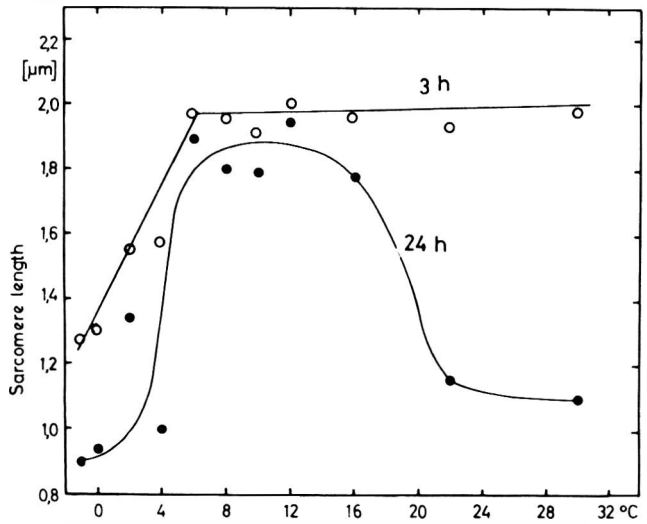


Fig. 2—Sarcomere length of bovine neck muscles at 3 and 24 hr postmortem at different incubation temperatures.

3 hr and 24 hr postmortem by a laser technique. As Figure 2 shows, at incubation temperatures below about 6°C a remarkable prerigor (3 hr postmortem) shortening occurred as compared with muscles incubated at temperatures above 6°C. By increasing the temperature from 16° to 30°C no prerigor shortening but a remarkable increase of rigor shortening (24 hr postmortem) was observed. At 30°C, rigor shortening was more far-reaching than at 0.5°C. We also measured changes in the length of unloaded muscle strips, incubated postmortem at different temperatures. At 0°C the unloaded muscle shortened to about 45% of its original length until 5.9 was reached; during the subsequent rigor development (decrease in extensibility) little or no further shortening occurred. At 21° and 30°C no shortening in the prerigor state (pH > 5.9) occurred but rigor shortening of 7% and 15%, respectively, was observed. These results agree quite well with the alteration in sarcomere length. So, decrease of the length of sarcomeres, and muscle, at temperatures below 6°C was apparently mainly due to prerigor shortening ("cold-shortening") whereas at temperatures above 16°C it was caused by rigor shortening.

Increased shortening of sarcomeres and muscle, caused by raising the tissue temperature above about 16°C (Fig. 2) was also accompanied by an accelerated decrease of pH postmortem (Fig. 1). Contrary to the cold-shortening effect, however, this acceleration of postmortem metaboliims did not result in prerigor shortening (Fig. 2), probably because not yet enough free Ca^{++} was released from calcium accumulation organelles. This assumption is supported by the fact that addition of EGTA to muscle tissue in the prerigor state in the temperature range between −1° and +27°C lowers the rate of glycolysis and ATP turnover within the first 3 hr postmortem at temperatures below 6-10°C only (Honikel and Hamm, 1978). Only after depletion of ATP (pH = 5.9) which results in an inactivation of the calcium pump of the SR and release of Ca^{++}, does rigor shortening occur (Fig. 2), as described also by Locker and Daines (1975). The accelerated postmortem metabolism at low temperatures lasted for a few hours postmortem only; then the further decrease of pH at 0°C was much slower than at 7.5°C or higher temperatures (Fig. 1). Winger et al. (1979) confirmed these results.

We explain these observations with the fact that after a few hours postmortem at all temperatures between 0° and 30°C similar amounts of Ca^{++} are released. Apparently then the rate of postmortem metabolism is determined by the normal influence of temperature on biochemical reactions. At 0.5°C the rate of pH fall decreased before pH 5.9 was reached. As an explanation we suggest that Ca^{++} ions released from mitochondria are pumped into the SR.

Influence of incubation temperature on WHC of muscle pieces and unsalted or salted muscle homogenates at different times postmortem

Muscle samples were removed from the carcass immediately after slaughter and incubated at different temperatures. After the tissue pH had reached certain values (6.8, 6.1, 5.9, 5.5) the WHC of the intact tissue was determined by measuring the cooking loss. Furthermore, from the same tissue, homogenates without and with added salt (2% NaCl) were prepared, and the cooking loss of samples from these homogenates was measured. The results of these experiments are listed in Table 1.

For intact muscle, temperature dependent differences in cooking loss were not observed regardless of whether the muscle was in the prerigor state (between pH 6.8 and 6.1) or at the onset of rigor mortis (pH 5.9), or in the early postrigor period (pH 5.5). This was also the case for the cooking losses of unsalted muscle homogenates. It should be pointed out that incubation temperature affects cooking

Table 1—Percent cooking loss of intact muscle, unsalted and salted muscle homogenates at different incubation temperatures and different pH values of the intact tissues, reached during postmortem glycolysis

Incub. temp °C	pH 6.8			pH 6.1			pH 5.9			pH 5.5		
	Intact muscle	Homogenate[a]		Intact muscle	Homogenate[a]		Intact muscle	Homogenate[a]		Intact muscle	Homogenate[a]	
		Unsalted	Salted		Unsalted	Salted		Unsalted	Salted		Unsalted	Salted
0.5	34	52	24	42	58	36	44	59	40	–	–	55
4	34	50	16	40	53	31	41	55	34	44	60	51
5	36	53	20	41	56	32	42	56	37	45	57	50
7.5	37	51	28	40	56	29	42	57	35	45	60	49
10	27	49	22	38	55	28	40	55	35	45	55	51
14	33	53	22	39	54	22	41	55	22	44	58	53
17	32	49	21	40	57	36	43	57	41	44	58	54
20	37	48	27	42	58	36	43	58	40	44	60	51
20	35	42	16	39	50	24	41	52	27	43	57	48
23	33	55	31	40	57	32	42	59	33	44	59	54
24	37	47	26	44	58	33	46	59	34	53	59	50
27	37	53	28	39	57	32	40	58	34	42	60	47
30	35	52	26	41	56	32	42	57	34	45	60	56
$\bar{x}$	34.4	50.3	23.6	40.4	55.8	31.0	42.1	56.7	34.3	44.8	58.6	51.5
SD	2.8	3.4	4.6	1.6	2.3	4.3	1.7	2.0	5.2	2.7	1.6	2.7

[a] Homogenates were prepared after reaching the required pH in intact muscles. The salted homogenates contained 2% NaCl. Cooking loss was determined as described in methods.

loss if compared at the same time postmortem because the pH drops at different incubation temperatures with different velocities (Fig. 1). With falling pH cooking loss increased (Table 1). In the intact muscle tissue between pH 6.8 and 5.9 the cooking loss increased constantly with a rate of 8.5 %/pH unit (Table 1, Fig. 3) independent of the temperature of postmortem incubation (Table 1). After the onset of rigor mortis (pH < 5.9) the rate was 6.7 %/pH unit. Unsalted muscle homogenates showed a similar pattern with cooking losses of 9.1%/pH unit between pH 6.8 and 6.1 and about 4.6%/pH unit between pH 6.1 and 5.5 (Fig. 3). This observation is in agreement with the results obtained and presented in Figure 1 by Honikel et al. (1981). It is also in agreement with the fact of Jolley et al. (1980) that postmortem change in WHC measured by a filter-paper press-method of raw intact neck muscle, was not influenced by the development of rigor mortis.

Quite different results were obtained for the cooking loss of salted muscle homogenates (Table 1, Fig. 3). The cooking loss of the salted homogenates prepared from pre-rigor muscles (pH > 5.9) were much lower than those of the unsalted homogenates as could be expected according to Hamm (1960, 1972, 1975). Between pH 6.8 and 6.1 the rate of change in cooking loss was 10.6 %/pH unit, i.e. similar to that of unsalted muscle pieces and homogenates, but after pH 5.9 was reached, cooking losses increased strongly up to 43 %/pH unit. Again there was no influence of temperature on incubation and contraction at pH values measured in the intact muscle prior to homogenization (Table 1). Apparently, the onset of rigor mortis (pH 5.9) cuased a remarkable decrease in WHC of salted muscle homogenates but not of unsalted meat in either the intact or the minced state, as was observed by Honikel et al. (1981) for muscle held at 20°C.

It should be pointed out that in these experiments cooking loss was measured not later than about 24 hr postmortem. Results (Honikel, unpublished) indicate that cold-shortening influences water retention of intact unheated neck muscle (drip loss) after a longer period of aging. A comparison of the results obtained by Honikel et al. (1981) with those presented in this paper reveals that all phenomena concerning the influence of postmortem changes (pH fall, rigor development) on WHC of meat in the range between 0 and 30°C are independent of tempera-

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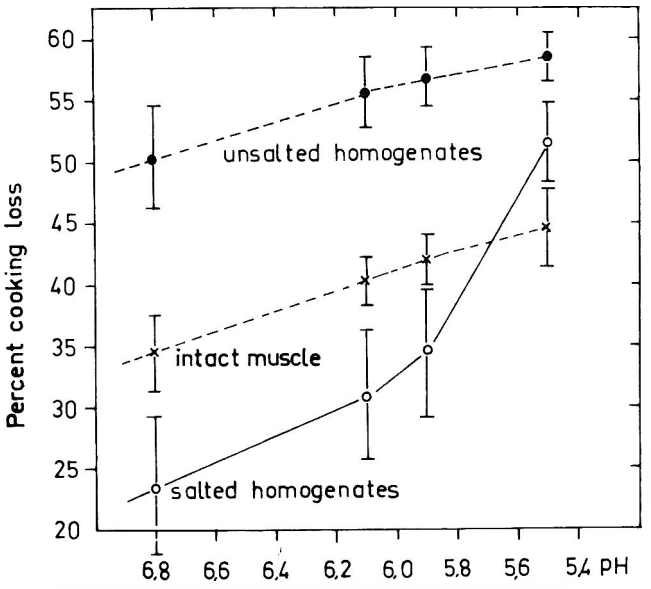


Fig. 3—Cooking loss of intact muscle, unsalted and salted homogenates in dependence on the postmortem pH fall in the intact muscle at temperatures between 0 and 30°C. The bars indicate the standard deviation.

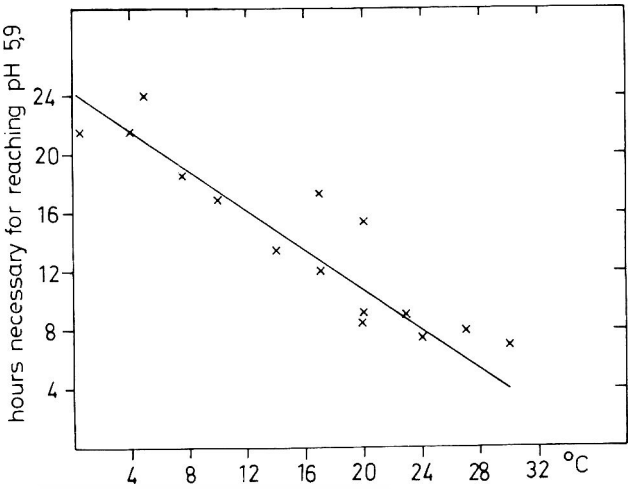


Fig. 4—Influence of temperature of incubation of bovine neck muscles on the time necessary for reaching pH 5.9.

Evaluation of the Contribution of Flavor Volatiles to the Aroma of Beef by Surface Response Methodology

K. O. BODRERO, A. M. PEARSON, and W. T. MAGEE

ABSTRACT

The contribution to beef aroma of some compounds previously identified in the flavor volatiles from cooked beef was estimated by surface response methodology. In general, heterocyclic compounds containing oxygen, nitrogen and/or sulfur and unsaturated alcohols gave higher predicted scores and had lower optimum concentrations than ketones, aldehydes, alkanes or saturated alcohols. It was further demonstrated that hydrophilic compounds are more likely to contribute to meat aroma than hydrophobic compounds, thus, verifying a more important role for water-soluble compounds in contributing to meat flavor. On testing mixtures of compounds, the optimum concentrations were generally lower, but there was a greater range in maximum scores. Special significance is given to the fact that highest scoring two-component mixture (2-acetyl-3-methylpyrazine and H_2S) resulted from combining the two single compounds that scored highest alone. Furthermore, the reaction mixture of H_2S with 4-hydroxy-5-methyl-3(2H)-furanone gave the highest predicted score in the entire study. Thus, results indicate that the surface response method offers promise for unraveling the complexity of the meat flavor system.

INTRODUCTION

ALTHOUGH MEAT FLAVOR has been extensively studied as summarized in recent reviews (Herz and Chang, 1970; Dwivedi, 1975) failure to find a single character impact compound (a single compound responsible for most of the flavor) suggests that it is due to a delicately balanced blend of many compounds (von Sydow, 1971). Characterization of such complex flavor mixtures requires three main steps: (1) identification of the flavor percursors present in raw meat; (2) identification of the compounds that contribute to the taste and aroma of cooked meat; and (3) determining the combinations and concentrations of compounds (both precursors and flavor compounds per se) that contribute to both taste and aroma of meat.

Crocker (1948) showed that cooking converted the blood-like serumy flavor of meat into the characteristic cooked flavor, suggesting certain flavor precursors react during heating to form flavor compounds. By fractionation of raw meat into successively smaller fractions followed by heating, several research groups (Batzer et al., 1960, 1962; Wasserman and Gray, 1965; Zaika et al., 1968) have identified glycoproteins, simple sugars, amino acids, 5'-nucleotides and lipids as possible flavor precursors.

Beef extract was first prepared from ox muscle by von Liebig (1847), during preparation of which he isolated and identified creatine, creatinine, sarcosine, tyrosine, and inosinic acid. MacLeod and Coppock (1976, 1977) have reported over 300 different compounds are found in the flavor volatiles of beef. The list has been further enlarged upon considering meat from other species, recent work with beef (Dwivedi, 1975; Chang and Peterson 1977) and with model systems. Boelens et al. (1974) have demonstrated that saturated aldehydes can react with both gaseous and liquid H_2S and produce a number of organic sulfur compounds, some of which have been identified as constitutents of meat flavor volatiles. Studies by van den Ouweland and Peer (1975) have shown that H_2S and 4-hydroxy-5-methyl-3(2H)-furanone can react and produce a mixture of compounds with a meaty aroma. Shibamoto and Russell (1976) have also demonstrated that model systems containing D-glucose, H_2S and NH_3 can react to produce heterocyclic sulfur compounds, which may also contribute to meat flavors.

In order to confirm the contribution of some volatile components to meat flavor, Parliament and Scarpellino (1977) have combined sensory analysis with gas-liquid chromatography (GLC) fractionation. In this technique smaller and smaller fractions are related to panel evaluation until narrowed down to single compounds. MacLeod and Coppock (1977) compared sensory descriptions with identifications made by GLC-mass spectroscopy and showed that concentrations for certain classes of compounds varied in the volatiles of beef cooked by roasting and boiling. Powers and Keith (1968) working with coffee volatiles were able to categorize it into flavor categories by correlation of aromas with GLC peak areas. Using a linear model system, Persson et al. (1973a, b) were able to correlate the levels of several chemical compounds with the odor qualities of canned beef. Subsequent studies then showed that it was possible to change the acceptability of the product by manipulating the levels of the different compounds in the product (Persson and von Sydow, 1974a, b; von Sydow, 1974).

The surface response method (Box and Wilson, 1951; Chang et al., 1960) has been applied to optimizing the ingredients in food systems (Pearson et al., 1962; Stubblefield and Hales, 1977) and would appear to be useful in solving complex flavor problems, such as determining the best combination and concentrations of flavor compounds or precursors needed for optimal meat flavor. Thus, the present investigation was undertaken to determine the contribution and concentrations of some compounds that have previously been identified in the volatiles from cooked beef. The compounds were tested for their contribution to meat aroma as single compounds or as mixtures of two or three components using surface response methodology to predict maximum levels.

EXPERIMENTAL

Compounds tested

The compounds tested were either purchased or obtained as gifts from commercial sources, or in some cases were synthesized in our laboratory. Although most of the compounds tested had been previously identified in meat flavor volatiles, some others were tested because they were available and similar in structure to compounds identified in the volatiles from meat.

The compounds tested were prepared in deionized, tasteless, glass distilled water. Compounds not soluble in water at the concentrations tested were dissolved in diethylphthalate (DEP). Some compounds were tested in both solvents and comparisons were made on threshold levels as will be reported later herein.

All solutions were prepared using standard glass pipettes and

Author Pearson is with the Dept. of Food Science & Human Nutrition and Author Magee is with the Dept. of Animal Husbandry, Michigan State Univ., East Lansing, MI 48824. Author Bodrero, formerly with Michigan State Univ., is now associated with Sigma Chemical Company, Box 14508, St. Louis, MO 63178.

volumetric flasks. All glassware was rinsed with water, followed by acetone, washed with Alconox and water, and rinsed thoroughly with deionized glass distilled water. All clean glassware was carefully checked to see if any residual odor was present before being used.

Panel scoring system

A scoring system based on the similarities and dissimilarities to meat aroma was developed as follows: 0 = aroma not different from solvent; 1 = does not resemble or contribute to meat aroma; 2 = probably does not contribute to meat aroma; 3 = undecided if sample resembles meat aroma; 4 = probably contributes to meat aroma; 5 = faintly resembles meat aroma; 6 = slightly resembles meat aroma; 7 = moderately resembles meat aroma; 8 = generally full, meaty aroma; and 9 = exceptionally good, full meaty aroma.

At each session, all panelists were provided a reference sample of pure solvent, either water or DEP. In addition, unknown reference samples were interspersed among the test samples to check the individual panelists' ability to correctly score the test compounds. All samples were presented to the panel at room temperature (about 21°C).

Presentation to panel

The panel consisted of a minimum of 5 panelists from a pool of 12, who had previously gained experience in evaluating meat flavor by comparing microwave and conventionally cooked beef roasts (Bodrero et al., 1980). No more than 10 samples were presented to the panel at one session, with no more than two sessions in one day.

Samples of about 30 ml were presented in a specified order to the panel in stoppered 100 ml wide mouth bottles. Participants were instructed to remove the stoppers, assess the aroma and mark the score card. In testing single compounds, two series of four concentrations along with the unidentified solvent control were presented at a single session. The lowest level tested was usually at the approximate odor threshold and the concentration was generally increased by factors of 10, 100, and 1,000. Thus, a typical test might include concentrations of 1, 10, 100, and 1,000 ppm. For testing mixtures of two compounds, a single series of 9 samples and an unidentified solvent control were presented at a single session. Mixtures were always arranged from lowest to highest concentrations, since testing of the highest concentration first often rendered the panelists insensitive to lower concentrations. In testing mixtures, samples included all nine combinations of two compounds and three concentrations of each compound.

Surface response predictions

The average panel score for each sample was calculated and used as a profile of the expected results for the computer calculations; i.e., whether a maximum score was to be expected or not. If not, the sample was retested after changing the concentrations to give a new set of scores. The data were then punched on cards and analyzed by the surface response method of least squares on a CDC 6500 computer (Pearson et al., 1962), which predicted the maximum score and the concentration at the maximum score for each compound or mixture.

The surface response method can be expressed for a two component system in terms of the following least squares estimating equation, which considers the linear and quadratic effects of each compound and the interaction between the two compounds, as follows:

$$\hat{Y} = a + bX + cZ + dX^2 + eZ^2 + fXZ$$

where: $\hat{Y}$ = estimated panel score; X = level of one compound tested in system; Z = level of other compound tested in system; a, b, c, d, e, and f = partial regression coefficients of panel score on levels of the two compounds considering linear and quadratic effects for each and the interaction between them.

The first derivative of $\hat{Y}$ with regard to X and then with regard to Z set equal to zero gives the following equations:

$$2dX + fz = -b$$
$$fX + 2eZ = -c$$

For a trial involving two compounds, numerical values for the five regression coefficients are calculated and substituted into the above equations. The solution of the numeric values for X and Z gives the estimated levels of X and Z that produce the maximum panel scores. If only one compound is tested in the system all factors involving Z are deleted from the equations; otherwise the same procedure is followed.

Table 1—Computed optimum concentration and predicted score for compounds tested individually

Compound	Solvent[a]	Conc (ppm) at the computed max	Predicted score at the max
2-acetyl-3-methylpyrazine	H_2O	559	5.98
hydrogen sulfide	H_2O	0.379	5.80
2,3-dimethylpyrazine	H_2O	539	5.79
3-heptanone	DEP	5389	5.46
1-octen-3-ol	DEP	5060	5.15
pyridine	H_2O	540	5.03
2,4-pentanedione	DEP	5380	4.92[b]
3-octanol	DEP	5210	4.87
1-penten-3-ol	DEP	574	4.82
2,5-dimethylpyrazine	H_2O	526	4.49
ethyldimethylpyrazines (mixture of 2-ethyl-3,5-dimethyl-and 2-ethyl-3,6-dimethylpyrazines)	H_2O	510	4.46
methanethiol	H_2O	5.34	4.40
2-methylpyrazine	H_2O	565	4.33
hexanal	DEP	5540	4.30
2-pentanone	DEP	5273	4.25
2-acetyl-3-methylpyrazine	DEP	490	4.21[b]
2-butanone	H_2O	528	3.89
2,4-pentanedione	H_2O	537	3.82
acetone	H_2O	5308	3.75
n-butyric acid	H_2O	556	3.58
2-heptanone	DEP	5390	3.55
3-hexanone	DEP	5890	3.54
heptanal	DEP	544	3.44
3-octanone	DEP	5670	3.40
4-hydroxy-5-methyl-3(2H)-furanone	H_2O	594	3.35
2-octanol	DEP	5040	3.34
trans-2 hexenal	DEP	539	3.30
tetramethylpyrazine	H_2O	644	3.06
2,3-pentanedione	H_2O	4.1	2.99
2,3,5- trimethylpyrazine	H_2O	609	2.96
furfural	H_2O	547	2.88
methional	DEP	655	2.87
benzaldehyde	H_2O	510	2.86
4-hydroxy-2,5-dimethyl-3(2H)-furanone	H_2O	635	2.76
methylthioethanethiol	H_2O	0.54	2.74
2,3-butanedione	H_2O	38	2.72
n-hexanoic acid	H_2O	570	2.66
1-undecanol	DEP	7710	2.63
3-methyl-2-butanone	DEP	5930	2.56
2-ethylpyrazine	H_2O	533	2.53
propanal	H_2O	58	2.53
pentanal	DEP	4640	2.42
3-methylbutanal	DEP	540	2.42
octanal	DEP	5088	2.35
2-hexanone	DEP	5425	2.31
4-methyl-3-pentanone	DEP	5800	2.15
ethanethiol	H_2O	5.40	2.08
n-butanol	H_2O	4633	2.02
2-methylpropanal	H_2O	563 (min)	1.84[b]
2-methylbutanal	DEP	5650	1.68
dimethyl sulfide	H_2O	69.0 (min)	0.930[b]
diethyl sulfide	H_2O	5.20 (min)	0.499[b]

[a] H_2O = deionized, distilled water; DEP = diethylphthalate.
[b] Not plotted on figures.

RESULTS & DISCUSSION

ON TESTING individual chemical compounds for their resemblance to meat aroma, it was found that very few components had high predicted scores as is shown in Table 1. Most compounds had predicted scores between 2 (probably does not contribute to meat aroma) and 4 (probably contributes to meat aroma). The highest predicted scores fell in a range of 5 (faintly resembles meat aroma) to 6 (slightly meaty aroma). The relatively low scores predicted by computer analysis support the concept that meat flavor

is probably not closely dependent upon any single compound, but rather is due to blending a number of compounds (von Sydow, 1971). The data in Table 1 also show that the range at the computed maximum concentration was extremely broad, varying from a low of less than 1 ppm for H_2S in H_2O to over 7700 ppm for undecanol in DEP.

Several compounds tested did not give a maximum at a low enough concentration (<10,000) to be considered as meat aroma contributors and are listed in Table 2. These compounds were not tested further, either alone or in mixtures. Some compounds were found to give a minimum instead of a maximum (Table 3), which generally is interpreted as meaning that the wrong concentrations were tested since higher scores would be found on both sides of the minimum. Most of these compounds were retested at other concentrations and did give a maximum. Not finding a maximum does not mean that the compounds do not have a maximum, but merely that the proper level was not tested in this study.

It was frequently, but not always, found that the concentration at the computed maximum was predicted to occur approximately half way between the highest and next highest concentration tested. If this is true, it follows that one would automatically limit the maximum by the choice of concentrations tested. However, the fact that mixtures tended to have lower predicted scores than either component tested alone would suggest that the above mentioned observation was due to chance selection of the concentrations tested and not the result of a systematic error.

Examination of the data in Table 1 more closely reveals that the highest scoring compounds were either nitrogen heterocycles, sulfur compounds, alcohols and/or carbonyls possessing additional functional groups. To examine the data in greater detail, the compounds were grouped into three categories: (1) those containing nitrogen, sulfur or oxygen as a heteroatom; (2) carbonyl compounds, including ketones, aldehydes and their derivatives; and (3) alcohols and their derivatives. Figure 1 shows a histogram of the number of compounds by classes plotted against predicted score at the maximum concentration. Results demonstrate that a fairly broad range in scores was obtained for each class of compounds, with some fairly high scoring compounds and some fairly low scoring compounds for each category. However, the nitrogen, sulfur and oxygen heteroatom compounds gave the highest scores on average and also had the highest proportion of high scoring compounds. This substantiates the conclusions made by other researchers that heteroatom compounds are the most important contributors to meat flavor (Dwivedi, 1975; Chang and Peterson, 1977).

Since some compounds that are possible contributors to meat aroma were not soluble in H_2O, DEP was sometimes used as the solvent. Thus, Figure 2 shows a comparison by a histogram of the water-insoluble, water-soluble and all compounds relative to their predicted scores at their maximum concentrations. Although the water-soluble compounds appeared to have slightly higher predicted scores, the differences are not great and the total range is essentially the same. Figure 3 also presents a histogram showing the water-insoluble, water-soluble, and all compounds on the basis of the calculated concentration at the maximum scores. The plot shows that the majority of the water-soluble compounds have predicted concentrations below 1,000 ppm, while those tested in DEP (water-insoluble compounds) have predicted concentrations at the maximum scores in the range of 4,500-6,000 ppm. This is borne out of observing that there are two distinct groups under all compounds; i.e., those reaching their maximum predicted scores at concentrations < 1,000 ppm, and those reaching their maximum predicted scores at 4,500-6,000 ppm.

Examination of the data in Table 1 in light of the concentrations at the maximum predicted scores shows that most compounds tested in DEP gave higher maximum concentrations than those tested in aqueous solution. This would suggest that higher concentrations were necessary to achieve the aroma thresholds for compounds tested in DEP.

Table 2—Compounds which did not give a maximum at <10,000 ppm

ethanol	heptane
methanol	2-undecanone
glycerol	2-tridecanone
1,3-propanediol	acetic acid
pentane	propanoic acid

Table 3—Compounds that gave a minima

heptanal	2-acetyl-3-methylpyrazine
2-methylpropanal	dimethyl sulfide
3-methylbutanal	diethyl sulfide
2,3-dimethylpyrazine	

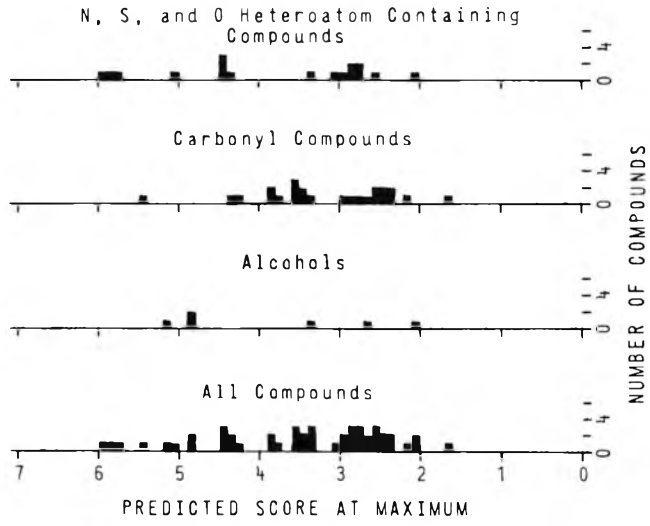


Fig. 1—Histogram showing distribution of compounds by classes at predicted maximum scores.

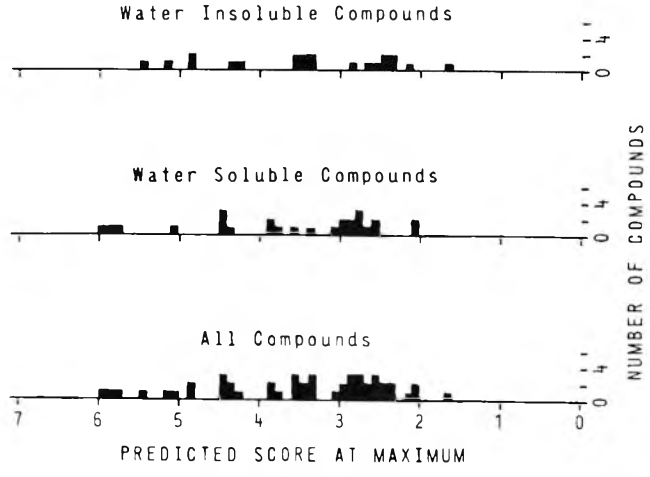


Fig. 2—Histogram showing distribution of water-insoluble and water-soluble compounds relative to all compounds at predicted maximum scores.

If this is true, the optimum concentrations would be lower for the water-soluble compounds than for the water-insoluble compounds. It is suggested that there may be two reasons for this phenomena. First, the hydrophobic compounds probably have relatively low vapor pressures as compared to hydrophilic compounds. Second, DEP per se may lower the vapor pressure of a solute in comparison to the same solute in aqueous solution. To test the latter theory, a number of compounds were tested in both DEP and H_2O. Confirmation of this theory was obtained by testing of 2,4-pentanedione which had an optimum predicted score at a predicted concentration of 537 ppm in H_2O but increased to 5,380 ppm in DEP—a 10-fold increase; however, 2-acetyl-3-methylpyrazine had a predicted maximum concentration at 559 ppm in H_2O and 490 ppm in DEP, thus changing in the opposite direction, but still less than 1-fold.

Results indicate that the solvent in some cases does alter the vapor pressure of the solute, but its effects appear to vary depending upon the individual compound. Thus, it was not possible to clearly tell what effect the solvent had on our results, since many compounds could not be tested in both solvent systems due to their solubility characteristics. Results do, however, substantiate the fact that water-soluble compounds are more likely to contribute to meat aroma than water-insoluble compounds, not only because their predicted scores were slightly higher, but also because they had lower concentrations at their maximum predicted scores.

Figure 4 presents a graph of the predicted maximum scores against the predicted optimum concentrations by classes of compounds. The plot shows that predicted maximum scores fell largely into three distinct concentration ranges, namely, those at <100 ppm, those at 400-600 ppm, and at 4,500-6,000 ppm. All of the sulfur compounds, and the nitrogen and oxygen heterocycle compounds fell into the lower two concentration ranges, due most likely to their lower aroma thresholds. Since the contribution of a compound to meat aroma tended to be greatest for those with the highest predicted scores and the lowest optimum concentration, the importance of compounds to meat aroma generally increases along a diagonal line running from the upper left to the lower right hand corner of the graph (Fig. 4). This supports the generalization that sulfur compounds and nitrogen and oxygen heterocycles are the most important contributing groups to meat aroma. It also bears out the fact that other compounds contributing to meat aroma generally include the unsaturated alcohols and the carbonyls that contain additional functional groups. This approach substantiates the role of these classes of compounds to meat flavor as suggested earlier by other researchers (Herz and Chang, 1970; Boelens et al., 1974; Dwivedi, 1975; Chang and Peterson, 1977).

When mixtures were tested, the optimum concentration predicted on testing each compound alone was generally used as the highest of the three concentrations tested. The optimum concentration of a compound tested in a mixture frequently was not closely related to its concentration on testing alone. In general, the optimum concentrations found for compounds in a mixture were lower than that of each compound tested alone. Furthermore, the predicted maximum scores for mixtures also covered a greater range than for single compounds. A total of 97 different mixtures were tested, including different combinations of high and low scoring compounds. Thus, results reported herein are not necessarily the only ones or the best possible ones.

Table 4 presents a list of mixtures of compounds having a higher predicted score than either of the compounds tested alone. The predicted optimum concentrations, predicted scores at the maximum and the solvents utilized in testing are also given. A total of 12 different mixtures were found to fit this category, with 10 of them being tested in H_2O and two in DEP. With one exception, the concentrations at the maximum score were low, being <300 ppm. Since one of the compounds in three of the mixtures gave a minimum rather than a maximum concentration, the compounds should be retested altering the concentration. However, it is sometimes difficult to interpret results obtained on retesting.

Table 5 presents a list of mixtures which gave higher predicted scores than one of the components in the mixture, whereas, the other compound had a higher predicted score

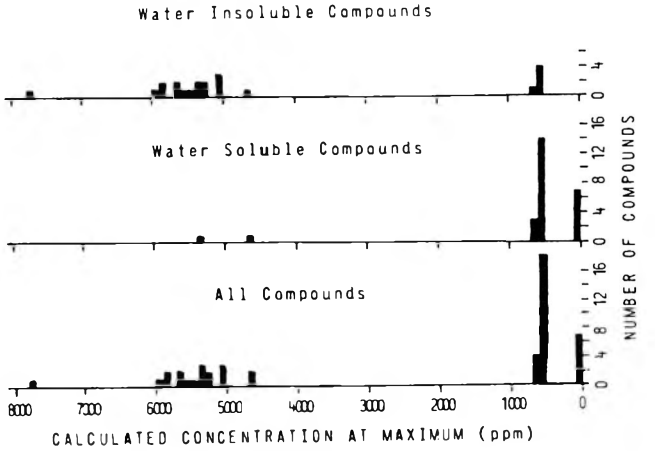


Fig. 3—Histogram showing distribution of water-insoluble and water-soluble compounds relative to all compounds at predicted concentration at the maximum scores.

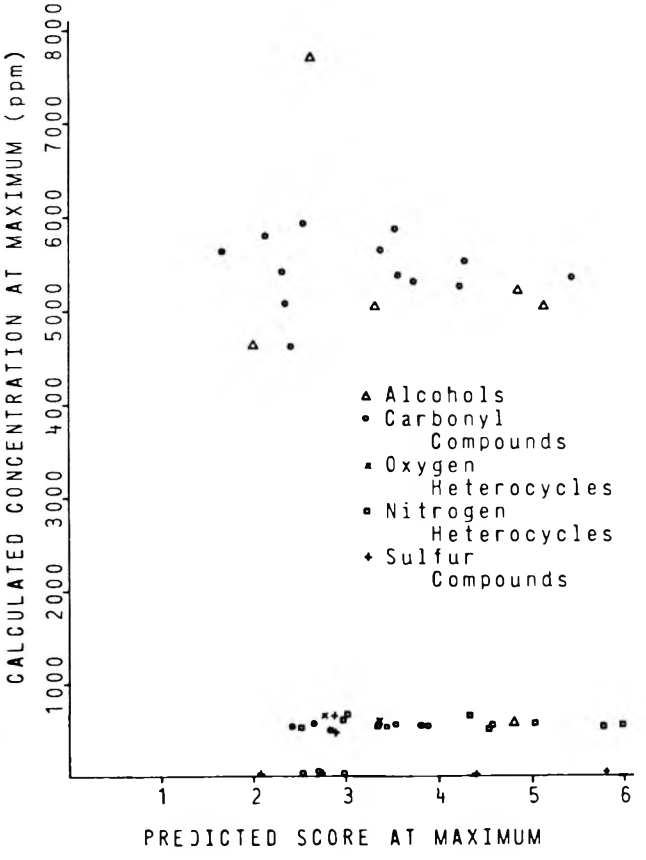


Fig. 4—Plot of alcohols, carbonyl compounds, oxygen heterocycles, nitrogen heterocycles and sulfur compounds showing distribution at predicted maximum scores versus calculated concentrations at maximum.

Table 4—Mixtures scoring higher than both components tested alone

Compounds	Solvent	Calculated conc at max (ppm)	Predicted score at max for mixture
1. 2-acetyl-3-methylpyrazine hydrogen sulfide	H_2O	5.55 0.424 (min)	10.6
2. 2,5-dimethylpyrazine furfural	H_2O	266 25.7	7.33
3. 2,3-dimethylpyrazine furfural	H_2O	260 26.9	6.44
4. 2,4-pentanedione n-butyric acid	H_2O	264 257	4.41
5. 4-hydroxy-5-methyl- 3(2H)-furanone methylthioethanethiol	H_2O	0.00 (min) 0.0217	4.37
6. acetone n-butyric acid	H_2O	2160 275	4.15
7. 2,3-pentanedione benzaldehyde	H_2O	289 26.5	3.96
8. 2-octanol *trans*-2-hexenal	DEP	264 267	3.78
9. 2,3-pentanedione furfural	H_2O	2.60 230	3.60
10. methional *trans*-2-hexenal	DEP	277 109 (min)	3.54
11. methylthloethanethiol 3-methylbutanal	H_2O	0.236 267	3.23
12. methylthloethanethiol ethanethiol	H_2O	0.234 0.0966	2.79

Table 5—Mixtures scoring higher than only one of the single components tested alone

Compounds	Solvent	Calculated conc at max (ppm)	Predicted score at max for mixture
1. 2-octanol 1-octen-3-ol	DEP	2680 372	4.80
2. acetone methanethiol	H_2O	2510 13.8 (min)	3.78
3. 2-octanol 3-heptanone	DEP	2730 2610	3.74
4. 4-hydroxy-5-methyl- 3(2H)-furanone 2-butanone	H_2O	336 307	3.39
5. *trans*-2-hexenal 3-octanol	DEP	266 259	3.31
6. 2-hexanone 1-penten-3-ol	DEP	2820 242	315
7. methional 1-penten-3-ol	DEP	523 188	3.10
8. tetramethylpyrazine 2,3-pentanedione	H_2O	471 (min) 2.71	3.05
9. 3-methylbutanal 2,5-dimethylpyrazine	H_2O	278 (min) 272	2.80
10. propanal 2,5-dimethylpyrazine	H_2O	27.6 349	2.72
11. 3-methyl-2-butanone 1-undecanol	DEP	2740 2440 (min)	2.58

than the mixture itself. In contrast to the mixtures in Table 4, six out of 11 mixtures were tested in DEP (Table 5). Furthermore, seven compounds in the mixtures gave predicted maximum concentrations greater than 2,000 ppm (Table 5) in comparison to only one in Table 4.

The mixtures in Table 4 yielded higher predicted scores than those in Table 5, with six scoring over 4 in the first group, but only one in the latter group. This clearly shows that one should concentrate on testing high scoring rather than low scoring compounds, although an occasional low scoring compound could conceivably contribute to meat aroma. Also, the results shown in Tables 4 and 5 further support the concept that water-soluble components are more likely contributors to meat aroma than water-insoluble compounds. Furthermore, the data in these two tables also support the general concept that sulfur, nitrogen, and oxygen heteroatomic compounds, the unsaturated alcohols and carbonyl compounds containing additional functional groups have the greatest resemblance to meat aroma, even when tested in mixtures.

Of special interest is the fact that the highest predicted score was for a mixture of 2-acetyl-3-methylpyrazine and H_2S with a predicted score of 10.6, which was above the maximum score on the ranking scale. In spite of this, the concentration of H_2S was calculated to have a minimum rather than a maximum which suggests that an even meatier aroma could be produced by obtaining a maximum. It is also encouraging to note that the two compounds having the highest predicted scores on testing alone, also gave the highest predicted score for the mixtures.

One additional mixture was tested, namely, the reaction mixture of 4-hydroxy-5-methyl-3(2H)-furanone and H_2S, which is modeled after that reported by van den Ouweland and Peer (1975). When tested essentially as a single component mixture it gave a predicted score of 11.6, the highest score obtained in this study. Actually this reaction produces a complex mixture of compounds, which means our best result came from a complicated mixture of compounds, which is probably the case for meat aroma and/or taste (von Sydow, 1971).

Results of this study demonstrate that the surface response procedure is a useful technique for determing the contribution of individual chemical compounds and their mixtures to meat aroma. This gives support to previous work from our laboratory that reported the surface response method to be useful in developing a synthetic meat flavor system (Hsieh et al., 1980). Further work using this technique should be helpful in elucidating the compounds that contribute to meat flavor.

SUMMARY

RESULTS SUGGEST that some compounds tested in this study may contribute to meat aroma but that others do not. In general, water-soluble compounds, heterocyclics and heteroatom compounds containing sulfur, nitrogen, and oxygen were found to be the most likely contributors to meat aroma. Application of the surface response method was shown to be useful in assessing the contribution of different compounds, either alone or in mixtures, to meat aroma. Testing of several mixtures by this procedure gave encouraging predicted scores.

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ture; cold-shortening has no effect. The probable causes for these phenomena, particularly for the different behavior of unsalted and salted muscle homogenates, have been discussed in the preceding paper of Honikel et al. (1981).

We may speculate from our experiments that longitudinal changes of muscle that occur during contraction have much less influence on WHC than transversal alterations as caused by attraction between oppositely charged groups of adjacent protein molecules and particulary by cross-linking between myofilaments during development of rigor mortis.

Influence of incubation temperature on the time at which the onset of rigor mortis (pH 5.9) occurs

The time taken to reach the onset of rigor mortis in the sternomandibularis muscle, i.e. a tissue pH of 5.9, increased with falling temperature (Fig. 1). In Figure 4 results obtained with muscle samples from 15 animals are presented. Within the temperature range between 0 and 30°C a linear function was observed with a slope of −6.7 hr/10°C. The temperatures indicated are the actual values measured in the tissue. Muscle reached pH 5.9 after 4 hr postmortem at 30°C, or 10.7 hr at 20°C.

In the prerigor state, the WHC of salted muscle homogenates is much higher (lower cooking losses) than that of unsalted homogenates (Fig. 3). As it was mentioned earlier by Hamm (1972) this high WHC of prerigor salted meat is maintained for several days and can be used for the production of sausage emulsions with excellent binding characteristics by different types of processing. For this "presalting effect" it is decisive that the beef is minced and salted before onset of rigor mortis. Therefore it is of practical importance to know the time of postmortem storage after which rigor occurs at a given temperature. The length of this period depends on the temperature of muscle because the time necessary for reaching pH 5.9 (onset of rigor) increases linearly with falling temperature (Fig. 4).

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Kinetics of Vitamin A Degradation in Beef Liver Puree on Heat Processing

S. A. WILKINSON, M. D. EARLE, and A. C. CLELAND

ABSTRACT

The effects of heating on Vitamin A (measured as trans-retinol) in beef liver puree were investigated. The liver puree was heated in capillary tubes at five temperatures in the range 103-127°C, the typical canning temperature range for meat products. It was found that in this system and over the temperature range studied, the observed rate of degradation followed first order kinetics. The activation energy for the temperature dependence of the rate constant was 112 ± 9 kJ/mole.

INTRODUCTION

VITAMIN A is one of the fat-soluble vitamins. Although no coenzyme role has been demonstrated, it is necessary for the maintenance of proper vision, bone development and growth, epithelial tissue, and for reproduction. It occurs naturally only in animals, being present in highest concentrations in the liver. Its precursors, the carotenoids, do however occur more widely being found in green and yellow pigmented plant foods. Pennington (1976) listed vitamin A as one of the seven index nutrients used in dietary evaluation. Because of the nutritional significance of vitamin A, it is important to know the losses occurring in the processing of foods.

Two recent reviews stated that vitamin A has good stability during cooking and processing operations, but losses do occur when the foods are heated in the presence of oxygen (Lang, 1970; Barratt, 1973). Retentions of vitamin A between 50 and 100% were found for heating of vitamin enriched food products such as cereal products (Bauernfeind and Cort, 1974; Harper, 1978); peanut butter (Willich et al., 1954); and vitamin preparations (Benterud, 1977). Braising liver to an internal temperature of 77°C resulted in 90-100% retention (Kizlaitis et al., 1964) whereas mincing and cooking resulted in 87% retention (Hannukainen and Niinivaara, 1974).

Widely differing retentions for vitamin A in food processing have been reported. Negligible losses of vitamin A were reported in the pasteurisation, sterilization and spray-drying of milk (Ford et al., 1969; Thompson, 1969), and in the spray-drying of whole egg; but there was only 67% retention on the spray-drying of egg yolk (Denton et al., 1944). For canning, Labuza (1974) reported that in soups containing meat, retention was between 80-85% after sterilization, whereas Hellendoorn et al. (1971) showed that greater losses (0-100%) occurred on the canning of meat and vegetable meals. During the preparation and preservation of canned baby foods, it was found that of the six vitamins studied, A and C were the most labile during processing, and after canning the average vitamin A retention was 46 ± 8.5% (Auffray et al., 1978).

Obviously, the differences found in vitamin A retention were caused by different processing conditions, but there are no data in the literature on the effect of vitamin concentration and temperature on the rate of thermal destruction of vitamin A in food systems. Analysis by the present authors of some published data on the heating of ghee (Maqsood et al., 1963) gave a first order reaction for vitamin A degradation at both 100 and 200°C. The rate constants were $k = 109 \times 10^{-5}\ s^{-1}$ (heating ghee in boiling water) and $k = 1870 \times 10^{-5}\ s^{-1}$ (frying ghee at 200°C). Garrett (1956) determined an activation energy for vitamin A in liquid multivitamin preparations of 61 kJ/mol (14.6 kcal/mol). Slater et al. (1979) measured the shelf-life stability of vitamin A in multivitamin tablets and determined that degradation was pseudo-first order. Analysis by the present authors of Slater's data gave an activation energy of 118 kJ/mol (28.1 kcal/mol).

The lack of kinetic data for food systems therefore justified research into the rate of vitamin A degradation during thermal processing. If rate data existed for different food systems, the extent of destruction of this vitamin during commercial heat processing operations could be predicted. Important conditions to study were therefore those liable to be encountered in the sterilization of meat products in the temperature range 103-127°C. Experimental work to determine and characterize the kinetics of vitamin A degradation at five temperatures in this range was therefore done.

EXPERIMENTAL

Sample preparation

Fresh beef liver (pH = 5.81 ± 0.01) was cut into 1.0 cm cubes and pureed in a Waring Blendor for ca 90 sec. Puree (0.5g) was filled in boro silicate glass vials (3 mm internal diameter, 1 mm thick and 130 mm long) with a 45 mm headspace. The vials were heat sealed under vacuum.

Thermal processing system

The vials were heated at specified temperatures (102.9, 111.0, 118.3, 122.1 and 126.7°C) in a thermostatically controlled oil bath (± 0.2°C). Consideration of the heat transfer characteristics through the glass vial indicted that the temperature difference between the center of the liver and the glass-liver interface during the initial heating period was ca 20°C after 3 sec and ca 3°C after 30 sec. In 15 sec, 90% of the desired temperature was reached and so nearly instantaneous heating and cooling was assumed. For each temperature, three vials were removed at five different times and immediately cooled in a crushed ice/water bath. After cooling, the vials were washed and stored in the dark at −18°C until analyzed for retinol content. Control samples, which were not heated were treated in a similar manner to the test ones.

Determination of total trans-retinol in beef liver puree

The assay method used was basically that described by Haggett (1976). This procedure was a development of methods used in the Vitamin Assay Laboratories of Roche Products Pty Ltd., Australia.

The frozen vial was opened and the beef liver puree blown out under a stream of compressed air into a round bottom flask. The beef liver puree and glass tubing were saponified with ethanolic potassium hydroxide (aldehyde free) for 30 min. After cooling, the unsaponifiable matter was extracted with peroxide free diethyl ether and then with petroleum ether (40-60°C). The extract was then washed with ether saturated water and the ether evaporated off to give a concentrated extract.

The retinol was separated from the carotenoids and other inter-

Authors Wilkinson and Earle are with the Dept. of Food Technology, and Author Cleland is with the Dept. of Biotechnology, Massey Univ., Palmerston North, New Zealand.

fering substances by thin-layer chromatography. Kieselgel G (Merck) thin-layer plates were activated at 100°C for 2 hr. The concentrated extract (ca 1 cm^3), dissolved in 1 cm^3 spotting solvent (petroleum ether:absolute ethanol:triethylamine, 20:1:1), was applied as a band to the plate. The flask was washed with 1 cm^3 of spotting solvent and the washings were then loaded onto the plate just below the band. The plate was developed in the dark with cyclohexane: ethyl acetate (3:1) and the trans-retinol band indentified with U.V. light. Trans-retinol was quantitatively recovered from the separated band with chloroform, and was colorimetrically measured according to the Carr-Price method at 610 nm (Carr and Price, 1926).

Treatment of data

The order of the degradation of trans-retinol in beef liver puree on heat processing was explored graphically by plotting different functions of the concentration against time of heating at constant temperature. A plot of $\log_e C_o/C$ (to normalize data) versus time generated a straight line, thus indicating first order kinetics for degradation of trans-retinol during thermal processing. Such a first order reaction is defined as:

$$\log_e(C_o/C) = kt$$

The reaction rate constant was determined from the slope of the line (calculated by regression analysis with all the lines forced through zero as instantaneous heating and cooling was assumed).

The activation energy and the frequency factor were derived from the Arrhenius equation defined as:

$$k = Ae^{-E_a/RT}$$

where k = rate constant (s^{-1}); A = frequency factor (s^{-1}); E_a = activation energy (J mol^{-1}); R = gas constant (J K^{-1} mol^{-1}); T = absolute temperature (K).

To determine the activation energy, $\log_e k$ was plotted against the inverse of absolute temperature and E_a deduced from the slope of the line. The frequency factor was determined from the intercept of the above plot.

RESULTS & DISCUSSION

THE CONCENTRATION relationships with time are shown in Figure 1. The reaction rate constants calculated from the results are shown in Table 1. The high correlation coefficients indicate that over the temperature range studied the kinetics of vitamin A degradation in beef liver puree were consistent with first order kinetics. Compared with the reaction coefficients calculated from the data of Maqsood et al. (1963), degradation of vitamin A was more rapid in heating ghee at 100°C than heating beef liver puree at 100°C.

To estimate the error in the raw data, a probability plot was constructed of % residuals, defined as:

$$(C_{pre} - C_{obs})/C_{pre}$$

where C_{pre} = predicted concentration, and C_{obs} = observed concentration. The probability plot was a straight line indicating that all deviation from the line was random. The deviations were assumed to be due to experimental uncertainty only. The major contributing factors could be variation in composition of the liver between each vial and the inherent error in the assay method.

The activation energy determined from the Arrhenius plot (Fig. 2) was 112 ± 9 kJ/mol (26.9 ± 2.1 kcal/mol) with 95% confidence. The activation energy for vitamin A in multivitamin tablets of 118 kJ/mol (calculated from the data of Slater et al., 1979) fell within the 95% confidence limits so there was no significant difference between the two values.

Differences in activation energies were reported for other vitamins when heated in different media. In the case of thiamin, an activation energy of 123 kJ/mol (29.4 kcal/mol) was reported for phosphate buffer and 114 kJ/mol (27.3kcal/mol) for beef puree (Mulley et al., 1975).

The frequency factor, A, was found to be 8.13 × $10^{11}s^{-1}$. The frequency factor is affected by the molecular structure of the reactants, and consequently there are considerable variations in it from system to system due to changes in structures of the reactants. This is exemplified by comparison of the present data with the pharmaceutical data of Slater et al. (1979). For vitamin A in beef liver puree, A had the value 8.13 × $10^{11}s^{-1}$ whilst for the multivitamin tablets, A was 4.98 × $10^{11}s^{-1}$. This difference could be due to several factors such as difference in component composition and other intrinsic properties such as pH.

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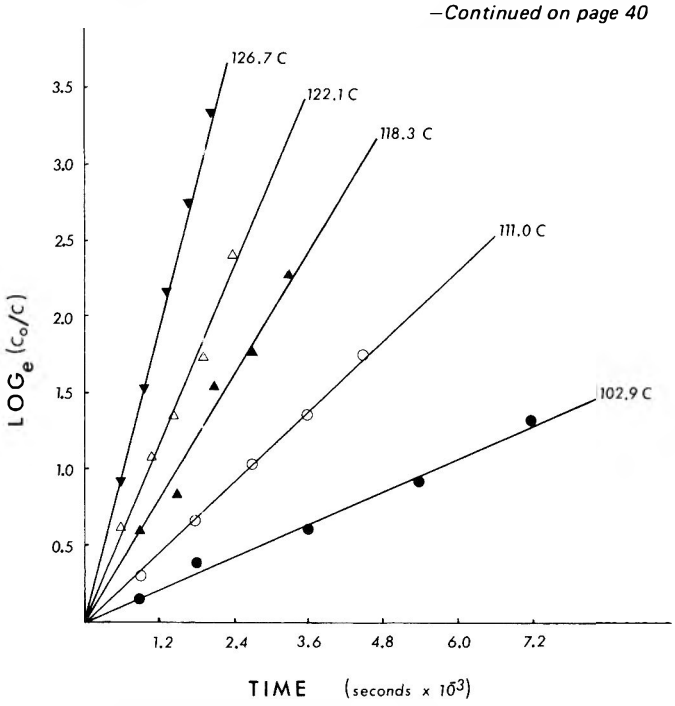


Fig. 1—Degradation rates for vitamin A in beef liver puree on heating, showing the natural logarithm of the concentration ratio as a function of time for various temperatures.

Table 1—First order reaction rate constants for trans-retinol with 95% confidence limits

Temp (°C)	k(s^{-1})	Correlation coefficient
102.9	(17.9 ± 0.7) × 10^{-5}	0.992
111.0	(38.6 ± 1.3) × 10^{-5}	0.993
118.3	(68.0 ± 2.8) × 10^{-5}	0.989
122.1	(96.1 ± 2.6) × 10^{-5}	0.995
126.7	(162.3 ± 1.7) × 10^{-5}	0.999

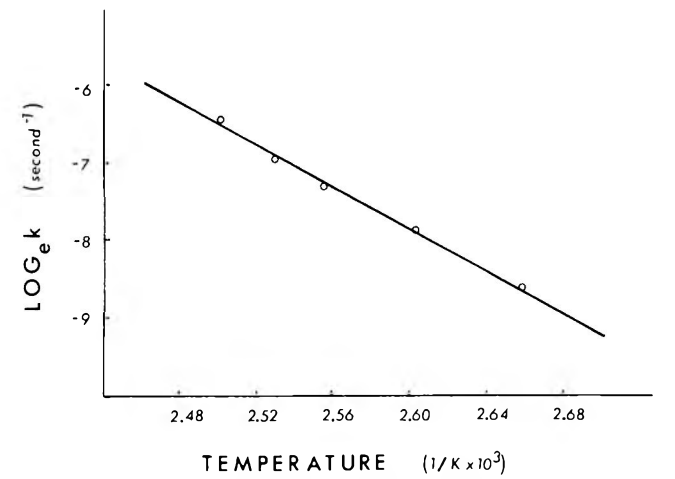


Fig. 2—Arrhenius plot for degradation of vitamin A in beef liver puree on heating, showing the natural logarithm of the first order rate constant as a function of the inverse of absolute temperature.

Effect of Salt and Tripolyphosphate on Acceptability of Flaked and Formed Hamburger Patties

D. L. HUFFMAN, H. R. CROSS, K. J. CAMPBELL, and J. C. CORDRAY

ABSTRACT

Utility chucks and Choice plates were coarse ground and mixed to provide raw material with a fat content of about 20%. Four 23 kg meat blocks were assigned, at random, to treatments: (1) control, (2) salt, (3) tripolyphosphate (TPP), and (4) salt and TPP. Each treatment was flaked, blended, and hand formed into logs which were PVC wrapped, freeze-tempered, pressed, and sliced to a 12 mm thickness. Patties were freezer wrapped and stored for evaluation initially (time) and after 30 and 60 days storage. The entire experiment was replicated three times. Sensory evaluation showed that cohesiveness, flavor, and juiciness of patties containing salt (treatments 2 and 4) were more desirable ($P<0.01$) than the control or TPP treatments. Differences over the storage period were not significant. Instron values were lower for the control patties than other treatment groups. Patties stored 60 days had higher ($P<0.05$) TBA values than those evaluated initially or after 30 days of freezer storage.

INTRODUCTION

AS MEAT PRICES continue to rise, better utilization of the lower quality grades of beef will become necessary. Ground beef has been an excellent market for the lower grades of beef as shown by the tremendous growth in the fast food industry. A method of comminution becoming well established in the industry is flaking.

Comminution of meats by flaking involves the use of a high speed centrifugal impeller which forces the meat against stationary precision honed shearing heads. The result of this process is uniform sized flakes of meat. The size and shape of these meat "flakes" may be adjusted by the size of the various cutting heads, the speed of the impeller, and the temperature of the raw material (Mandigo et al., 1972; Anon, 1973; Popenhagen et al., 1973).

Comminution by flaking and forming by pressing yields a tender comminuted product with a "bite" approximating an actual cut of meat (Randall and Larmond, 1977). Flaking has been shown to improve texture, retention of juices, binding characteristics, better cohesive properties, less cooking losses, and improved sensory characteristics (Ferren, 1972; Anon, 1973). Flaking also has a major benefit in that it eliminates cylindrical pellets of gristle and connective tissue so often associated with grinding (Anon, 1973). Chesney and Mandigo (1973) reported that taste panels preferred flaked pork products over ground pork products because of greater cohesiveness of the flaked product.

Many factors may influence the sensory and textural properties of the final product. Water-holding capacity may be increased by long mixing periods (Belahlavy and Mandigo, 1974). Addition of salt and/or polyphosphates to meat products increases water-binding ability (Hellendoorn, 1962; Shults and Wierbicki, 1973). Neer and Mandigo (1977) have shown that increasing amounts of salt increase cooking yields, tenderness, and water-binding capacity in flaked cured pork products. As salt was increased, color, appearance, texture and general acceptability were rated higher by the taste panel. When tripolyphosphate and salt were used in combination, a synergistic effect was noted and the taste panel ratings became more favorable for texture and general acceptability of the flaked cured pork (Neer and Mandigo, 1977).

Theno et al. (1978a) confirmed previous observations that the addition of salt and phosphate in conjunction with massaging, contributes to the disruption of muscle fibers, with subsequent production of an exudate rich in solubilized myofibrillar proteins rather than just broken pieces of muscle. Sectioned and formed hams were found to possess good binding characteristics with a high degree of alignment with a salt and phosphate level greater than or equal to 2% and 0.5%. respectively (Theno et al., 1978b).

This investigation was designed to show the effect of addition of salt and tripolyphosphate on physical and sensory properties of flaked and formed hamburger patties initially and after storage at $-20^{\circ}C$ for 30 and 60 day time intervals.

EXPERIMENTAL

THE RAW MATERIALS used in this study were obtained from three lots of cattle slaughtered at the Auburn University Meats Laboratory. Boneless USDA Utility cow chucks and Choice plates were removed from carcasses 5 days postmortem, ground twice through a 3.2 mm plate, sampled and analyzed for approximate fat content using the Modified Babcock procedure as described by Koniecko (1979). Ninety kilograms of the two raw materials were mixed to provide a final fat content of approximately 20 ± 2%. The batch was sub-divided into 22.5 kg blocks which were wrapped with (0.50 mm thick, oxygen permeability of 900 $cc/m^2/24$ hr/atmosphere) PVC film and frozen at $-26^{\circ}C$ for 24 hr. After tempering at $-4^{\circ}C$ for 48 hr, blocks were assigned at random to one of the four treatment groups (Table 1).

Treatments were mixed in a Hobart-horizontal mixer and passed through the Urschel Comitrol (model 3600) twice. The initial flaking was through the 6.86 mm head (0.270 in. opening). Individual batches were placed in the mixer (Butcher Boy horizontal) for 5 min with additives introduced at the beginning of the mixing cycle. The subsequent products were hand-formed into 1.6-1.8 kg logs, wrapped with 0.50 mm thick PVC film and frozen at $-26^{\circ}C$. Prior to pressing and slicing, logs were tempered for 24-36 hr at $-3^{\circ}C$ to an internal temperature of -3° to $-4^{\circ}C$. Tempered logs were unwrapped and pressed at 344 Newtons/cm^2 on a hydraulic meat press (Bettcher model 70), using a 350 die (this die is round with a diameter of 9 cm). Formed logs were sliced 12 mm thick (Model 39 Bettcher power cleaver). Each treatment consisted of 160 patties placed four to a package in plastic foam trays, PVC (0.50 mm thick) overwrapped, then overwrapped twice with wax-coated freezer paper and stored at $-26^{\circ}C$.

Approximately 50 samples were removed initially from each treatment group for chemical, physical, and sensory analysis at the end of storage periods of 0, 30, and 60 days at $-26^{\circ}C$.

Proximate analysis

Percent moisture and fat were determined at the day 0 time interval for all three lots of cattle. The oven drying method (AOAC, 1975) and a diethyl ether extraction method using a soxhlet-type

Authors Huffman, Campbell, and Cordray are affiliated with the Dept. of Animal & Dairy Science, Agricultural Experiment Station, Auburn Univ., Auburn, AL 36849. Author Cross, formerly with the USDA Meat Science Research Lab, SEA-FR, Beltsville, is now with the USDA Meat Animal Research Center, SEA-FR, Clay Center, NE 68933.

apparatus (AOAC, 1975) were the respective procedures used. The 2-thiobarbituric acid (TBA) test described by Tarladgis et al. (1960), was used to determine degree of lipid oxidation. All analyses were run in triplicate for each of the three replications and at all time intervals.

Cooking

Patties were cooked from the frozen state for Instron and trained sensory panel evaluations on a Hotpoint Grill, Model HG-2 and cooking losses determined. The consumer panel samples were prepared on a General Electric Model CG415 grill. All samples were cooked to an internal temperature of 70°C according to the procedures outlined in *Meat in the Food Service Industry* (Anon., 1975). Patties were allowed to cool for 60 min after cooking before sampling for Instron readings.

Mechanical measurements

Shear tests were carried out with the Instron Universal Testing Machine (type TM). Each pattie was cut into one 4.2 mm diameter circle and two 3.15 cm squares to be sheared by the circle blade shear and the single blade shear as described by Cross et al. (1978a).

Panel selection and training

Trained panelists were selected from the Animal & Dairy Science Dept. staff at Auburn Univ., Auburn, Ala. Panel members were selected and trained according to the procedure outlined by Cross et al. (1978b). Triangle tests were conducted for all comparisons to determine if differences existed between each of the treatments. Since panelists were able to detect a difference between all comparisons except one (control vs TPP) a scaling technique was justified to determine which sensory traits differed.

The patties were evaluated under red lights by the trained 6-member sensory panel for cohesiveness, juiciness, and flavor on an 8-point scale (8 = extremely desirable, juicy, flavorful, and 1 = extremely undesirable, dry, unflavorful). Overall color-desirability and degree of discoloration were evaluated on two patties from each treatment group using an 8-point scale (8 = extremely desirable, 10-0% discoloration and 1 = extremely undesirable, 100% discolored). All trained panel evaluations were conducted at all storage time intervals, for all treatments and replications.

Consumer panelists were selected from the staff at the Beltsville Agriculture Research Center, Beltsville, Md. The evaluations were conducted in a cafeteria at the steam tables as it came off the grill. No flavorings were used by panelists and they had no knowledge of treatments. An 8-point Hedonic scale was used to evaluate appearance, aroma, tenderness, juiciness, flavor, connective tissue, and overall acceptability (8 = extremely desirable and 1 = extremely undesirable). A color photo scale where 8 = rare and 1 = well done was used to determine degree of doneness (a rating of 3 approximated an internal temperature of 70°C). Consumer panel evaluations were conducted at all storage time intervals, for all treatments on only one replication.

Statistical analysis

Data were treated by analysis of variance (Snedecor and Cochran, 1967). Where significant differences were found, means were separated by Duncan's multiple range test (Steel and Torrie, 1960).

RESULTS & DISCUSSION

PROXIMATE ANALYSIS was not found to be significantly different among treatment groups (Table 2), therefore it was assumed that all treatment groups had essentially identical raw material make-up.

Physical properties of the four treatments are reported in Table 2. Samples containing salt showed a lower degree of doneness and a lower cooking loss. Controls showed the greatest degree of doneness and the greatest cooking loss. A serious effort had been made to cook all patties to the same degree of doneness; however, it is apparent that retention of moisture by salt addition is a facotr in cookery. This method of cookery (griddle broiling) necessitates frequent turning and movement of patties to assure uniform cookery, therefore, constant time is the best endpoint to use.

Table 1—Formulation of experimental batches

Treatment	Salt[a]	TPP[a]	Water[a]
Control	–	–	2.0
Salt	0.75	–	2.0
Tripolyphosphate (TPP)	–	0.30	2.0
Salt + TPP	0.75	0.30	2.0

[a] Percent by weight

Table 2—Effect of treatment variation with time intervals and replications combined

Item	Control	Salt	TPP	Salt + TPP
Moisture (%) (n=39)	58.47	58.28	58.83	59.15
Fat (%) (n=39)	23.51	24.10	23.45	24.00
Cooking loss (%) (n=88)	39.8^{c}	26.8^{a}	33.7^{b}	31.4^{ab}
Instron (sq. cm) (n=90)				
single blade	37.9^{a}	45.4^{b}	44.6^{b}	42.1^{ab}
circle blade	56.4^{a}	67.8^{b}	69.7^{b}	70.3^{b}
Trained panel (n=54)				
color desirability	5.2^{a}	6.3^{b}	6.1^{ab}	6.6^{b}
discoloration	6.8^{a}	7.7^{ab}	7.7^{ab}	8.0^{b}
cohesiveness	5.1^{a}	6.1^{c}	5.6^{b}	6.3^{c}
juiciness	4.3^{a}	5.4^{b}	4.7^{a}	6.3^{c}
flavor	4.6^{a}	5.4^{b}	4.7^{a}	6.3^{c}
Consumer panel (n=120)				
degree of doneness	2.6^{a}	3.3^{b}	2.9^{ab}	3.3^{b}
appearance	5.8	5.6	5.6	5.8
aroma	5.9	5.5	5.7	5.8
tenderness	5.8	5.9	5.6	6.0
juiciness	5.9	5.7	5.5	6.1
flavor	5.8^{c}	5.4^{ab}	5.3^{a}	5.9^{c}
connective tissue	5.6	5.5	5.6	5.9
overall acceptability	5.7	5.3	5.1	6.0

[abc] Means in the same row with different superscripts are significantly different ($P<0.05$).
[a] Force required to shear samples.

Instron readings on the single and circle blade shear were lowest in the control samples, thus the control samples required the least amount of shear force. Samples containing salt, TPP, or both required more shear force due to the increase in binding and cohesiveness.

Trained panel evaluations (Table 2) showed that, with the addition of salt, a more desirable color was obtained. When salt and/or TPP were added a lower percent discoloration developed than was found in the control. Panel evaluations for cohesiveness indicate that salt was the primary factor in development of this property. Juiciness and flavor of the patties were also increased with the addition of salt; however, when both salt and TPP are incorporated into the patties, a synergistic effect was noted, thus increasing the juiciness and flavor beyond those levels attained by salt or TPP alone.

Triangle test results (Table 3) indicate that the addition of salt increased the differences between samples and increased the correct number of samples as determined by a significant number of panel members. It is important to note that the TPP samples were not found to be significantly different from the control in this evaluation.

Consumer sensory panel evaluations (Table 2) showed similar results to the trained panel for flavor, preferring those samples with salt or TPP over the control. No significant difference could be determined from the consumer panel evaluations for appearance, aroma, tenderness, juiciness, connective tissue, or overall acceptability.

The storage of patties up to 60 days, shown in Table 4, had little effect on any of the sensory attributes. Both trained and consumer panel ratings revealed a nonsignificant storage effect. TBA values did indicate that lipid

Table 3—Triangle tests for difference[a]

Comparison	Observations No. correct	Observations % correct	Significance
Control vs salt	91	84	P<0.001
Control vs TPP	54	50	N.S.
Control vs salt + TPP	96	89	P<0.001
Salt vs TPP	79	73	P<0.01
Salt vs salt + TPP	72	67	P<0.05
TPP vs salt + TPP	96	89	P<0.001

[a] Means of 108 observations

Table 4—Effect of storage time with treatments and replications combined

Item	0 days	30 days	60 days
Trained panel (n=72)			
color desirability	6.3	5.8	6.0
discoloration	7.7	7.5	7.5
cohesiveness	5.7	5.7	5.9
juiciness	5.4	5.0	5.2
flavor	5.4	5.1	5.2
Consumer panel[c]			
appearance	5.5[b]	5.8[ab]	5.9[a]
aroma	5.6	5.9	5.9
tenderness	5.7	6.0	5.9
juiciness	5.7	6.1	6.0
flavor	5.6	5.6	5.7
connective tissue	5.5	6.0	5.8
overall-acceptability	5.5	5.8	5.5
TBA values[d] (n=24)	0.42[a]	0.42[a]	1.27[b]

[ab] Means in the same row with different superscripts are significantly different (P<0.05).
[c] n = 0 days 196; 30 days 140; 60 days 147 for all taste traits.
[d] mg malonaldehyde/1000g meat

oxidation had occurred to a significant extent by day 60, but not sufficient for detection by a sensory panel.

CONCLUSION

THE FOLLOWING conclusions can be drawn from this study; (1) the addition of salt altered the sensory, color, and physical properties while TPP had little effect on the sensory, color, and physical properties of beef patties; (2) the combination of salt and TPP improved the sensory properties over TPP alone, and salt alone in all properties except juiciness and flavor; (3) color desirability as well as lack of discoloration were improved with the addition of salt and TPP alone or in combination; and (4) freezer storage for up to 60 days did not significantly affect the sensory properties of the beef patties, even though lipid oxidation occurred, as indicated by increased TBA values.

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Color Stability of Radappertized Cured Meat

A. R. KAMAREI, M. KAREL, and E. WIERBICKI

ABSTRACT

Porcine semimembranosus muscles were cured (with different levels of nitrite, nitrate, and their combination), smoked, and irradiated with 3.2 Mrad gamma radiation at −40°C. Subjective studies by a trained panel, objective studies of total color difference (calculated from tristimulus values), and behavior of residual nitrite and nitrate show that radiation alters the pink color of cured meats (radiation-induced fading). Contrary to prior reports, nitrate affects neither color development nor post-irradiation fading. Color of nitrate-cured samples, like uncured samples, become bright red when irradiated, due to reduction of globin myohemichromogen (indistinguishable from pink color of cured meat), and therefore are more desirable.

INTRODUCTION

PROCESSING cured meats with sterilizing doses of ionizing radiation reduces the need for nitrites. Most of the allowable nitrite added to cured meats is used to prevent development of *Clostridium botulinum* (Ingram, 1974; Grever, 1974). Only a fraction is required to develop the product's characteristic color, flavor, and taste (Roberts, 1975). Consequently, in radappertized cured meats, nitrite can be reduced to the level needed only for development of color, flavor, and taste. However, radiation sterilization with minimum levels of nitrite causes an undesirable color fading. Previous studies have shown that small amounts of sodium nitrate may be used along with reductions of added nitrite ("mixed cure") to prevent fading of the cured meat color (Wierbicki et al., 1977). When exposed to air and light, radappertized cured meat, like nonirradiated meat, fades further.

Understanding cured meat color's behavior when irradiated and nitrate's effects (if any) on color stabilization is helpful in establishing conditions for obtaining the optimal color in radappertized cured meats.

MATERIALS & METHODS

CHOICE-GRADE fresh ham was selected at random from a commercial market. Pretreatment included deboning, defatting, and trimming meat. Semimembranosus muscles (average weight 800g) were carefully separated. Nitrite and nitrate concentrations were the only variable factors in irradiated and nonirradiated samples. We found experimentally that 15% of the meat weight can easily be added to meat as "curing pickle," although this extra weight is later removed during smoking (or mild drying thereafter). Therefore, the concentrations of ingredients in curing pickle should be 6.66 times more than desired final concentrations in ham (Table 1).

Sodium tripolyphosphate (TPP) and sodium chloride were dissolved in cold deionized water to prepare "bulk cure." Ascorbate was dissolved in "bulk cure" shortly before the meat was cured. To prepare the "curing pickle," we dissolved the desired amounts of pure sodium nitrite and sodium nitrate in the "bulk cure" just before the "curing pickle" was injected into the meat. Table 2 gives the sodium nitrite and nitrate concentrations of differently treated groups. We applied 12% of "curing pickle" by stitch-pumping, using hollow perforated needles attached to a pump with an injection pressure of 25 lb/in^2. In this manner, the pressurized pickle was infused throughout the meat. Muscles were cut into smaller (about 100g) pieces, and the remaining 3% of "curing pickle" was added. The whole system was then mixed in an evacuated (to avoid air entrapment in the sample) stainless steel mixer for 15 min. Cured meat was stuffed into regenerated cellulose casings and as much entrapped air as possible was let out through small holes in the casing. These holes also allow water evaporation during smoking and drying. The filled casings were then placed in stainless steel screen cages and kept at 2°C for 48 hr.

Smoking was conducted in a multifloored smokehouse, using hickory chips, so that autolytic enzymes, which do not get inactivated by sterilizing doses of ionizing radiation, were inactivated with a mild heat treatment. Table 3 shows the smoking schedule. Weight changes at different steps were monitored and recorded. The smoked ham was cooled overnight and sliced (under indirect, low intensity light) into rectangular pieces weighing 70 ± 5g, with approximate dimensions of 13 x 8.5 x 0.63 cm. Using a Multivac packaging machine (Standard Packaging Corp., Jersey City, N.J.), we immediately double-sealed samples into flexible pouches under vacuum and stored them overnight at −40°C.

Frozen packages were irradiated at −40°C with 3.2 Mrad (32K Gray) gamma radiation from a Cobalt-60 source which has been described in detail elsewhere (Karel, 1975). Post-irradiated samples were kept at −40°C for all the following experiments.

A Diano-Hardy II recording spectrophotometer (exploiting CIE standard illuminant C) was used for visible reflectance spectra of meat samples.

—Continued on next page

Table 1—Concentration of ingredients of curing pickle and ham

Ingredient	Curing pickle (%)	Ham (%)
NaCl, food grade	16.000	2.4
TPP, food grade	2.000	0.3
Sodium ascorbate, food grade	0.366	0.055
Water	81.634	
Total	100.000	

Table 2—Final concentration of sodium nitrite and nitrate ingredients in sectioned and formed ham[a]

Group	Sodium nitrite	Sodium nitrate
1 (control)	0	0
2	25	0
3	50	0
4	75	0
5	0	25
6	0	50
7	0	75
8	25	25
9	25	50
10	25	75

[a] Containing 2.4% NaCl, 0.3% TPP, and 550 ppm sodium ascorbate.

Authors Kamarei and Karel are with the Dept. of Nutrition & Food Science, Massachusetts Institute of Technology, Cambridge, MA 02139. Author Wierbicki is with the U.S. Army Natick Research & Development Command, Natick, MA 01760.

EXPERIMENTAL

MEAT SAMPLES were thawed at refrigeration temperature and then their colors were evaluated subjectively at room temperature and under direct fluorescent light by a 15-member trained panel, immediately after the pouch was opened and 90 min later. Panel members gave their appraisal on a scale of 1 to 9 (1 = extremely poor, 9 = excellent; Peryam and Pilgrim, 1957).

Objective evaluation was conducted on five replicates of each of 10 groups of irradiated and nonirradiated samples. Reflectance spectra, from 380-700 nm, were obtained after 0, 20, and 90 min exposure to air and light at room temperature.

For spectrophotometric assays, the most homogeneous (without fat and air pockets) parts of the meat samples were chosen and cut into approximately 3 x 4 cm pieces. A piece of white paper board (with average reflectivity of 90%) at the back of each sample assured uniformity of reflectance measurements.

Along with reflectance spectra, CIE (Commission International de l'Eclairage) tristimulus values (X, Y, Z) were obtained. These values were then converted to total color difference (ΔE) according to 1976 color difference L* a* b* equations (CIE, 1976):

$$L^* = 25\left(\frac{100\,Y}{Y_o}\right)^{1/3} - 16, \quad 1 \leq Y \leq 100$$

$$a^* = 500\left[\left(\frac{X}{X_o}\right)^{1/3} - \left(\frac{Y}{Y_o}\right)^{1/3}\right]$$

$$b = 200\left[\left(\frac{Y}{Y_o}\right)^{1/3} - \left(\frac{Z}{Z_o}\right)^{1/3}\right]$$

Table 3–Schedule for smoking sectioned and formed ham

Smoke presence	Duration (hr)	Controlled parameters[a] (in smokehouse) T_{DB}(°F)	T_{WB}(°F)	Calculated parameters[b] R.H. (%)	A.H. lb water/ lb dry air
–	1	150	120	41	0.07
+	1	150	120	41	0.07
+	1	160	130	42	0.10
+	3	170	135	40	0.12
+	?[c]	180	150	48	0.20

[a] T_{DB} = dry bulb temperature; T_{WB} = wet bulb temperature.
[b] R.H. = Relative humidity; A.H. = Absolute humidity.
[c] Time to reach internal temperature of 164°F (73°C), recorded by inserted thermocouples.

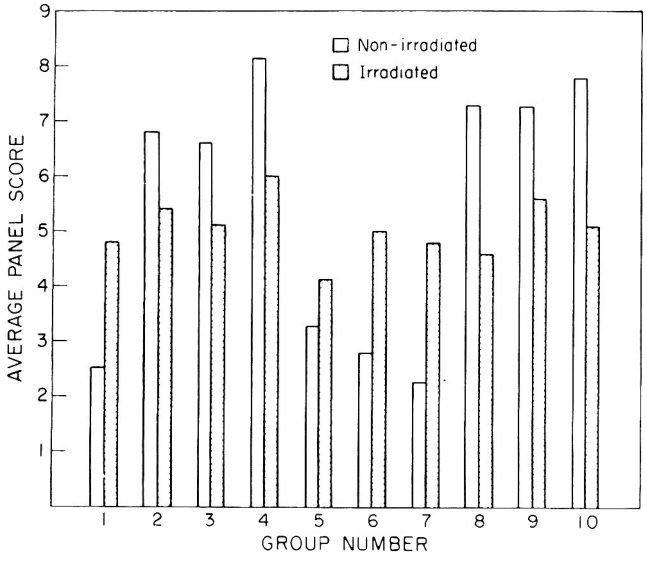


Fig. 1–Effect of 3.2 Mrad gamma radiation at –40°C on color rating of cured ham upon opening the pouch (0 min exposure).

X_o, Y_o, and Z_o are the tristimulus values of the chosen standard C illuminant (98, 100, 118). In each group, average control values were assumed as baselines for measuring this color difference. The total difference between two colors, each given in terms of L* a* b*, is calculated from:

$$\Delta E_{CIE}\,(L^*\,a^*\,b^*) = [(L^*)^2 + (a^*)^2 + (b^*)^2]^{1/2}$$

We determined residual nitrite and nitrate of all 10 groups, using modified AOAC method (1975) at the pre-smoking, post-smoking, and post-smoking/irradiation stages. Post-smoking and post-smoking/irradiation samples were chosen from the center of the cured meat rolls, because nitrite stability is affected considerably by temperature.

RESULTS & DISCUSSION

FIGURES 1-3 show the average scores of the 15-member panel on color preference of nonirradiated and irradiated samples after 0 and 90 min exposure. The color of nonirradiated cured and cooked ham is highly desirable (Fig. 1) only when it contains 25-75 ppm sodium nitrite and/or

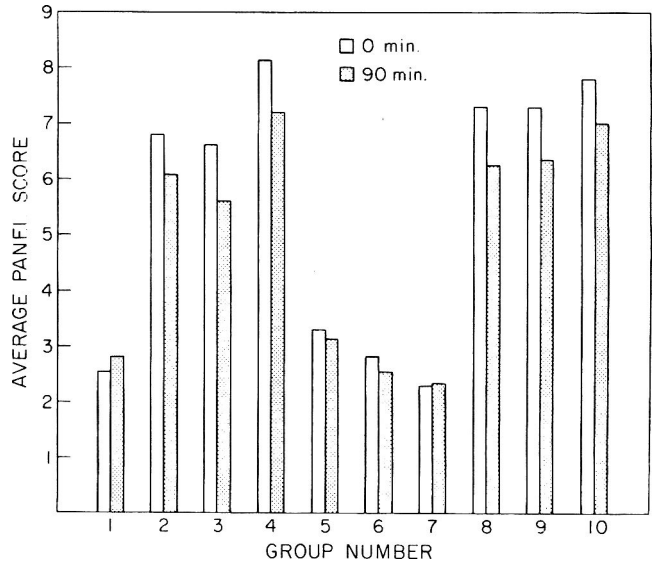


Fig. 2–Effect of exposure for 90 min to air and light at room temperature on color of cured, nonirradiated ham.

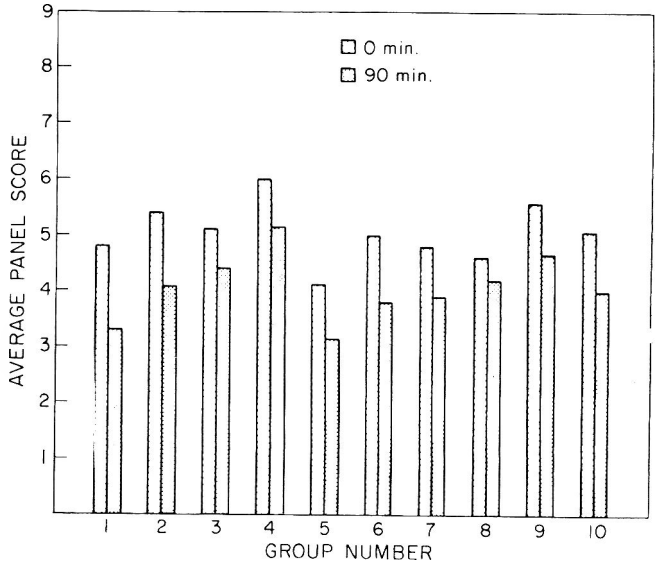


Fig. 3–Effect of exposure for 90 min to air and light at room temperature on color of cured, irradiated (3.2 Mrad at –40°C) ham.

combined sodium nitrite and sodium nitrate. Nitrate alone was ineffective. The present finding that degree of color acceptability to panel members generally decreases as nitrite concentration decreases below 75 ppm agrees well with the findings of others (Eakes and Blumer, 1975; Sebranek et al., 1977). It is difficult to establish a single level of nitrite addition below which color would be unacceptable because the required amount varies with product and process. However, in this study, 25 ppm nitrite seems to be a highly acceptable level (score = 6.80). When different groups of nitrite- and nitrate-cured hams are irradiated, the visual scores for samples containing nitrite or nitrate + nitrite decrease, and those for samples without any curing agent or with nitrate increase. Uncured samples (group 1) behave in accordance with conclusions of our previous work (Kamarei et al., 1979), in which we showed that brown globin myohemichromogen is formed upon enzyme inactivation, and bright red globin myohemochromogen is formed upon vacuum packaging and irradiation. It is therefore to be expected that uncured brown ham becomes more acceptable when irradiated, since panel members did not differentiate between the bright red radiation-induced color of uncured meats and the pink color of cured meats. Samples that contain only nitrate (groups 5-7) behave like uncured ham (group 1) pre- and post-irradiation. Nitrate does not affect color development pre- or post-irradiation. Samples containing nitrate alone should be considered *uncured* and be expected to behave as such, unless there is potential for reduction of nitrate to nitrite. Samples that contain only nitrite (groups 2-4) or nitrite + nitrate (groups 8-10) behave similarly pre- and post-irradiation.

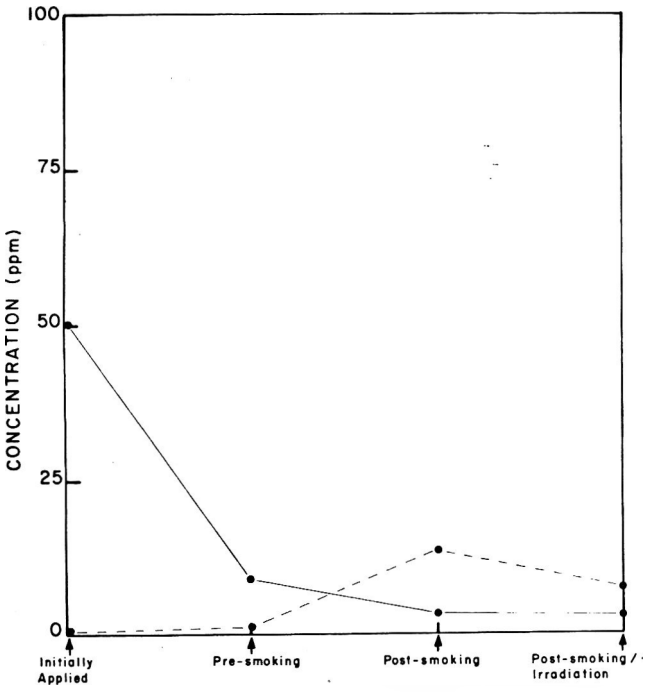


Fig. 4—Determination of residual sodium nitrite (——) and nitrate (- - -) by modified AOAC (1975) method, Group 3.

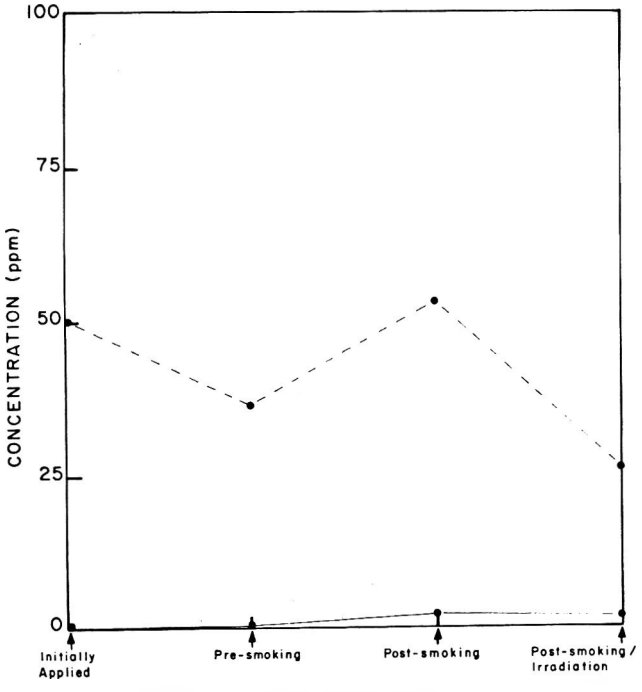


Fig. 5—Determination of residual sodium nitrite (——) and nitrate (- - -) by modified AOAC (1975) method, Group 6.

However, radiation alters the pink color of cured ham to a brownish color and therefore decreases the color score of these samples (radiation-induced fading). The mechanism of this fading is not clear and deserves further investigation.

Figure 2 shows the scores of nonirradiated samples after 90 min exposure to air and light. Decreases in panel scores are more pronounced for nitrite-cured (groups 2-4) and nitrite + nitrate-cured (groups 8-10) samples than for uncured (group 1) and nitrate-cured (groups 5-7) samples.

Figure 3 shows the scores for irradiated samples after 90 min exposure to air and light. The results are consistent with the conclusion that radiation-reduced pigments in groups 1 and 5-7 are oxidized by air to brown globin myohemichromogen (Kamarei et al., 1979). Subsequent fading of cured samples due to autooxidation and/or photooxidation is well known (Draudt and Deatherage, 1956; Hornsey, 1957; Walsh and Rose, 1956).

Objective studies using reflectance spectrophotometry confirm the subjective results.

We also conducted studies on residual nitrite and nitrate in all samples. Results in Figure 4 show that, after application of 50 ppm nitrite, most of the nitrite is used up. The actual fate of nitrite in meat has been very controversial. Cassens et al. (1977), in tests conducted over a period of 5 yr, recovered the following proportions of nitrite originally added to meat: in myoglobin 5-15, nitrate 1-10, nitrite 5-20, gas 1-5, sulfhydryl 5-15, lipid 1-5, and protein 20-

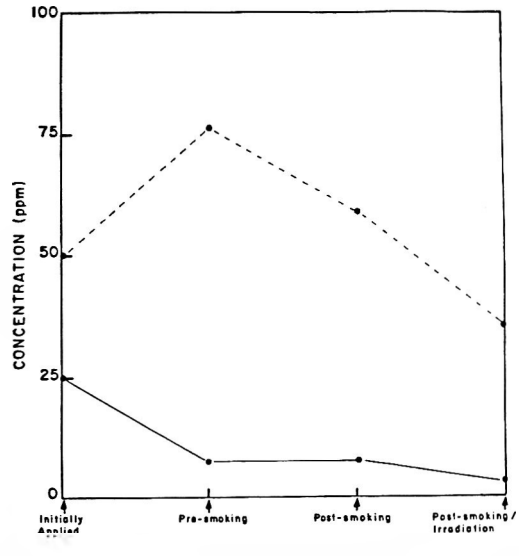


Fig. 6—Determination of residual sodium nitrite (——) and nitrate (- - -) by modified AOAC (1975) method, Group 9.

30%, representing a total recovery of 38-100% (usually 70-80%). However, upon smoking, some residual nitrite is oxidized to nitrate, which is destroyed further by radiation.

Nitrates have a somewhat uncertain role. It is generally believed that they may be a reservoir of nitrite formed by reduction in meat (Chichester and Tanner, 1975). Samples containing nitrate alone (e.g., group 6 with 50 ppm nitrate; Fig. 5) did not show important reduction of nitrate to nitrite at any stage. This finding confirms that, under our conditions, nitrate does not contribute to cured meat color. However, when nitrate itself is applied, about 15 ppm is reversibly complexed in all groups. Upon smoking, this nitrate, as well as meat's indigenous nitrate, can be measured as free residual nitrate. These higher nitrate concentrations are significantly destroyed by radiation. When nitrite and nitrate are used together (e.g., group 9 with 25 ppm nitrite and 50 ppm nitrate; Fig. 6), nitrite's behavior is the same as with 25 ppm nitrite alone. While higher nitrate concentration post-smoking (from oxidation of nitrite) and its destruction by radiation were expected, we did not expect the higher nitrate concentration pre-smoking in these groups. One explanation could be that the complexing sites of meat components are saturated in the presence of nitrite. The added nitrate, together with the indigenous nitrate and partially oxidized nitrite, will result in a higher nitrate concentration in the pre-smoking stage.

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VITAMIN A DEGRADATION ON HEAT PROCESSING . . . From page 33

The comparison of the experimental results with the pharmaceutical data of Slater et al. (1979) and the analogy of thiamin, indicate that other factors apart from those studied, affect the degradation of vitamin A on heat processing. Hence, further work is planned to quantify the variation in kinetics as a function of other factors such as product composition and pH.

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Isolation of Potentially Virulent *Yersinia enterocolitica* from Variety Meats

NORMAN J. STERN

ABSTRACT

Sixty-three samples of variety meats were examined for the presence of *Yersinia enterocolitica*. Surface swab samples in a modified selenite enrichment broth were incubated at 23°C for 2 and 3 days and then streaked onto MacConkey agar with Tween 80 or refrigerated bismuth sulfite agar, with and without a potassium hydroxide preplating treatment. The organism was isolated from 2 of 23 porcine throat swabs but was not isolated from 40 bovine tongue, kidney, heart, or liver swabs. The isolates from the throat swabs were classified into Nilehn and Wauters biotypes 3 or 4. One porcine isolate was of serotype 0:19, while another isolate from the second *Y. enterocolitica*-bearing porcine throat swab was nontypeable. The nontypeable isolates autoagglutinated in tissue culture when grown overnight at 35°C, suggesting that these isolates are potentially virulent. The results of this study support the contention of European researchers that swine may be a significant source of *Y. enterocolitica* involved in human infections.

INTRODUCTION

Yersinia enterocolitica is a zoonotic microorganism capable of causing foodborne enteritis in humans (Black et al., 1978). The organism is capable of growth in foods at refrigeration temperatures (Stern et al., 1980; Hanna et al., 1977) and may be passed to humans through direct or indirect contamination of food by feces or urine (Keet, 1974). The proximity of variety meats (hearts, livers, tongues, kidneys, etc.) to the enteron may be cause for concern, for variety meats can thus be contaminated by fecal materials during slaughter and dressing.

Several surveys have been conducted to recover *Y. enterocolitica* from meats and these are covered in reviews on the subject (Lee et al., in press; Stern and Pierson, 1979). The 0:3 serotype of *Y. enterocolitica*, which is often involved in human disease, has consistently been recovered from the cecal contents and mesenteric lymph nodes of healthy pigs from different parts of the world (Asakawa et al., 1979; Toma and Deidrick, 1975; Zen-Yoji et al., 1974). Limited data on the incidence of *Y. enterocolitica* in variety meats in the United States have been reported (Hanna et al., 1980). Therefore, I examined isolates of *Y. enterocolitica* from selected variety meats from beef and pork for biotype, serotype, and virulence potential, and also determined their incidence.

MATERIALS & METHODS

Variety meat samples

Thirty kidneys, tongues, and hearts (ten each) from freshly slaughtered and dressed beef carcasses were obtained from a local meat-packer in the Baltimore area. The organs were excised from the carcasses, washed, and graded by USDA inspectors. They were then placed into plastic freezer bags and taken (in less than 2 hours' time) to the laboratory for microbiological analysis. Additionally, 10 livers were sampled with 0.1% peptone moistened aliginate swabs (Lazarus et al., 1977) at the meat packing plant. Each swab was placed in a modified selenite enrichment broth after three template areas from each organ were swabbed; a total of 37 cm^2 was swabbed for each variety meat sample. The three areas of each kidney, heart and liver that were swabbed were chosen randomly from the surface of each organ. Tongue swabs were taken from the tonsil area of the beef carcasses because of reported European recoveries from this area in swine (Wauters and Janssens, 1976).

Surfaces in the general area of swine tongue and tonsils were swabbed. Obtaining samples from the same area in each swine carcass was difficult because samples were taken in the slaughterhouse after exsanguination but before scalding, when the tongue had not yet been excised from the animal. The samples were thus representative of the indigenous *Y. enterocolitica* present on swine tongue and tonsils.

Microbiological techniques

Modified selenite enrichment broth was made with 4 ml of 5% selenite solution and 0.05% beef extract (Lee et al., 1980; Stern and Oblinger, 1980). Surface swabs were placed in enrichment broth and incubated at 23°C for 2 and 3 days. After incubation, loopfuls of broth were streaked onto plates containing MacConkey agar with Tween 80 (Lee, 1977) or plates containing bismuth sulfite agar (Difco) that had been refrigerated for 2 to 3 days before use. In a modification of the method described by Aulisio and co-workers (1980), 0.5 ml of the enrichment broth was transferred to a tube containing 4.5 ml of potassium hydroxide solution, mixed with a Genie Vortexer, and streaked onto the two types of selective agar plates. Plates were incubated and colonies picked as described by Lee (1977). Biochemical analyses were carried out in accordance with the specifications of Nilehn (1969) and Wauters (1970). An isolate from each positive sample was submitted to T.J. Quan (Center for Disease Control, Fort Collins, Colo.) for serological determination.

Isolated strains were also tested for virulence potential according to methods described by Laird and Cavanaugh (1980). *Y. enterocolitica* isolates were streaked onto trypticase soy agar (BBL) and incubated 3 days at 23°C. Nine isolated colonies (from each isolate) were inoculated into pairs of tubes containing 2.0 ml of tissue culture medium (Microbiological Associates, Walkersville, Md.). Sets of tubes were incubated at 23°C or 35°C for 18 hr. Isolates which autoagglutinated at 35°C, but not at 23°C were considered potentially virulent. Strain TAMU 54, which has a positive reaction for keratoconjunctivitis in the guinea pig (Sereny) was used as a positive control while strain TAMU 75, which has a negative reaction for keratoconjunctivitis was used as a negative control.

RESULTS & DISCUSSION

TWELVE ISOLATES of *Y. enterocolitica* were recovered from 2 of 23 swine throat samples. Three were isolated on MacConkey agar with Tween 80 and four colonies were isolated on this medium with KOH pretreatment. Two colonies were isolated on prerefrigerated bismuth sulfite and three on this medium with KOH pretreatment. These isolates were either nontypeable or were classified into 0:19 serology. This 9% recovery rate from swine throat samples is lower than that reported in Europe. In 19 Belgian communities 53% of samples taken from swine throats contained *Y. enterocolitica* 0:3 serotype (Wauters and Janssens, 1976), whereas in Denmark, 30% of swine throat swabs yielded this serotype (Pederson, 1979). Recently, Hanna and co-workers (1980) isolated *Y. enterocolitica* from 8 of 27 pig tonsils samples. In this present study no *Y. enterocolitica* isolates were obtained from the 40 sam-

Author Stern is with the USDA-SEA-AR, Meat Science Research Laboratory, Beltsville Agricultural Research Center, Beltsville, MD 20705.

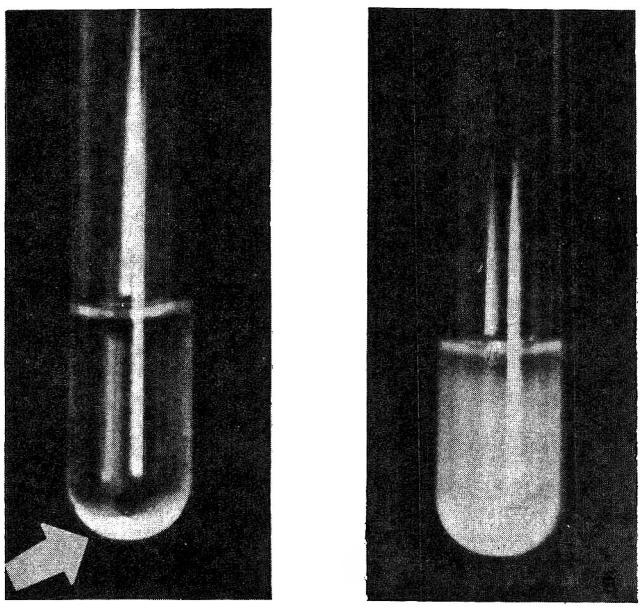
ples of variety meats from beef carcasses.

Several taxonomical characteristics of the 12 *Y. enterocolitica* isolates from swine throats (Table 1) are considered useful markers in discriminating environmental from clinical strains (Bottone et al., 1974; Bottone, 1978) and in biotyping the isolates. All 12 isolates demonstrated typical taxonomical characteristics of *Y. enterocolitica*: they were gram-negative rods that produced acid slants, acid butts, and no gas or H_2S after overnight incubation at 23°C in triple sugar iron agar slants, they demonstrated positive urease and negative phenylalanine deaminase activity, and were motile at 23°C but not at 35°C.

Table 1—Selected taxonomical characteristics of 12 Yersinia enterocolitica strains isolated from swine throat/tongue swabs[a]

Taxonomical characteristic	No. of positive strains
β-galactosidase (ONPG) activity	12/12
Esculin utilized	0/12
Indole produced	0/12
Lactose (O/F) utilized	0/12
Lecithinase activity	0/12
Nitrate reduced	12/12
Ornithine decarboxylase acitvity	12/12
Rhamnose utilizated	0/12
Salicin utilized	0/12
Sorbitol utilized	0/12
Sorbose utilized	12/12
Surcrose utilized	12/12
Trehalose utilized	12/12
Voges-Proskauer test	6/12

[a]Test conditions described by Nilehn (1969) and Wauters (1970).



Positive Pork Isolate

Negative Pork Isolate

Fig. 1—Appearance of virulent (positive) and nonvirulent (negative) pork isolates after 18 hr incubation at 35°C in tissue culture medium (Laird and Cavanaugh, 1980). Note flocculent sediment (arrow) with clear tissue culture in positive pork isolate. Turbidity of tissue culture is indicative of nonautoagglutinating (negative) Yersinia enterocolitica *isolate.*

The taxonomic characteristics of the isolates were uniform, excepting that isolates from the two infected pigs differed consistently in ability to give a positive Voges-Proskauer (VP) test, as well as in serology. Isolates from one pig giving a positive VP reaction were nontypeable, whereas isolates from the second pig were negative for the VP test and were categorized as having 0:19 serology. The isolates could not be differentiated clearly into either of the two major biotype schemes for *Y. enterocolitica* (Nilehn, 1969; Wauters, 1970). All isolates were lecithinase, indole, and lactose (O/F) negative but xylose positive. Because this combination of biochemical markers is not accounted for by either the Wauters or Nilehn schemes, the isolates cannot be set clearly into the biotype 3 or 4 classifications of either scheme.

The VP-positive, nontypeable isolates demonstrated the ability to autoagglutinate in tissue culture (Ag+), while the VP-negative 0:19 serotype isolates did not (Ag−; Fig 1). Laird and Cavanaugh (1980) reported that all 25 strains found to be Ag+ proved virulent to mice by the oral route. None of the Ag− strains they tested proved virulent to mice. They further stated, " . . . there is without question a correlation between the autoagglutination and virulence properties of yersiniae." Because use of human volunteers cannot be considered, this in vitro procedure appears to be a practical and accurate alternative in predicting disease-causing abilities of *Y. enterocolitica* strains. Therefore, the Ag+ isolates from the swine throat swabs in this study show potential pathogenicity. Zink and co-workers (1978) demonstrated that virulence of *Y. enterocolitica* (as measured by the Sereny test) correlates with the presence of a 41 Mdal plasmid. In this study none of the 0:19 serotype isolates autoagglutinated in tissue cultures, while 52 of 54 of the nontypeable isolates did. It is probable that some of the isolates which originally contained the 41 Mdal plasmid were cured through subsequent transfers. Therefore, the importance of picking 9 or 10 isolated colonies from each culture originally isolated from selective agars must be emphasized. If any isolated *Y. enterocolitica* colonies are Ag+, the original isolate must also be considered as potentially virulent.

Relatively large surface areas (37 cm^2) were sampled for each of the 63 variety meats tested. Stern and Oblinger (1980) have shown that in 25 of 40 trials, sampling methodology used in the present report is capable of detecting approximately 20 *Y. enterocolitica* cells/cm^2. Bacterial distribution is probably not uniform on variety meats and therefore the true incidence of the organism may not have been detected for each sample. Strains of *Y. enterocolitica* isolated from one porcine throat swab in this study demonstrate virulence potential, as measured by an autoagglutination test, and therefore this work lends supporting evidence to the hypotehsis that swine may be an important source of *Y. enterocolitica* in the human food chain.

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—Continued on page 51

Effect of Gamma-irradiation on Lipid Constituents of Autolysing Lamb Liver

BRIJ BHUSHAN, V. NINJOOR and G.B. NADKARNI

ABSTRACT

Alterations in lipid constituents of lamb liver in response to varying doses (0-2.0 Mrad) of gamma-radiation were ascertained in terms of total vitamin A, total phospholipid, and fatty acid content. Radiation exposure at 2.0 Mrad dose resulted in a five-fold increase in thiobarbituric acid reacting substances (TBRS) measured in terms of malonaldehyde and a 64% rise in carbonyls. The losses in lipid constituents were marginal. During the course of autolysis significant lipolytic activities persisted in irradiated lamb liver as evidenced by the decline in total phospholipid (50%) and neutral fat levels accompanied by a four-fold increase in fatty acid content. In irradiated lamb liver undergoing autolysis, a rapid hydrolysis of vitamin A Esters occurred leading to enhanced vitamin A alcohol/ester ratios. A comparison of radiation destruction of vitamin A in model systems, shark liver oil, and lamb liver revealed that similar doses of gamma-radiation cause more destruction in model systems than in others.

INTRODUCTION

IT IS NOW RECOGNIZED that action of tissue enzymes and bacteria are the major factors that contribute to spoilage of foods during storage (Keay and Hardy, 1978). In radiation processed foods, however, where microbiological spoilage is suppressed, the autolytic reactions may predominate (Rhodes, 1969). Although several autolytic enzymes in homogeneous solutions were found to be sensitive to radiation inactivation under in vitro conditions, they were reported to be least affected in meat foods (Rhodes, 1969).

The involvement of lysosomal hydrolases which could degrade practically all tissue constituents has been implicated in the spoilage of a variety of flesh foods (Feeney and Whitaker, 1977; Dutson and Lawrie, 1974; Warrier et al., 1972) including sea-foods (Warrier et al., 1972; Doke et al., 1978). Several physical and chemical treatments have been shown to act on lysosomal membrane leading to increased availability of autolytic enzymes (Ninjoor et al., 1969, 1973; Harikumar et al., 1978). Among the chemicals which cause lysosomal labilization, vitamin A is of particular interest since the ester form (retinyl palmitate) normally occurring in tissues, is inactive in causing the release of autolytic enzymes, while the alcohol form (retinol) is reported to be a potent labilizer of lysosomes (Roels, 1969; Ninjoor et al., 1978). Earlier work from our laboratory has demonstrated that the radiation degradation products (RDP) of vitamin A exhibited a very high lysosome labilizing activity and caused accentuated autolysis of lamb liver (Ninjoor et al., 1973).

In irradiated lamb liver, besides the formation of RDP-like compounds, the autolytic rates could also be altered for the conversion of vitamin A ester to retinol. This aspect has not been examined so far. The present paper reports on the effect of gamma-radiation on autolysis of lamb liver. This tissue was particularly chosen because it is a rich source of vitamin A and also of hydrolytic enzymes.

Authors Bhushan, Ninjoor, and Nadkarni are affiliated with the Biochemistry & Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, India.

EXPERIMENTAL

Materials

Fresh lamb liver obtained from a local slaughter house was brought to the laboratory in iced condition. The shark liver oil and coconut oil were purchased from commercial sources. Neutral alumina, silica gel G, and sodium diethyldithiocarbamate were the products of E. Merck, Germany. Antimony trichloride was procured from May and Baker, England. Pure crystalline vitamin A acetate was a gift from Roche Products (India) Ltd. 1,1,3,3,-Tetraethoxypropane was purchased from Fluka, Switzerland. All other chemicals and solvents used in the present investigation were of 'Analar' grade. The solvents were distilled before use.

Methods

Preparation of lamb liver slurry. After removing blood and connective tissues the scissor minced liver slices were subjected to a dip treatment in oxytetracycline solution (15 ppm) for 30 min. After blotting the slices over layers of filter paper, a uniform slurry of liver was prepared in a Waring Blendor at low speed for 2 min. The entire operation was carried out at 0-4°C.

Irradiation. Weighed quantities of lamb liver slurry was transferred to screw-capped glass tubes and exposed to varying doses of gamma-radiation (0.5-2.0 Mrad) at 0-4°C in a Gamma Cell-220 (AECL, Canada; dose rate 0.56 Mrad/hr). Unirradiated samples served as contro..

Autolysis. Both control and irradiated lamb liver slurry were incubated at 37°C for varying intervals of time. Thereafter the tubes were withdrawn,chilled (0-4°C) and the slurry divided into three portions for lipid extraction, TBRS estimation, and vitamin A measurement. respectively.

Lipid extraction. Lipids were extracted from liver slurry using chloroform:methanol (2:1) mixture as solvent (Folch et al., 1957).

Thin-layer chromatography (TLC). A known amount (100 μg) of lipid solution in petroleum ether was applied to silica gel G plates along with oleic acid and coconut oil as reference standards for fatty acids and triglycerides (TG), respectively. The chromatogram was developed with a solvent system containing petroleum ether (60-80), diethyl ether, and acetic acid (80:10:1). The spots were detected by spraying with 40% H_2SO_4 followed by charring in an oven set at 140°C.

Fatty acids. Total content of long chain fatty acids was measured by the colorimetric method of Duncombe (1963).

Total phospholipids. A portion of lipid extract was digested repeatedly with concentrated nitric acid and perchloric acid until free from color (Frampton et al., 1948). Lipid phosphorus was quantitatively estimated colorimetrically (Fiske and Subbarow, 1925). The amount of total phospholipids was computed on a weight basis as equivalent of lecithin for lipid phorphorus values by employing a conversion factor of 25.

Thiobarbituric acid reacting substances (TBRS) and carbonyls. A modified method of Turner et al. (1954) was employed. A 5-ml portion of 10% liver homogenate was treated with equal volume of 4% trichloroacetic acid. The precipitated protein was removed by centrifugation and the supernatant fluid was used for the colorimetric determination of TBRS. 1,1,3,3-Tetraethoxypropane was employed as the standard for malonaldehyde.

Carbonyls were estimated according to the method of Lappin and Clark (1951).

Vitamin A. Total vitamin A was extracted from lamb liver slurry according to the method of Ames et al. (1954). Carr-Price color reaction (1926) was employed for the determination of vitamin A.

Vitamin A alcohol/ester ratio. The column chromatographic method of Ganguly et al. (1952) was employed for the separation

of ester and nonesterified forms of vitamin A on alumina by using 5 and 25% diethyl ester in petroleum ether as eluants.

RESULTS

Influence of gamma-radiation on lipid constituents of lamb liver

The data included in Table 1 indicate that carbonyls and TBRS levels increased concomitant with the dose of irradiation. Other minor alterations included some reduction in vitamin A and fatty acid content. There was no quantitative change in total phospholipid levels. It is worth noting that while TBRS showed a 5-fold increase, the enhancement in carbonyls was only 64% at 2.0 Mrad dose. The losses in vitamin A and fatty acids were less than 20%. Similarly the alcohol/ester ratio of vitamin A did not show a measurable change immediately after irradiation. The radiation stability of vitamin A in the freely dissolved state, as compared to a complex in biological system was assessed as shown in Figure 1. It was observed that naturally occurring vitamin A in lamb liver and shark liver oil was more radio-resistant than synthetic vitamin A acetate irradiated as solutions in coconut oil or in isooctane. A linear logarithmic relationship was observed between the radiation dose and destruction of vitamin A. In isooctane or coconut oil, the radiation responses were similar and concentration dependent (Fig. 1).

Post-irradiation autolytic changes in lamb liver

The results presented in Figure 2 show qualitative changes in lipids for unirradiated lamb liver, prior to initiation of autolysis (A), after 8 hr (B), after 18 hr (F), and after 24 hr (G) of autolysis at 37°C. Prior to the initiation of autolysis, lipids from lamb liver separated into 6 components (A). They were identified from the point of application as phospholipids, cholesterol, cholesterol ester, fatty acids and triglyceride (TG). The component with highest migration rate was identified as hydrocarbons. A gradual decrease in TG spot with concurrent increase in fatty acid was observed during the progress of autolysis (B, F and G). Immediately after irradiation lipids from lamb liver gave a TLC pattern identical to that of the control (Fig. not shown). However, during the course of autolysis, the spots corresponding to total phospholipids and fatty acids showed detectable changes.

The effects of gamma-radiation on quantitative changes in phospholipids and fatty acid levels of autolysing lamb liver slurry are summarized in Table 2. A gradual loss of total phospholipids and an increase in fatty acid levels was observed during the progress of autolysis. Autolysis for 24 hr resulted in more than a three-fold increase in fatty acid level which was not parallel to the loss in total phospholipids. Moreover, while fatty acid levels exhibited a lag phase during the initial period of autolysis of lamb liver irradiated at 2.0 Mrad, the phospholipid disappearance

Table 1—Effect of gamma-radiation on the lipid constituents in lamb liver slurry[a]

Constituents	Radiation dose, Mrad 0	0.5	1.0	2.0
TBRS	1.24	3.57	4.95	6.6
Carbonyls	47	55	60	79
Fatty acids	3.11	2.83	2.83	2.54
Total phospholipids	19.38	19.38	19.38	19.38
Total vitamin A	452	452	430	380
Vitamin A alcohol/ester	0.19	0.18	0.18	0.17

[a] Lamb liver slurry was irradiated at 0-4°C with different doses of gamma-radiation and analyzed for various indices as per the procedures described in the text. TBRS and cabonyls were calculated as μmoles/100g wet tissue whereas fatty acids and total phospholipids were computed as mg/g wet tissue. Vitamin A was expressed as IU/g. The values are the mean of 3 experiments. The range from the mean values did not vary ± 3.7% for TBRS, ± 4.2% for total phospholipids, ± 3.2% for carbonyls, ± 2.3% for fatty acids, and ± 5.5% for total vitamin A.

Table 2—Post-irradiation levels of fatty acids and total phospholipids in autolysing lamb liver slurry[a]

Constituents	Treatment	Time of incubation, hr 0	2	8	18	24
Fatty acids	Unirradiated	3.11	7.63	7.35	9.32	10.17
	0.5 Mrad	2.83	7.63	7.35	8.78	9.32
	1.0 Mrad	2.83	6.22	6.22	8.48	10.17
	2.0 Mrad	2.54	3.11	3.67	8.48	10.17
Total phospholipds	Unirradiated	19.38	19.38	12.79	14.49	10.39
	0.5 Mrad	19.38	19.22	11.94	11.94	9.84
	1.0 Mrad	19.38	17.48	11.55	11.08	9.38
	2.0 Mrad	19.38	15.81	11.08	11.09	10.39

[a] Lamb liver slurry was allowed to autolyse for varying intervals of time at 37°C. The lipid constituents were analysed as described in the text. The values expressed as mg/g wet tissue are the means of 3 experiments.

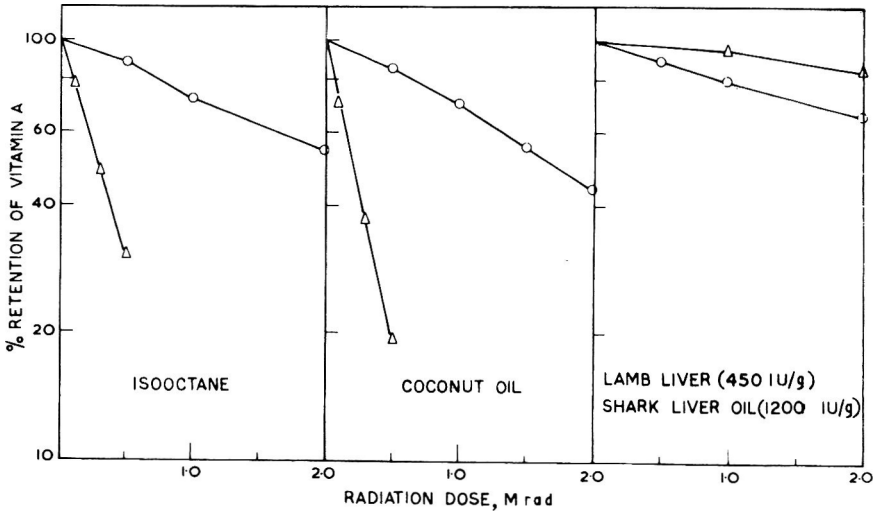


Fig. 1—Comparative radiation stability of vitamin A in lamb liver, shark liver oil, coconut oil and in isooctaine. Shark liver oil (1200 IU/g) and lamb liver slurry (450 IU/g) were exposed to varying doses of γ-radiation and examined for the retention of vitamin A. Isooctane and coconut oil solutions contained 450 IU/ml (△) and 1200 IU/ml (○) vitamin A.

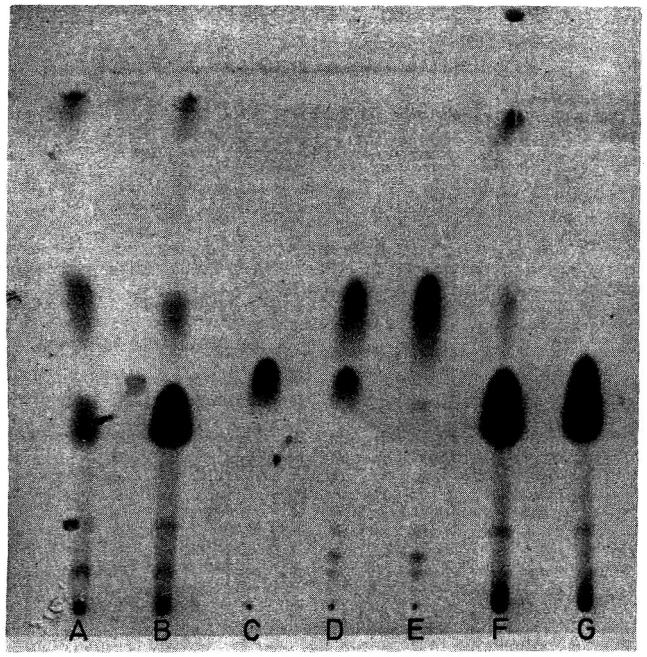


Fig. 2—TLC separation of lipids from lamb liver slurry. Lipids extracted from the lamb liver slurry were separated on silica gel G coated plates as described in the text. The position of cholesterol was ascertained in a separate experiment. Spots C, E and D show separation of oleic acid, coconut oil and the mixture of the two, respectively. A, B, F and G are profiles of lipids prior to autolysis and after 8, 18 and 24 hr of autolysis at 37° C, respectively.

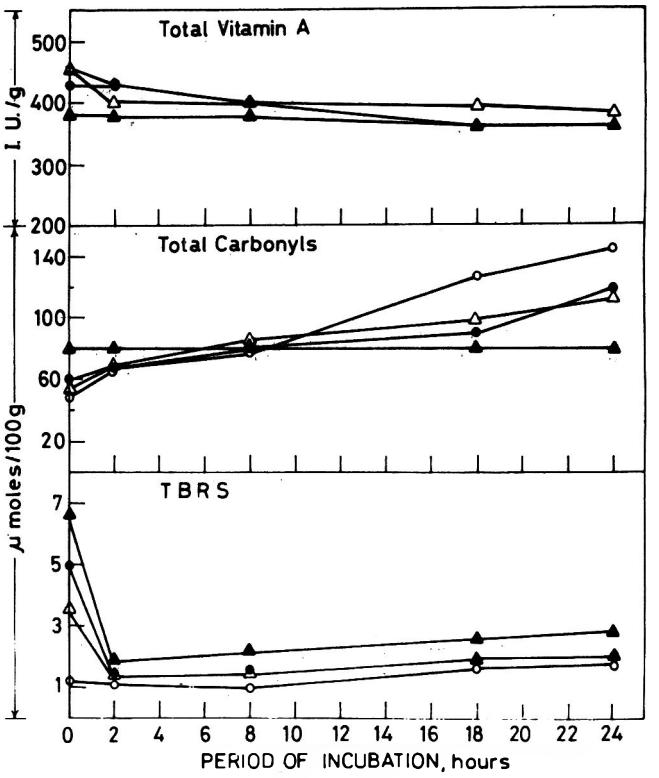


Fig. 3—Post-irradiation levels of vitamin A and the extent of lipid peroxidation in autolysing lamb liver. The accumulation of carbonyls and TBRS was monitored along with the retention of vitamin A in autolysing lamb liver as per the procedures described in the text. The autolysis was carried out at 37° C. Unirradiated (○ — ○); Irradiated, 0.5 Mrad (△ — △); 1.0 Mrad (● — ●) and 2.0 Mrad (▲ — ▲).

remained the least affected by gamma-radiation. The profile of total vitamin A, carbonyls, and TBRS during the progress of autolysis is illustrated in Figure 3. During the initial phase of autolysis the TBRS exhibited a drop and then a marginal increase; more in irradiated liver than in control. No significant loss of total vitamin A occurred during autolysis of both irradiated and control lamb liver. However, when the vitamin A content was computed in terms of alcohol/ester, an increase in the ratio was observed (Fig. 4). The change in ratio was primarily due to the conversion of retinyl esters to retinol during the autolysis of irradiated lamb liver (Fig. 4a).

DISCUSSION

THE FOREGOING RESULTS indicate rapid hydrolysis of vitamin A esters leading to the formation of retinol as a result of irradiation in lamb liver undergoing autolysis. In liver, while vitamin A in the form of palmitate derivative is stored in Kupffer cells, parenchymal cells contain minor amounts of vitamin A alcohol. The vitamin A esters could be hydrolyzed either by microsomal enzymes which are

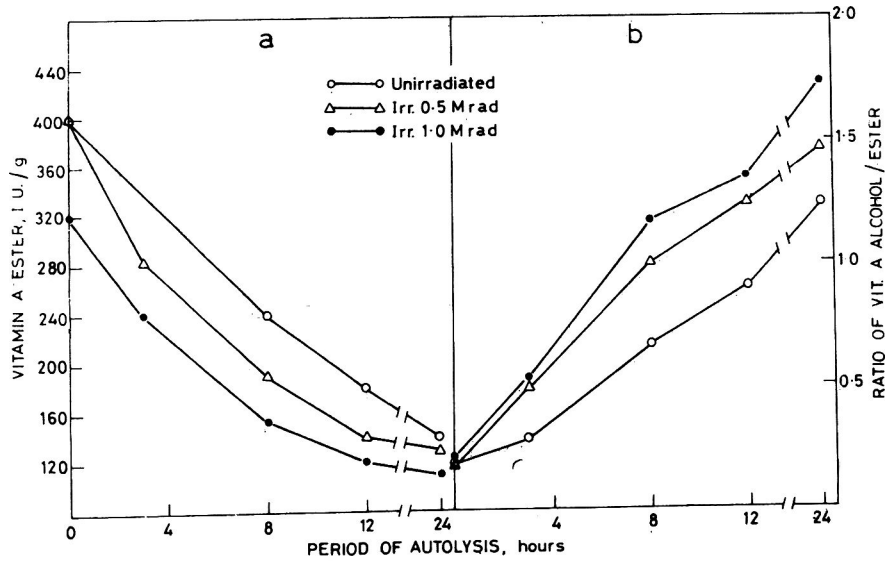


Fig. 4—Post-irradiation hydrolysis of vitamin A esters in autolysing lamb liver. The vitamin A extracted from the lamb liver slurry was resolved into esters (a) and alcohol forms of vitamin A by column chromatographic separation. The forms were quantitated colorimetrically and the ratio of alcohol to ester of vitamin A was computed (b). Unirradiated (○ — ○); Irradiated at 0.5 Mrad (△ — △) and 1.0 Mrad (● — ●).

specific to short chain fatty acid esters or those acting mainly on esters of long chain fatty acids (Mahadevan et al., 1966). The latter enzymes are reported to be associated with mitochondria-lysosome (ML) fraction (Mahadevan et al., 1966). Although the high pH requirement at 8.6 suggests nonlysosomal origin of these enzymes, their possible association with cell membrane cannot be ruled out (Olson, 1967). The increase in alcohol/ester ratio of vitamin A observed during autolysis of irradiated lamb liver in the present investigation could therefore be attributed to the action of enzymes associated with the membrane. In this context, it is pertinent to note the dose-dependent accumulation of TBRS and carbonyl compounds (Table 1). Since exposure to gamma-radiation and lipid peroxides are known to cause membrane damage (Wills and Wilkinson, 1966) an elevation in the enzymes involved in the conversion of esterified vitamin A to alcohol form could be expected.

Increases in the activities of lysosomal enzymes and accentuated rate of autolysis in irradiated lamb liver homogenate have already been reported (Ninjoor et al., 1973). Further, it has been shown that in vitro addition of polar radiation degradation products (RDP) promoted lysosomal labilization (Ninjoor et al, 1978; Bhushan et al., 1977) resulting in rapid autolysis (Ninjoor et al., 1973). In the present investigation, however, the formation of such compounds (RDP) in liver is unlikely since the complexity of the system seems to reduce the vitamin A destruction during irradiation (Fig. 1). The lipolytic activities, however, did not seem to be affected in irradiated lamb liver, since a fourfold increase in fatty acids and 50% loss of phospholipids after 24 hr autolysis was observed. This might suggest that the accumulation of fatty acids was due to hydrolysis of triglycerides rather than of phospholipids. A reduction in fatty acid level during the initial phase of autolysis in lamb liver irradiated at 2.0 Mrad could be ascribed to the decline in enzyme activities as a result of irradiation (Rhodes, 1969) or by interaction with lipid peroxides (Tappel, 1973).

A logarithmic relationship between the radiation dose and percent retention of vitamin A could be ascribed to the primary radiochemical reactions rather than to indirect action of lipid peroxides. The relative stability of vitamin A in irradiated lamb liver could perhaps be attributed to various physicochemical factors as discussed elsewhere (Bhushan and Kumta, 1977; Bhushan et al., 1978). In a recent study, Diehl (1979) has demonstrated that the loss in vitamin A in sausage immediately after irradiation (5.0 Mrad) was only to the extent of 22% which increased to 61% after 4 wk of storage. While propagation of peroxidative chain reactions could cause accumulation of TBRS and carbonly compounds (Wills and Wilkinson, 1966), the interaction of these with tissue constituents (Tappel, 1973) would lower their levels as observed in the early phase of autolysis.

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Survey of Rabbit Skeletal Muscle Peptidases Active at Neutral pH Regions

AKIHIRO OKITANI, YUZURU OTSUKA, RYOICHI KATAKAI, YASUHIRO KONDO, and HIROMICHI KATO

ABSTRACT

A survey of oligopeptide hydrolases active at neutral pH regions responsible for increased free amino acids on aging of high ultimate pH muscles were performed examining chromatographic behavior and substrate specificity of rabbit skeletal muscle extract. The DEAE-cellulose chromatography of muscle extract revealed five major activity peaks respectively ascribable to a dipeptidase, an aminotripeptidase, an aminopeptidase, leucine aminopeptidase and the 160,000 dalton-aminopeptidase purified previously by us. Since the 160,000 dalton-aminopeptidase showed a broader substrate specificity than the two other aminopeptidases, it is most likely that this enzyme shares the largest part in the tetrapeptide hydrolysis in rabbit skeletal muscle at neutral pH regions.

INTRODUCTION

IT IS WELL KNOWN that free amino acids increase due to proteolysis on storage of muscle. Such increase in amino acids is generally considered to associate with the improvement in flavor obtained by the aging of meat. These amino acids are derived from oligopeptides mainly by the action of exopeptidases. Whereas the known muscle exopeptidases, cathepsins A(catheptic carboxypeptidase A), B2-(catheptic carboxypeptidase B) and C(dipeptidylaminopeptidase I) act optimally at acidic pH regions (Caldwell and Grosjean, 1971), we observed that nonprotein ninhydrin positive materials increased more rapidly in a high pH muscle (DFD muscle) than in a low pH muscle (normal postmortem muscle) (Okitani et al., 1973). Subsequently, we have purified a neutral aminopeptidase of 160,000 daltons which might make some contribution to the rapid proteolysis in a high pH muscle (Otsuka et al., 1976).

Few works have been made so far on the survey of the total oligopeptide hydrolase activity at neutral pH regions in skeletal muscle (Bury and Pennington, 1975; Parsons and Pennington, 1976; Parsons et al., 1979). In those works amino acid or dipeptide derivatives including amides and 2-naphthylamides have been used as substrates, and those substrates are only applicable to detect aminopeptidases. The present work has thus been conducted to survey the total oligopeptide hydrolase activity of rabbit skeletal muscle at neutral pH regions using several oligopeptides including di-, tri- and tetrapeptides.

MATERIALS & METHODS

Muscles

The muscles (longissimus dorsi) of female rabbits weighing about 3 kg were obtained from the carcasses immediately after slaughter and minced with a meat chopper.

Chemicals

Gly-Pro-Gly, Pro-Ala-Ala, Val-Val-Val-Ala, Lys-Gly-Ile-Ala, Pro-Ala-Leu-Ala, Gly-Gly-Ile-Ala, and Phe-Gly-Ile-Ala were synthesized by the method reported by Katakai (1975). Val-Tyr-Val and Ala-Ala-Ala were obtained from Miles Seravac Ltd. Gly-Gly and Gly-Leu were from Tokyo Kasei Kogyo Co. Bz(Benzoyl)-Gly-Lys, Leu-Gly-Gly and Gly-Gly-Gly were from Peptide Institute, Osaka. Cbz(Carbobenzoxy)-Gly-Leu was from Seikagaku Kogyo, Tokyo. Leu-Gly and Lys-Gly were from Cyclo Chemical Inc., California. The peptides were all constituted of L-amino acids. DEAE-cellulose(DE-52) was purchased from Watman Co. All other chemicals were of guaranteed reagent grade.

Extraction and chromatography

All operations were carried out at 4°C. Minced muscle (15g) was homogenized with 45 ml of 40 mM Tris-HCl buffer (pH 7.2) in a Waring Blendor for 1 min. The homogenate was centrifuged for 20 min at 20,000 x G. The supernatant was dialyzed overnight against 10 mM Tris-HCl buffer (pH 7.2) containing 4 mM 2-mercaptoethanol and 1 mM NaN_3 (Buffer A). The dialysate was centrifuged at 20,000 x G for 20 min and the supernatant was pooled as a muscle extract. A portion (20 ml) of the muscle extract was applied to a column (1.9 x 27 cm) of DE-52 previously equilibrated with Buffer A. After unadsorbed proteins (breakthrough proteins) were removed completely from the column by flushing with Buffer A, elution with a concentration gradient of NaCl in Buffer A was started. Fractions (10 ml) were collected and every fraction was assayed for hydrolysis of various peptides.

Enzyme activities

One-fifth ml of each fraction eluted from the column was incubated at 37°C for 3-120 min with 0.2 ml of 0.3 mM peptide in 10 mM Tris-HCl (pH 7.2) containing 1 mM NaN_3. Fractions of the breakthrough peak whose optical density at 280 nm exceeded 2 (Fractions 4-6 in Fig. 1) and the muscle extract were incubated after dilution with 9 and 19 volumes of Buffer A, respectively. Incubation time was chosen according to the readiness of each substrate to be hydrolyzed. On the basis of the data of the muscle extract indicated in Table 1, the longest incubation time was employed for the substrates hard to be cleaved. The incubation was stopped by adding 0.4 ml of the ninhydrin reagent prepared by the method of Cocking and Yemm (1954). After 0.2 ml of 0.2M citrate buffer (pH 5.0) was added to the reaction mixture, the whole solution was boiled at 100°C for 15 min and then cooled in running water. The optical density was measured at 570 nm. The unincubated enzyme solution of every fraction was mixed with ninhydrin reagent prior to substrates and used as control. The activity was expressed as μg of tyro-

Table 1—Hydrolysis of peptidase substrates by the extract of rabbit skeletal muscle

Substrate	Activity[a] (μg tyr equiv/g muscle/min)
Cbz-Gly-Leu	4.3 ± 4.0
Bz-Gly-Lys	3.2 ± 1.7
Leu-Gly	36.6 ± 16.2
Lys-Gly	55.1 ± 5.9
Leu-Gly-Gly	1093 ± 52
Pro-Ala-Ala	89.9 ± 24.5
Val-Tyr-Val	203 ± 48
Val-Val-Val-Ala	131 ± 43
Pro-Ala-Leu-Ala	179 ± 26
Phe-Gly-Ile-Ala	203 ± 25
Lys-Gly-Ile-Ala	68.6 ± 9.0
Gly-Gly-Ile-Ala	20.2 ± 8.1

[a]Given as means ± S.D. obtained from four different rabbits

Authors Okitani, Otsuka, Kondo, and Kato are with the Dept. of Agricultural Chemistry, Faculty of Agriculture, Univ. of Tokyo, Tokyo 113, Japan. Author Katakai is with the Dept. of Industrial Chemistry, College of Technology, Univ. of Gunma, Kiryu 376, Japan.

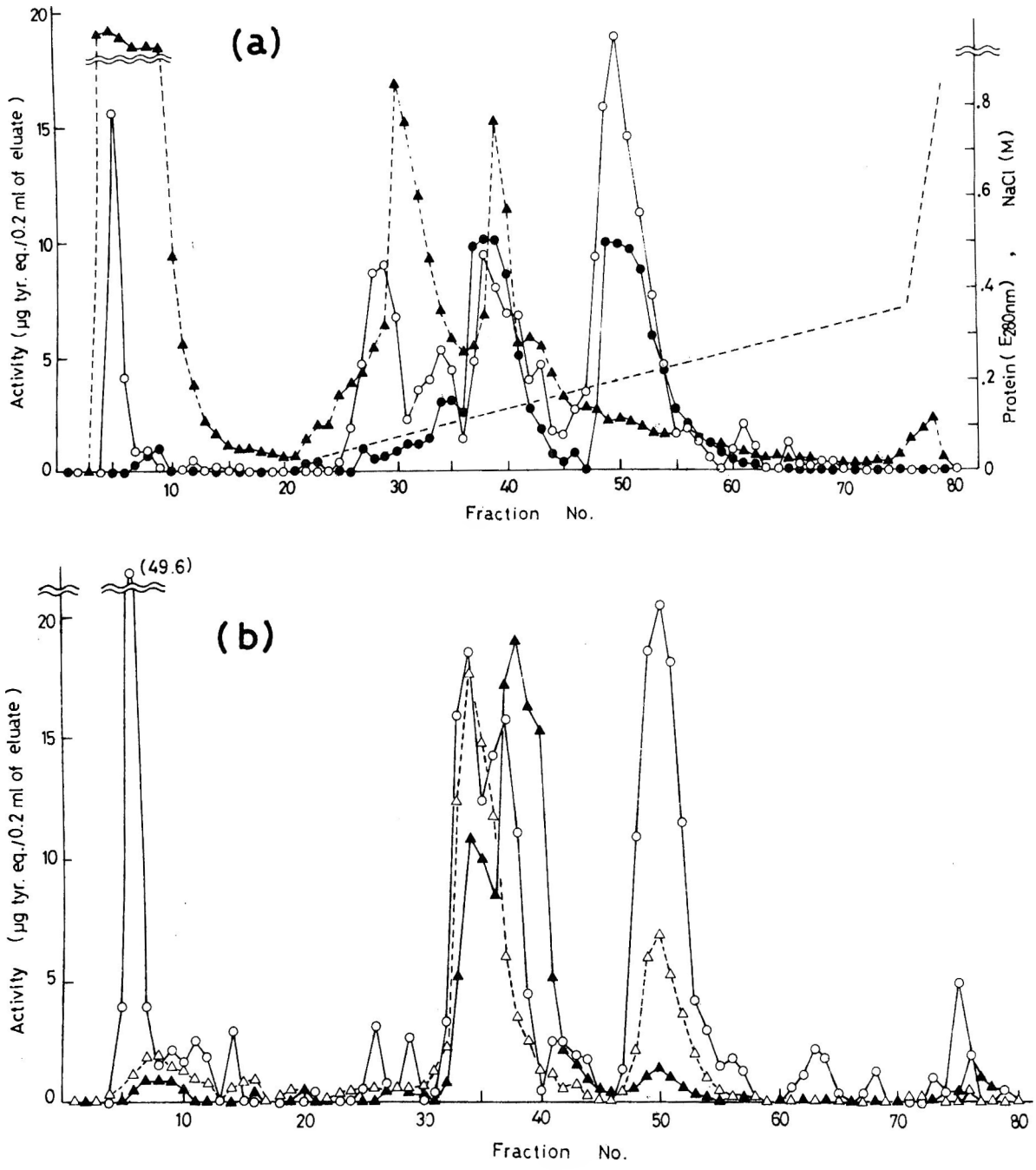


Fig. 1—Hydrolysis of peptidase substrates by fractions from DEAE-cellulose chromatography of rabbit muscle extract. The numbers in parentheses are the activities in the most active fractions: (a) ▲, Protein; - - - - NaCl; ●, Lys-Gly (incubation time, 1 hr); ○, Leu-Gly (2 hr). (b) △, Leu-Gly-Gly (3 min); ▲, Pro-Ala-Ala (40 min); ○, Val-Tyr-Val (40 min). (c) △, Val-Val-Val-Ala (1 hr); ●, Lys-Gly-Ile-Ala (1 hr); ○, Gly-Gly-Ile-Ala (2 hr). (d) ○, Phe-Gly-Ile-Ala (30 min); ●, Pro-Ala-Leu-Ala (40 min).

sine equivalent. The activity of the muscle extract against each substrate was determined by triplicate assays for each preparation, while the activity of each fraction obtained by chromatography was measured by a single assay.

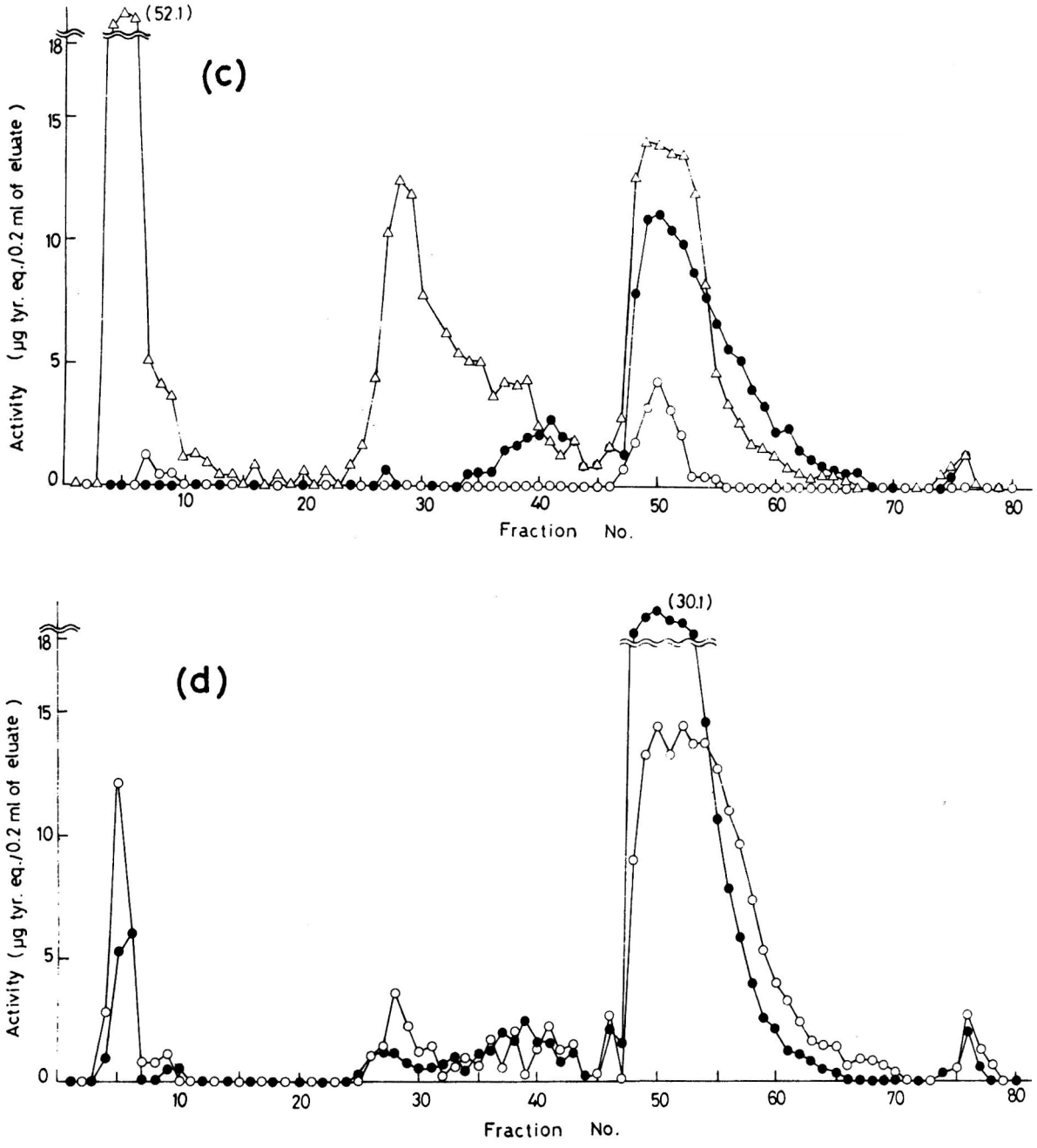
Protein determination

Absorbance at 280 nm was used to monitor the protein peaks on the chromatograph. The protein concentration of the muscle extract was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

RESULTS

Extraction of enzyme

About 40 ml of muscle extract with a protein concentration of 10-14 mg/ml was obtained from 15g of muscle. The extract possessed hydrolytic activities towards various substrates, as shown in Table 1. Although some values showed noticeable variances associating with the sample rabbits, a clear trend was observed as described below. The activities against the dipeptides with the blocked N-terminal amino acids were lower, as compared with those against several oligopeptides with the unblocked N-terminal residues. Tripeptides were hydrolyzed more readily than dipeptides, and the activity against Leu-Gly-Gly was extremely high. The activities against several tetrapeptides were comparable to those against tripeptides except Leu-Gly-Gly. However, the tetrapeptide with N-terminal glycine was



hardly hydrolyzed. These results indicate that tri- or tetrapeptide hydrolysis by the muscle extract is ascribable to the action of aminopeptidases rather than that of carboxypeptidases under the condition used here.

When the muscle extract prepared with an extracting solution containing 0.3% Triton X-100 was subjected to DEAE-cellulose column chromatography, the elution pattern of peptidase activities was similar to that obtained from the extract prepared without Triton; no new activity peaks appeared. Thus, the results on the latter extract were presented below.

Chromatography of muscle extract

Chromatography of muscle extract on a DEAE-cellulose column gave the results shown in Figure 1. These are the results obtained from one of six rabbits. The other five rabbits showed almost the same chromatographic patterns of activities as in Figure 1. Figure 1a indicated that about 75% of the loaded protein was eluted as an unadsorbed breakthrough peak. Almost all the adsorbed protein was eluted until NaCl concentration reached 0.2M. The activities of every fraction eluted from the column against four dipeptides, three tripeptides and five tetrapeptides were measured.

Dipeptide hydrolyzing activities. The activities against Cbz-Gly-Leu and Bz-Gly-Lys were extremely low in all the fractions and no distinct activity peaks were observed (not shown in Figure). Figure 1a illustrates that the activity against Leu-Gly appeared in five separate peaks eluting at 0, 0.06, 0.1, 0.12, and 0.2M NaCl. The last peak was largest among them. Lys-Gly hydrolyzing activity showed three peaks at 0.1, 0.12, and 0.2M NaCl, the latter two being major.

Tripeptide hydrolyzing activities. The activity against Leu-Gly-Gly was recognized in two peaks: the larger one at 0.1M NaCl and the smaller one at 0.2M NaCl (Fig. 1b). Two major peaks for Pro-Ala-Ala hydrolysis were eluted at 0.1 and 0.12M Nacl. Val-Tyr-Val hydrolyzing activity consisted of four peaks eluting at 0, 01., 0.12, and 0.2 M NaCl.

—Continued on next page

Tetrapeptide hydrolyzing activities. As shown in Figure 1c, three major peaks of the activity against Val-Val-Val-Ala appeared at 0, 0.06, and 0.2M NaCl. A sizable level of the activity was also observed between 0.07 and 0.13M NaCl, forming no distinct peaks. The greater part of the activity against Lys-Gly-Ile-Ala was eluted in a peak at 0.2M NaCl. Most of the activity against Gly-Gly-Ile-Ala was also eluted at 0.2M NaCl, while its peak was small. Both activities against Pro-Ala-Leu-Ala and Phe-Gly-Ile-Ala were eluted mostly in a peak at 0.2M NaCl and partly in a peak at 0M NaCl (Fig. 1d).

Assignment of the activity peaks

All the results obtained above indicated that the chromatography of muscle extract gave five distinguishable peaks of oligopeptide hydrolyzing activity eluted at 0, 0.06, 0.1, 0.12, and 0.2M NaCl. Assignment of the main enzymes responsible in those five peaks was conducted on the basis of the substrate specificity and some other properties, as mentioned below. The fact that the activity of Cbz-Gly-Leu and Bz-Gly-Lys hydrolysis was low or undetectable in those peaks ruled out the possibility that those peaks were ascribable to carboxypeptidases.

Activity peak eluted at 0M NaCl. Since the activity was sizable towards Leu-Gly, Val-Tyr-Val, and Val-Val-Val-Ala, an aminopeptidase seems to be more or less responsible for it. However, it is unclear whether or not all the activity is due to a single enzyme, because many cathionic proteins might coexist in this breakthrough protein peak.

Activity peak eluted at 0.06M NaCl. The activity was appreciable towards a dipeptide and two tetrapeptides with hydrophobic N-terminal amino acids, i.e. Leu-Gly, Val-Val-Val-Ala, and Phe-Gly-Ile-Ala. The activity against Leu-Gly was enhanced about four times with 1 mM $MgCl_2$, while any other peaks of Leu-Gly hydrolyzing activity were not affected. These results indicate that most of the activity eluted at 0.06M NaCl is attributable to leucine aminopeptidase.

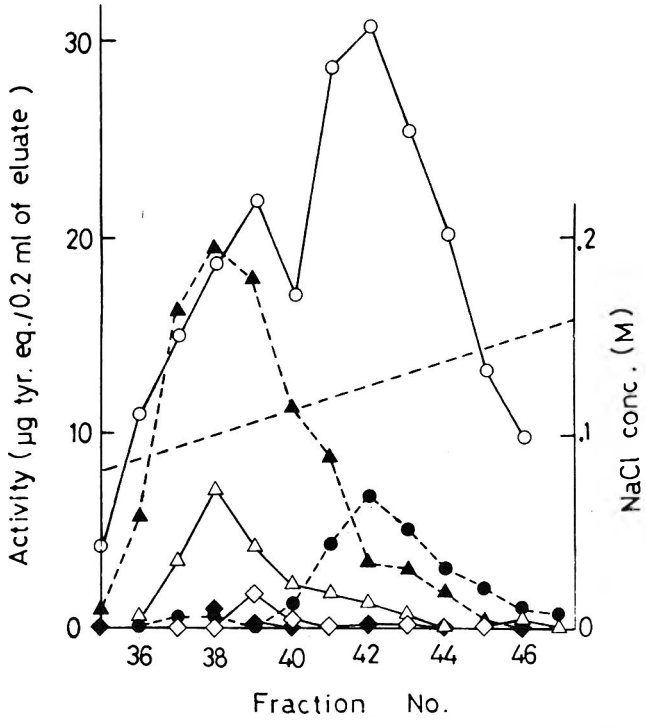


Fig. 2—Hydrolysis of di- and tri-peptides by fractions from DEAE-cellulose chromatography of rabbit muscle extract.
○, Ala-Ala-Ala (incubation time, 1.5 hr);
▲, Leu-Gly-Gly (3 min); △, Gly-Gly-Gly (10 min);
◇, Gly-Pro-Gly (30 min); ◆, Gly-Gly (1 hr)
●, Gly-Leu (1 hr); ---- , NaCl.

Activity peaks eluted at 0.1 and 0.12 M NaCl. Leu-Gly, Lys-Gly, Pro-Ala-Ala, and Val-Tyr-Val were split by the both peaks. No tetrapeptides appeared to serve as substrates for these two peaks. The activity against Leu-Gly-Gly was eluted at 0.1M NaCl but not at 0.12M NaCl. These results suggested that these two activity peaks were ascribable to dipeptidase and/or aminotripeptidase. Therefore, some other di- and tripeptides were applied to these peaks (Fig. 2). Activity against Gly-Gly-Gly was observed at 0.1M NaCl but not at 0.12M NaCl, while activity against Gly-Leu was observed at 0.12M NaCl and not at 0.1M NaCl. No activity was detected at 0.1 and 0.12 NaCl towards Gly-Pro-Gly. This peptide has been demonstrated not to be cleaved by aminotripeptidase of swine kidney (Chenoweth et al., 1973). Activity against Gly-Gly was found neither at 0.1M nor 0.12M NaCl. On the basis of all these results it is most reasonable to conceive that the major enzyme eluted at 0.1M NaCl is an aminotripeptidase and the major one at 0.12M NaCl is a dipeptidase. This conception is not incompatible with the fact that a distinct activity peak towards the tripeptides except Leu-Gly-Gly and Gly-Gly-Gly was observed around 0.12M NaCl where a dipeptidase is predominant. The result on Gly-Gly-Gly hydrolysis indicates that the aminotripeptidase was eluted at 0.1M NaCl with some tailings of the activity around 0.12M NaCl (Fig. 2). Thus, the eluates around 0.12M NaCl contain the aminotripeptidase as a contaminant, which cleaves some tripeptides producing suitable substrates for the dipeptidase predominant in those eluates. When Leu-Gly-Gly or Gly-Gly-Gly was applied to those eluates, no activity peaks were observed at 0.12M NaCl. This might be because Gly-Gly liberated from those peptides is not split by the dipeptidase predominant at 0.12M NaCl.

A small but distinct peak of activity against Leu-Gly detected at 0.1M NaCl suggested that the occurrence of another dipeptidase with the substrate specificity different from that of the enzyme eluted at 0.12M NaCl.

Activity peak eluted at 0.2M NaCl. The eluates around 0.2M NaCl showed high activity against all the substrates with unblocked N-terminal amino acids except Pro-Ala-Ala and Gly-Gly -Ile-Ala. The results implied that the responsible enzyme was an aminopeptidase. Our previous work has shown that a neutral aminopeptidase of 160,000 daltons in rabbit skeletal muscle is eluted at 0.2 NaCl under the same chromatographic condition as employed in the present work (Otsuka et al., 1976). Furthermore, our other experiment has revealed that the 160,000-dalton aminopeptidase accounts for all the activities towards Val-Val-Val-Ala eluted at 0.2M NaCl on the DEAE-cellulose chromatography of the rabbit muscle extract (Otsuka et al., 1980). Therefore, the hydrolyzing activities against a variety of peptides eluted at 0.2M NaCl are mostly attributable to a single enzyme, ie., the 160,000-dalton aminopeptidase, since this enzyme has been shown to possess a broad substrate specificity (Otsuka et al., 1976). The fact that the activity peak against Gly-Gly-Ile-Ala was low at 0.2M NaCl is in good accordance with our finding that the 160,000-dalton aminopeptidase hardly cleaves N-terminal glycine (Otsuka et al., 1976).

DISCUSSION

THE PRESENT WORK demonstrates that the oligopeptide hydrolysis by muscle extract at neutral pH regions is ascribable to at least five major enzymes: a dipeptidase (eluted at 0.12M NaCl), an aminotripeptidase (0.1M), an aminopeptidase (0M), leucine aminopeptidase (0.06M) and 160,000 dalton-aminopeptidase (0.2M) eluted from a DEAE-cellulose column at pH 7.2.

The dipeptidases detected in muscle include glycylglycine dipeptidase (Smith, 1948a), glycylleucine dipeptidase (Smith, 1948b), aminoacylhistidine dipeptidase (Wood, 1957), prolinase and prolidase (Smith, 1948c), although none of them has been purified. Since the dipeptidase eluted at 0.12M NaCl is active towards Gly-Leu, Leu-Gly, and Lys-Gly but not Gly-Gly, it seems to have a broad specificity. However, it is uncertain whether or not this enzyme corresponds to one of the known dipeptidases described above.

Aminotripeptidase has been also detected in muscle but not yet isolated (Smith, 1948c). Chenoweth et al. (1973) have purified swine kidney aminotripeptidase and shown that the enzyme hydrolyzes a variety of tripeptides but not Gly-Pro-Gly. Such properties seem common to the muscle aminotripeptidase eluted at 0.1M NaCl.

The major peptidases cleaving tetrapeptides were elucidated to be an aminopeptidase, leucine aminopeptidase, and 160,000 dalton-aminopeptidase. The latter enzyme has a broader substrate specificity than the former two enzymes. These results suggest that 160,000 dalton-aminopeptidase shares the largest part in oligopeptide hydrolysis in muscle at neutral pH regions.

The observation that no additional peaks of peptidase activity appeared on the chromatography of the muscle extract by the addition of Triton X-100 into the extractant seems to indicate that membrane- or particulate-bound neutral peptidases do not occur in skeletal muscle. Parsons and Pennington (1976) have reported that neutral arylamidases of rat skeletal muscle are likely to be present largely or wholly in the cytoplasm. They also have shown that a large part of the arylamidase activity is eluted beyond 0.2M NaCl on the DEAE-Sephadex chromatograph. Our other work has indicated that such arylamidase activity is mostly attributable to the 160,000 dalton-aminopeptidase (Otsuka et al., 1980).

As limited conditions, e.g. media and substrates, were employed for activity assay, the present results are not enough to preclude the possibility that muscle contains other major tetrapeptide hydrolyzing enzymes active at neutral pH regions than the ones assigned here. It is possible for the same reason to conclude that muscle contains small amounts of neutral carboxypeptidases, although the low activity was observed towards Cbz-Gly-Leu and Bz-Gly-Lys. Gly-Gly-Ile-Ala was only one substrate hard to be hydrolyzed among all the peptides with unblocked N-terminal amino acids, suggesting the occurrence of an undetected peptidase cleaving N-terminal glycine. Therefore, a further survey study of neutral oligopeptide-hydrolases deserves to be continued.

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High Performance Liquid Chromatographic Determination of Organic Acids in Dairy Products

R.T. MARSILI, H. OSTAPENKO, R.E. SIMMONS, and D.E. GREEN

ABSTRACT

A simple isocratic HPLC technique was developed for the quantitative analysis of organic acids in dairy products. An Aminex HPX-87 column at 65°C, 0.0090N H_2SO_4 mobile phase and UV detection at 220 and 275 nm were utilized. Orotic, citric, pyruvic, lactic, uric, formic, acetic, propionic, butyric, and hippuric acids were quantitated for whole milk, skim powder, cultured buttermilk, sour cream, cottage cheese, yogurt, sharp Cheddar cheese, and blue cheese. Over 90% recoveries of acids added to whole milk were observed for all acids except butyric; the average recovery for butyric was 86%.

INTRODUCTION

ORGANIC ACIDS occur in dairy products as a result of the hydrolysis of butterfat (fatty acids), direct addition as acidulants (e.g., citric, lactic, and propionic acids), normal bovine biochemical metabolic processes (e.g., citric, hippuric, uric, orotic, and ascorbic acids), or bacterial growth (e.g., pyruvic, lactic, acetic, and propionic acids). Quantitative determinations of these acids in dairy products is important to flavor studies, for nutritional reasons, and as an indicator of bacterial activity.

Marshall and Harmon (1978) and Zanstra and deVries (1977), utilizing colorimetric analysis, suggested measurement of the pyruvate content in milk as an index of bacteriological quality. Using gas chromatography, Gray (1976) chromatographed lactic acid in whey after chemical derivitization to the butyl esters. Various wet chemistry analytical techniques for acids in dairy products have been reported for citric acid (AOAC, 1975), lactic acid (Davidson, 1948), hippuric acid (Patton, 1953), uric acid (Shahani and Sommer, 1951), and orotic acid (Larson and Hegarty, 1977).

Several workers have reported HPLC procedures for organic acids in foods. Palmer and List (1973) used anion exchange chromatography, a special precolumn injector technique, and a differential refractometer detector to quantitate 15 commonly occurring food acids in fruit juices and acidulated beverages. Turkelson and Richards (1978) presented an excellent summary of gas and liquid chromatographic techniques currently used to analyze the citric acid cycle acids. In addition, they have demonstrated a simple liquid chromatographic procedure to measure organic acids in foods (and urine) based on ion exclusion and partition chromatography, effluent monitoring by UV detection, and the use of dilute mineral acid as the mobile phase.

Considering the simplicity and speed of analysis inherent in HPLC, an investigation of various organic acids in dairy products was carried out using a modification of the method of Turkelson and Richards (1978). Typical analytical results are reported for orotic, citric, pyruvic, lactic, uric, formic, acetic, propionic, butyric, and hippuric acids in whole milk, cultured buttermilk, sour cream, cottage cheese, yogurt, sharp Cheddar cheese, and blue cheese. Finally, a comparative analysis of an acceptable quality skim powder sample and an inferior quality skim powder sample is presented as a practical application.

All authors are affiliated with Dean Foods Co., Rockford, IL 61101.

EXPERIMENTAL

Apparatus and operating conditions

A Varian Model 5020 Liquid Chromatograph was used. The chromatograph was equipped with a manual 10 ul loop injector, a Vari-chrom variable wavelength ultraviolet/visible detector and an insulated column oven. Column effluents were monitored at detector wavelengths of 220 and 275 nm and a slit width of 8 nm. Quantitation was based on peak height measurments using a Varian Model 9176 recorder. Analyses were performed isocratically at a flow rate of 0.7 ml/min and a temperature of 65°C.

Column

A 300 mm x 7.8 mm i.d. HPLC Organic Acid Analysis Column (Bio-Rad Laboratories, Richmond, CA) was used. The column contains Aminex HPX-87, a strong cation exchange resin (8% crosslinked and 9 μm diameter) which separates organic acids by ion exclusion and partition chromatography.

Reagents

The mobile phase used 0.0090N H_2SO_4 prepared by diluting reagent grade sulfuric acid with distilled water. Analytical grade organic acids were used as standards; HPLC grade acetonitrile (Waters Associates, Milford, Mass.) was used in sample preparation as the protein denaturant/solvent.

Calibration and calculations

Four aqueous organic acid calibration standards covering a broad concentration range were prepared from stock solutions. These standards contained orotic, citric, pyruvic, lactic, uric, acetic, propionic, butyric, and hippuric acids (see Fig. 1 for exact concentrations); duplicate 10 μl injections were made at 220 nm. The resulting peak heights were determined and plotted against concentration. The slopes of these lines are the response factors for each acid; the linear correlation coefficients and y-intercepts provide a measure of analytical precision. Quantitation was based on the external standard method.

Formic and uric acids exactly coelute (RT = 12.4 min) under the chromatographic conditions listed above. Chromatography at 50°C resolved the formic and uric acid peaks but resulted in unacceptable resolution of earlier eluting peaks.

Analyzing samples at two different wavelengths permitted quantitation of formic and uric acids despite their coelution. Three aqueous standards were prepared: 10 ppm uric acid, 500 ppm formic acid, and 10 ppm uric + 500 ppm formic acid. Each standard was chromatographed in duplicate at 65°C at both 220 and 275 nm. Therefore, the following calculations were applied to determine the peak heights (ph's) of both acids at 220 nm (std = aqueous standard with uric + formic acids and unk = dairy sample of unknown organic acid composition):

$$(\text{ph uric in unk})_{220} = \frac{(\text{ph uric in std})_{220}}{(\text{ph uric in std})_{275}} \times (\text{ph uric in unk})_{275}$$

$$(\text{ph formic in unk})_{220} = (\text{total ph in unk})_{220} - (\text{ph uric in unk})_{220}$$

Confirmation of analytical results for formic acid in dairy samples was made by rechromatographing at 50°C when the above technique indicated the presence of formic acid.

Sample preparation

Whole milk, cultured buttermilk, sour cream, cottage cheese, yogurt (plain), sharp Cheddar cheese and blue cheese were purchased and analyzed. The same procedure of sample preparation was followed for each dairy product (except buttermilk): 5.00g of sample, 5.0 ml distilled water, and 20 ml acetonitrile were added to a 50 ml glass centrifuge tube, shaken for 1 min, centrifuged at 7000 × G for 5 min. The supernatant was injected into the 10 µl loop with a 5 ml syringe fitted with a Swinney syringe filter holder (Gelman Sciences, Inc., Ann Arbor, Mich.) containing 0.2 µm Teflon membrane filters. The same procedure of sample preparation was used for buttermilk except 10.0g of sample and no distilled water was used.

In addition to the dairy products mentioned above, two skim powders were analyzed. Twenty percent (w/w) aqueous dilutions were made, and the samples were prepared as for buttermilk.

Complete duplicate analyses were performed on all samples to enable calculation of average deviations which were useful as a measure of the extraction and chromatographic reproducibility.

Recovery study

The recovery of acids added to whole milk was evaluated to determine the accuracy of the test. Whole milk was analyzed in duplicate before and after the addition of known amounts of acids. Two different concentration levels of spiked acids were analyzed; complete duplicate analyses were performed.

RESULTS AND DISCUSSION

Standard chromatograms

Initially aqueous standards of individual acids were chromatographed separately to determine the retention times of each acid. Mixtures of aqueous acids were then chromatographed and chromatographic conditions were altered to maximize resolution of peaks. The effects of changing pH of the eluent and temperature on the resolution of organic acids in wine for Aminex HPX-87 resin have been previously reported (Bio-Rad, 1979). Changes in chromatographic conditions were made based on this work. Initial chromatography was performed at ambient temperatures with 0.0045N H_2SO_4 mobile phase. Under these conditions, citric and pyruvic acids coelute and the retention time of hippuric acid is unreasonably long (over 60 min). Increasing the mobile phase concentration to 0.0090N H_2SO_4 at ambient column temperatures, resulted in coelution of orotic and citric acids but shortened the retention time of hippuric acid. Using 0.0090N H_2SO_4 mobile phase and increasing the column temperature to 65°C resolved orotic, citric, and pyruvic acids, permitted a greater flow rate, and further reduced the retention time of hippuric. The choice of 0.0090N H_2SO_4 acid mobile phase and 65°C, provided acceptable resolution for the organic acids found in dairy products.

An aqueous standard chromatogram containing a mixture of organic acids is illustrated in Figure 2. The smaller unresolved peaks eluting immediately before lactic acid are probably lactic acid isomers. They occurred in four different analytical grade lactic acid reagents considered for use in the preparation of the standard solutions.

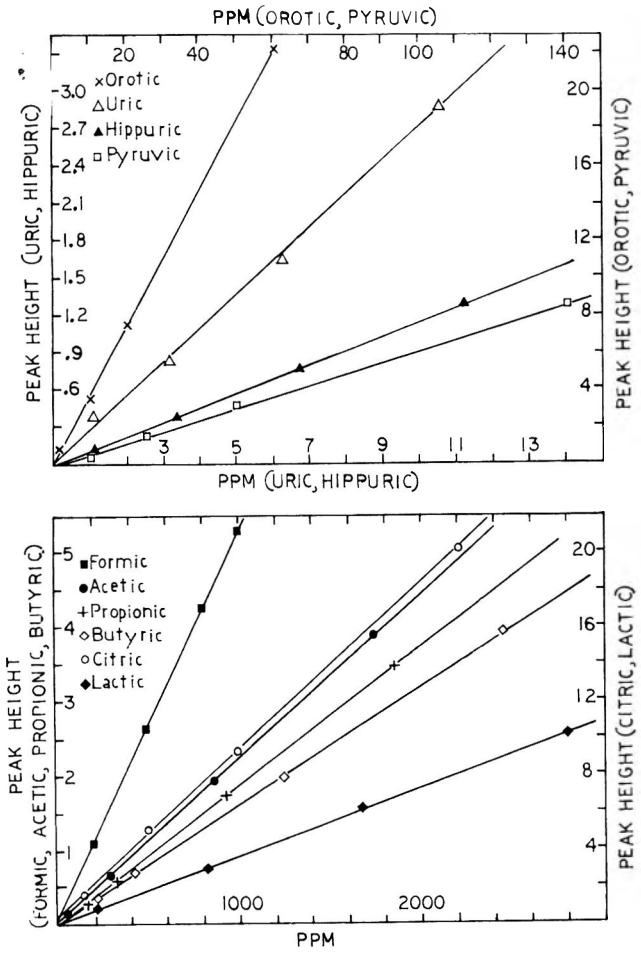


Fig. 1—Calibration curves for aqueous standards using the following chromatographic conditions: 10 µl injected, 0.0090N H_2SO_4 mobile phase, 65°C, UV detection at 220 nm, 0.7 ml/min flow rate.

Standard calibration curves

The calibration curve data are presented in Table 1 and clearly indicate excellent linear responses over wide concentration ranges of acids. In addition, capacity factors (k') and sensitivities (the concentration of acid which produces a peak height signal corresponding to twice the detector noise) were calculated and tabulated for the exact chromatographic conditions previously mentioned.

The actual calibration curves for each acid are illustrated in Figure 1. Duplicate determinations were made on each of the calibration standards; the peak height values that were plotted are average values. The relative percent average deviations of duplicates were less than 2% in all cases.

Typical analytical results and chromatograms for dairy products

Typical results and average deviations of duplicate determinations for a variety of commercial dairy products are

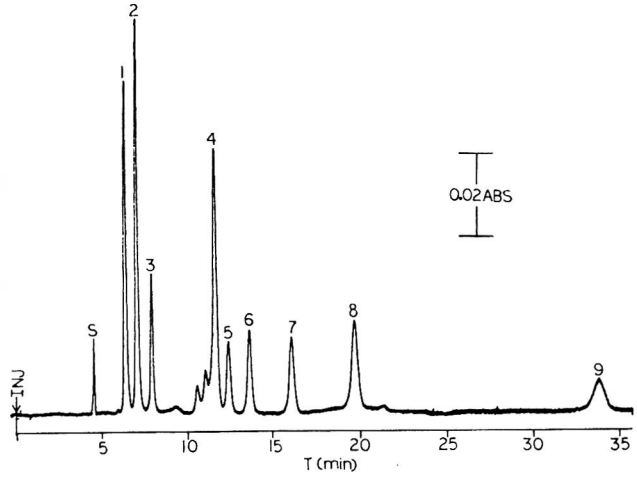


Fig. 2—Chromatogram of an aqueous standard solution containing the following acids: (1) 20.4 ppm orotic, (2) 1000 ppm citric, (3) 50 ppm pyruvic, (4) 1680 ppm lactic acid, (5) 6.3 ppm uric, (6) 880 ppm acetic, (7) 925 ppm propionic, (8) 1230 ppm butyric, (9) 6.7 ppm hippuric. Chromatographic conditions same as Fig. 1.

presented in Table 2. Results reported for orotic acid agree closely with results reported by Larson and Hegarty (1979) using colorimetric techniques.

Typical chromatograms for whole milk, sour cream, yogurt (plain), sharp Cheddar cheese, and blue cheese are illustrated in Figure 3. The initial solvent peaks for all chromatograms occur between 4 and 6 min and result from water, phosphates, and other unretained components. The negative peak occurring in all sample chromatograms at approximately 18 min is due to the acetonitrile denaturant/solvent. Several unidentified peaks occur in the chromatograms especially for the sharp Cheddar cheese and the blue cheese samples. In all samples except the sharp Cheddar and the blue cheeses, the uric acid peak (eluting at approximately 12.4 min) had an unidentified shoulder peak, barely detectable, eluting after the uric acid peak maxima. This shoulder peak disappeared when samples were rechromatographed at 275 nm; it is not formic acid. The coelution problem of uric and formic acids encountered at 65°C was overcome by chromatographing at two different detector wavelengths and, when necessary, rechromatographing at 50°C as previously described. Simply decreasing the column temperature to 50°C while maintaining other chromatographic conditions constant, resulted in excellent resolution (R = 1.5). Resolution of lactic, formic, uric, and acetic acids was acceptable at 50C. The k′ values for lactic, formic, uric, and acetic acids were 1.553, 1.745, 1.936, and 2.043, respectively.

The technique of chromatographing at two detector wavelengths was advantageous. It was used to qualitatively identify orotic and uric acids in sample chromatograms. Orotic and uric acids absorb UV radiation at both 220 and 275 nm but more strongly at 275 nm. A standard solution containing both acids was chromatographed at both wavelengths. The peak height ratios for the two wavelengths were calculated for each acid. Likewise, peak height ratios were calculated for peaks with the appropriate retention times in the sample chromatograms and compared to the ratios calculated for the standard. Agreement confirmed the identities of the peaks.

Uncharacteristic UV-spectroscopic behavior was exhibited by the peaks believed to be propionic acid in the sharp Cheddar and blue cheeses. The propionic acid peak was observed at 16.2 min at 220 nm but gave no detector response at 275 nm when standard solutions were injected. This was observed for all samples reported to contain propionic acid with the exception of the Cheddar and blue cheeses. For these samples, the characteristic propionic acid peak at 16.2 min was observed at 220 nm but did not disappear when rechromatographed at 275 nm. The detector responses for the 16.2 min peaks were approximately the same intensity at both wavelengths. This suggests that the peaks were not propionic acid, or, at least, that they were not solely propionic acid in the cheeses.

No fatty acids with molecular weights higher than propionic were observed in sample chromatograms. Undoubtedly this is due to the poor sensitivity of the detector to these acids by UV detection. As indicated in Table 1, orotic, uric, hippuric, and pyruvic acids are easily quantitated at low concentrations. A sample preparation procedure using 10.00g of sample and 20 ml acetonitrile in conjunction with 100 µl loop injections has been used to increase sensitivities in Table 1 by a factor of 20. No column overloading was observed. This technique could be used for low level detection of the acids with poor detector sensitivities including lactic, acetic, propionic, and butyric.

Table 1—Capacity factors (k′), sensitivities, and calibration curve data for aqueous calibration standards[a]

Acid	k′[b]	Sensitivity[c] (µg/g)	Calibration curve data: Response factor, slope (µg/g/ph)	Calibration curve data: y-intercept (µg/g)	Calibration curve data: Corr. coef.
Orotic	0.41	0.1	2.78	−0.40	0.9999
Citric	0.56	4.0	110	1.62	0.9999
Pyruvic	0.74	0.6	15.9	−0.21	0.9999
Lactic	1.54	10.	273.	8.03	0.9999
Uric	1.70	0.1	3.70	0.01	0.9985
Formic	1.70	6.6	185	2.51	0.9991
Acetic	1.97	17.	453.	7.06	0.9999
Propionic	2.50	19.	538.	−0.36	0.9999
Butyric	3.27	22.	621.	−0.16	0.9999
Hippuric	6.34	0.3	8.67	0.32	0.9998

[a] Data apply to the following chromatographic conditions; 10 µl injections, 0.0090N H_2SO_4 mobile phase, T = 65°C, Detector-UV @ 220 nm, flow rate — 0.7 ml/min.
[b] k′ = $(t_r - tm)/tm$ where t_r = retention time of the acid peak and tm = retention time of an unretained component.
[c] Sensitivity = 2N.

Recovery study

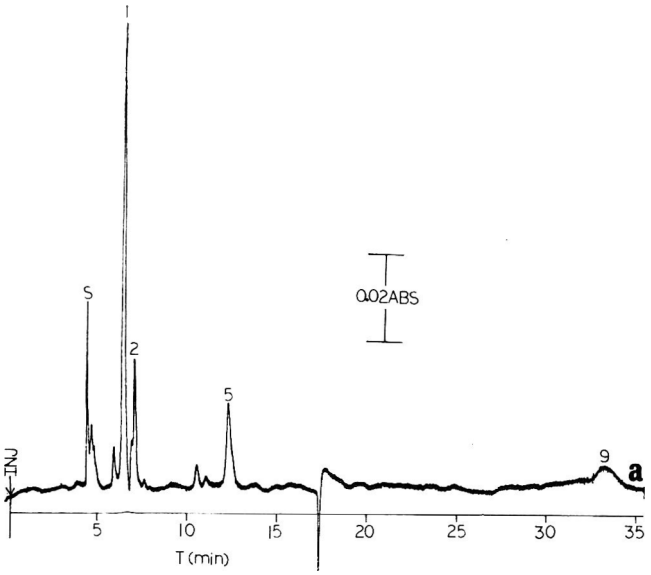
The recoveries of organic acids added to whole milk were over 90% for all acids except butyric. The average percent recovery for butyric was 85.6%. Recovery data are presented in Table 3.

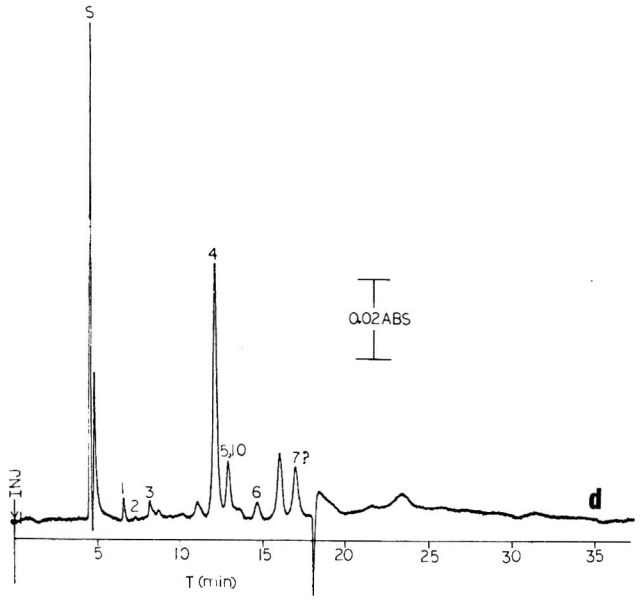
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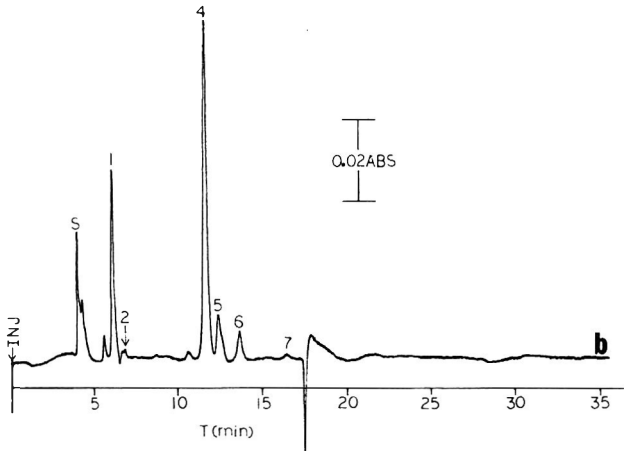
Table 2—Organic acid composition of typical commercial dairy products

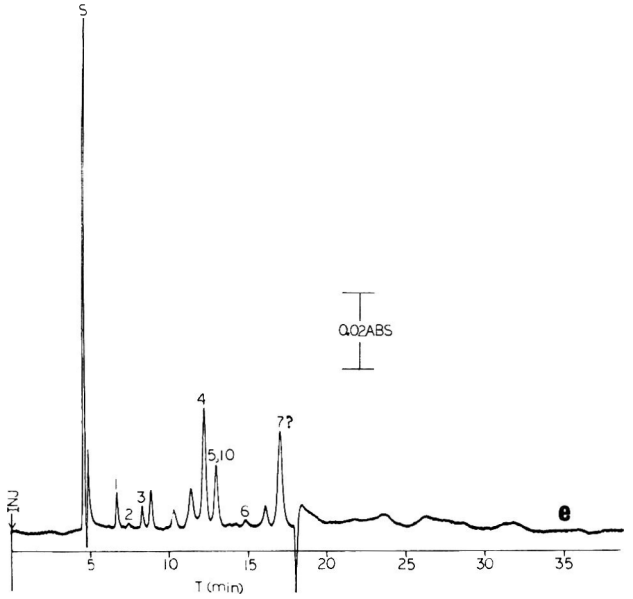
Dairy product	Concentration, µg/g[a]: Orotic	Citric	Pyruvic	Lactic	Uric	Formic	Acetic	Propionic	Hippuric
Whole milk	83.6±1.0	940±40	<4	<60	21.8±0.2	<40	<100	<120	15.4±0.9
Buttermilk (Cultured)	39.3±1.2	60±1	<2	5890±10	13.9±0.6	110±10	850±10	<60	<1
Sour cream	48.6±2.4	120±0	<4	8410±160	18.8±1.1	<40	900±30	180±20	<2
Cottage cheese	39.8±0.5	430±10	<4	650±10	11.1±0.2	<40	<100	<120	7.5±0.4
Yogurt (plain)	72.5±2.5	710±30	24±0	14550±150	31.8±0.0	<40	120±20	<120	<2
Sharp Cheddar cheese	4.9±0,2	25±2	26±2	5140±40	16.7±0.1	420±40	600±10	?[b]	<2
Blue cheese	7.3±0.1	40±2	27±1	3080±10	20.8±0.4	420±20	250±20	?[b]	<2

[a] Uncertainties represent average deviations of complete duplicate determinations.
[b] See text for explanation.









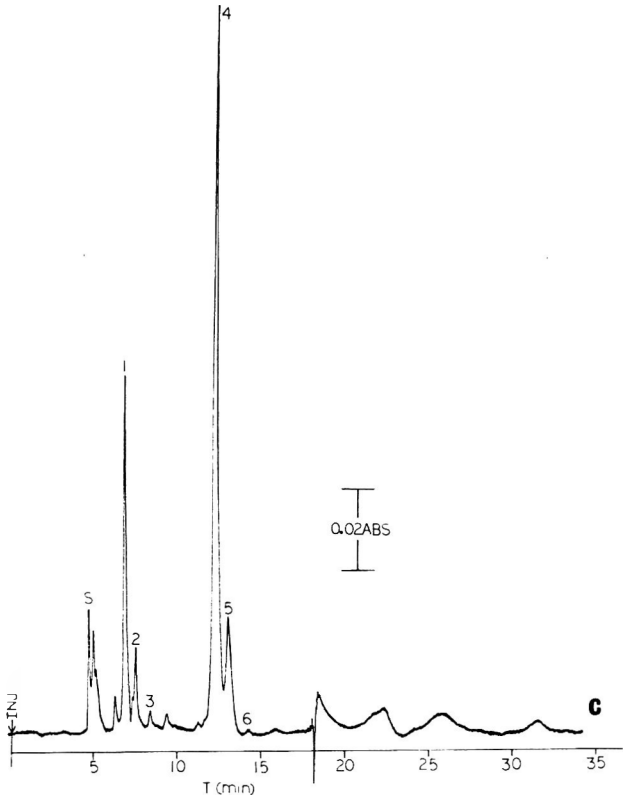


Fig. 3—Typical chromatograms of dairy products (same chromatographic conditions as Figure 1): (a) homogenized whole milk, (b) sour cream, (c) plain yogurt, (d) sharp Cheddar cheese, (e) blue cheese. The numbers correspond to the numbers in Fig. 2 with respect to peak identification.

Table 3—Recovery study of organic acids added to homogenized milk

Acid	Amount in milk mg/L	Trial I Added mg	Trial I Total Calc mg	Trial I Total Found mg	Trial I % Recovery	Trial II Added mg	Trial II Total Calc mg	Trial II Total Found mg	Trial II % Recovery
Orotic	81.8±0.5	20.4	102.2	100.8±1.9	98.6	61.3	141.3	132.6±2.5	92.7
Citric	1003±27	990.2	1993	1973±22	99.0	2971	3974	3905±50	98.3
Pyruvic	<4	47.1	47.1	45.2±0.8	96.0	141.4	141.4	135.8±1.4	96.0
Lactic	<60	1623	1623	1595±7	98.3	4869	4869	4772±35	98.0
Uric	22.3±0.4	21.3	43.6	40.9±1.2	93.8	64.0	86.3	82.2±0.9	95.2
Formic	<40	666.0	666.0	659.0±20	98.9	1998	1998	1994±47	97.3
Acetic	<100	1855	1855	1773±19	95.6	5565	5565	5448±62	97.9
Propionic	<120	1957	1957	1893±27	96.7	5871	5871	5572±16	94.9
Butyric	<140	1112	1112	964.1±35	86.7	3336	3336	2816±45	84.4
Hippuric	14.5±0.6	20.9	35.4	32.0±0.3	90.4	62.8	77.3	76.9±0.1	99.5

Skim powders; an applications example

The ash alkalinity of a low heat, Grade A skim powder and a similar control sample tested 170 and 39 ml 1N HCl/100g of powder, respectively. The unusually high ash alkalinity suggested the possibility that the sample had spoiled before drying and that the acidic bacterial metabolites had been neutralized. Titratable acidities, solubility index results, and microbiological testing indicated no significant differences in the two samples. As indicated by the comparison of Figures 4a and 4b and by the analytical data in Table 4, excessive fermentation had definitely occurred. This is just one example of how HPLC analysis can be used as a problem-solving tool.

Ascorbic acid

Low level quantitation of ascorbic acid in dairy products appears promising by this technique. Ten microliter injections of a 1 ppm aqueous solution of ascorbic acid were detectable using a detector wavelength of 220 nm, a column temperature of 25°C, and 0.0090N H_2SO_4 eluent (RT was approximately 10.4 min). Prior treatment with H_2S was necessary before ascorbic acid could be detected in milk. The usual metaphosphoric acid/acetic acid extracting solution was used instead of acetonitrile to precipitate proteins. Chromatography of ascorbic acid could not be conducted at elevated column temperatures because of chemical degradation. Approximately 88% of the ascorbic acid was lost

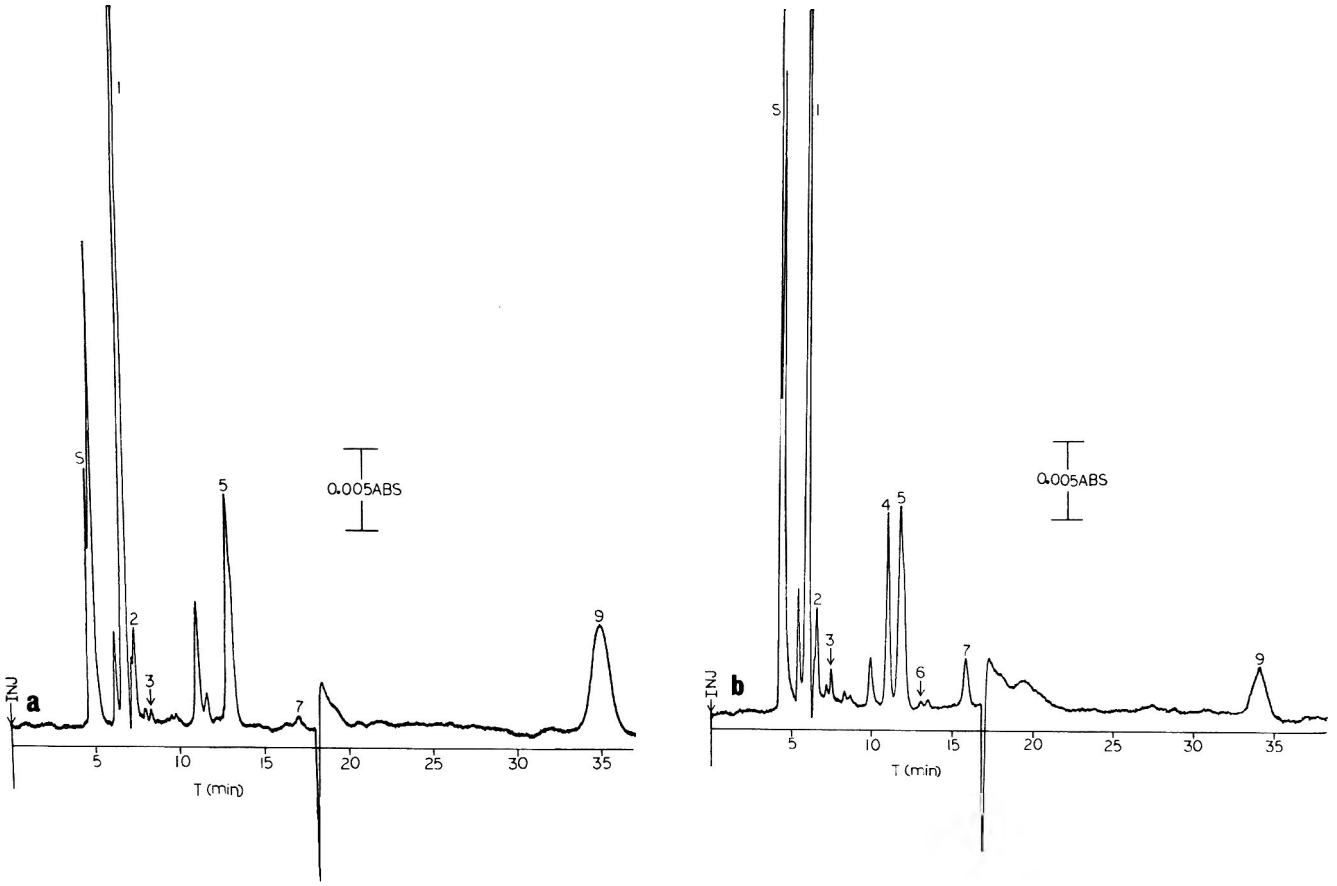


Fig. 4—Chromatograms of normal (control) skim powder (a) and of an inferior quality skim powder (b). Chromatographic conditions same as Fig. 1. The numbers correspond to the numbers in Fig. 2 with respect to peak identification.

Table 4—Comparative organic acid profiles for control and inferior quality skim powder

Acid	μg/g	
	Control sample	"Bad" sample
Orotic	479	457
Citric	1630	1220
Pyruvic	25.0	44.8
Lactic	<150	4320
Uric	103	75.3
Formic	<100	<100
Acetic	<200	305
Propionic	497	2010
Butyric	<300	<300
Hippuric	85.2	42.7

at column temperatures of 45°C while as much as 96% was lost at 65°C.

CONCLUSION

The use of two different UV wavelengths for detection was important in optimizing analyte responses and in discriminating against interferences. In addition, varying column temperature and the pH of the mobile phase were utilized to optimize resolution.

The Bio-Rad HPX-87 column has been used to analyze dairy samples for 9 months on a regular basis. No deterioration of separation capabilities has been noticed. Earlier attempts at chromatographing aqueous standard solutions of lactic and citric acid were unsuccessful when anion exchange Micro Pak AX-10 columns (Varian Associates) were tried. Chronic, irreversible clogging of the columns occurred after only a few injections (despite the use of precolumns and filtration of the mobile phase). Furthermore, extreme nonlinear responses for citric acid peaks were observed. Analyses were performed at ambient temperatures employing aqueous solutions of KH_2PO_4 (0.015-0.50M) as the mobile phase. Three different Micropak AX-10 columns were evaluated.

One potentially useful application of the HPLC procedure is to supply support data to microbiological studies by quantitating bacterial metabolites. Production of pyruvic, lactic, formic, acetic, and propionic acids is easily monitored by this HPLC technique. In addition, several workers have reported orotic acid to be readily utilized by various bacteria used in the fermentation of dairy products (Larson and Hegarty, 1979; Chen and Larson, 1971; Empie and Melachouris, 1977). This observation was confirmed by results of orotic acid analyses presented here for the various cultured products. Bacterial activity reduced the concentrations of hippuric acid and, in some cases, citric acid.

This modified Turkelson and Richards' HPLC procedure is well suited to the analysis of organic acids in dairy products based on the following considerations: (a) a total analysis time of less than 1 hr per sample is required; (b) less than 0.01% of each organic acid can be detected; (c) linear calibration curves for aqueous standards were observed over a broad concentration range; (d) the relative standard deviation for all results in Table 2 calculated by pooling variances was ± 4.75%; (e) over 90% recoveries of organic acids added to milk were observed for every acid except butyric.

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Glandless Cottonseed, Peanut and Soy Protein Ingredients in Ground Beef Patties: Effect on Rancidity and Other Quality Factors

Y. A. ZIPRIN, K. S. RHEE, Z. L. CARPENTER, R. L. HOSTETLER, R. N. TERRELL, and K. C. RHEE

ABSTRACT

Defatted flours and protein concentrates and isolates produced from glandless cottonseed, peanut, and soybean were used in rehydrated state (1:2 dry material/water) to replace 10% of the meat in ground beef patties. Fat content of both all-beef and extended patties was adjusted to 20%. Patties were baked on racks to varying final internal temperatures. Extended patties were compared with all-beef patties for rancidity development, cooking properties, and sensory quality. All the oilseed protein ingredients retarded oxidative rancidity development in cooked refrigerated patties, with the highest antioxidant potential shown by the cottonseed protein ingredients. Extended patties had higher cooked yields than all-beef patties, contained less fat, and were not significantly different from all-beef patties in sensory quality.

INTRODUCTION

THE VALUE of oilseed proteins, especially soy proteins, as extenders in comminuted meat products is well recognized. Several studies have been reported on cooking properties and eating quality of ground beef patties extended with a limited number of oilseed protein ingredients (Anderson and Lind, 1975; Bowers and Engler, 1975; Drake et al., 1975; McWatters, 1977; Molonon and Bowers, 1975; Smith et al., 1976). However, most of these studies, with a few exceptions, included only textured soy protein. Different types of protein ingredients, i.e., flour, concentrate, isolate, and textured protein, prepared from different oilseeds have never been compared in one study for their effect on various quality aspects, including oxidative rancidity development, of meat products.

No studies exist reporting the use of glandless cottonseed and peanut protein ingredients to prolong the keeping quality of meat products. Antioxidant activity of soy protein products has been reviewed recently by Hayes et al. (1977). Pratt (1972) found aqueous extracts of soy flour and concentrate to inhibit rancidity development in cooked beef slices when used as cover solutions. Recently, Rhee et al. (1979) reported on antioxidant activity of aqueous extracts of glandless cottonseed, peanut and soy protein ingredients in model lipid oxidation systems where the oxidation of linoleate was catalyzed by metmyoglobin, Fe^{++}–EDTA (1:1), and fresh beef homogenates. Antioxidative effectiveness of the aqueous extracts was shown to vary depending on the kind of oilseed, type of protein preparation, and catalytic system used. Antioxidant effect of soy protein ingredients has been reported in raw ground beef patties (Kotula et al., 1976; Thompson et al., 1978), cooked beef loaves (Sato et al., 1973) and vegetable-beef soup (Sanger and Pratt, 1974).

The purpose of the present study was to determine antioxidant effect of various oilseed protein ingredients by comparing the extent of oxidative rancidity development in all-beef patties and in patties extended with oilseed protein ingredients. In addition, cooking properties and sensory quality of the patties were evaluated. Oilseed protein ingredients included were defatted flours and protein concentrates and isolates prepared from glandless cottonseed, peanut and soybean, and a textured soy flour.

Authors K.S. Rhee, Carpenter, Hostetler, and Terrell are with the Dept. of Animal Science, Texas Agricultural Experiment Station, Texas A&M Univ., College Station, TX 77843. Author K.C. Rhee is with the Food Protein Research & Development Center, Texas Engineering Experiment Station, Texas A&M Univ. Author Ziprin is at 3515 Greenridge Circle, Bryan, TX 77801.

EXPERIMENTAL

Oilseed proteins

Glandless cottonseed (McNair variety) and peanut (Spanish variety) protein ingredients were produced on pilot-plant scale at the Food Protein Research & Development Center, Texas A&M University. Defatted cottonseed flour was prepared by hexane extraction and the isolate (a classical isolate with both storage and nonstorage protein included; Martinez et al., 1970) by alkali extraction of defatted flour followed by isoelectric protein precipitation. Peanuts were prepressed to remove a bulk of oil before extraction with hexane for defatted flour; the isolate was prepared by alkali extraction of defatted flour and isoelectric protein precipitation. Defatted soy flour (Soyafluff 200W), soy concentrate (Promosoy 100), soy isolate (Cenpro M), and textured soy flour (Centex 200) were obtained from Central Soya. All these oilseed protein ingredients had been tested previously for water-soluble antioxidant activity in model lipid oxidation systems (Rhee et al., 1979) and stored at −18°C until used in the present study.

Beef patties

Lean beef chuck and U.S. Choice grade beef plates were ground separately through a 1/2 inch plate of a Hobart meat grinder and sub-sampled for fat analysis. The modified Babcock method (Salwin et al., 1955) was used to determine the percentage of fat. Based on the results of the fat analysis, appropriate amounts of the ground chuck and the ground beef plates were mixed to yield two batches of ground beef with expected fat contents of 20 and 22%. The ground beef with 20% fat was to be used for all-beef patties while that with 22% fat was for patties extended with 10% rehydrated oilseed protein ingredients. This would result in 20% fat content for both all-beef and extended patties. (Since fat contents of 15-30% have been used in most studies dealing with ground beef patties extended with nonmeat protein ingredients, the 20% fat content was chosen arbitrarily.) Then, each batch of ground beef was reground separately through a 3/16-inch grinder plate, subsampled for fat and adjusted with either the lean or fat, if necessary, followed by grinding a second time.

To incorporate oilseed protein ingredients into the ground beef, a measured amount of an oilseed ingredient and the hydration water (1:2 oilseed protein/distilled water), together equivalent to 10% replacement of the meat, were added alternately in small aliquots to the ground beef while mixing lightly by hand. (The reason for not rehydrating the dry ingredients prior to mixing with the meat was that it was not possible to completely recover the rehydrated paste of certain oilseed protein ingredients from the container used for rehydration and also was difficult to adequately mix the rehydrated paste with the meat.) Final mixing was done in a Kitchen-Aid mixer with dough hook for 2-3 min. At this point, the meat appeared to have been mixed thoroughly with oilseed ingredient.

Patties weighing 115g (9.2 cm diameter, 1.7 cm thickness) were prepared from the all-beef and beef-oilseed protein batches, both containing 20% fat. Patties were baked on oven racks in preheated ovens at 177°C to various final internal temperatures depending on the purpose of the experiments. Copper-constantan thermo-

couples connected to a recording potentiometer were used to monitor the temperature of patties during cooking.

Oxidative rancidity

In order to evaluate antioxidative effectiveness of the oilseed protein ingredients, patties were prepared from fresh raw ground beef and raw ground beef stored 3 days at 4°C. Patties were cooked to final internal temperatures of 70°, 74° or 77°C. The extent of oxidative rancidity development was determined by the thiobarbituric acid (TBA) test of cooked patties immediately after cooking and after 3 and 6 days of storage at 4°C. The distillation TBA method of Tarladgis et al. (1960) was used with the addition of 5 ml of a 0.5% solution of propyl gallate and EDTA for each 10-g sample in the blending process (Rhee, 1978).

To minimize variations due to sampling the following steps were taken for the TBA test: (1) two cooked patties for each product were crumbled and ground through a 3/16-inch meat grinder plate and mixed thoroughly; (2) 60g of the ground meat sample were blended with 90 ml of distilled water and 30 ml of the propyl gallate-EDTA solution; and (3) 30g of the slurry (= 10g meat sample) were taken for each distillation. The duplicated distillation results of ground beef slurries prepared in this manner showed very little variations between the duplicates (Rhee, 1978). Each meat sample slurry was, therefore, distilled in one replicate in the present study but the TBA color development was carried out in duplicate with each distillate. Data presented in Table 1 are the means of the duplicate color determinations.

Cooking properties and sensory quality

Patties were preapred from freshly ground beef and cooked to an internal temperature of 74°C. Cooking properties were determined in two replications (two separate experiments). Cooking yield was determined by weight difference between the raw and cooked patties, with at least eight patties per product within each replication. Fat and moisture contents of the raw and cooked patties were determined by the AOAC method (1970). Two patties per product were ground through a 3/16-inch grinder plate and mixed thoroughly before sampling for fat and moisture analyses. An incomplete block design (Plan 11.4a, page 476, Cochran and Cox, 1957) was used for sensory evaluation. Eleven experienced panel members, who had routinely served as sensory judges for a variety of meat products including ground beef patties, evaluated the cooked patties for flavor, juiciness, texture, and overall satisfaction in a total of five sessions held on three consecutive days–one session each in the mid-morning and mid-afternoon for the first and second days and one mid-morning session for the third day. Each panelist was given one-half patty each of a reference sample (all-beef patty, but labeled "R") and two coded samples, and asked to determine whether each sample was more desirable than, equal to, or less desirable than "R" and to mark the amount of difference (none; slight; moderate; much; extreme). All-beef patties were also included as one of the eleven groups of coded samples.

For statistical analysis of the sensory data, numerical scores were assigned to the ratings with no difference from "R" equalling 5, extremely more desirable than "R" equalling 9, and extremely less desirable than "R" equalling 1. Data were evaluated by analysis of variance, and the Student-Newman-Keuls' test (Steel and Torrie, 1969) was used for mean separation when the treatment differences were significant ($p < 0.05$).

RESULTS & DISCUSSION

Antioxidant effect

All the oilseed protein ingredients effectively prevented rancidity development in cooked patties stored at 4°C for 3 and 6 days, when patties had been prepared from freshly ground beef (Table 1). This effect was observed with patties cooked to 74°C as well as with those cooked to 77°C. All-beef patties progressively increased in TBA value during storage whereas extended patties changed only slightly.

A few studies have been reported on the antioxidative effect of textured soy protein in raw ground beef patties. Thompson et al. (1978) observed antioxidant effect of textured soy protein in refrigerated storage of raw ground beef patties of the same fat content. Kotula et al. (1976) conducted a 12-month frozen storage study of raw beef patties with the same fat level and found TBA values to increase at a faster rate for all-beef patties than for patties containing textured soy flour or soy concentrate. No systematic controlled studies have been reported relative to antioxidative nature of any oilseed protein ingredient in cooked ground beef patties. Although Bowers and Engler (1975) found lower TBA values in beef-soy (textured) patties than in all-beef patties upon cooking or reheating the precooked patties, their patty formulations were such that fat content was lower in beef-soy patties even before cooking.

When patties had been prepared from raw ground beef stored 3 days at 4°C in the present study, TBA values after cooking of all-beef patties were higher than those of extended patties even on "Day 0" (immediately after cooking), regardless of the cooking endpoint temperature

Table 1–TBA values[a] of all-beef and extended beef patties cooked to different final internal temperatures and stored at 4°C

	Patties prepared from freshly ground beef						Patties prepared from ground beef stored at 4°C for 3 days					
	74°C			77°C			70°C			74°C		
Product	0	3 (Days)	6	0	3 (Days)	6	0	3 (Days)	6	0	3 (Days)	6
All-beef	1.43	2.32	3.00	1.66	1.83	2.53	4.14	5.83	7.63	4.15	7.60	10.04
Beef + defatted flour												
Cottonseed, glandless	1.48	1.44	1.44	1.46	1.41	1.64	2.50	2.56	2.50	2.99	2.28	2.41
Peanut	1.51	2.15	2.06	2.17	2.17	1.68	3.51	4.05	4.91	3.15	2.74	4.48
Soy	1.48	1.49	1.46	1.65	1.58	1.76	3.90	4.14	4.61	3.74	4.56	5.64
Soy-textured	1.57	1.58	1.59	1.66	1.60	1.71	3.09	4.02	3.42	2.45	2.61	3.24
Beef + concentrate												
Cottonseed, glandless	1.41	1.42	1.36	1.49	1.39	1.67	2.47	2.42	2.46	2.66	2.49	2.16
Peanut	1.66	1.41	1.41	1.66	1.54	1.69	2.57	2.55	2.47	2.66	2.74	4.40
Soy	1.50	1.66	1.58	1.74	1.74	1.87	3.39	3.64	4.08	2.49	2.08	3.03
Beef + isolate												
Cottonseed, glandless	1.49	1.44	1.36	1.62	1.45	1.68	2.57	2.52	2.56	2.82	2.08	2.41
Peanut	1.66	1.41	1.41	1.72	1.66	1.79	3.20	3.56	5.12	3.32	3.65	4.73
Soy	1.91	2.08	2.01	2.08	2.02	2.42	3.22	3.32	3.59	3.30	4.44	5.02

[a] mg Malonaldehyde/kg sample.

(Table 1). In addition, distinctive differences in antioxidant effect were shown among the oilseed protein ingredients. All the cottonseed products were superior to other oilseed protein products in antioxidative effectiveness (cf., Rhee et al., 1981); cottonseed flour, concentrate, and isolate were all equally effective for complete inhibition of oxidative rancidity development in patties cooked to either 70° or 77°C. Varying degrees of antioxidative effectiveness were shown by different types of protein preparations from peanut and soybean.

An obvious difference was found between the results of the present study on beef patties and those of our previous study (Rhee et al., 1979) on antioxidant activity of aqueous extracts of the same oilseed protein ingredients; whereas none of the three oilseeds was uniquely superior to others in regard to water-soluble antioxidant activity in model lipid oxidation systems, cottonseed had a greater antioxidant potential than peanut and soybean in cooked beef patties. Therefore, a parallel study was conducted to determine the antioxidant activity of methanolic extracts of these oilseed protein ingredients and the results are published as a separate paper (Rhee et al., 1981).

Yield

All the extended beef patties had significantly higher yields than all-beef patties (Table 2). Oilseed type, with the exception of concentrates, had little effect on the yield of extended patties. Among the patties extended with concentrates, cooking yield was lowest for those containing peanut concentrate, higher for patties containing soy concentrate, and highest for patties containing cottonseed concentrate.

Several studies of beef patties extended with soy or peanut protein ingredients also showed a beneficial effect for yield by the oilseed proteins (Anderson and Lind, 1975; Ayres and Davenport, 1977; Bowers and Engler, 1975; Drake et al., 1975; McWatters, 1977; Smith et al., 1976). With patties cooked from the frozen state, however, Thomas et al. (1978) found a higher yield for all-beef patties than for beef-soy (textured) patties.

A few studies have dealt with cooking yield of meat products extended with cottonseed protein ingredients. Molonon and Bowers (1976) reported that cooking losses of beef patties were unaffected by textured cottonseed flour (not tested in the present study), whereas Lawhon et al. (1972) found lower cooking losses with meat loaves containing cottonseed protein concentrate as compared with all-meat loaves. The difference in findings between the two studies may be attributable to the type of cottonseed protein ingredient (textured flour vs. concentrate), kind of meat product (beef patty vs. meat loaf), method of cooking (baking on a wire rack vs. in a baking dish), or all these factors combined.

Fat and moisture

The results of fat and moisture analysis are shown in Table 2. Raw patties, all-beef as well as extended patties, had about 20% fat (ether-soluble) as intended and 60% moisture. The percentage of fat in cooked patties was significantly lower for extended patties than for all-beef patties. Among extended patties neither the kind of oilseed nor the type of protein preparation had a significant effect on the amount of fat in cooked patties.

No general agreement has been found in the literature in regard to the effect of oilseed protein ingredients (mostly soy products) on the fat content of cooked beef patties. It is likely that differences in the patty formulation method, size and thickness of patties, cooking method, degree of doneness, etc. may have caused the differences.

Drake et al. (1975) found no significant differences in the amount of fat lost during cooking when beef patties containing 0, 15, 20 and 25% of rehydrated textured soy protein and 15, 20, 25, and 30% fat were grilled in an electric frypan. They also found that the amount of textured soy protein incorporated had no effect on fat losses. The amount of fat lost during grilling depended upon the level of fat originally incorporated into the raw ground beef patties before cooking regardless of the level of textured soy protein.

On the contrary, Anderson and Lind (1975) reported that, regardless of fat levels of raw patties, ground beef patties extended with 25% rehydrated textured soy protein retained a lesser percentage of fat in cooking than did all-beef patties of comparable fat levels. This was so whether the patties were cooked on the grill or broiled in the oven. Thomas et al. (1978) also showed a lower fat content in beef-soy (textured) patties than in all-beef patties. Bowers and Engler (1975) investigated beef patties containing 15 and 30% rehydrated textured soy protein and found extended patties to have lower fat content than all-beef patties after cooking; however, the initial fat content before cooking was lower for extended patties than for all-beef patties in their study. Similar results were reported for beef patties extended with textured cottonseed flour (Molonon and Bowers, 1976), but again, all groups of raw patties were not of the same fat level.

McWatters (1977), conversely, reported a slightly higher but variable fat retention for beef patties extended with 5, 10, and 15% peanut, soy, or pea meals than for all-beef patties. Since the meals were hydrated only in 1:1 ratio of meal/water, the affinity for fat could have been greater with the extended patties than the patties without meal.

As shown in Table 2, moisture content of extended patties was generally comparable to that of all-beef patties in the present study, except the patties containing cottonseed concentrate and isolate which were slightly lower in moisture than all-beef patties. Since extended patties had significantly higher cooking yields and lower fat contents after cooking in comparison with all-beef patties, higher moisture contents were anticipated of cooked extended patties but were not observed. Anderson and Lind (1975)

Table 2—Cooking yield and fat and moisture content of raw and cooked beef patties[a]

Product	% Yield	% Fat[b]		% Moisture	
		Raw	Cooked	Raw	Cooked
All-beef	70.1e	20.2a	22.5a	61.2a	55.8a,b,c
Beef + defatted flour					
Cottonseed, glandless	72.6c	21.7a	19.9b	61.0a	55.1c,d
Peanut	72.2c,d	18.8a	19.8b	61.0a	55.6b,c
Soy	73.1b,c	21.1a	20.4b	59.9a	56.3a,b
Soy-textured	73.2b,c	20.9a	20.3b	60.6a	55.8a,b,c
Beef + concentrate					
Cottonseed, glandless	74.4a	20.1a	20.6b	60.5a	54.6d
Peanut	71.4d	20.9a	20.0b	61.4a	56.0a,b,c
Soy	73.5b	20.4a	19.6b	59.7a	55.7b,c
Beef + isolate					
Cottonseed, glandless	71.6d	20.4a	20.8b	61.8a	54.5d
Peanut	72.2c,d	19.5a	20.2b	60.8a	55.2c,d
Soy	71.2d	20.0a	17.7c	60.9a	56.7a

[a] Mean values of duplicate determinations of two replications. Means in a column which are not followed by the same letter are significantly different ($P < 0.05$).
[b] Wet basis.

and Drake et al. (1975), however, reported a greater moisture retention in beef-soy (textured; 1:2 hydrated) patties as compared with all-beef patties.

Sensory quality

At 10% substitution of rehydrated oilseed proteins, or in the presence of 3.3% dry oilseed ingredients, the flavor, juiciness, texture and overall satisfaction scores of extended patties were not significantly different ($p < 0.05$) from those of all-beef patties (Table 3). No significant differences were found among the various extended patties in all the sensory attributes evaluated with the exception of texture; the texture scores were significantly higher for the patties containing textured soy flour in comparison with those containing defatted cottonseed flour. These findings suggest that, at 10% level of substitution, glandless cottonseed, peanut and soy protein ingredients can be used in beef patties without significantly affecting their sensory quality.

Drake et al. (1975), Kotula (1976), and Smith et al. (1976) reported higher ratings for all-beef patties in certain sensory attributes than for beef-soy patties. However, the levels of substitution tested in their studies were higher than the 10% used in the present experiments. According to McWatters (1977), flavor, texture, and aroma of beef patties extended with peanut, soy, and pea meals were influenced to a greater degree by meal concentration rather than type of meal. Textured cottonseed flour was reported to affect the texture of beef patties at 30% level of substitution but not at 15% (Molonon and Bowers, 1976).

Table 3—Adjusted mean sensory scores for all-beef and extended beef patties[a]

Product	Flavor	Juiciness	Texture	Overall satisfaction
All-beef	4.4a	4.9a	5.3a,b	4.7a
Beef + defatted flour				
Cottonseed, glandless	3.9a	4.8a	3.0b	3.6a
Peanut	2.8a	5.0a	4.8a,b	3.5a
Soy	4.7a	5.2a	4.6a,b	4.6a
Soy-textured	4.7a	5.2a	6.7a	4.9a
Beef + concentrate				
Cottonseed, glandless	4.2a	4.9a	4.3a,b	4.4a
Peanut	2.8a	4.1a	5.2a,b	2.9a
Soy	3.4a	4.3a	4.1a,b	3.4a
Beef + isolate				
Cottonseed, glandless	4.6a	4.9a	5.2a,b	4.5a
Peanut	2.8a	5.1a	5.0a,b	3.5a
Soy	4.4a	4.1a	3.5a,b	3.6a

[a] The data represent the results from five sensory evaluation sessions (two observations per product in each session). Means were adjusted for the incomplete block in experimental design. Means in a column which are not followed by the same letter are significantly different ($P < 0.05$).

CONCLUSION

FOOD PROTEIN ingredients produced from glandless cottonseed, peanut and soybean can be used as antioxidant ingredients in ground beef patties and perhaps in other comminuted meat products. They have beneficial effects on cooking properties, even at a low level (10%) of substitution with rehydrated products. The amount used to exert the antioxidant effect does not seem to significantly affect the sensory quality of unseasoned ground beef patties.

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Effect of Temperatures on Fish Alkaline Protease, Protein Interaction and Texture Quality

J. C. DENG

ABSTRACT

The effect of the activity of fish alkaline protease and protein denaturation on texture changes in mullet muscle during heating were studied. The proteolytic enzyme and the urea denatured hemoglobin substrate were incubated at different pH values and various temperatures. The enzyme activity was optimal at pH 8 and at 65°C. The extent of protein-protein interactions in mullet actomyosin solutions increased with increasing temperature. The rate of protein-protein interactions increased sharply as temperature increased from 35°C to 45°C at pH 6. When mullet muscle was heated at various temperatures ranging from 55°-85°C, the shear force value increased (toughening) during the initial stage of heating and then decreased (softening). The toughening of fish flesh is probably due to muscle protein interactions, and the later tenderizing of fish flesh may be explained by alkaline protease activity (hydrolysis) masking the protein denaturation effect. When fish flesh was cooked at 100°C, no tenderizing effect was observed. This was probably due to loss of enzyme activity and only protein denaturation had significant effect on texture quality.

INTRODUCTION

A CONSIDERABLE amount of work has been done concerning temperature effects on tenderization of muscle food (Machlik and Draudt, 1963; Makinodan et al., 1963a,b; Hamm, 1966; Bouton et al., 1975; Davey and Gilbert, 1976). Most of the reports are related to red meat or mammalian muscle, and only a small amount of work has been done on thermally induced changes on seafood texture.

It was reported that shear-force values are generally greater for beef muscle samples cooked at 50°C than at 60°C. The difference was attributed to temperature effects on connective tissue (Machlik and Draudt, 1963; Bouton and Harris, 1972). However, it is generally believed that collagen, a connective tissue, shrinks (toughening effect) at a temperature around 63°C and may be partially transformed to gelatin at higher temperatures (Hamm, 1966). Bouton et al. (1975) cooked fresh and aged (0-1°C) veal muscle and found that increasing the temperature from 50 to 60°C had a greater effect on decreasing shear force value in aged veal than for fresh veal. They believed that additional factors are involved in the cooking effect at 50 and 60°C than just changes in connective tissue alone. Davey and Gilbert (1976) studied the tenderization rate of beef sternomandibularis muscle aged at temperatures ranging from 0-70°C. The maximum rate of tenderization was observed at approximately 60°C. They postulated that the proteolytic enzyme cathepsin C (relatively heat stable) may be responsible for the greater tenderization effect at 60°C.

Higher gel strength was found in fish muscle paste cooked at 30-40°C and above 70°C than at 60°C (Makinodan et al., 1963a). It was postulated that the lower gel strength at 60°C was due to muscle alkaline protease which was found in the fish flesh. The protease had optimum activity at pH 8.0 and 65°C (Makinodan and Ikeda, 1969) which differs from the properties of cathepsins.

Recently, Deng et al. (1976) studied the effects of temperature and pH on actomyosin interaction. Actomyosin, the main component of myofibrillar protein, was prepared from beef and mackerel muscle. In general, if the pH remained constant, then both the interaction rate and extent of interaction increased with increasing temperature. At the same temperature and pH (except at pH 5.8), more change was observed in the beef actomyosin with increasing temperature resulted in a decrease in the water-holding capacity (Deng et al., 1981) of communited flesh and an increase in the toughness of the heated flesh.

Since heating the muscle causes interaction of muscle proteins, enzyme hydrolysis, and change of connective tissue, this study was designed to investigate the role of proteolytic enzyme and protein interactions in texture quality change of mullet muscle cooked at various temperatures. The amount of connective tissue in fish flesh is very small (Hammoir, 1955), therefore, minimal changes could be attributed to the effect of connective tissue.

EXPERIMENTAL

Preparation of muscle alkaline protease

Fresh mullet was obtained from Homosassa, Fla., packed in ice and shipped to Gainesville, Fla., where it was immediately eviscerated and filleted. The enzyme preparation was similar to the procedure of Makinodan and Ikeda (1969). Muscle tissue (boneless fillet containing a small amount of dark muscle) was cut into small sections. The enzyme was extracted by blending two parts of cold 0.5%, KCl with one part of small sectioned tissue for 1 min, and the homogenate was centrifuged at 13,000 × G for 30 min. The supernatant was dialyzed in the cold 0.5% KCl overnight. The dialyzed homogenate was again centrifuged at 13,000 × G for 20 min. The extract was mixed with an equal volume of pH 8.0 buffer (0.05M $Na_2B_4O_7$-0.1M HCl) and heated in 20 ml Pyrex tubes at 65°C (internal temperature) for 5 min. The heated suspension was centrifuged at 13,000 × G for 20 min. The supernatant was fractionated with ammonium sulfate. Proteins precipitated with 40-65% ammonium sulfate were used for further analysis. In all cases, the salt was added slowly with continuous stirring. After addition of ammonium sulfate, the solution was centrifuged at 13,000 × G for 20 min. The precipitate was dissolved in cold 0.5% KCl and dialyzed overnight against 0.5% KCl. The dialysate was centrifuged at 13,000 × G for 20 min and the supernatant was kept at −35°C until used. All steps of enzyme preparation were conducted at 2°C with exception of the heating treatment.

Assay of enzyme activity

A procedure similar to that of Deng and Lillard (1973) with slight modification was used to assay for enzyme activity. The reaction mixture contained 3.0 ml of buffer solution (0.2M) of the desired pH, 1.0 ml of the enzyme solution, and 1 ml of 3M urea denatured 2% hemoglobin. Citrate buffer at pH 2, 3, and 4, phosphate buffer at pH 5, 6, 7, and 8, and glycine-NaOH buffer at pH 9 and 10 were used. The frozen enzyme was thawed at 2°C for 16 hr before incubation. A 3M urea denatured 2% hemoglobin solution was prepared according to the methods of Sliwinski et al. (1959). The substrate was buffered to various pH's. The reaction mixture was incubated in a shaking water bath of 60 rpm and various temperatures. After 2 hr incubation, 5 ml of 5% trichloroacetic acid (TCA) was added. Controls were prepared in the same way with the exception of incubating the substrate and buffer-enzyme sepa-

Author Deng is with the Food Science & Human Nutrition Dept., IFAS, Univ. of Florida, Gainesville, FL 32611.

rately for 2 hr. The reaction mixture was combined after adding TCA. The reaction mixture was filtered through No. 4 Whatman filter paper and absorbance of the filtrate was measured at 280 nm. Activity was calculated as the absorbance at 280 nm of the reaction mixture incubated at various conditions (pH and temperature) for 2 hr and compared against a control. The highest activity at 65°C and pH 8 was expressed as 100%. Enzyme assays were conducted in triplicate.

Preparation of actomyosin

Minced mullet flesh was extracted with 6 volumes of 0.6M KCl containing 0.01M $NaHCO_3$ (pH 8.0) for 12 hr with slow stirring. The solution was centrifuged at 16,000 × G for 1 hr. The ionic strength of supernatant was decreased to 0.2 by mixing with 0.2 mM $NaHCO_3$ and the mixture was centrifuged at 9,750 × G for 20 min. The precipitate was dissolved in 2 volumes of 1M KCl with slow stirring and centrifuged at 16,000 × G for 1 hr. The supernatant was again mixed with 0.2 mM $NaHCO_3$ until its ionic strength decreased to 0.1 and the mixture was centrifuged at 9,750 × G for 30 min. The precipitate was dissolved in 1.0M KCl containing 0.1 M Tris (hydroxymethyl) aminomethane-maleate (Tris-maleate) buffer (pH 6.0) and centrifuged at 16,000 × G for 1 hr. The supernatant was diluted with 0.6M KCl containing 0.2M Tris-maleate buffer (pH 6.0). The pH of the final protein solution was 6.0, which is similar to the pH of mullet flesh.

Protein-protein interaction at different temperatures

The procedure was similar to the method of Deng et al. (1976). The protein aggregation was studied using an ultraviolet technique by measuring the changes in the spectra of the chromophores. Protein-protein interaction was monitored by measuring optical density changes attributable to light scattering. The experiments were carried out in a Shimadzu 200 UV spectrophotometer at a wavelength of 400 nm. The spectrophotometer was equipped with a thermostable cell compartment and adapters which allowed the sample compartment to be maintained at various temperatures. In order to keep all solutions at the same temperature before beginning measurments and to keep the initial extent of interaction to a minimum, the sample was kept in an ice chest and maximum preparation time of transferring sample from ice chest to the cuvette was maintained within 20 sec for all cases. The results reported are averages of duplicate experiments.

Texture measurements

Boneless fillets were cut into samples 1 inch (long) × 1 inch (wide) × 3/16 inches (thick). The sliced fish flesh (ratio of light color flesh to dark color flesh = 6:1), were sealed in Cryovac bags or heated in a water bath at various temperatures for different periods of time, and cooled in ice water immediately. When fillets were directly immersed in water at 100°C, the heated fillets were allowed to cool at room temperature and sliced to the size indicated above. The shear force of heated samples was measured using a Kramer shear with 2,500-lb ring. Heat penetration was determined by placing copper-constantan thermocouples into the geometric center of samples. The thermocouples were linked to an automatic temperature recorder (Westronics, Inc., No. M11B) to record the heat penetration.

RESULTS & DISCUSSION

Protein-protein interaction

In general the relative light scattering absorbance of the protein solution increased as the duration and temperature increased (Fig. 1). At 25°C, the increase was almost negligible. The changes in the actomyosin was detectable at 35°C but at a much reduced level compared to the higher temperatures. The interaction of the proteins showed a sharp increase from 35 to 45°C but a small change accompanying an increase in temperature from 45 to 65°C. The change pattern in actomyosin solution of mullet was different from that of mackerel and beef (Deng et al., 1976). The interaction of mackerel actomyosin increased sharply as temperature changed from 30 to 35°C at pH 6.0. Beef actomyosin started to interact at a faster rate when temperature increased from 40°C to 45°C at pH 6.0.

Arrhenius-type plots of the reaction are shown in Figure 2. This suggests that different denaturation mechanisms occur in mullet actomyosin at various temperatures.

Alkaline protease

The effect of hydrogen ion concentration on mullet muscle alkaline protease is shown in Figure 3. Maximum activity of mullet muscle alkaline protease was at pH 8 and 65°C. This pH optimum was also found in the enzyme prepared from other fish muscle (Makinodan and Ikeda, 1969; Makinodan et al., 1963a, b; Cheng et al.,

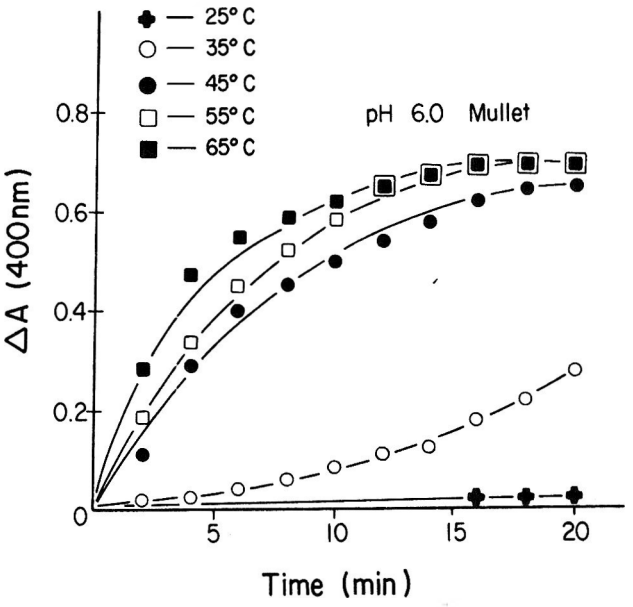


Fig. 1—Protein-protein interaction in mullet actomyosin solution at pH 6.0 at various temperatures.

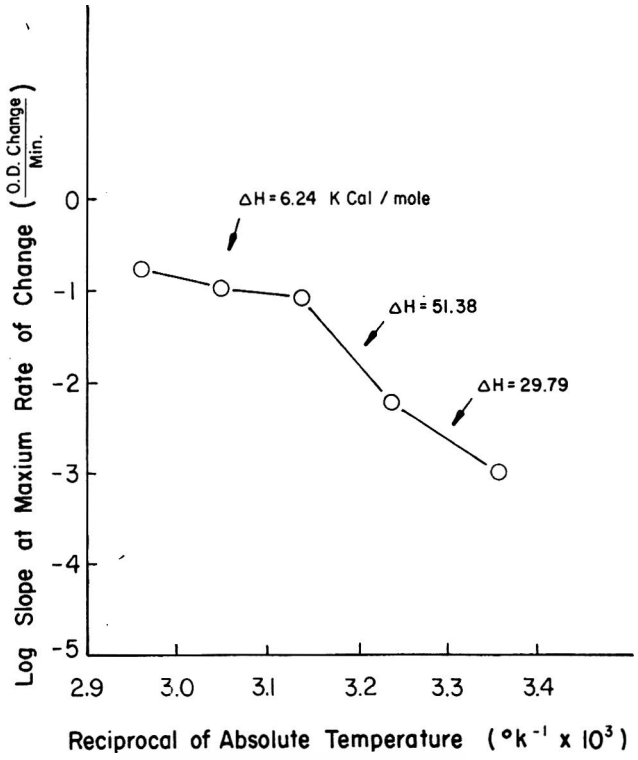


Fig. 2—Arrhenium plots of protein-protein interaction in mullet actomyosin solutions.

1979). Makinodan and Ikeda (1977) applied a denaturation agent to carp muscle alkaline protease and found that the optimum pH remained the same (pH 8) but the optimum temperature shifted to a temperature lower than 65°C depending on the amount of denaturation agent used. It is of interest that at a hydrogen ion concentration close to muscle pH (6.0), the enzyme activity was still relatively high (Fig. 3). This result has a very significant implication in that the muscle enzyme may be active during the actual heating process. Cathepsin D, generally, has minimum activity at a pH of 6 (Ting et al., 1968; Deng and Lillard, 1973). Makinodan et al. (1963b) also reported relative high activity of muscle alkaline protease at a pH of 6 in Siaena schlegeli (Bleeker), Sphyraena pinguis (Gunther) and Saurida argyrophanes (Richardson).

Texture quality at various temperatures

When mullet flesh was heated at 35°C, the texture change was almost negligible (Fig. 4) with time of heating.

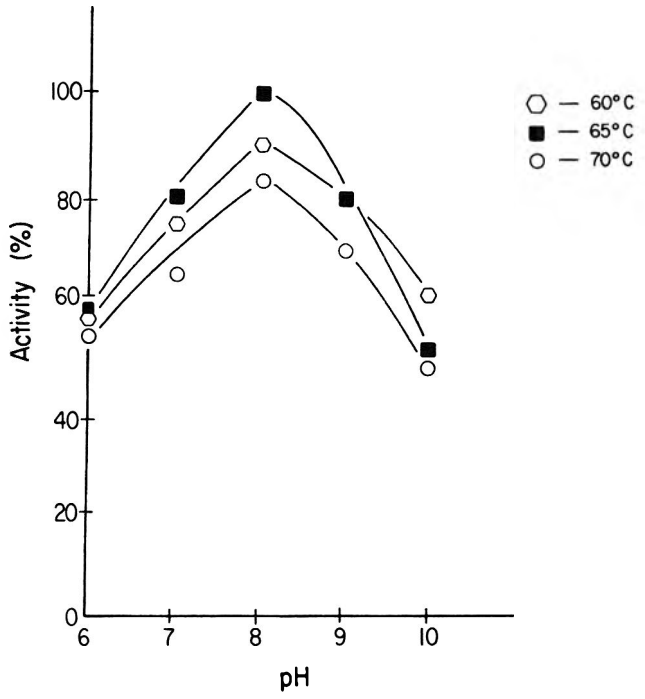


Fig. 3—Optima pH and temperature of mullet alkaline protease.

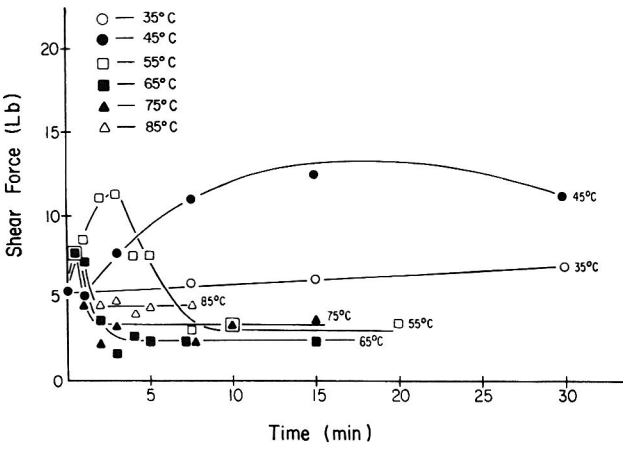


Fig. 4—Effect of temperatures (35-85°C) on shear force value of mullet flesh.

At 45°C shear force data showed a 1 min initial lag followed by a steady toughening reaching a maximum at about 15 min of heating and a slight decrease with prolonged heating. At 55-85°C, shear force increased sharply immediately upon start of heating and peaked with 1-3 min. At 55°C, the shear force value decreased as heating time increased from 3 to 8 min and then leveled off. At 65, 75, and 85°C, the shear force value decreased from 1-2 min and then leveled off. When fish muscle was heated at 100°C, the toughness of the fish increased sharply initially and leveled off after 15 min heating (Fig. 5). Lee and Toledo (1976) reported that lower shear strength was detected in comminuted mackerel flesh precooked at 50-80°C when compared to those preheated at other temperatures. Cheng et al. (1979) also indicated that minced croaker flesh with rapid heating to 85°C internal temperature had greater shear strength compared to those slowly heated to 70°C internal temperature.

The increase of shear force value initially during heating at temperatures greater than 35°C (Fig. 4) was probably due to protein-protein interaction (Fig. 1). The rate of toughening of fish flesh (the maximum slope of the shear force curve) with heating increased with increasing temperature (Fig. 4), a characteristic that parallels that of protein-protein interaction (Fig. 1 and 2). This relation is strongly supported by the fact that both shear force value and protein interaction sharply increased when heating temperature changed from 35 to 45°C (Fig. 1 and 4).

The decrease in shear force value of fish flesh after cooking for a short time at higher temperatures (Fig. 4) could be attributed to two possible mechanisms. One is the thermal dissociation of muscle proteins, and the other is the enzyme hydrolysis of muscle proteins. When fish flesh was cooked at 100°C, the shear force value increased and leveled off but did not drop. This indicates that thermal dissociation was not the factor causing the decrease of shear force value of fish flesh cooked at temperatures between 55 and 85°C. Enzyme hydrolysis probably played the major role in reducing the shear force value of fish flesh heated at temperatures ranging from 55-85°C.

Cathepsin C, although suggested by Davey and Gilbert (1976) as a possible enzyme in tenderization of meat at 60°C, can only catalyze the hydrolysis of certain peptides, such as deamidating glycyl-l-phenylalaninamide. In addition, cathepsin C can act as an dipeptidyl transferase and catalyze the polymerization of glycyl-l-phenylalaninamide and formation of decapeptide. Cathepsin D, however, is able to hydrolyze protein molecules to small fragments (e.g., peptides and amino acids). This enzyme is heat labile (Barrett and Dingle, 1971). The mullet muscle alkaline protease has optimum activity at 65°C and relatively high activity at 60 and 70°C (Fig. 3). The fish flesh cooked at 65°C showed the most effect of the enzyme among all

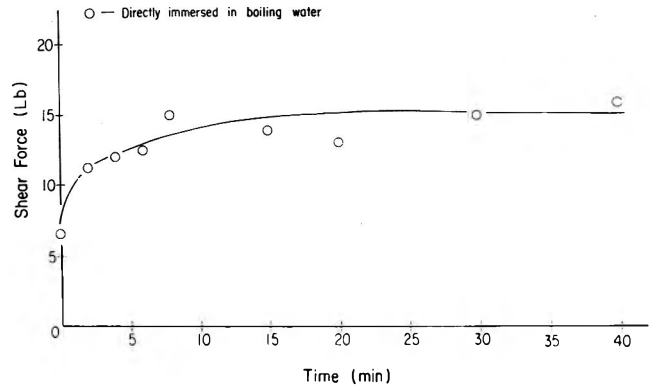


Fig. 5—Effect of temperature at 100°C on shear force value of mullet flesh.

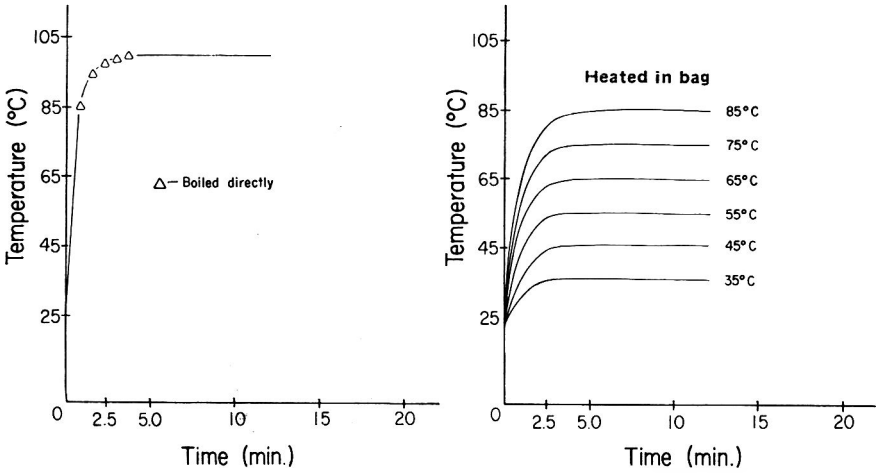


Fig. 6—Effect of time of heating on internal temperature of mullet flesh.

the samples as manifested by the lowest shear force values (Fig. 4). When fish flesh was heated at various temperatures, the internal temperature reached the medium temperature after 3-4 min heating (Fig. 6). During the initial temperature coming-up period at 75 and 85°C, the fish flesh would pass through the temperature zone, 60-70°C, optimum for enzyme activity for approximately 1 min. This probably allowed the enzyme to catalyze protein hydrolysis to a large extent and to mask the protein interaction-toughening effect. At 55°C, toughening was significantly faster because the enzyme catalyzed hydrolysis of proteins that cause softening was less than at 65, 75, and 85°C. The enzyme induced softening was barely detectable after 15-20 min heating at 45°C. The coming-up period for fish flesh cooked at 100°C was very short. It took less than 1 min for the flesh (internal temperature) to reach 85°C. The enzyme activity at this cooking temperature probably became limited or was not high enough to mask the increased effect of protein-protein interaction.

In summary, when fish flesh was heated at various temperatures, the texture quality was influenced by protein-protein interaction and enzyme hydrolysis. At 100°C, the protein interaction was the predominant factor influencing the texture. At temperatures between 55 and 85°C, the protein interaction was more significant initially but was masked by enzyme induced protein hydrolysis as heating time increased. When fish flesh was heated at 35 and 45°C, the protein interaction was the primary factor affecting texture quality, but only very slight effect at 35°C. The enzyme influence was barely detectable in a later stage of heating at 45°C.

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Vibrio cholerae in Marine Foods and Environmental Waters: A Literature Review

ANGELO DePAOLA

ABSTRACT

Seafoods have been implicated in a number of cholera outbreaks in the United States and abroad. The incidence of *Vibrio cholerae* in the environment may be attributed to its extended survival in seawater or to short- or long-term carriers. Strains of *V. cholerae* which do not agglutinate in O group 1 antiserum (NAG) have been associated with a number of gastroenteritis, septicemia, and meningoencephalitis cases in recent years, and genetic studies illustrate a close relationship between the deoxyribonucleic acid (DNA) of NAG and *V. cholerae*. The ability of NAGs to elicit responses in toxigenicity testing systems similar to those caused by *V. cholerae* has also been demonstrated. The incidence of NAGs and *V. cholerae* in U.S. coastal waters and seafoods, their survival, and the conditions that promote genetic changes and increase NAG virulence must be fully evaluated to determine the public health significance of these organisms. These studies are needed to reevaluate the effectiveness of the National Shellfish Sanitation Program (NSSP) growing area standards and criteria so that consumers may be protected against shellfish-associated vibrio diseases.

INTRODUCTION

AN OUTBREAK of cholera occurred after the consumption of home-cooked crabs in Louisiana in 1978; a cholera-like disease caused by strains of *Vibrio cholerae* which do not aggultinate in O group 1 antiserum (NAG) also occurred after the consumption of raw oysters in the same area. Recent data indicate that NAG strains of *V. cholerae* may be ubiquitous in estuarine waters and are not necessarily associated with domestic sewage.

These situations have resulted in a renewed interest in the public health significance of the vibrio group as a cause of seafood-associated gastroenteritis in the United States and an urgent need to reevaluate the effectiveness of National Shellfish Sanitation Program (NSSP) standards and guidelines for the classification of shellfish growing area waters.

CHOLERA IN SEAFOOD AND THE ENVIRONMENT

Epidemiology of seafood-related outbreaks

Seafood products have been increasingly incriminated in cholera outbreaks since the 1960s, especially in the Western Hemisphere. An explosive outbreak of *Vibrio cholerae* El Tor in the Philippines during 1961 and 1962 was reported by Joseph et al. (1965), who suggested that the initial infection was transmitted principally by shrimp that were consumed raw. Dutt et al. (1971) investigated the role of shellfish in vibrio infections during the 1969 cholera outbreak in Kelantan, Malaysia. El Tor and NAG strains were isolated from water and shellfish and from the stools of suspected patients and contacts. In 1973 a cholera epidemic caused by the El Tor biotype began in Naples, Italy, and resulted in 25 deaths among the 278 bacteriologically confirmed cases (Baine et al., 1974). The suspected vehicle of transmission was raw seafood, particularly mussels washed with dockside water that may have been contaminated with *V. cholerae*. One year later in Portugal, 2467 bacteriologically confirmed cases in which 48 deaths occurred were reported (Blake et al., 1977). *V. cholerae* biotype El Tor was isolated from 42% of the shellfish samples tested, and consumption of raw or partially cooked cockles was significantly more common among cholera patients than paired controls. During the same period six cases of cholera occurred in Guam and were associated with home-preserved fish (Merson et al., 1977). In the United States the first case of cholera since 1911 was reported in Texas in 1973. Although the source of the infection was undetermined, the individual had consumed raw oysters (Weissman et al., 1974). In 1977 a similar case occurred in Alabama in an individual who had eaten large quantities of raw oysters for many years (Cameron et al., 1977). *V. cholerae* El Tor Inaba was isolated from the stools of 11 persons who displayed 8 symptomatic and 3 asymptomatic infections in Louisiana in the fall of 1978 (Bradford and Caraway, 1978). A relationship was noted between infected individuals and consumption of boiled and steamed crabs, and *V. cholerae* was isolated from some leftover crab. A single raw shrimp sample was also found to be positive for El Tor organisms. In a Food & Drug Administration (FDA) investigation, samples of blue crab from the suspect areas revealed numerous nonagglutinable strains of *V. cholerae*, but O-1 serotypes were not found (Twedt, personal communication). In October 1978, FDA began monthly monitoring of shellfish, water, and muds from 14 sampling sites from Florida to Texas for the presence of *V. cholerae* (Hunt, 1978).

A summary of these cholera outbreaks (Table 1) suggests a westward migration of El Tor from Asia to Europe and finally to North America. If this trend continues future outbreaks can be predicted in North and South America. Most of the infections were caused by ingestion of raw or partially cooked shellfish and crustacea; however, a fin fish was incriminated in the Guam outbreak. In the Eastern Hemisphere cholera has usually infected impoverished people who live in unsanitary conditions. However, in the West, cholera often occurs among the middle class and is usually more severe in individuals with gastric disorders. Most of the outbreaks occur in the late summer and peak in August. Although isolates of *V. cholerae* from the environment were difficult to find during the outbreaks in Italy and Louisiana, they were common in Portugal (42% in shellfish; 24% in water), Guam, Malaysia, and the Philippines. The difficulty in determining the source of *V. cholerae* in outbreaks suggests the organism's prolonged survival periods or perhaps the presence of asymptomatic carriers.

SURVIVAL AND PROLIFERATION IN SEAFOOD AND ENVIRONMENT

THE SURVIVAL of *V. cholerae* under varying conditions has been investigated by a number of researchers. Pollitzer (1959) reviewed the literature on *V. cholerae* and reported a wide range of survival periods depending on experimental conditions. Research conducted in India

Author DePaola is with the Gulf Coast Technical Services Unit, Fishery Technology Branch, Bureau of Foods, U.S. Food & Drug Administration, Dauphin Island, AL 36528

illustrated greater viability in stool samples collected at cooler ambient temperatures. Pollitzer (1959) cited a number of investigations conducted on the survival of *V. cholerae* in seawater during the early part of the century. In general, survival was enhanced by intermediate salinities, lower temperature, high organic content, near neutral pH, dark storage, and absence of competing microflora. Studies were made on the survival of *V. cholerae* in artificially contaminated ocean water and bay water from New York. Survival time in unsterilized bay water was 47 days compared to 7 days for ocean water, but survival for more than 285 days was observed when both ocean and bay water were sterilized before being inoculated with *V. cholerae*.

Pollitzer (1959) also reviewed investigations of the survival of *V. cholerae* in seafoods. Japanese researchers reported uptake of *V. cholerae* by oysters and clams kept in cholera-polluted seawater. The vibrios entered the gastrointestinal tract of the shellfish and survived for 1.5 months at 0-5°C and 15-20 days at 22°C. In shucked oysters smeared with cholera vibrios and stored at 20°C, the number of organisms first decreased, then increased, with the maximum number occurring after 68 hr. A gradual decrease followed and most vibrios disappeared by 171 hr. Vibrio survival increased to 20 days on oysters and clams that had been sterilized or boiled before contamination. *V. cholerae* survived only a few days on fish stored at room temperature but persisted for more than 3 wk when refrigerated. Felsenfeld (1963) compared the survival time of classical and El Tor biotypes in shallow well water and in the stomachs of fish. Mean survival time for El Tor biotypes was 19.3 days, while classical cholera organisms survived for 7.5 days in water; both biotypes disappeared from fish stomachs within a mean time of 5.3 days. Felsenfeld (1965) determined survival under "natural" contamination conditions by inoculating various foods with vibrio-free stool material to which a saline suspension of cholera organisms was added. Survival periods of less than 1 wk in raw and cooked catfish stored at room temperature increased up to 3 wk when the product was stored at 2°C. Pesigan et al. (1967) contaminated various foodstuffs and water directly with stool material from cholera patients and carriers to determine viability of El Tor vibrios. Vibrios in raw seafoods survived 2-4 days when stored at 30-32°C and 4-9 days when stored between 5 and 10°C. Likewise, El Tor vibrios survived in seawater for 10-13 days at 30-32°C and 58-60 days at 5-10°C. Venkatraman and Ramakrishnan (1941) reported that *V. cholerae* survived for 198 days in 2% sea salt solution. Miyaki et al. (1967) observed a survival time for El Tor vibrios of more than 1 month in various foodstuffs frozen at −20°C and much longer in foods frozen at −72°C. Optimum conditions for growth and survival were pH 6-8 and osmotic pressure 250-700m Osm.

V. cholerae strains other than those agglutinable in cholerae O group 1 antiserum were reported by Colwell et al. (1977) in water samples from the Chesapeake Bay. In a later publication Kaper et al. (1979) reported that these vibrios are indigenous to Chesapeake Bay waters, sediments, and shellfish and usually occur when the salinity is between 5 and 15 ppt. Kaneko and Colwell (1973) reported that vibrio species and related bacteria associate with zooplankton in summer months and that chitinase activity in vibrios plays a role in this association. The chitinase activity may increase the affinity to crustacea, thus explaining why *V. cholerae*, a chitin digester, is found more frequently in crustacea (crabs and shrimp) than in other seafoods. The chitinase activity may also prolong the survival of *V. cholerae* by providing a maintenance or growth substrate. Some strains of *V. cholerae* can survive for long periods in estuarine environments with moderate salinities (5-15 ppt) where most seafood is harvested. Survival is further enhanced in cool seasons and when the amount of organic matter is high.

Cholera carrier state

The presence of *V. cholerae* organisms in waters of areas which have been free from cholera infection for decades could be explained by the existence of asymptomatic carriers. Sayamov (1963) injected El Tor and classical cholera organisms into the gall bladder of rabbits and noted that the cholera multiplied in the bile system throughout the experiment (15 wk). Cholera carrier studies in the Philippines showed a prevalence rate of 21.7% among household contacts of cholera patients and 8.4% for next door neighbors compared to 0.35% in the general population (Dizon et al., 1967). During the carrier state of 5-19 days, the vibrio concentration in stool samples was between 10^2 and 10^5/ml in carriers compared to 10^6-10^9/ml in cholera cases. Azurin et al. (1967) reported a long-term carrier of El Tor cholera who excreted vibrios intermittently from 1962 through 1966. Duodenal intubation demonstrated that the vibrios were lodged in the biliary tract. Barui et al. (1972) compared detection methods of *V. cholerae* in inapparent infections collected by purgation and multiple rectal swabs. The detection rate was 94% with rectal swab cultures and only 54% with a single purge.

Because of the wide geographical spread of cases and the lack of previous and subsequent cases in the area, it is doubtful that a long-term cholera carrier was responsible for the Louisiana outbreak. Asymptomatic carriers among Southeast Asian immigrants living in the area were considered as a possible source, but no isolates were recovered by Moore swabs in sewers downstream from residences and no clinical cases were reported in the group. The *V. cholerae* strains from Louisiana and Port Lavaca, Texas, were more hemolytic than those from the Eastern Hemisphere, but Ganguly et al. (1966) have reported that the hemolytic ability of *V. cholerae* increases after exposure of the organism to low levels of chlorine, which is used more extensively in the United States than in Southeast Asian countries.

—Continued on next page

Table 1—Epidemiology of seafood related outbreaks of cholera

Location	Year	Month(s)	Strain	Cases	Deaths	Food	Source
Philippines	1961	Nov	El Tor	330	–	Raw shrimp	Unknown
Malaysia	1969	–	El Tor: Inaba Ogawa NAG	135	–	Shellfish	Water
Italy	1973	Aug-Oct	El Tor: Ogawa	278	25	Raw mussels	Tunisia
Portugal	1974	May-Nov	El Tor: Inaba	2467	48	Raw shellfish	African colonies
Guam	1974	Jul-Aug	El Tor: Ogawa Inaba	6	0	Raw fish	Sewage
Texas	1973	Aug	El Tor: Inaba	1	0	Raw oysters	Unknown
Alabama	1977	Apr	El Tor: Inaba	1	0	Raw oysters	Unknown
Louisiana	1978	Apr-Sep	El Tor: Inaba	11	0	Crab	Unknown

VIBRIO CHOLERAE–NON-O GROUP 1

Epidemiology

In the past, isolates of *V. cholerae* which failed to agglutinate in O group 1 antiserum (NAGs) have been considered to be of little public health significance. International quarantine regulations require vaccination against cholera for persons leaving areas infected with *V. cholerae* O group 1. Serotypes within the O group 1 organisms include Inaba, Ogawa, and Hikojima. However, other vibrios have been associated with cholera-like diarrhea (McIntyre and Feely, 1965; Aldova et al., 1968; El-Shawi and Thewaini, 1969; Dutt et al., 1971; Zafari et al., 1973; Dakin et al., 1974; De Gerome and Smith, 1974; Hughes et al., 1978). McIntyre and Feeley (1965) isolated NAGs from cases of diarrhea in East Pakistan and noted a rise in agglutinating antibody titer to these organisms in approximately 1/3 of the cases. Non-O group 1 vibrios in Heiberg group II were implicated in a gastroenteritis outbreak in Czechoslovakia in 1965 (Aldova et al., 1968). French potatoes were the suspected vehicle of 56 of these diarrheal cases in which no fatalities occurred. Zafari et al. (1973) surveyed Iranian pilgrims returning from Saudi Arabia and found less than one percent to be infected with NAGs, although more than half of the infected individuals had mild to severe diarrhea. El-Shawi and Thewaini (1969) studied NAGs isolated from patients with clinical cholera during the 1966 epidemic of cholera in Iraq and reported cultures belonging to Heiberg groups I, II, IV, and V. Dutt et al. (1971) investigated the role of shellfish in the transmission of vibrio infection in the 1969 Kelantan cholera outbreak and found NAGs in shellfish and stools of suspected cholera patients. Sixty-four passengers aboard an international aircraft from London to Sydney suffered acute gastroenteritis shortly after eating the suspected food vehicle, chopped egg on asparagus (Dakin et al., 1974). Examination of stool cultures yielded NAGs in Heiberg groups I and II. De Gerome and Smith (1974) noted the isolation of a NAG from an American who had prolonged diarrhea after eating raw oysters in New Orleans. Between 1972 and 1975, 26 NAG infections were investigated by the Center for Disease Control (CDC) in Atlanta, GA (Hughes et al., 1978). Fifty percent of the isolates were obtained from stool samples of patients with acture diarrhea. Frequently these patients had recently eaten shellfish (Table 2).

NAGs have also been incriminated in cases of septicemia and meningoencephalitis (Back et al., 1974; Fearrington et al., 1974; Prats et al., 1975; Hughes et al., 1978). Back et al. (1974) reported NAG infections in two persons shortly after they were exposed to marine waters in Sweden: vibrios were isolated from a middle ear infection in one and from a leg wound in the other. NAGs were isolated from cultures of blood and cerebrospinal fluid from a North Carolina man who sustained insect bites that were later exposed to polluted river water (Fearrington et al., 1974). This case was fatal, but the patient had a history of hematemesis caused by esophageal varices. A similar case in Spain was reported by Prats et al. (1975) in which a man with hepatic cirrhosis was found to have noncholera vibrio septicemia and meningoencephalitis. Hughes et al. (1978) noted that nine of the NAG infections reported to CDC from 1972 to 1975 were from tissues or fluids other than the gastrointestinal or biliary tracts. Of this figure, which represents 35% of all reported NAG infections in the U.S. during this period, four deaths occurred. Some patients with systemic NAG infections had recently been exposed through occupation or recreation to salt water. Blake et al. (1979) studied the clinical characteristics and epidemiology of disease caused by an unnamed halophilic, lactose-fermenting vibrio from 1964 to 1977. Septicemia in 39 persons was associated with consumption of raw oysters, exposure to seawater, or injury during the handling of crabs. Twelve deaths occurred in this group, usually with preexisting hepatic disease. The NAG outbreaks are summarized in Table 3.

In general, NAG outbreaks are similar to those caused by O-1 *V. cholerae* but are less severe. Consumption of seafood and exposure to salt water are often associated with NAG infections. Outbreaks usually occur during late summer and early fall. Heiberg group II organisms are most commonly isolated from non O-1 cholera infections, but Heiberg groups I, IV, and V have been reported. The implication of sucrose (–) or more specifically Heiberg group V organisms in diarrheal cases illustrates the need for greater consideration of green colonies on thiosulfate citrate bile salt sucrose (TCBS) medium. The reports of NAG septicemia and meningoencephalitis point to a deviation from the clinical picture shown by O-1 *V. cholerae* and raise the question of the invasive potential of NAGs.

LABORATORY STUDIES ON SEROLOGY, TOXIGENCITY, AND GENETICS OF *V. CHOLERAE* AND NAGS

LABORATORY STUDIES have been conducted to determine the public health significance of NAGs. Smith and Goodner (1965) collected 1426 strains of NAGs from diarrheal outbreaks around the world and classified them in 424 catagories based on Heiberg fermentation patterns, serologic evaluation, and "GIN" reactions (gelatin degradation, indole production, and nitrate reduction). They concluded that this group of organisms exhibited extreme changes in morphology which were closely linked to environmental conditions.

In vitro experiments demonstrated conversion of classical *V. cholerae* to El Tor biotypes when the organisms were subjected to repeated exposures of chlorine (Ganguly et al., 1966). Gangarosa et al. (1967) isolated three serotypes of *V. cholerae* (Ogawa, Inaba, and Hikojima) from the stool of a diarrheal patient who lived in an area where only Ogawa had been reported previously. It was suggested that Inaba and Hikojima types were in vivo mutations. This claim was further substantiated by Sheehy et al. (1966) who reported laboratory infections of *V. cholerae* Inaba in two

Table 2–Review of outbreaks of diarrhea caused by V. cholerae-non-O group 1

Location	Year	Season	Strain	Cases	Deaths	Food	Source
East Pakistan	1965	–	Heiberg I, II	19	–	–	–
Czechoslovakia	1965	Fall	Heiberg II	56	0	French potatoes	–
Iran	1965	–	–	96	–	–	Saudi Arabia
Iraq	1966	–	Heiberg I, II, IV, V	22	–	–	–
Malaysia	1969	–	–	10	0	Raw seafood	–
Louisiana	1972	July	Heiberg II	1	0	Raw oysters	–
Iraq	1973	June	Heiberg I, II	64	0	Chopped egg asparagus	–
United States	1972-1975	–	–	13	0	Seafood	Foreign travel

Table 3—Review of nonenteric diseases in man caused by V. cholerae-non-O group 1

Location	Year	Season	Strain	Cases	Deaths	Manifestation	Source	Predisposing factors
Sweden	1972-1973	Aug-Sep	Serotype 107 and 22	2	0	Middle ear and wound infection	Baltic Sea water	Hematemesis
North Carolina	1973	Oct	—	1	1	Septicemia and meningoencephalitis	River water	Esophageal varices
Spain	1973	Aug	Heiberg II	1	1	Septicemia and meningoencephalitis	—	Hepatic cirrhosis
United States	1972-1975	—	—	9	4	Invasion of biliary tract soft tissue and fluids	Foreign travel, salt water	Significant underlying disease

technicians in the United States. As the infections progressed Ogawa types appeared and were the predominant serotype of *V. cholerae* isolated from the stools of one of these patients. Sack and Miller (1969) demonstrated progressive changes of vibrio serotypes in germ-free mice infected with *V. cholerae*, noting reciprocal conversions of both Inaba and Ogawa serotypes and rough to smooth and smooth to rough transformations. Changes in serotype were correlated with the appearance of serum-agglutinating antibody, and serotypic changes seemed to result from the selective effect of antibody within the intestinal lumen. However, Gallut and Quiniou (1970) studied interactions of classical, El Tor, and NAG *V. cholerae* and hypothesized that bacteriophage could be involved in serological and biochemical changes of *V. cholerae*.

The genetic relationship among organisms within the genus *Vibrio* has also been studied. Citarella and Colwell (1970) examined the polynucleotide relationship among vibrio species by means of deoxyribonucleic acid (DNA) reassociation reactions and chromatography hydroxyapatite. While the complementary polynucleotide sequence of *V. cholerae* and some of the NAG strains was nearly identical, there was little relationship between these organisms and *V. parahaemolyticus*, *V. alginolyticus*, or *V. marinus*. In serological studies of the proteases and alkaline phosphatases of NAGs, Hsieh and Liu (1970) found that these enzymes were indistinguishable from those produced by classical strains of *V. cholerae*, but were distinct from those produced by *V. metschnikovii* and *Aeromonas* species.

The public health significance of NAGs should be determined by their ability to cause disease in humans. Dutta et al. (1963) used the infant rabbit model to determine toxicity of El Tor and NAG vibrios isolated from clinical cholera cases and from cholera-like water vibrios isolated from tanks or rivers. Diarrheal symptoms were noted in rabbits infected intraintestinally by some of the strains in each of these groups, but only the El Tor biotype caused death. However, when these strains were inoculated intraintestinally in rabbits and recovered from the large intestine (rabbit passaged), then used to infect infant rabbits, the virulence was enhanced. The infective dose of "rabbit passaged" strains was reduced from 10^9 to 10^6, and NAGs or water vibrios were able to cause death in infant rabbits. This work was further substantiated by McIntyre et al. (1965) who noted that low inoculum levels of NAGs isolated from diarrheal cases in Pakistan produced diarrhea in in infant rabbits. In comparing the toxins produced by *V. cholerae* and NAGs, Ohashi et al. (1972) found that both toxins evoked positive ileal loop reactions in rabbits, caused fatal diarrhea in suckling mice, and increased permeability in guinea pig skin. Although volume to length ratios in ileal loops were less for NAGs than *V. cholerae*, higher toxicity was produced by NAG toxin in suckling mice. A heat stable "hemorrhagic principle" elaborated by some serotypes of *V. cholerae* and NAGs was also reported. Zinnaka and Carpenter (1972) noted that NAGs isolated from human diarrheal disease caused fluid accumulation in the rabbit ileal loop test and increased vascular permeability in the rabbit skin. Kaper et al. (1979) reported that most NAGs isolated from the Chesapeake Bay produced fluid accumulation in rabbit ileal loops and a positive response in Y-1 mouse adrenal cells.

Although laboratory studies have illustrated similarities between O-1 and non-O-1 *V. cholerae*, the public health significance of the non-O-1 organisms is still controversial. Their ability to produce toxin raises questions about the mechanisms involved. A number of environmental factors, such as elevated temperature or animal passage, may be involved. Although other possibilities may not involve toxigenicity, they could be linked to virulence factors such as motility and adhesion to the gut wall. Finkelstein et al. (1977) that a cell-bound hemagglutinin plays a role in attachment of *V. cholerae* to the epithelial surface of the small intestine. They hypothesized that El Tor vibrios, which have cell-bound hemagglutinin, are better able to establish infection in man than classical biotypes, which produce more toxin but have only a soluble hemagglutinin. The literature does not indicate that animals serve as reservoirs for *V. cholerae* or NAGs. However, a large concentration of wildlife exists in southern Louisiana, and many of these animals, such as birds and raccoons, eat seafood and would therefore offer a mechanism for survival and proliferation as well as a route for serotype changes and increased toxicity.

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Functional Properties of the Great Northern Bean (*Phaseolus vulgaris* L.) Proteins: Emulsion, Foaming, Viscosity, and Gelation Properties

S. K. SATHE and D. K. SALUNKHE

ABSTRACT

Functional properties of the Great Northern bean (*Phaseolus vulgaris* L.) flour, albumins, globulins, protein concentrates, and protein isolates were investigated. Protein concentrates had the highest water and oil absorption capacity (5.93 and 4.12 g/g, respectively) among all the samples studied. Protein concentrates registered the highest emulsion capacity (72.6g oil emulsified/g) while albumins had the highest emulsion stability (less than 5 ml separation of phase in 780 hr at room temperature of 21°C). Foaming ability of the Great Northern bean proteins was fair. Foamability of the proteins was concentration dependent.

INTRODUCTION

IN THE U.S., the beans of *Phaseolus vulgaris* are an important food crop, both nutritionally and economically. The Great Northern beans (*Phaseolus vulgaris* L.) rank third in production among the five major bean cultivars of *Phaseolus* in the U.S. (Change and Satterlee, 1979).

Numerous researchers have reported the preparation and functional properties of protein concentrates and protein isolates from plant, animal, and microbial sources (Satterlee et al., 1975; Vananuvat and Kinsella, 1975; Hutton and Campbell, 1977; Ramanatham et al., 1978; Zakaria and McFeeters, 1978; Gwiazda et al., 1979; Burgess and Kelly, 1979). Such efforts are often aimed at effective utilization of inexpensive proteins for nutritional and functional purposes. Precision in defining "functionality" appears to be lacking (Martinez, 1979; Hermansson, 1979). Excellent reviews on functional properties of proteins have appeared (van Kleef et al., 1979; Schroder, 1979; Chou and Morr, 1979; Craig, 1979) elucidating several parameters affecting the functional properties. Hydration of proteins is vital for several of the functional properties such as emulsion capacity, foaming, viscosity, and gelation. Carbohydrates deserve consideration while evaluating functional properties of proteins (Kinsella, 1979) as they are known to be important for functionality. Recently, Susheelamma and Rao (1979) reported the role of arabinogalactan (a polysaccharide) of black gram (*Phaseolus mungo*) in the texture of leavened foods (steamed puddings).

In a previous paper we (Sathe and Salunkhe, 1981) reported the solubilization and electrophoretic characterization of Great Northern bean proteins. The purpose of the present investigation was to study certain functional properties of Great Northern bean proteins.

MATERIALS & METHODS

THE SOURCE of the beans, protein fractionation and composition were reported earlier (Sathe and Salunkhe, 1981). All the analyses were performed in triplicate and means reported. All the chemicals (unless mentioned otherwise) were of reagent grade.

Scanning electron microscopy

The bean flour and the freeze-dehydrated albumins, globulins, protein concentrates, and protein isolates were prepared for scanning electron microscopic observations as follows. An appropriate dry sample was sprinkled on an aluminum stub (with a double-stick tape on it) and was coated completely with gold-palladium alloy in a Polaron E 5000 Sputter Coater (U.K.). The specimens were then observed and photographed in an AMR 1000B Scanning Electron Microscope (Cambridge, Mass.) at an accelerating voltage of 20 KV and suitable magnification.

Viscosity

The appropriate sample was dispersed in distilled water and magnetically stirred for 2 hr at room temperature (21°C) prior to viscosity measurements. Each sample was prepared at concentrations of 1, 2, 3, 4, 5, 7, and 10% (w/v) and viscosity measured at room temperature (21°C) employing an Ostwald type viscometer (Sargent-Welch Scientific Co., U.S.A.; size # 200, approximate constant = 0.1 centistokes/s). All the measurements were performed in triplicate and means (relative to distilled water) were reported.

Gelation

The method of Coffmann and Garcia (1977) was employed with slight modifications. Appropriate sample suspensions of 2, 4, 6, 8, 10, 12, 14, 16, 18, and 20% (w/v) were prepared in 5 ml distilled water. The test tubes containing these suspensions were then heated for 1 hr in a boiling water bath followed by rapid cooling under running cold tap water. The test tubes were then further cooled for 2 hr at 4°C. The least gelation concentration was determined as that concentration when the sample from the inverted test tube did not fall down or slip.

Oil and water absorption

For oil (Crisco Vegetable Cooking oil, Procter & Gamble, Cincinnati, Ohio) and water absorption determinations, the method of Beuchat (1977) was followed. One gram of the sample was mixed with 10 ml distilled water or oil for 30 sec in a mixer (Vari-Whirl, mixing control-"fast"). The samples were then allowed to stand at room temperature (21°C) for 30 min, centrifuged at 5000 x G for 30 min, and the volume of the supernatant noted in a 10 ml graduated cylinder. Density of water was assumed to be 1 g/ml and that of oil was determined to be 0.88 g/ml. Results were expressed on a dry weight basis.

Emulsion capacity and stability

Emulsions were prepared according to the method by Beuchat (1977). The sample (2g) was blended in a Waring Blendor with 100 ml of distilled water for 30 sec at "HI" speed. Oil was added in 5-ml portions continuing blending. The drop in consistency (from a maximum) judged by a decrease in resistance to blending (subjectively) was considered to be the point of discontinuation of oil addition. The amount of oil added upto this point was interpreted as the emulsifying capacity of the sample. The emulsion so prepared was then allowed to stand in a graduated cylinder and the volume of water separated at time intervals of 10, 20, 35, 60, 100, 120, 140, 160, 180, 200, and 780 hr was noted in each case, to study the emulsion stability. All the experiments were conducted at room temperature (21°C).

Foaming capacity and stability

Foaming capacity and stability of the samples was studied according to the method of Coffmann and Garcia (1977). One gram of sample was whipped with 100 ml distilled water for 5 min in a

Authors Sathe and Salunkhe are with the Dept. of Nutrition & Food Sciences, Utah State Univ., Logan, UT 84322.

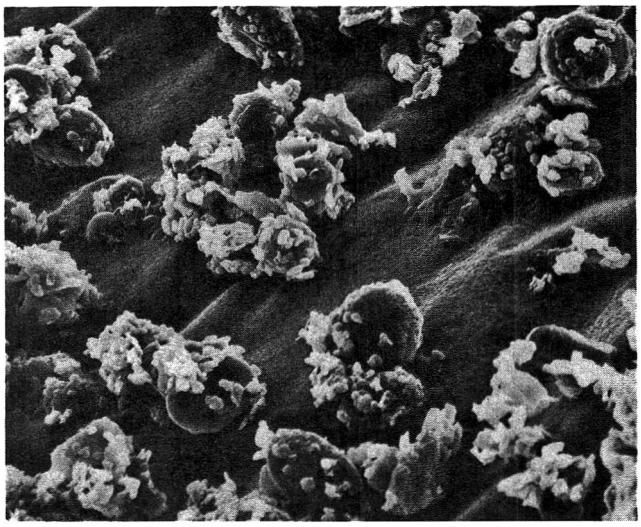


Fig. 1—Scanning electron photomicrograph of Great Northern bean flour (1500 X).

Waring Blendor at speed setting "HI" and was poured into a 250 ml cylinder and the total volume at time intervals of 0.0, 0.5, 1.0, 2.0, 2.5, 3.0, 8.0, and 36 hr was noted. Specific volume and volume increase (%) were calculated according to the following equations:

$$\text{Specific vol.} = \frac{\text{Vol. after whipping (ml)}}{\text{Wt. after whipping (g)}}$$

$$\text{Vol. increase (\%)} = \frac{\text{(Vol. after whipping - Vol. before whipping) (ml)}}{\text{Vol. before whipping (ml)}} \times 100$$

Egg albumin (Fisher Scientific Co., Fairlawn, N.J.) powder was employed as a reference standard. All the experiments were conducted at room temperature (21°).

To study the effect of concentration on foamability; 1, 2, 3, 4, 5, 7, and 10% (w/v) aqueous suspensions were whipped identically as described above and the final volume was noted in each case in a 250 ml cylinder.

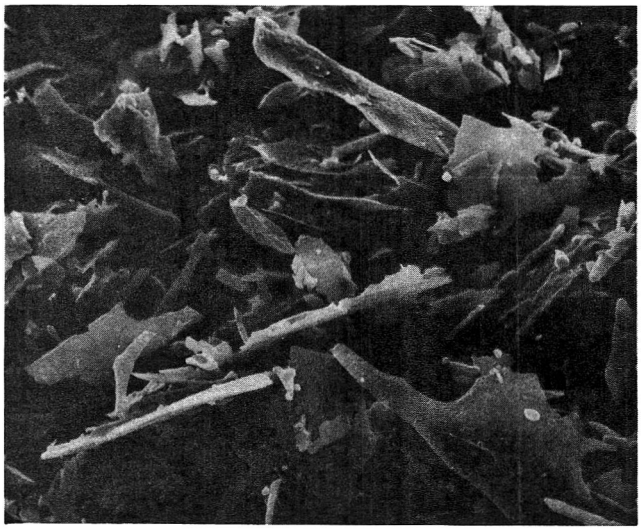


Fig. 2—Scanning electron photomicrograph of Great Northern bean albumins (1500 X).

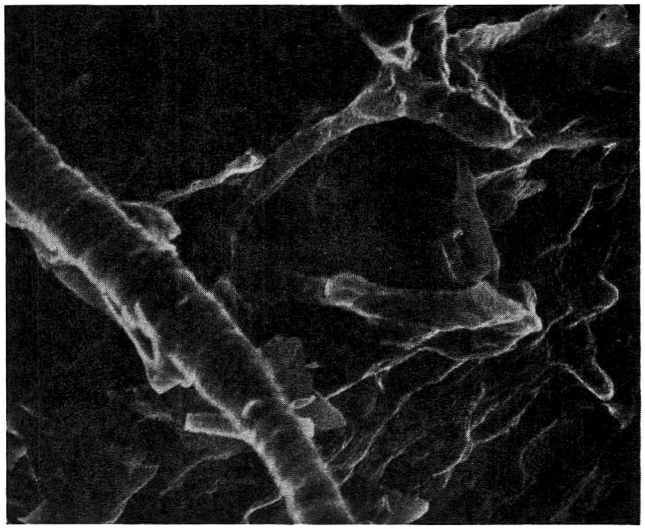


Fig. 4—Scanning electron photomicrograph of Great Northern bean protein concentrates (3750 X).

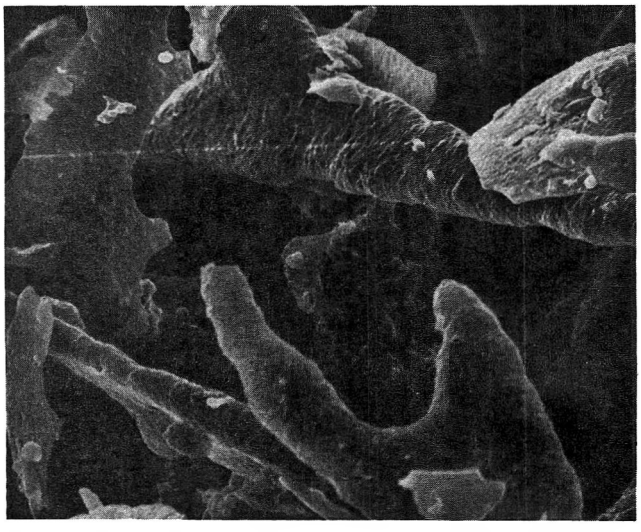


Fig. 3—Scanning electron photomicrograph of Great Northern bean globulins (3750 X).

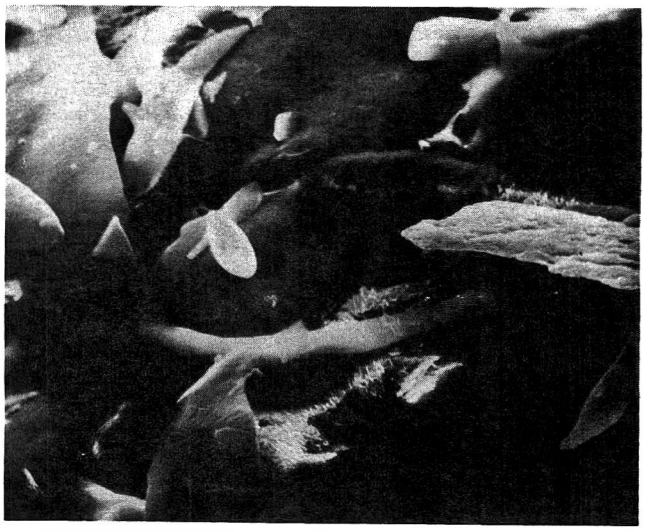


Fig. 5—Scanning electron photomicrograph of Great Northern bean protein isolates (3750 X).

RESULTS & DISCUSSION

THE CRUDE PROTEIN CONTENT of the bean flour, albumins, globulins, protein concentrates, and protein isolates was 26.10, 81.70, 92.30, 85.40, and 92.40% (dry weight basis) respectively (Sathe and Salunkhe, 1981).

Scanning electron microscopy

The scanning electron microscopic observations (Fig. 1-5) indicated that albumins had rod-like structures with relatively smooth surface topography while globulins consisted of different shapes and sizes with relatively rougher surfaces than albumins. Protein concentrates and protein isolates, as expected, consisted of both albumins and globulins, and hence possessed characteristics of both.

Viscosity

The results of viscosity measurements are presented in Table 1. As can be seen from this table, viscosity appeared to be a function of not only the solids concentration but also of the type of proteins. Albumins registered comparable viscosity to that of globulins at corresponding concentrations. Protein concentrates and protein isolates had comparable viscosities at corresponding concentrations. Protein concentrates and isolates, however, were more viscous than both the albumins and globulins at a particular concentration suggesting that when albumins and globulins were together, they behaved in different manner than when they were separate. These data further support our earlier observations on electrophoretic mobility and column chromatographic behavior of the Great Northern bean proteins (Sathe and Salunkhe, 1981).

Gelation

The least gelation concentrations for the bean flour, albumins, protein concentrates, and protein isolates were 10, 18, 8, and 12% (all w/v) respectively. Globulins did not form a firm gel up to 20% (w/v) concentration range. These results further suggest that gelation is not only the function of quantity of protein but also the type of protein(s) and the nonprotein component(s) as well.

Oil and water absorption

Results of the oil and water absorption are embodied in Table 2. Protein concentrates registered the highest oil and water absorption (5.93 and 4.12 g/g, respectively) among all the samples studied. The bean flour had the lowest oil and water absorption (1.67 and 1.0 g/g, respectively). Sosulski and Fleming (1977) reported water absorption by soybean flour, soybean concentrate, sunflower flour, and sunflower concentrate to be 2.4, 3.6, 1.8, and 3.9 g/g. Water absorption by the Great Northern bean flour observed in the present investigation was lower than those by soybean and sunflower flour reported by Sosulski and Fleming (1977). However, water absorption by the Great Northern bean protein concentrates noted in the present investigation (5.93 g/g) was higher than those by soybean and sunflower protein concentrates (Sosulski and Fleming, 1977); succinylated peanut flour (Beuchat, 1977); sunflower protein concentrate (control, succinylated, and acetylated) (Canella et al., 1979); soyflour, soy concentrate, and soy isolate (Lin et al., 1974).

It is known that water binding by proteins is a function of several parameters including size, shape, conformational characteristics, steric factors, hydrophilic-hydrophobic balance of amino acids in the protein molecule, lipids and carbohydrates associated with the proteins, thermodynamic properties of the system (energy of bonding, interfacial tension, etc.), physicochemical environment (pH, ionic strength, vapor pressure, temperature, presence/absence of surfactants etc.), solubility of the protein molecules, and others (Chou and Morr, 1979). However, polar amino groups of protein molecules are the primary sites of protein-water interactions. Cationic, anionic, and nonionic sites bind different amounts of water (Kuntz, 1971). The difference in water absorption by albumins and golbulins (3.18 and 2.77 g/g dry sample, respectively), may have been due to the protein concentration and possibly the conformational characteristics of these proteins. Water absorption by the protein isolates and the globulins (2.73 and 2.77 g/g dry sample, respectively) were comparable.

Oil absorption trends were similar to those for water absorption. Protein concentrates registered the highest oil

Table 1—Viscosity data on Great Northern bean proteins[a]

Sample	Conc % (w/v)	Relative viscosity[b]
Albumins	1	1.21
	2	1.83
	3	2.55
	4	3.66
	5	4.38
	7	9.51
	10	12.11
Globulins	1	1.29
	2	1.65
	3	2.06
	4	3.15
	5	3.71
	7	5.47
	10	10.99
Protein concentrates	1	1.65
	2	2.59
	3	4.59
	4	7.81
	5	12.15
	7	23.25
	10	28.79
Protein isolates	1	1.61
	2	2.57
	3	4.11
	4	6.34
	5	11.34
	7	21.49
	10	32.35

[a] Mean of triplicate analyses
[b] Relative viscosity (relative to distilled water) at room temperature (21°C). The time required for water or sample dispersion to traverse the fixed distance between the two marks on the viscometer was measured.

Table 2—Water and oil absorption capacity of Great Northern bean proteins

Sample	Water absorbed[a] (g/g)	Oil absorbed[a] (g/g)
Bean flour	1.67	1.00
Albumins	3.18	3.29
Globulins	2.77	3.23
Protein concentrates	5.93	4.12
Protein isolates	2.73	1.57

[a] Mean of triplicate analyses

Table 3—Emulsion capacity and stability of Great Northern bean proteins

Sample	Oil emulsified (g/g)	Initial vol. of emulsion (ml)	Vol. of water separated (ml) at room temp (21°C) after time (hr)										
			10	20	35	60	100	120	140	160	180	200	780
Bean flour	39.6	208	104	104	116	120	120	120	120	120	120	120	120
Albumins	63.8	230	0	0	0	0	trace	trace	1	1	1	1	5
Globulins	44.0	218	26	30	32	32	32	32	32	32	32	32	32
Protein conc	72.6	270	0	0	0	0	0	trace	1	1	1	1	30
Protein isolates	63.8	250	150	150	155	155	155	155	155	155	155	155	155

Table 4—Foaming capacity and stability of Great Northern bean proteins

Sample	Wt after whipping (g)	Vol. after whipping (ml)	Vol. increase (%)	Specific vol. (ml/g)	Vol. (ml) at room temperature (21°C) after time (hr)							
					0.5	1.0	1.5	2.0	2.5	3.0	8.0	36.0
Egg albumin	91.50	140	40	1.53	140	135	130	125	125	125	125	115
Bean flour	99.80	132	32	1.32	132	128	110	106	104	102	100	100
Albumins	98.98	180	80	1.82	170	164	160	156	154	150	114	100
Globulins	96.44	140	40	1.45	130	126	120	118	116	114	104	100
Protein conc	99.67	164	64	1.65	150	122	107	104	104	102	100	100
Protein isolates	98.27	106	6	1.10	100	100	100	100	100	100	100	100

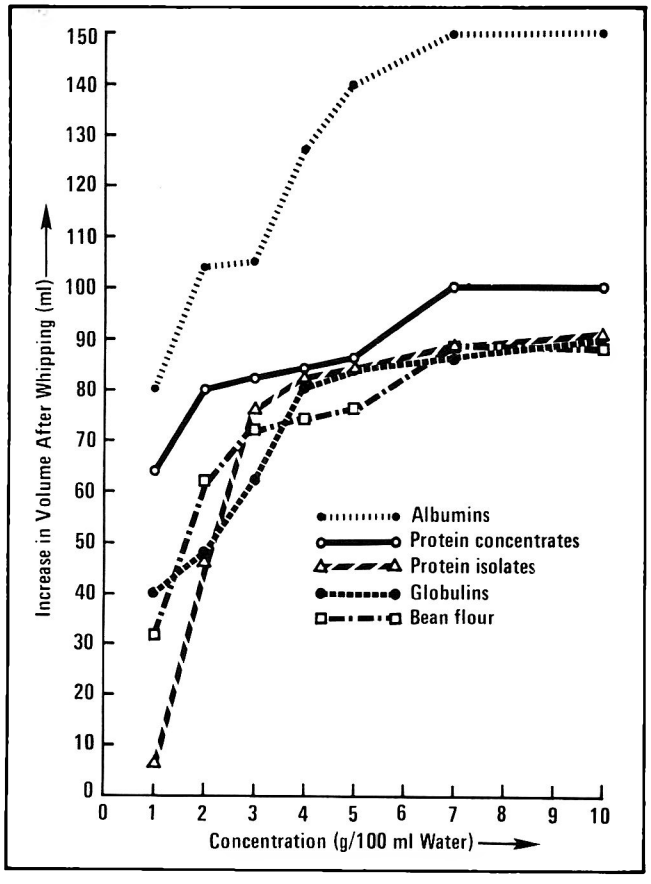


Fig. 6—Effect of concentration on foaming ability of Great Northern bean proteins.

absorption (4.12 g/g dry sample) among all the samples studied, and was higher than the corn oil absorption by the soybean protein concentrates and isolates reported by Lin et al. (1974).

Emulsion capacity and stability

Emulsion capacity of the Great Northern bean flour, albumins, globulins, protein concentrates, and protein isolates was, respectively, 39.6, 63.8, 44.0, 72.6, and 63.8g oil/g dry sample (Table 3). Satterlee et al. (1975) studied the emulsion capacity of the Great Northern bean proteins and found the emulsion capacities of the bean protein concentrates, albumins, and globulins to be 131, 204, and 113 ml oil emulsified/g dry sample. They also found that globulin emulsion was more stable than the albumin emulsion. The present investigations indicated that the albumins were better emulsifiers than globulins in relation to both capacity and stability. Albumins registered the highest emulsion stability (5 ml phase separation ater 780 hr standing at a room temperature of 21°C); protein concentrates, however, had the highest emulsion capacity among all the samples investigated. The differences observed in the emulsion behavior in the present studies and those of Satterlee et al. (1975) may have been predominantly due to the methods of preparation of the protein fractions.

Foaming

The foaming capacity of the albumins was the highest while that of the protein isolates was the lowest among the samples studied (Table 4). Protein concentrates performed better than egg albumin powder in foamability and globulins were comparable to the egg albumin powder. However, none of the samples studied equalled egg albumins in foam stability. Therefore, the foaming ability of Great Northern bean proteins was rated as fair.

Foamability of the Great Northern bean proteins was concentration dependent (Fig. 6). All the samples registered increasing foamability with the increase in concentration of solids, reaching a maximum at 10% (w/v) solids concentration.

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Antioxidant Activity of Methanolic Extracts of Various Oilseed Protein Ingredients

KI SOON RHEE, YOLANDA A. ZIPRIN and KHEE CHOON RHEE

ABSTRACT

Antioxidant activity (AA) of methanolic extracts of defatted flours, concentrates, and isolates produced from glandless cottonseed, peanut, and soybean was determined against linoleate oxidation catalyzed by metmyoglobin (MetMb), Fe^{++}-EDTA (1:1 molar ratio) and fresh beef homogenates and against autoxidation of safflower oil. Total phenolic content of the extracts was also determined. Methanolic extracts of glandless cottonseed protein ingredients showed uniquely and consistently higher AA than those of peanut and soy protein ingredients. Total phenolic content was also markedly higher for the cottonseed protein extracts. Methanol-soluble AA was significantly correlated with total phenolic content for the extracts of defatted flours and concentrates but not for the extracts of isolates. The comparative methanol-soluble AA values of these oilseed protein ingredients were indicative of their antioxidation potential in cooked meat products.

INTRODUCTION

WE REPORTED previously that aqueous extracts of defatted flours, concentrates, and isolates produced from glandless cottonseed, peanut, and soybean showed varying degrees of antioxidative effectiveness for lipid peroxidation catalyzed by metmyoglobin (MetMb), Fe^{++}-EDTA (1:1) and fresh beef homogenates (Rhee et al., 1979). Our investigation of lipid peroxidation in beef patties extended with the same oilseed protein ingredients (Ziprin et al., 1981) suggested that the antioxidant activity (AA) of aqueous extracts of these protein ingredients may not be a reliable predictor of the antioxidative potential of the ingredients when used either as extenders or as functional ingredients in meat products. To be more specific, none of the oilseeds was singularly superior to others in water-soluble AA (Rhee et al., 1979) whereas cottonseed protein ingredients were more antioxidative than peanut and soy protein ingredients when incorporated in beef patties (Ziprin et al., 1981). We speculated that this discrepancy might be attributable to: (1) ineffectiveness of water as an extraction medium of antioxidant compounds in the oilseed protein ingredients; (2) inappropriate choice of model systems to predict lipid peroxidation in meat; or (3) combination of both. With these taken into consideration, we have conducted a further study of oilseed protein ingredients using methanol as the extraction solvent. AA was determined in the three model catalytic systems employed in the previous study (Rhee et al., 1979) and also in an autoxidation system. Phenolic content of the methanolic extracts was assayed to determine the extent of the contribution of phenolic compounds to the observed AA.

Author K.S. Rhee is with the Dept. of Animal Science, Texas Agricultural Experiment Station, Texas A&M Univ., College Station, TX 77843. Author K.C. Rhee is with the Food Protein Research and Development Center, Texas Engineering Experiment Station, Texas A&M Univ. Author Ziprin, formerly with Texas A&M Univ., is now at 3515 Greenridge Circle, Bryan, TX 77801.

EXPERIMENTAL

Preparation of extracts

The same batches of those oilseed protein ingredients tested in the previous study (Ziprin et al., 1981) were used again in the present study. Soy protein ingredients were from commercial sources. Glandless cottonseed (McNair variety) and peanut (Spanish variety) ingredients were prepared on a pilot-plant scale by solvent extraction of the oilseeds and alkali extraction and isoelectric precipitation of proteins, as already described by Ziprin et al. (1981).

The procedure used for preparing methanolic extracts of the protein ingredients was essentially the same as that for the aqueous extracts (Rhee et al., 1979), except that absolute methanol was used in place of distilled water as the extraction medium and the final suspension was filtered by suction instead of centrifugation.

Determination of AA

AA against lipid oxidation catalyzed by MetMb, Fe^{++}-EDTA, or fresh beef homogenates was determined as described by Rhee et al. (1979) with an amount of methanolic extract representing 0.1% (w/v) oilseed protein ingredient in the final O_2 uptake system (60 ml)—an equivalent amount of methanol alone was used for controls. Each sample was determined in duplicate in each separate set of experiments. Within each set, duplicate controls were determined at the beginning and after every other protein extract sample.

To determine AA against autoxidation, 10 ml of an extract (12%) were mixed with 10g safflower oil without additives, the solvent was removed from the oil, and the oil containing the extract residue was stored in a 100-ml beaker at 50°C for 3 days—a control was prepared by mixing 10 ml of methanol with 10g oil. Peroxide value of the oil was determined in duplicate before and after storage according to the AOAC (1975) method and the increase in peroxide value calculated.

Where necessary, data were evaluated by analysis of variance and the Student-Newman-Keuls' test (Steel and Torrie, 1960).

Determination of total phenolic content

Methanolic extract was first hydrolyzed by acid to free bound phenols. An aliquot (0.5 ml) of methanolic extract was placed in a small test tube immersed in a water bath at 45°C and evaporated to dryness under a stream of nitrogen. A few drops of methanol were added to the test tube to redissolve the residue and 1.5 ml of 6% HCl were added. The test tube was then capped and heated in a boiling water bath for 45 min. Two milliliters of diethyl ether were then added to the tube with shaking. The upper layer (ether) was removed and evaporated to dryness under a stream of nitrogen. The residue was redissolved in methanol.

Phenolic content of the hydrolyzed extract (test solution) was determined in duplicate assays by adapting the procedure of Bray and Thorpe (1954) using the Folin-Ciocalteau reagent. Test solution (0.2 ml) was combined with 2.0 ml of 2% Na_2CO_3. After 2 min, 0.2 ml of 50% Folin-Ciocalteau reagent was added and the mixture incubated at room temperature for 30 min. The color was read at 750 mμ against a blank for which 0.2 ml methanol replaced 0.2 ml test solution. Chlorogenic acid was used for constructing a standard curve.

RESULTS & DISCUSSION

FIRST OF ALL, a special remark should be made in regard to the three lipid peroxidation catalytic systems used to determine antioxidant activity of the oilseed product extracts. Since heme and/or nonheme irons have been considered as primary prooxidants in muscle tissue, measure-

ments of linoleate oxidation catalyzed by inorganic iron (as a nonenzymic nonheme iron), MetMb (as a heme iron), or tissue homogenates (providing heme and nonheme iron catalysts; Wills, 1966) have been used in many studies to investigate the mechanism of lipid peroxidation in meat and fish (Fischer and Deng, 1977; Kendrick and Watts, 1969; Kwoh, 1971; Lee et al., 1975). The rate of oxygen uptake of the systems has been measured in those studies to determine the linoleate peroxidation by the catalysts. Fe^{++}-EDTA (1:1 molar ratio) has often been used as a nonheme iron catalyst since the rate of linoleate peroxidation catalyzed by Fe^{++} alone is not fast enough to result in measurable amounts of O_2 uptake within a relatively short period (<10 min) of polarographic measurements of O_2 uptake (Rhee, 1978). Iron chelates of EDTA have been used to produce free radicals in various oxidative reactions (Norman and Smith, 1964; Orr, 1967; Udenfriend et al., 1954).

From our experience with the previous study (Rhee et al., 1979) and the present one, we have concluded that the fresh beef homogenate catalytic system is difficult to use for comparative antioxidant activity studies when the number of test materials within a set of experiments is such that duplicate assays of the materials cannot be completed within several hours using the same batch of beef homogenate. The concentration as well as proportions of myoglobin, MetMb, and oxymyoglobin in a selected fresh beef cut (semitendinosus muscle in the present study) purchased from the retail market varied considerably from day to day. Accordingly, the lipid peroxidation rate of controls (without any oilseed protein extract) with an equal concentration of muscle tissue also varied with the meat. Taking these into consideration we took the following steps to use the beef homogenate system: (1) the concentration of the homogenate used in each set of experiments was so adjusted as to bring the O_2 uptake rates of the controls within a predetermined range, and (2) the cottonseed, peanut and soy products within each protein preparation category (flour, concentrate, or isolate) were tested with the same batch of beef homogenate in each set (replication) of experiments.

AA data of the methanolic extracts are summarized in Table 1. The glandless cottonseed product showed a much higher methanol-soluble AA than the peanut and soy products within the same type of protein preparation (defatted flour, concentrate, or isolate) and with each lipid peroxidation system employed excepting beef homogenate system in which the three isolates were not significantly different in methanol-soluble AA. The relative order in AA of the peanut and soy protein extracts varied with the type of protein preparation and the lipid peroxidation system used for AA determination.

The results of methanol-soluble AA of these oilseed protein ingredients are in agreement with the findings of our previous study (Ziprin et al., 1981) of cooked, ground beef patties extended with the same protein ingredients in that a higher antioxidant potential was shown by the glandless cottonseed protein ingredients over the protein ingredients of the other two oilseeds. When extracted with water, however, none of the oilseeds had a distinctively higher AA (Rhee et al., 1979).

AA of methanolic extract of each oilseed protein ingredient, as shown in Table 1, is much higher than that of the corresponding aqueous extract (Rhee et al., 1979) when the activities are compared on the basis of the amount of oilseed protein material represented in the final oxygen uptake system. Philip (1974) also found a greater AA for petroleum ether extracts, rather than for aqueous extracts, of a textured soy protein product.

An attempt has been made to relate the observed AA to total phenolic content of the methanolic extracts, since most oilseed protein products contain considerable amounts of phenolic compounds (Sosulski, 1979) and many of these compounds are known to possess strong antioxidant properties (Naim et al., 1976; Pratt and Birac, 1979). As shown in Table 2, defatted glandless cottonseed flour had a slightly higher phenolic content than defatted soybean and peanut flours, whereas cottonseed concentrate and isolate were much higher in phenolic content than the correspond-

Table 1—Antioxidant activity[a] of methanolic extracts of various oilseed protein ingredients against linoleate peroxidation catalyzed by MetMb, Fe^{++}-EDTA, and fresh beef homogenates and against autoxidation of safflower oil

Oilseed protein	% Inhibition of linoleate oxidation catalyzed by[b]			% Inhibition of autoxidation of safflower oil[c]
	MetMb	Fe^{++}-EDTA	Beef homogenates	
Flours				
Cottonseed, glandless	67a	88a	51a	92
Peanut	43b	57b	43b	81
Soybean	35b	61b	46b	87
Concentrates				
Cottonseed, glandless	49a	91a	51a	82
Peanut	37b	47b	28b	74
Soybean	24c	31c	32b	68
Isolates				
Cottonseed, glandless	53a	58a	53a	60
Peanut	35b	32c	51a	25
Soybean	23c	47b	48a	52

[a] Expressed as % inhibition of oxidation, 100-(oxidation of sample/oxidation of control) × 100.
[b] Extract equivalent to 0.1% (w/v) product in the final oxygen uptake system. The data represent the means of two independent series (duplicate determinations in each series) of experiments within each protein category and catalytic system. Means in the same column, within a protein type (flour, concentrate, or isolate), which are not followed by the same letter are significantly different ($P < 0.05$).
[c] Extract equivalent to 1.2g product/10g safflower oil.

Table 2—Total phenolic concentration

Oilseed	μg Phenol/ml of 12% extract[a]		
	Defatted flour	Conc	Isolate
Cottonseed, glandless	383a	393a	536a
Peanut	207b	105b	107c
Soybean	366a	25c	190b

[a] Each value represents the mean of two replicates. Means within a column which are not followed by the same letter are significantly different ($P < 0.01$).

Table 3—Correlation coefficients between antioxidant activity (AA) determined in different lipid peroxidation systems and total phenolic content

Type of protein ingredients incl. in computation	Lipid oxidation system used for AA determination			
	MetMb catalysis	Fe^{++}-EDTA catalysis	Beef-homogenate catalysis	Autoxidation
All types (N=9)	0.71*	0.73*	0.70*	0.38
Without isolates (N=6)	0.71	0.91**	0.93**	0.91**
Isolates only (N=3)	0.83	0.91	0.68	0.80

*Significant at $P < 0.05$
**Significant at $P < 0.01$

ing soybean and peanut products.

Correlation coefficients were computed to determine the association between the AA determined in different lipid peroxidation systems and total phenolic content. They were computed in three different ways for each lipid peroxidation system used for evaluating AA: (1) including all types (flours, concentrates, and isolates) of protein preparations; (2) without including isolates; and (3) including isolates only. The reason for this was that isolates data points formed a line independent of that formed by the flours-plus-concentrates ("without isolates") data points in the scatter diagrams (not shown herein) of AA versus phenolic content.

When the data of all types of oilseed protein preparations were included in computation, AA was significantly ($P < 0.05$) correlated with total phenolic content except the AA determined in autoxidation system. The correlation between AA and total phenolic content was highly significant ($P < 0.01$) when the isolates data were excluded; one exception was the correlation between the AA measured in MetMb system and phenolic content. When the data of isolates only were computed, no significant correlation was found between AA and total phenolic content. In general, isolates had lower AA for a given unit of phenolic content than defatted flours and concentrates.

Of the phenolic compounds in oilseeds, flavonoids and phenolic acids may be the most important antioxidant compounds. Many studies directly or indirectly proved that the antioxidative effectiveness of organic solvent extracts of soy protein products are attributable to flavonoids (Hammerschmidt and Pratt, 1978; Hayes et al., 1977; Naim et al., 1976; Pratt and Birac, 1979) and phenolic acids (Pratt and Birac, 1979). Glanded cottonseed contains gossypol, a toxic glandular pigment that is also highly antioxidative. The cottonseed protein ingredients used in the present study, however, were prepared from the kernels of a glandless variety. Glandless and deglanded cottonseed protein ingredients contain flavonoid compounds in sufficient concentrations to discolor food products containing the ingredients (Blouin et al., 1979; Wan et al., 1979). Further research is needed to elucidate the nature of antioxidant compounds in cottonseed, peanut, and other less researched oilseed protein ingredients. In addition, it is desirable to determine any varietal differences in AA of each oilseed.

Finally, it may be concluded that: (1) methanolic extracts of glandless cottonseed protein ingredients are highly antioxidative, more antioxidative than those of peanut and soy protein ingredients; (2) AA of methanolic extracts of these oilseed protein ingredients other than the isolates is significantly correlated with their total phenolic content; and (3) methanol-soluble AA of these ingredients as measured against lipid peroxidation catalyzed by MetMb and Fe^{++}-EDTA may predict the antioxidative potential of the ingredients when used in cooked meat products.

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Characterization of Red Bean (*Phaseolus radiatus* var. *Aurea*) Starch and Its Noodle Quality

CHENG-YI LII and SHUH-MING CHANG

ABSTRACT

Red bean (*Phaseolus radiatus* var. *aurea*) starch was isolated by a wet-milling process and steeping in 0.1% sodium hydroxide which gave the best starch purity. Physical properties of the starch including granule sizes, gelatinization temperature range, iodine affinity, X-ray diffractogram, swelling and solubility pattern, Brabender amylogram, gel strength and degree of syneresis, were examined. Starch noodles were prepared from red bean starch and compared with those from mung bean. Noodle quality was exmained by solid loss during cooking, tensile strength and organoleptic tests. The results indicated that red bean starch noodles gave fairly good quality, although not as good as mung bean starch noodles.

INTRODUCTION

RED BEAN (*Phaseolus radiatus* var. *aurea*) is mainly used as one of the popular ingredients in oriental style desserts (*e.g.*, cake and pie fillings), bean jams (Watanabe and Hando, 1955; Takahashi et al., 1954; Tsuyuki and Itoh, 1976; Tsuyuki et al., 1976; Shiota et al., 1976; Nagasawa et al., 1977) and Japanese confectionery (Matsuhashi and Shimada, 1971). Very few studies have been reported on the starch (Kawamura, 1969; Kawamura and Fukuba, 1957; Tada, 1959) of red bean, although starch is one of the major components and may influence the functional properties of red beans. Hence, investigations of the physicochemical properties of red bean starch and the quality of red bean starch noodles were initiated.

MATERIALS & METHODS

Isolation of starch

Red bean (*Phaseolus radiatus* var. *aurea*), grown in the Southern part of Taiwan, was purchased from the local market. The starch was isolated using the method of Schoch and Maywald, (1968) with some modifications. Red beans were first steeped overnight respectively in three different solutions: water, 0.1% sodium hydroxide, and 0.2% sodium sulfite. The steeped beans were washed with distilled water and ground in a Waring Blendor at low speed for 2 min. The slurry was filtered through polymon bolting cloth (132μ pore size). The filtrate was settled for enough time to sediment the prime starch. The prime starch was reslurried in water and sedimented several times until the upper layer was substantially free of color. The recovered prime starch was then dried in an air oven at 40°C.

Chemical analysis

Moisture, crude protein, ash, crude fat and crude fiber contents of both red beans and starch were determined as described previously (Lii and Chang, 1978). The starch content of the sample was determined according to the method of Hassid and Neufeld (1964). The method of Smith and Caruso (1964) was used to measure the phosphorus content in starch.

Physical properties of the starch

Gelatinization temperature ranges were determined using a polarizing microscope equipped with a Kofler hot stage (Lii and Chang, 1978). Temperatures were recorded that corresponded to loss of birefringence by 2, 50, and 98% of the starch granules observed in the field, which were taken as initial, midpoint, and completion values, respectively.

Iodine affinities were determined, after being defatted with methanol for 24 hr, by potentiometric titration (Schoch, 1964) using a Digital pH meter with a platinum electrode calibrated with a voltage reference cell.

X-ray diffraction patterns were obtained with an X-ray diffractometer (Model ADG-301E, Tokyo Shibaura Co.) after the starch was saturated with water vapor overnight in a closed container (Zobel, 1964).

Swelling power and solubility were determined from 65-95°C, at 10°C intervals, by the method of Leach et al. (1959). Four grams of starch were used for the measurement.

The degree of syneresis of starch gels was determined from the volume of water separated from the gel after storage at 4°C for 24 hr. The amount of water was measured by centrifugation at 8,000 rpm for 15 min (Yang et al., 1980).

Gel strength was measured by an Autograph S-100 with a 10-kg loading cell (Shimadzu Seisakusho Co. Ltd, Kyoto, Japan) following the method described previously (Yang et al., 1980).

Brabender viscosity curves were obtained with a Brabender viscoamylograph, using 6, 7, and 8% starch concentration, according to the procedure of Mazurs et al. (1957).

Noodle preparation (Chen, 1978)

95% Starch was mixed with 5% gelatinized starch, prepared by heating a starch slurry at 95°C in a water bath, to form a dough with a glossy, smooth surface. The dough, containing approximately 54% moisture, was placed in a 20-ml syringe and extruded directly into a hot water bath (90-95°C) for 10-20 sec. The noodles were immediately transferred to cool water, kept there for 3-5 min, hung on a bamboo pole, and frozen at −10°C for 24 hr. After thawing in cool water for 2 hr, the noodles were air-dried at 40°C in an oven. The dried noodles were placed in a polyethylene bag and stored in a desiccator. This is the most common process for commercially manufacturing starch noodles, except that a syringe is used in this study instead of extruder.

Physical properties of starch noodles

Solid loss during cooking. Noodles (5g), cut into 3-5 cm lengths, were added to a beaker containing about 200 ml of boiling, distilled water on a hot plate. The beaker was covered with a watch-glass, and cooking was continued for 2 hr. Boiling, distilled water was added every 30 min to compensate for evaporation losses; the mixture was stirred slightly with a glass rod. The cooked noodles were filtered through a nylon screen; the beaker, noodles, and screen were washed with distilled water. The combined filtrate and washings were placed in a washed, dried, tared beaker (W_1), and dried at 80°C, then 110°C, in an oven to constant weight (W_2). The moisture content of the noodles was also determined (W_3). The solid loss during cooking was calculated by the equation:

$$\text{Solid loss (\%) during cooking} = \frac{W_2 - W_1}{5 - W_3} \times 100$$

Tensile strength. Noodles were cooked in boiling, distilled water and removed at definite intervals (5, 10, and 30 min). After cooling at room temperature, the tensile strength was measured using an Autograph S-100 with a 1-kg loading cell. The crosshead speed was 20 mm/min.

Organoleptic test. Noodles made from mung bean starch, red bean starch and mixed bean starch (50% red bean and 50% mung

Authors Lii and Chang are with the Institute of Chemistry, Academia Sinica, Nankang, Taipei, Taiwan, R.O.C.

bean) were cooked with the proper amount of water for 10 min. Texture was evaluated by 10 experienced judges.

RESULTS & DISCUSSION

Proximate composition of red bean

The proximate compositions of red beans are listed in Table 1. Red beans contained about 46% starch and 25% crude protein. These values were approximately the same as those of other legume seeds (Yang et al., 1980; FIRDI, 1973).

Starch isolation

As shown in Table 2, the three different steeping solutions gave similar starch products. Since the amount of protein is usually considered as an index of the purity of legume starch, the starch isolated with sodium hydroxide was used for further studies. The phosphorus content of red bean starch was 0.10 mg/g which was less than that in kidney bean starch (Yang et al., 1980).

Physical properties of the starch

Microscopically most red bean starch granules had irregular shapes with deep fissures (Fig. 1A), which may be due to the way in which the granules were packed within the protein matrix in the endosperm (Lii and Lineback, 1977). Clearly centric birefringence was observed when the granules was examined under the polarized light (Fig. 1B). The sizes of the granules ranged from 25-67μ which were relatively larger than those of other legume starches (Kawamura, 1969; Yang et al., 1980; Chen, 1978; Lai and Varriano-Marston, 1979; Lineback and Ke, 1975).

The gelatinization temperature range of red bean starch was 63-66.5-70°C (Table 3). The range is close to those of chick pea and horse bean starches (Lineback and Ke, 1975), but is lower than those of most other legume starches, such as mung bean (Chen, 1978), kidney bean (Yang et al., 1980), and black bean (Lai and Varriano-Marston, 1979). As measured by potentiometric titration, the iodine affinity value of the starch was 4.83%. The value is much lower than those of other legume starches which are generally in the range 6.0-7.5% (Schoch and Maywald, 1968; Tolmasquim et al., 1971).

The swelling power and solubility patterns of the starch are shown in Figure 2. The swelling power of red bean starch is of the restricted type, like mung bean starch (Chen, 1978), but is less restricted than that of black bean, yellow pea and navy bean (Lai and Varriano-Marston, 1979; Schoch and Maywald, 1968). The solubility pattern, as usual, paralleled the swelling power.

The Brabender viscosity patterns of the starch pastes from 6-8% concentrations gave no pasting peak during cooking. Neither did they show a breakdown of the hot paste. Such a pattern is similar to those of most legume starch pastes and could be classified into type C, according to Schoch's Classification (Schoch and Maywald, 1968). The iodine affinity value of the red bean starch was not high (4.83%), but the starch had high hot paste stability. This phenomenon seems peculiar. The results also indicated that the different steeping solutions used during starch isolation did not affect the viscosity pattern (Fig. 3). However, they did influence the pasting viscosities.

The strength and the degree of syneresis of red bean starch gels at various concentrations are shown in Table 4. The results indicated that the degree of syneresis of red bean starch gel is higher than that of mung bean and less than those of kidney bean and pea starch gels (Yang et al., 1980). The degree of syneresis increased as the concentration of the starch gel decreased, which is similar to other legume starches being studied (Lii, unpublished results). The gel strength of red bean starch was much weaker than those of mung bean, pea and kidney bean starches (Yang

Table 1—Proximate composition of red bean

Composition	% of dry weight basis
Crude protein	24.83
Crude fat	2.75
Crude fiber	4.84
Ash	3.66
Starch	46.43

Table 2—Composition of Red Bean Starch

Composition (%, Dry wt basis)	Steeping solution		
	Water	Sodium hydroxide	Sodium sulfite
Crude protein	0.20	0.13	0.15
Crude fiber	0.01	0.01	trace
Crude fat	0.02	0.01	0.04
Ash	0.06	0.05	0.02
Phosphorus (mg/g)	–	0.10	–

Table 3—Properties of red bean starch[a]

Shape	Irregular
Size (μ)	25-67
Gelatinization temp range (°C)	63-66.5-70
I_2 affinity (%)	4.83

[a] Isolated after steeping in 0.1% sodium hydroxide

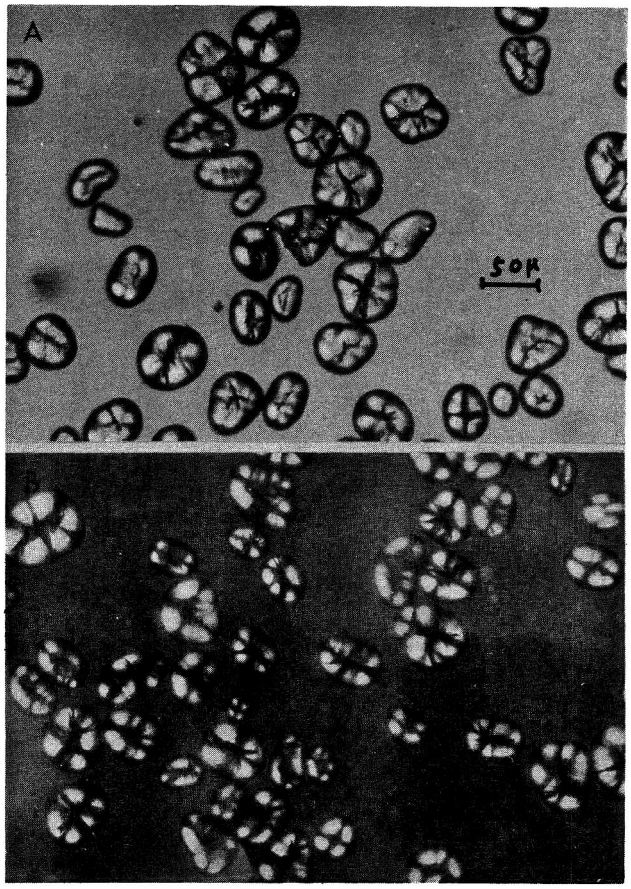


Fig. 1—Red bean starch granules: (A) under normal light; (B) under polarized light.

et al., 1980). This may be attributed to its low iodine affinity value and resulting lower amylose content. The lower amylose content may cause less retrogradation of the starch during the gel formation, and it resulted in the weaker gel structure.

The X-ray diffractogram of the native starch granule of red bean showed an A-pattern (Zobel, 1964).

Noodle qualities

Noodle qualities were evaluated by the amount of solid loss during cooking, the tensile strength, and a taste panel. Since mung bean starch has been the best raw material for starch noodle preparation (Chen, 1978), mung bean starch noodles were prepared as a reference. Solid loss was higher for red bean starch noodles than for mung bean starch noodles and noodles prepared from mixed (1:1) red bean and mung bean starches (Table 5). However, the 5.77% solid loss was still far below the acceptable 10% level set by the Chinese National Standards for starch noodles (CSB, 1963).

As Table 6 shows, the tensile strengths of the noodles decreased in the order: mung bean, mixed 1:1 red bean and mung bean, and red bean. This may be explained by the lower content of linear fractions in red bean starch which may cause less retrogradation of the starch in the noodle. Consequently, it resulted in a higher amount of solid loss and less tensile strength of the red bean starch noodles.

Organoleptic evaluation (Table 7) indicated that noodles made from mung bean, and mixed bean starches had the similar scores based on texture. Red bean starch noodles were slightly softer in texture.

From previous studies (Lii et al., 1979), we suggested that an ideal starch for noodle manufacturing should have high amylose content or high iodine affinity value (6-7%), restricted swelling, and a C-type Brabender viscosity curve. Mung bean starch possess these three characters. Red bean starch has restricted swelling and a C-type Brabender amylogram, but its iodine affinity (4.83%) was not as high as that of mung bean. Consequently, starch noodles pre-

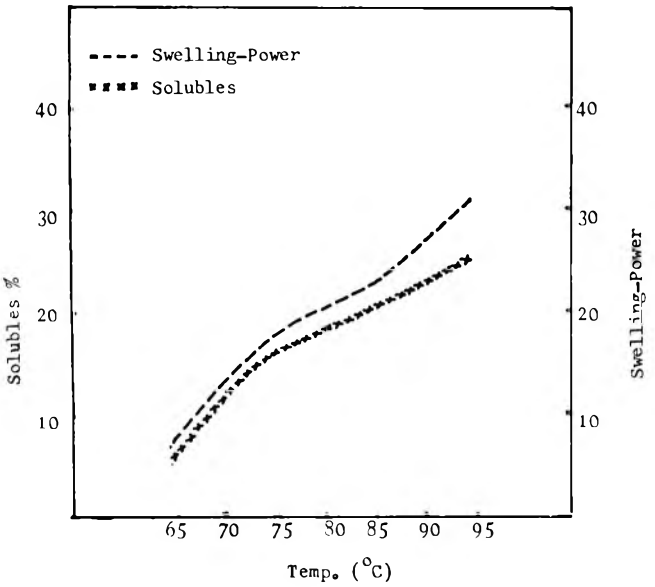


Fig. 2—Swelling power and solubility of red bean starch.

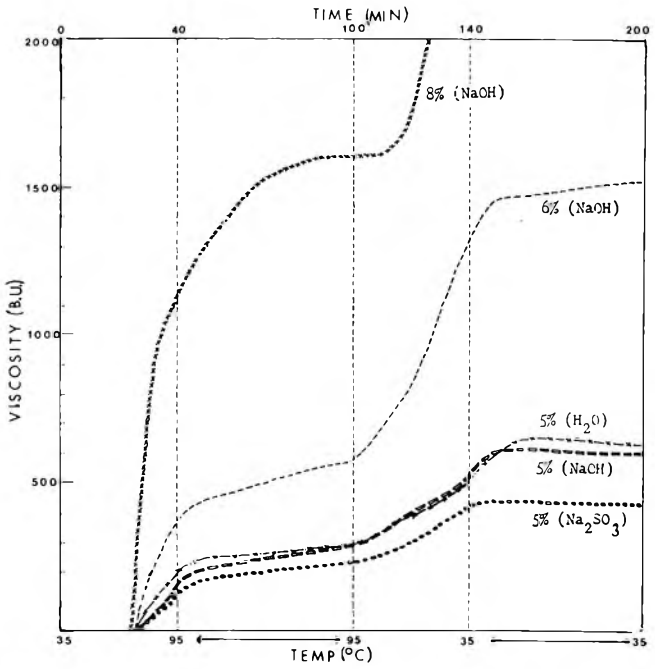


Fig. 3—Brabender viscograms of red bean starch at different concentrations as isolated from different steeping solutions.

Table 4—Gel strength and degree of syneresis of red bean starch at various concentrations

Conc	Gel strength (g) At room temp	Gel strength (g) After 24 hr at 4°C	Degree of syneresis[a] (ml H_2O)
7%	120	218	6.7
9%	768	1090	0.4

[a] The degree of syneresis was determined as the volume of water separated from 30 ml of the starch gel after storage at 4°C for 24 hr.

Table 5—Solid loss of various bean starch noodles[a]

Sample	Solid loss %
Red bean starch noodle	5.77
Mung bean starch noodle	3.20
50% red bean and 50% mung bean starch noodle	3.85

[a] The diameter is approximately 1 mm.

Table 6—Tensile strength of various bean starch noodles[a]

Cooking time (min.)	Tensile strength (g) Red bean starch noodle	Tensile strength (g) Mung bean starch noodle	Tensile strength (g) 50% red bean and 50% mung bean starch noodle
5	3.8	6.6	4.2
10	1.6	13.0	4.0
30	ND[b]	7.4	3.6

[a] The diameter is approximately 1 mm.
[b] The noodle is too soft to measure.

Table 7—Organoleptic evaluation of various bean starch noodles[a]

Noodle	Score[b]
Red bean starch	4.8
Mung bean starch	3.7
50% red bean and 50% mung bean starches	3.6

[a] Cooking time was 10 min.
[b] Score 1-7: 1 = toughness; 4 = optimum; 7 = softness.

pared from red bean were relatively good, but not as good as from mung bean.

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Solubilization and Electrophoretic Characterization of the Great Northern Bean (*Phaseolus vulgaris* L.) Proteins

S.K. SATHE and D.K. SALUNKHE

ABSTRACT

Protein content of dry beans (*Phaseolus vulgaris* L.) cultivar Great Northern, was 26.10% (dry weight basis). The isoelectirc pH of the NaCl extractable proteins was about 4.4. Several salts, NaOH, and HCl were employed to solubilize the Great Northern bean proteins. Amongst all the protein solubilizing agents, Na_2CO_3, K_2SO_4, sodium dodecyl sulfate (SDS), and NaOH at respective concentrations of 0.5, 5, 5% (all w/v), and 0.02N were found to be better protein solubilizers than the rest; solubilizing 93.6g of Lowry protein per 100g of Kjeldahl protein. Albumins and globulins accounted for 21.18 and 73.40% respectively, of the total bean proteins. Protein content of albumins, globulins, protein concentrates, and protein isolates was 81.68, 92.26, 85.44, and 92.43% (dry weight basis) respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the bean flour, albumins, globulins, protein concentrates, and protein isolates revealed the presence of 22, 14, 10, 14, and 11 subunits, respectively. The bean flour, albumins, globulins, protein concentrates and protein isolates were characterized by the predominance of subunits with apparent molecular weights of 294,000, 266,000, 123,000, 146,000, and 135,000 daltons, respectively. Isoelectric focusing of the bean flour, albumins, globulins, protein concentrates, and protein isolates indicated 15, 13, 15, 16, and 11 subunits, respectively. Molecular sieve chromatography of the bean flour proteins, albumins, and globulins followed by SDS-PAGE was also employed to study the complexities of these proteins.

INTRODUCTION

THE DIETARY IMPORTANCE of legume proteins is well recognized. The economics of legumes is significant. In the U.S., the dry bean, *Phaseolus vulgaris* L. is an important food crop in view of both nutrition and economics. The western states contribute about 40% of the U.S. crop of dry beans, 30% of the processed beans, and all of the seeds of dry and green beans. The availability of food legumes on a global scale shows a wide range of 1.1 g/day/person in Denmark to 135.6 g/day/person in Burundi (Milner, 1973). Remarkably, in contrast to the Western European countries, dry bean consumption in the U.S. has remained at a fairly constant level of 8.5 g/day/person over the past 70 years (Hellendoorn, 1979). Although legumes are rich protein sources (20-40% protein on dry weight basis) their underutilization is evidenced, which can be attributed primarily to three factors: viz. (a) presence of several antinutrients (trypsin, chymotrypsin, amylase inhibitors, lectins); (b) low protein quality (deficiency of sulfur containing amino acids); and (c) presence of flatulence causing agents (oligosaccharides of raffinose family, i.e., stachyose, verbascose, and raffinose).

Legume proteins are complex in nature, but the majority of proteins are globulins and albumins. Due to the ease and rapidity, polyacrylamide gel electrophoresis had been a method of choice to study seed proteins. Numerous investigators employed electrophoretic techniques to study several legume proteins, e.g., *Phaseolus* (Derbyshire and Boulter, 1976; Rubin and Cousins, 1976; Padhye and Salunkhe, 1977; Bollini and Chrispeels, 1978; Romero and Ryan, 1978). Due to uniformity, stability, and additive nature of seed protein profiles, electrophoresis of seed proteins has proved to be an important tool for taxonomic and evolutionary studies of legumes. Extraction procedures (Ladizinsky and Hymowitz, 1979); maturity (Robinson and Megarrity, 1975); intrinsic changes in plant (chromosomal rearrangement, doubling of the chromosome numbers) (Nakai, 1977) do not have significant effects on the conspicuously species specific seed protein profile.

The purpose of the present investigations was to study the solubility and electrophoretic behavior of proteins of *Phaseolus vulgaris* L. cultivar Great Northern.

Authors Sathe and Salunkhe are with the Dept. of Nutrition & Food Sciences, Utah State Univ., Logan, UT 84322.

MATERIALS & METHODS

GREAT NORTHERN BEANS were purchased from Bean Growers' Warehouse, Filer, Idaho, and stored at 4°C until experiments were conducted. Beans were ground to 20 mesh (bean flour) in a Fitz mill (The W.J. Fitzpatrick Co., Chicago, Ill.) and were employed throughout.

Chemicals

Following were the sources of chemicals. Acrylamide, N,N'-methylene-bisacrylamide, N,N,N',N'-tetramethylethylenediamine, Naphthol blue black (CI 20470) from Eastman Kodak Co., Rochester, N.Y.; Urea (ultrapure), Sucrose (density gradient grade) from Schwarz/Mann, Orangeburg, N.Y.; Ampholine (pH 3.5-10) from LKB, Stockholm, Sweden; Bio-Gel A 0.5 from Bio-Rad Laboratories, Richmond, Calif.; Tris hydroxymethyl aminomethane (Tris), enzyme grade, from United States Biochemical Corp., Cleveland, Ohio; Ribonuclease A (MW 13,700), Chymotrypsinogen A (MW 25,000), Ovalbumin (MW 45,000), and Aldolase (MW 158,000) as molecular weight markers from Pharmacia Fine Chemicals, Piscataway, N.J. All other chemicals were of reagent grade.

Protein fractionation and determination

Albumins, globulins, protein concentrates, and protein isolates were prepared from the bean flour by the process outlined in Figure 1. The bean flour to the solvent ratio was 1:10 (w/v), throughout. The protein contents of the bean flour, albumins, globulins, protein concentrates, and protein isolates were determined by the Kjeldahl method (N X 6.25). All the analyses were conducted in triplicate and means reported.

Moisture determination

Moisture content of the bean flour, albumins, globulins, protein concentrates, and protein isolates was determined (in triplicate) by drying at 115°C for 6 hr (until the sample reached constant weight) and means reported.

Protein solubilization

To 1g of bean flour 25 ml of the respective extractant was added, mixed thoroughly, and allowed to stand at room (21°C) temperature for 12 hr (flasks covered with parafilm). The slurries were then centrifuged at 5000 X G for 45 min and protein contents of supernatants determined by Lowry's method (Lowry et al., 1951). The pH of each supernatant was also determined.

Protein solubility

The bean flour (25 g) was extracted with 250 ml of 2% NaCl by blending for 2 min (a Waring Blendor at "HI" speed) followed by magnetic stirring (1 hr). The slurry was then centrifuged (10,-

000 X G, 30 min) and pH of portions (10 ml) of supernant was adjusted to a desired value by 0.1N HCl or 0.1N NaOH. The portions (pH adjusted) were magnetically stirred for additional 15 min and centrifuged (10,000 X G, 30 min). The absorbance of the supernants was measured at 280 nm.

Polyacrylamide gel electrophoresis

SDS-PAGE was carried out according to the procedure of Weber and Osborn (1969) employing Ribonuclease A (MW 13,700), Chymotrypsinogen A (MW 25,000), Ovalbumin (MW 45,000), and Aldolase (MW 158,000) as molecular weight markers. The freeze-dehydrated samples (10 mg) were dissolved in 0.01M sodium phosphate buffer (pH 7.0) containing 2% of each of SDS and beta-mercaptoethanol. Gels, 11 X 0.5 cm (length X diameter) (10% monomer concentration) were loaded with about 100 microliters of sample (incubated at 50°C for 12 hr, prior to electrophoresis) and electrophoresis conducted for 10.5 hr with a 6 mA current per gel. Staining and destaining was accomplished respectively by naphthol blue black (CI 20470) and 10% acetic acid containing methanol (10:1 v/v). Phosphate buffer (0.02M, pH 7.2) served as electrode buffer (for both cathode and anode).

Phenol-acetic acid-mercaptoethanol-urea polyacrylamide gel electrophoresis (PAMU-PAGE) was conducted according to the procedure of Catsimpoolas et al. (1968). Electrophoresis was carried out for 8 hr at 6 mA per gel (11 X 0.5 cm, length X diameter).

Isoelectric focusing was performed according to the method of Llewellyn and Flaherty (1976). Gels (5% monomer concentration), 11 X 0.5 cm (length X diameter) were employed. Freeze-dehydrated samples (10 mg) were dissolved in 10 ml of distilled water 6M in urea, 2% in beta-mercaptoethanol, and 5% in sucrose. Twenty-five microliters of the samples were loaded on gels and electrophoresis conducted for 4 hr during which current of 1 mA per gel was reduced to 0.28 mA per gel. Gels were stained (2 hr) with 0.2% bromophenol blue and destained in ethyl alcohol-acetic acid-water (6:1:13 v/v/v). The pH gradient across the gel was constructed as follows. Five gels (blanks electrophoresed under same conditions) were cut in 1 cm length sections and soaked in distilled water (2 ml) for 24 hr at room (21°C) temperature and pH measured. Means were employed for construction of the pH gradient.

Column chromatography

The bean flour (5g), albumins (1g), and globulins (1g) were extracted in 20 ml Tris buffer (0.02M, pH 8.2) each, at room (21°C) temperature for 2 hr and centrifuged at 12,000 X g for 30 min and supernatants applied on column (92 X 2.6 cm, length X diameter) packed with Bio-Gel A 0.5 (range 10,000-500,000 daltons). The eluent was 0.02M Tris buffer (pH 8.2). A total of 140 fractions (100 drops i.e. about 4.7 ml each) were collected and absorbance read at 280 nm in a Gilford dual beam spectrophotometer (Gilford Instruments, Oberlin, Ohio). The fractions (corresponding to respective peaks) were pooled, dialyzed against distilled water for 24 hr at 4°C with 4 changes of distilled water and freeze-dehydrated. The dry fractions were then electrophoresed (SDS-PAGE) for 5 hr at 8 mA per gel (6 X 0.5 cm, length X diameter). The samples were prepared by dissolving 2-5 mg of fraction in 1 ml of the sample solution and 50 microliters applied on gels.

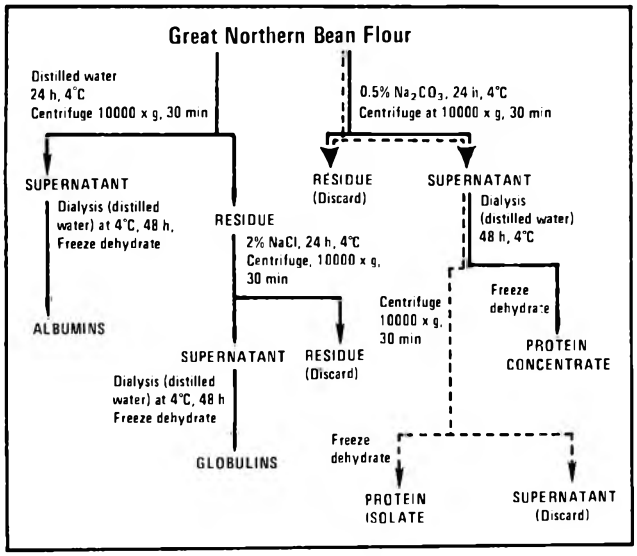


Fig. 1—Fractionation of the Great Northern bean proteins.

Table 1—Proteins and moisture contents of Great Northern bean flour, albumins, globulins, protein concentrates, and protein isolates

Sample	Moisture %	Crude protein (as is basis) %	Crude protein (dry wt basis) %
Bean flour	15.20	22.17	26.10
Albumins	10.36	73.22	81.68
Globulins	6.18	86.89	92.26
Protein concentrates	8.59	78.00	85.44
Protein isolates	2.82	89.82	92.43

RESULTS AND DISCUSSION

Protein and moisture analyses

The proteins and moisture contents of the bean flour, albumins, globulins, protein concentrates, and protein isolates are represented in Table 1. The protein content of the bean flour, albumins, globulins, protein concentrates, and protein isolates was 26.10, 81.68, 92.26, 85.44, and 92.43% (dry weight basis), respectively. Extractable albumins and globulins accounted for 21.80 and 73.40% of the total bean proteins, respectively.

Protein solubility

The protein solubility (proteins extractable by 2% NaCl) was minimum in the pH range 4.2-4.4 (Fig. 2). The protein solubility pattern indicated higher protein solubilization at alkaline pH compared to acid pH with a minimum at a pH of about 4.4. Similar observations were reported by Fan and Sosulski (1974) and Thompson (1977).

Protein solubilization

Solubilization of the Great Northern bean proteins by different extractants is summarized in Tables 2-5. Extrac-

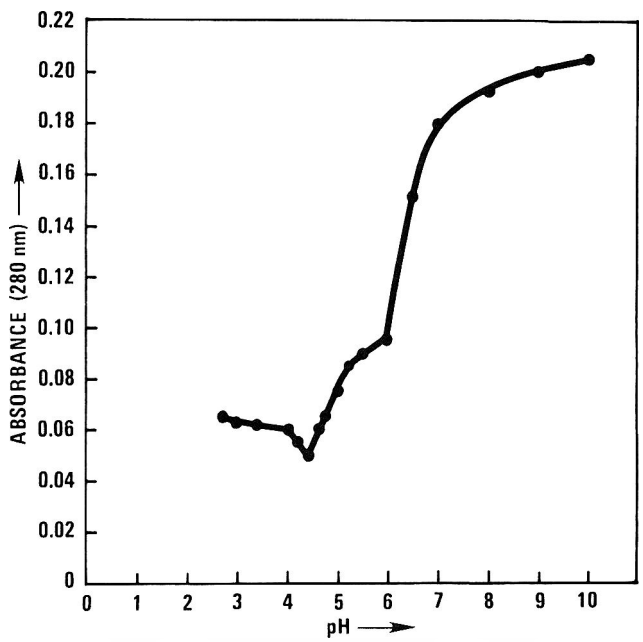


Fig. 2—Solubility profile of the Great Northern bean proteins

tion was performed only once (by respective extractant) as the purpose was to determine the relative efficiency of the extractants to solubilize the bean proteins. Data were grouped on the basis of type of solubilizing agents employed. Na_2CO_3 (0.5%), K_2SO_4 (5.0%), SDS (5.0%), and NaOH (0.02N and 1N) were found to be the most effective protein solubilizers amongst all the solubilizing agents employed (all of them solubilized 93.6g of Lowry protein per 100g of Kjeldahl protein). Dimethyl formamide was the poorest protein solubilizer among the solubilizing agents studied (82.50-92.50 mg of Lowry protein solubilized per 100g of Kjeldahl protein). These observations are consistent with those of Padhye and Salunkhe (1977). The differences in protein solubilizing capacity between and within the extractants, observed in the present investigation are not surprising. It is well known that the solubility of proteins is a function of several parameters including type of proteins, proteins to solvent ratio, particle size of the bean flour, temperature and length of time of extraction, pH, ionic strength, type of solubilizer, mechanical treatments during extraction, and others. Recently, Kazazis and Kalaissakis (1979) studied the nitrogen extraction from vetch seeds (*Vicia sativa*) by employing solutions of neutral and alkaline salts. They found that sodium hydroxide extracted most nitrogen (93.6%) at 0.01 molarity in 120 min at a flour to solvent ratio of 2% (w/v). They also reported that stirring was slightly more efficient than shaking. Nitrogen solubility ranged from 35.8% (by 0.01M KH_2PO_4 at pH 6.2) to 83.8% (by 0.01M NaOH at pH 8.8) at bean flour to solvent ratio of 6% (w/v) in their studies which included distilled water, NaCl, Na_2SO_4, Na_2HPO_4, KH_2PO_4, $CaHPO_4 \cdot H_2O$, NaOH, and $Ca(OH)_2$. Wu and Sexson (1975) reported that particle size was increasingly important at higher pH values (pH 11.2) in case of wheat proteins solubilization. Miers et al. (1977) observed similar dependence of protein solubilization on particle size in California small white beans (*Phaseolus vulgaris* L.). Similar observations on several legumes including soybean (*Glycine max*), white lupine (*Lupinus albus*), small fababean (*Vicia faba* minor), white pea bean (*Phaseolus vulgaris*), mung bean (*Vigna radiata*), field pea (*Pisum sativum* arvense), baby lima bean (*Phaseolus lunatus*), lentil (*Lens culinaris*), and chickpea (*Cicer arietinum*) employing 0.02% NaOH for extraction purpose were reported by Fan and Sosulski (1974).

Table 2—Effects of salts and their concentrations on solubilization of Great Northern bean proteins

Salt	%	pH	Solubilized protein mg/g bean flour
NaCl	0.5	6.20	137.50
	1.0	6.20	122.50
	2.0	5.70	150.00
	5.0	5.90	157.50
	10.0	5.80	83.75
Na_2SO_4	0.5	6.30	145.00
	1.0	6.00	145.00
	2.0	5.95	161.25
	5.0	5.80	155.00
	10.0	6.00	145.00
Na-acetate	0.5	6.50	117.50
	1.0	6.50	125.00
	2.0	6.70	121.25
	5.0	6.80	155.00
	10.00	7.00	136.25
Na_2CO_3	0.5	10.00	207.50
	1.0	10.10	185.00
	2.0	10.40	187.50
	5.0	10.60	185.00
	10.0	10.80	175.00
KCl	0.5	6.75	127.50
	1.0	6.90	175.00
	2.0	6.35	175.00
	5.0	6.28	187.50
	10.0	6.20	170.00
K_2SO_4	0.5	5.92	168.75
	1.0	6.00	185.00
	2.0	6.20	197.50
	5.0	6.00	207.50
	10.0	6.10	177.50

Table 3—Effects of phosphates and their concentrations on solubility of Great Northern bean proteins

Salt	%	pH	Solubilized protein mg/g bean flour
Sodium dihydrogen phophate	0.5	5.10	122.50
	1.0	5.50	82.50
	2.0	5.20	158.75
	5.0	4.80	152.50
	10.0	4.58	175.00
Sodium metaphosphate	0.5	5.00	85.00
	1.0	5.90	175.00
	2.0	5.70	175.00
	5.0	5.50	170.00
	10.00	5.30	135.00
Tetrasodium pyrophosphate	0.5	7.40	168.75
	1.0	7.30	175.00
	2.0	8.00	175.00
	5.0	8.90	175.00
	10.00	8.80	175.00
Sodium tripolyphosphate	0.5	7.50	198.75
	1.0	6.90	168.75
	2.0	7.05	165.00
	5.0	7.00	137.50
	10.0	7.00	155.00

Table 4—Effects of dissociating agents and their concentrations on solubility of Great Northern bean proteins

Salt	%	pH	Solubilized protein mg/g bean flour
Urea	0.5	7.20	155.00
	1.0	7.30	147.00
	2.0	7.60	160.00
	5.0	8.16	156.25
	10.0	8.18	165.00
SDS	0.5	7.10	133.75
	1.0	7.20	133.75
	2.0	7.20	196.25
	5.0	7.32	207.50
	10.0	7.35	185.00
Dimethyl formamide	0.5	5.98	92.50
	1.0	6.45	83.75
	2.0	6.30	92.50
	5.0	6.40	82.50
	10.0	6.50	82.50

Table 5—Effects of alkali/acid and their concentrations on solubility of Great Northern bean proteins

Alkali/acid	Normality	pH	Solubilized protein mg/g bean flour
NaOH	0.02	9.20	207.50
	0.10	10.15	168.75
	1.00	12.50	207.50
HCl	0.10	1.50	168.75
	1.00	0.50	133.75

Padhye and Salunkhe (1977) observed that lower concentrations of polyphosphates solubilized more proteins than at higher concentrations in case of black gram (*Phaseolus mungo*) proteins. Thompson et al. (1976) found 2% sodium hexametaphosphate level to be optimum for rapeseed protein solubilization. Combination of NaCl with phosphates (in the form of buffer) has also been employed for solubilizing the globulins of pigeon pea (*Cajanus cajan* Mill sp.) by Gopalakrishna et al. (1977). They reported 78% solubilization of proteins by employing 0.15M phosphate buffer (pH 7.2) containing 10% NaCl at 4°C (bean flour to solvent ratio being 5:1) as solubilizing agent. In the present investigation, phosphates were found to be less effective in solubilizing the Great Northern bean proteins than Na_2CO_3, NaOH, SDS, and K_2SO_4; were comparable to NaCl, KCl; and more efficient than Na-acetate, urea, and dimethyl formamide. Within the phosphates, sodium tripolyphosphate at 0.5% concentration registered maximum (198.75 mg/g bean flour) protein solubilization. Lower concentrations (1-2%)of phosphates favored more solubilization of the Great Northern bean proteins with the exception of sodium dihydrogen phosphate which solubilized 175 mg/g bean flour at 10% concentration. These observations are in close agreement to those reported by Padhye and Salunkhe (1977) and Thompson et al. (1976).

Although alkaline pH proved to be an effective solubilizer of the Great Northern bean proteins, the changes such as destruction of lysine and cystine, formation of lysinoalanine, racemization which may occur during extraction, may reduce the protein quality. Nashef et al. (1977) have recently reported effects of alkali on proteins in a comprehensive manner. They found that the alkali treatment of proteins (10^{-5}M protein in 0.1M NaOH at 50°C for 24 hr) resulted in loss of lysine and cystine and also the formation of new amino acids such as, lysinoalanine, ornithoalanine, beta-aminoalanine, lanthionine, and unknown amino acids through reactions between dehydroalanine and histidine and tryptophan. The mechanism of action of alkali involved beta elimination of the disulfides resulting in production of dehydroalanine which in turn reacted with epsilon-amino groups of lysine, sulfur of cystine, and nitrogen of ammonia; through its double bond leading to the formation of lysinoalanine, lanthionine, and beta-aminoalanine, respectively.

The solubility of proteins depends upon hydration. Legume proteins are difficult to solubilize due to two factors: (a) globular proteins are predominant in legumes, and (b) the hydrophobicity of globular proteins. Denaturation of globular proteins would theoretically increase the access to polar residues for hydration which in turn would result in better hydration (and then solubilization) of globular proteins. Perutz (1978) discussed denaturation of proteins by acid and alkali. He stated that the free energy of a spherical polyelectrolyte is proportional to the square of its net surface charge which implies that the stability of such molecule decreases on either side of its isoelectric point. Ionization of nonpolar groups that lie in the interior of the molecule by alkali or acid leads these groups to attract hydration shells which are misfits in the native structure thus shifting the equilibrium toward the unfolded form. He further stressed that the electrostatic interactions (ionization of interior nonpolar groups) are more important in hydration of proteins than the surface charge. This may partially explain the improved protein solubilization obtained in the present investigation, at the alkaline pH.

SDS-PAGE and PAMU-PAGE

Eletrophoresis of the bean flour, albumins, globulins, protein concentrates, and protein isolates in the SDS-PAGE system (Fig. 3) brought about better resolution of protein subunits as compared to the PAMU-PAGE system (Fig. 4). The SDS-PAGE electrophoresis revealed that the bean flour, albumins, globulins, protein concentrates, and protein isolates (solubilized by SDS, beta-mercaptoethanol, $NaH_2PO_4 \cdot H_2O$, and $Na_2HPO_4 \cdot 7H_2O$; solvent system)

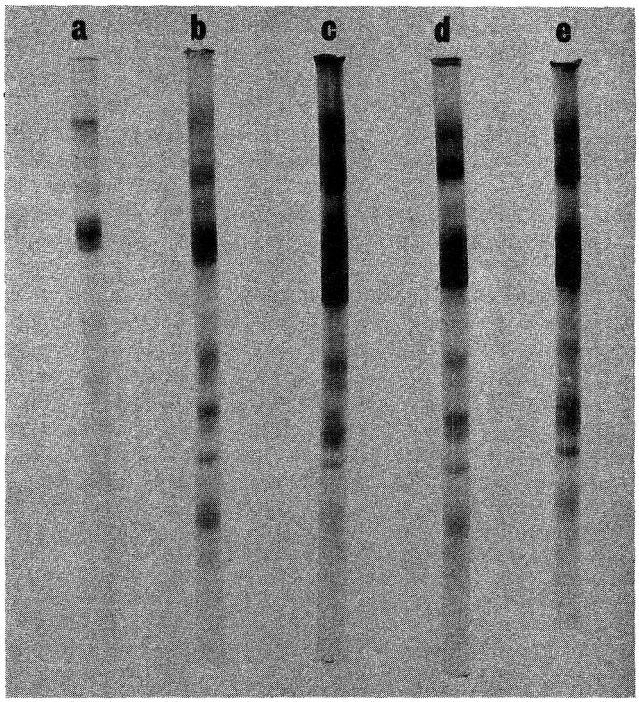


Fig. 3—Electrophoretic separation (SDS—PAGE system) of the Great Northern bean proteins: a—Bean flour, b—albumins, c—Globulins, d—Protein concentrates, e—Protein isolates.

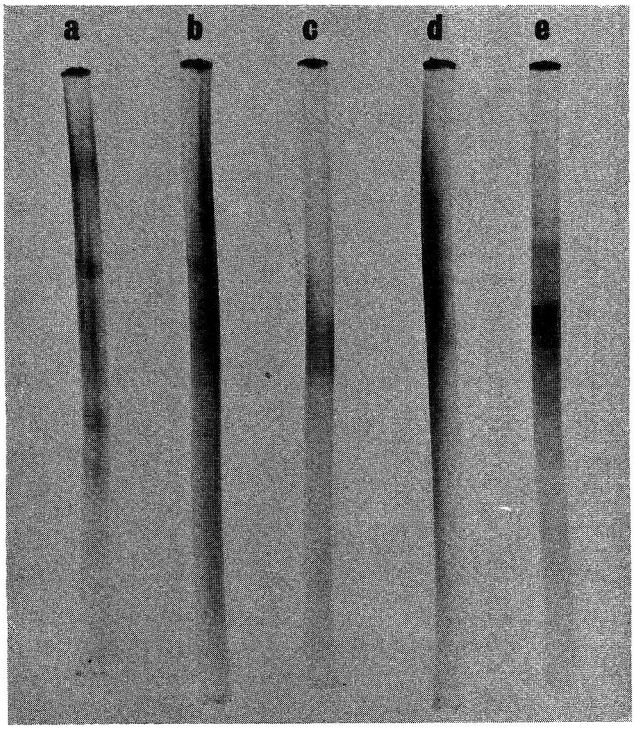


Fig. 4—Electrophoretic separation (PAMU-PAGE system) of the Great Northern bean proteins: a—Bean flour, b—albumins, c—Globulins, d—Protein concentrates, e—Protein isolates.

were composed of at least 22, 14, 10, 14, and 11 subunits, respectively; with corresponding apparent molecular weight ranges of 14,700-550,000, 14,000-554,000, 19,300-450,-000, 19,500-432,000, and 19,400-460,000 daltons. The predominant subunits (judged on the basis of band width) in bean flour, albumins, globulins, protein concentrates, and protein isolates, respectively, had apparent molecular weights of 294,000, 266,000, 123,000, 146,000, and 135,000 daltons. As expected, protein concentrates and protein isolates had similar subunit profiles particularly in lower apparent molecular weight ranges (135,000 daltons). The bean flour proteins and albumins were similar in terms of apparent molecular weight profiles in that, both showed 50% subunits with apparent molecular weights greater than 100,000 daltons while globulins had 3 subunits with apparent molecular weights greater than 123,000 daltons. The significance of these observations is obscure at present, but may be of value to the plant breeders. Globulins were composed of 10 subunits, similar to the number of subunits reported by several workers in globulins of legumes including black gram (Padhye and Salunkhe, 1977) and pigeon pea (Gopalakrishna et al., 1977). The conspicuous presence of subunits with apparent molecular weights of about 20,-000 and 40,000 daltons is strikingly similar to the one reported in pea seeds (Thomson et al., 1976). Advantages, limitations, and other pertinent aspects of SDS-PAGE for molecular weight determination of proteins had been discussed extensively by several investigators (Weber and Osborn, 1969, 1975; Seagrest and Jackson, 1972; Reynolds and Tanford, 1976; Lambin, 1978).

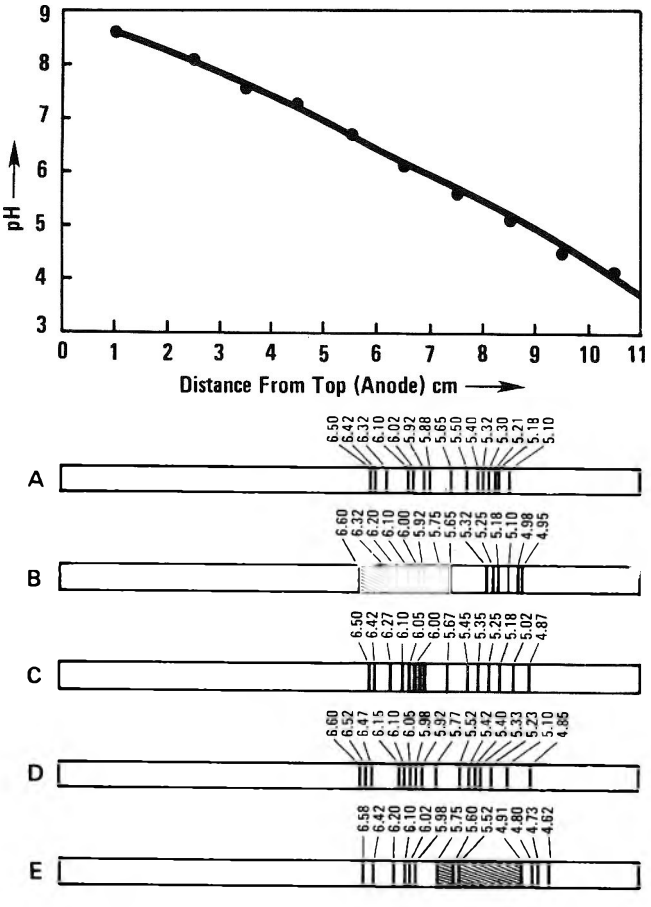


Fig. 5—Isoelectric focusing of the Great Northern bean proteins: A—Bean flour, B—Albumins, C—Globulins, D—Protein concentrates, E—Protein isolates. The graph demonstrates the construction of the pH gradient.

Isoelectric focusing

The bean flour, albumins, globulins, protein concentrates, and protein isolates consisted of 15, 13, 15, 16, and 11 subunits, respectively, with corresponding apparent isoelectric pH ranges of 6.50-5.10, 6.60-4.95, 6.50-4.87, 6.60-4.85, and 6.58-4.62 (Fig. 5). Difficulties were encountered in focusing of albumins (diffused band between apparent pI of 6.60-6.32) and protein isolates (diffused bands between apparent pI ranges of 5.75-5.60 and 5.52-4.91). These diffused bands probably arose through aggregation of proteins induced by solvent employed for their solubilization. Bean flour proteins contained 7 subunits in the apparent pI range 6.50-5.88 and 8 subunits in the pI range 5.65-5.10. Albumins had 2 clusters of 6 subunits each in the apparent pI ranges 6.20-5.65 and 5.32-4.95. Globulins registered a much more complex pattern consisting of 2 closely spaced triplets (apparent pI ranges 6.10-6.00 and 5.45-5.25) and a doublet (apparent pI range 5.95-5.90). Protein concentrates had 8 subunits in apparent pI ranges

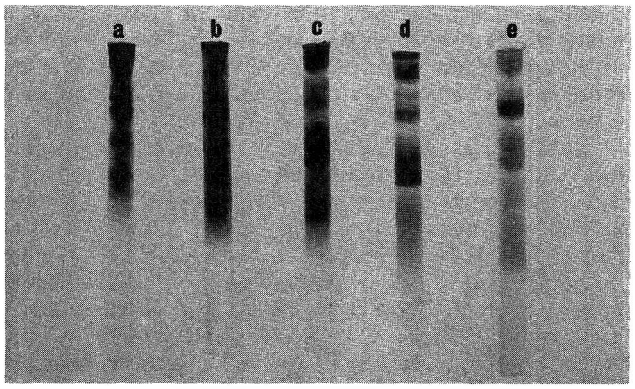


Fig. 6—SDS-PAGE of the bean flour proteins separated by molecular sieve chromatography (Bio-Gel A 0.5). The proteins were eluted with 0.02M Tris buffer (pH 8.2). A total of 140 fractions (4.7 ml each) were collected. The gels a through e correspond to the pooled fractions under each peak: a—Tube # 35-50, b—Tube # 51-75, c—Tube # 76-80, d—Tube # 81-100, e—Tube # 101-135.

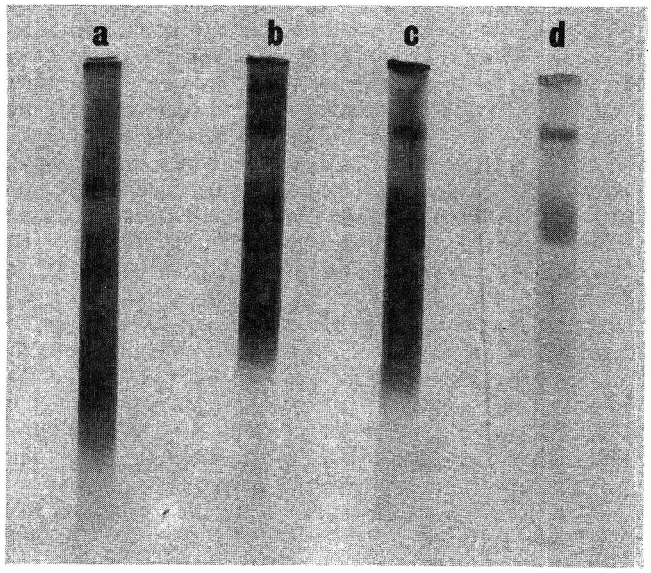


Fig. 7—SDS-PAGE of the albumins separated by molecular sieve chromatography (Bio-Gel A 0.5). The proteins were eluted with 0.02M Tris buffer (pH 8.2). A total of 140 fractions (4.7 ml each) were collected. The gels a through d correspond to the pooled fractions under each peak: a—Tube # 43-55, b—Tube # 56-68, c—Tube # 69-85, d—Tube # 86-100.

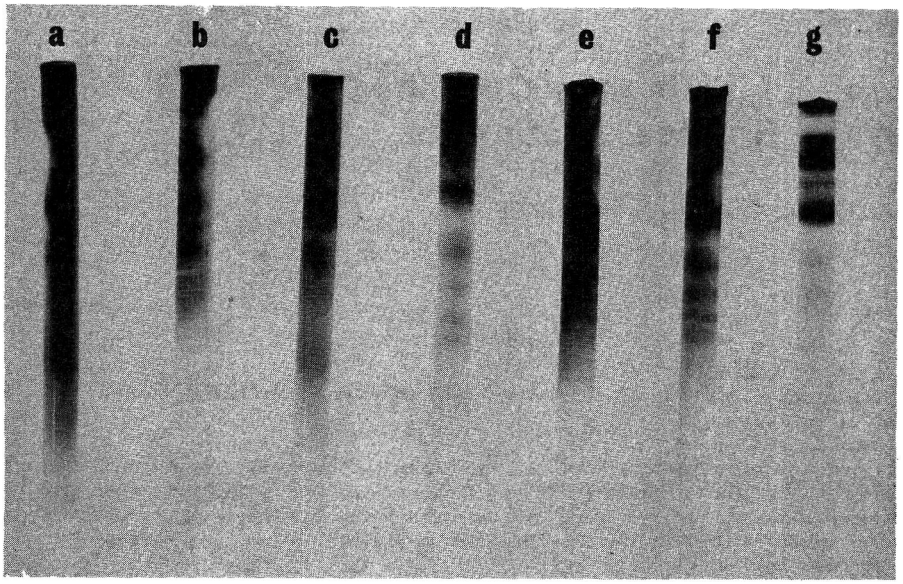


Fig. 8—SDS-PAGE of the globulins separated by molecular sieve chromatography (Biol-Gel A 0.5). The proteins were eluted with Tris buffer (pH 8.2). A total of 140 fractions (4.7 ml each) were collected. The gels a through g correspond to the pooled fractions under each peak: a—Tube # 37-56, b—Tube # 57-67, c—Tube # 68-72, d—Tube # 73-81, e—Tube # 82-91, f—Tube # 92-100, g—Tube # 105-111.

6.60-5.92 and 5.77-4.85, while protein isolates consisted of 6 subunits between 6.58-5.98 and 5 subunits between 5.75-4.62 pH ranges.

Column chromatography

The complexity of the Great Northern bean proteins was further evident when bean flour, albumins, and globulins were first separated by molecular sieve chromatography followed by SDS-PAGE. The bean flour, albumins, and globulins had 5, 4, and 7 peaks (column chromatography) respectively. The SDS-PAGE of each of these fractions demonstrated several subunits in each. The bean flour proteins (Fig. 6) had a total of 35 subunits (10, 7, 5, 5, and 8 subunits in fractions a through e respectively), while albumins (Fig. 7) consisted of 31 subunits (8, 6, 9, and 8 fractions a through d respectively), and globulins of 35 subunits (5, 5, 3, 2, 5, 9 and 6 subunits in fractions a through g respectively). The combined total of albumins and globulins subunits (66) was approximately 2 times that of the subunits (35) in the bean flour proteins. This suggests that when albumins and globulins were present together they behaved in a different manner than when they were separate.

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Pisum sativum and *Vicia faba* Carbohydrates: Structural Studies of Starches

P. COLONNA, A. BULEON, and C. MERCIER

ABSTRACT

The fine structure of laboratory purified broadbean and smooth pea starches, with an amylose content of 32-34%, has been studied by pullulanase debranching, before or after beta-amylolysis, and by the properties of the chemically fractionated amylose and amylopectin. The enzymatic study has shown the presence of the three chain populations ($\overline{DP}$ > 60, 45 and 15) observed with other starches. The linear DP 15 and 45 chains occur in a ratio of 8.5 for broadbean and 9.75 for pea, which indicates an amylopectin similar to cereal starches. The λ_{max}, beta-amylolysis limit and intrinsic viscosity of the two amylopectins confirm the cereal-like nature. The two amylose components are not completely linear according to their beta-amylolysis limit of 81.5% which corroborates the incomplete debranching of the total starch. The physical structure, studied by X-ray diffractometry, is of C-type. By submitting legume starch granules to mild acid hydrolysis (lintnerization), a residue has been obtained from both starches, which has an increase in the crystalline fraction, with a tendency towards the A-type pattern for broadbean and the B-type for pea. The crystallites are mainly formed of linear chains ($\overline{CL}$ 15) with some singly branched material ($\overline{DP}$ 25). Gelatinization of starch granules occurs at 44-65-86°C for broadbean with a heat of gelatinization of 3.8 cal g^{-1} and at 48-61-80°C for pea with a heat of gelatinization of 3.2 cal g^{-1}.

INTRODUCTION

ISOLATION and purification of starches from broadbean and pea flours have been described previously (Colonna *et al.*, 1980). Although some physicochemical properties of the two legumes starches have been studied (Greenwood and Thomson, 1962a, b; Kawamura, 1969; Banks *et al.*, 1971; Biliaderis *et al.*, 1979), the literature contains little information on their granular structure and the fine structure of their components. Their pasting behavior indicates a higher resistance to swelling and rupture (Schoch and Maywald, 1968; Lineback and Ke, 1975; Naivikul and D'Appolonia, 1979) than normal cereal and tuber starches.

Physical methods, such as X-ray diffractometry, have allowed the differentiation of cereal and tuber starches due to their typical patterns (Katz, 1930; Kainuma and French, 1971, 1972; Duprat *et al.*, 1974). Sequential enzymatic studies on native dispersed starch, based on successive action of specific enzymes and fractionation by gel chromatography (Lee *et al.*, 1968; Gunja-Smith *et al.*, 1971; Mercier, 1973; Lii and Lineback, 1977; Hood and Mercier, 1978) coupled with mild acid hydrolysis of the granule (Robin *et al.*, 1974, 1975; Maningat and Juliano, 1979) have yielded informations on the fine structure of the amylose and amylopectin components.

The aim of this study was to determine granular structure and fine structure of the amylose and amylopectin components with special attention to point out differences between legume starches and cereal or tuber starches. The investigation was limited to broadbean and smooth pea starches since their amylose content is similar (31-33%) (Colonna and Mercier, 1979).

Authors Colonna and Mercier are with Laboratoire de Biochimie des Aliments, and Author Buleon is with Laboratoire de Biophysique des Aliments, Centre de Recherches Agro-Alimentaires, Chemin de la Géraudière, 44072 Nántes, Cedex, France.

MATERIALS & METHODS

Materials

Native starches were isolated and purified from flours of broadbean (*Vicia faba minor, Ascott* variety) and smooth pea (*Pisum sativum, Frimas* variety) according to Colonna *et al.*, (1980).

Lintnerized starch granules were prepared as described by Robin (1976) at 35°C at a concentration of 10g (dry matter) per 100 ml of 2.2N HCl with daily hand shaking to resuspend the starch granules. After 45 days, residual starch was obtained by filtering on a porous glass filter (5-15μ) under reduced pressure, repeated washing with distilled water to remove chloride ions, residual acid and soluble carbohydrates. The residue was dried to constant weight in an oven at 25°C. The residue was called "lintnerized starch." The extent of starch hydrolysis with time was followed by measuring the carbohydrate content in the filtrate using the orcinol-sulfuric acid method (Tollier and Robin, 1979). Results were expressed in percent of the initial starch.

Pullulanase (EC 3.2.1.41) of *Enterobacter aerogenes (Aerobacter aerogenes)* (Mercier *et al.*, 1972), barley beta-amylase (EC 3.2.1.2) (Fluka AG, Buchs SG, Switzerland), *Aspergillus niger* glucose oxidase (EC 1.1.3.4) (grade II) and horse radish peroxidase (EC 1.11.1.7) (grade I) (Sigma Chemical Company, St. Louis, Mo.), *A. niger* amyloglucosidase (EC 3.2.1.3) (E. Merck, Darmstadt, R.F.A.) and ABTS [2,2'-azino-di(3-ethyl-benzothiazoline sulfonic acid)] (Boehringer Mannheim Gmbh, Mannheim, R.F.A.) were used. Sephadex G-50 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden.

Solubilization of native and lintnerized starches was accomplished by boiling a 1% starch suspension in distilled water for 3 min and then autoclaving for 1 hr at 130°C.

Enzymatic methods

Debranching of the native and lintnerized starches was performed essentially as by Hood and Mercier (1978). The digests (P_1) contained per ml: starch (5 mg), acetate buffer (0.04M, pH 5.4) and pullulanase (8.32 nKatals). Beta-amylolysis of the debranched starches (P_1 β_1) was carried out after inactivation of pullulanase by heating the digest for 20 min at 100°C and addition of 10 nkatals of beta-amylase per ml of P_1 digest. Beta-limit dextrins (β_1) were prepared in a mixture containing per ml: starch (5 mg), acetete buffer (0.04M, pH 5.4) and beta-amylase (5.17 nkatals). After dialysis, the maltose-free beta-limit dextrin was debranched (β_1 P_1) and subsequently hydrolyzed with beta-amylase (β_1 P_1 β_2) under the conditions already described for P_1 and β_1.

The total concentration of starch-type polysaccharide was measured by the successive action of amyloglucosidase (Lee and Whelan, 1966) and glucose oxidase-peroxidase (Lloyd and Whelan, 1969). The latter procedure was modified as follows. Glucose oxidase-peroxidase reagent was prepared by dissolving 50mg of ABTS, 3 mg of peroxidase and 100 mg of glucose oxidase in 100 ml of 0.3M Tris-phosphate buffer, pH 7.0, and was stored in a dark bottle. The sample (1 ml containing 5-45 μg of glucose) was mixed with 2 ml of reagent and 2 ml of distilled water, and kept exactly 30 min in the dark. The absorbance was read at 560 nm.

The rates of hydrolysis by pullulanase and beta-amylase were monitored by measuring the reducing power according to Nelson (1944).

The degree of debranching was measured as glucose reducing power liberated. The average degree of polymerization ($\overline{DP}$) was calculated as:

$$\overline{DP} = \frac{\text{polysaccharide in digest (glucose equivalents)}}{\text{reducing capacity (glucose equivalents)}}$$

The term average chain length ($\overline{CL}$) was used when the material was proven to be linear.

The percent of beta-amylolysis was expressed as maltose equivalent and calculated as:

$$\frac{\text{reducing capacity (maltose equivalents)}}{\text{polysaccharide in digest (glucose equivalents)}}$$

Solutions of native and lintnerized starch, before and after enzymatic action, were chromatographed on a column of Sephadex G-50 (2.5 x 100 cm) at 20–25°C. Elution was carried out with an upward flow at 20 ml h^{-1} using 10 mM phosphate buffer (pH 7.0) containing 0.02% sodium azide (Hood and Mercier, 1978) as eluent. The elution profiles represent the weight percent (mg 100 mg^{-1}) recovered in each fraction plotted against the elution volume. Recoveries from the columns varied from 94 to 115%.

Physicochemical methods

Chemical fractionation. Starches were fractionated into amylose and amylopectin according to Banks and Greenwood (1967). Amylose was recrystallized once from 1-butanol, isolated by precipitation with acetone and vacuum-dried at 20°C. Amylopectin was isolated from the gel after extraction of amylose.

Iodine-absorption spectra were determined on mixtures containing 1 ml of 0.02% starch solution (starch solubilized 30 min in 1N KOH), 1 ml of IN HCl, 2.1 ml of 0.33M KCl and 0.208 ml of aqueous iodine solution (0.2% iodine + 2.0% potassium iodide).

Amylose determination. Amylose content of the starches was measured by an amperometric method (Larson *et al.*, 1953) at 25°C, assuming that "pure" amylose has an iodine-binding capacity of 19%.

Intrinsic viscosity [η] was determined in 0.2M KOH at 25°C with an automatic Oswald viscometer (Banks and Greenwood, 1969).

Birefringence end-point temperature range. Measurements were determined using a polarizing microscope (Leitz) with a Kofler hot stage. Temperatures were recorded which correspond to the loss of birefringence by 2, 50, and 98% of starch granules, observed in the field, designating them as initial, midpoint and end-point values, respectively (Watson, 1964).

Physical methods

X-ray diffractometry. X-ray patterns were obtained using a spectrophotometer type Sigma 2080 from the "Compagnie Générale de radiologie," equipped with a Guinier monochromator and using Cu Kα radiation. The patterns of starch powders were recorded, following the Debye-Scherrer method, over the range of Bragg angles from 3.5-26 degrees, using a scintillation counter. Hydrated samples were obtained after equilibration for 3 days in a flask containing a saturated solution of potassium bichromide corresponding to a relative humidity of 98%. Moisture content was determined by drying to constant dry weight over phosphoric anhydride at 50°C.

Thermal analysis. Differential Scanning Calorimetry (DSC) was carried out by using a Perkin-Elmer DSC-2B differential scanning calorimeter with a heating rate of 10°C min^{-1}, a sensitivity of 2 mcal s^{-1} and a chart speed of 40 mm mn^{-1}. About 20 mg wet starch (ratio dry starch:water = 1:2) was sealed in an air-tight aluminimum cell and weighed accurately (sensitivity: 1 μg.). The scanning temperature range was 7°C through 95°C. The thermogram was recorded with water as reference. Each peak was characterized by the temperature of initial deflection, the temperature of peak maximum, and the conclusion temperature. Indium was used for calibration. The heat of each endotherm was estimated by integrating the area between the thermogram and a base-line under the peak and was expressed in terms of calory per unit weight of dry starch (cal g^{-1}) (Richardson, 1978).

RESULTS

Characteristics of starch components

The amylose content of broadbean and pea starches is 32.9 and 32.5% respectively. The wavelength of maximum absorption (λ_{max}) of the iodine-starch complex for both starches is 595-600 nm.

Based on the recovery of amylose after starch fractionation according to Banks and Greenwood (1967), the amylose content of broadbean and pea starches is 31.8% and 33.2%, respectively. The amylose and amylopectin components of pea and broadbean starches have very similar properties (Table 1). The λ_{max} of the iodine-polysaccharide complex is 540 nm for both amylopectins, 630 nm for broadbean amylose, and 625 nm for pea amylose. The beta-amylolysis limits are 56.1% and 56.7% for broadbean and pea amylopectins, 81.5% and 81.6% for broadbean and pea amyloses, respectively. On the other hand, the intrinsic viscosities are different: 200 ml g^{-1} and 264 ml g^{-1} for broadbean and pea amyloses, 131 ml g^{-1} and 126 ml g^{-1} for broadbean and pea amylopectins, respectively.

Enzymatic analysis of native starch

The elution profiles of pullulanase-debranched (P_1) broadbean (Fig. 1) and pea starches are similar (Table 2). Three peaks are visible: the peak at the void volume (Vo) represents 37.3% of total polysaccharide for broadbean and 39.0% for pea, two peaks are located at $\overline{DP}$ 45 and 15. Beta-amylase converts the total debranched polysaccharide digest into maltose and maltotriose, except for a small amount of material present at the Vo:0.35% for broadbean and 0.67% for pea. These results indicate that the material of $\overline{DP}$ 45 and 15 and most of the material present at the void volume are completely linear. The two components of $\overline{CL}$ 45 and 15 are in the relative concentration of 1 to 2.8 for broadbean and 1 to 3.25 for pea. Calculated on a molecular weight basis, the ratio of $\overline{CL}$ 15/$\overline{CL}$ 45 chains corresponds to 8.5 for broadbean and 9.75 for pea.

Beta-amylolysis limits of broadbean and pea starches were 66.7 and 67.5 respectively (Table 3). The elution pro-

Table 1—Characteristics of amylose and amylopectin components from broad bean and pea starches

Component	Broadbean starch	Pea starch
Amylopectin		
λ max (nm)	540	540
Beta-amylolysis %. .	56.1	56.7
[η] (ml.g^{-1})	131	126
Amylose		
λ max (nm)	630	625
Beta-amylolysis %. .	81.5	81.6
[η] (ml.g^{-1})	200	264

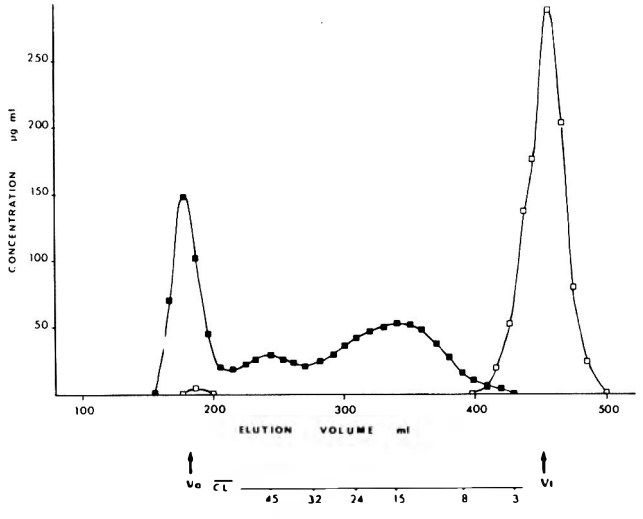


Fig. 1—Elution profiles from Sephadex G-50 of broadbean starch. Polysaccharide in each fraction was determined by the amyloglucosidase-D-glucose oxidase method. Debranched (P_1) (—■—■—), P_1 hydrolyzed with beta-amylase (P_1 β_1) (—□—□—□—□).

files of the debranched beta-limit dextrins (β_1 P_1) are similar and are shown for broadbean starch in Figure 2. Three peaks are observed and are located at DP 30-35, 10-12 and 2. Further beta-amylolysis of the debranched beta-limit dextrin (β_1 P_1 β_2) results in disappearance of the $\overline{DP}$ 30-35 and 10-12 material. A small amount of material, 0.8% and 0.5% for broadbean and pea, respectively, is still present at the void volume: this material was not evident after β_1 P_1 due to dispersion of the peak of $\overline{DP}$ 30-35.

Hydrolysis kinetics and characteristics of lintnerized starches

The kinetics of acid hydrolysis of the two starches are similar. A plot of the extent of hydrolysis (X) vs time (Fig. 3) leveled off after 7-9 days at 35°C in 2.2N HCl. Expressed as $\log_{10}$ [100/100-X], a two stage corrosion was evident (Fig. 3). The first stage requires 10 days for broadbean starch and 8 days for pea starch. The slope was greater for broadbean in the first stage, but no difference was observed in the second stage. Lintnerization for 45 days resulted in 22% of acid residual dextrin for broadbean starch and 20% for pea starch. In the second stage, the dependence of $\log_{10}$ [100/100-X] with time has a correlation coefficient of +0.978 for broadbean starch and +0.964 for pea starch.

The intercept of this regression line with time enables calculation of the easily degradable fraction of the starch granules which represent 48.7% and 53.2% of total starch for broadbean and pea, respectively.

The elution pattern from Sephadex G-50 of aqueous dispersed lintnerized broadbean starch shows a main peak at $\overline{DP}$ 15 with a weak shoulder at $\overline{DP}$ 25 (Fig. 4), the latter being less pronounced in the case of lintnerized pea starch. After pullulanase action, the material located under the shoulder ($\overline{DP}$ 25) disappears whereas DP 15 increases, indicating that the DP 25 fraction is probably singly branched with two chains of DP 15. Further beta-amylolysis (β_1 P_1) transforms the $\overline{DP}$ 16 material into maltose and maltotriose with an enzyme resistant fraction for broadbean (3% of total polysaccharide), which is confirmed by an incomplete beta-amylolysis (93.3%). With pea starch, this fraction is negligible, which is confirmed by a beta-amylolysis limit of 99%.

X-ray diffractometry

X-ray patterns of native broadbean starch, at 14.0% and 34.3% moisture content (Fig. 5.1 and 5.2), are characteristic of C-type starch (Sterling, 1960), which is a mixture of A-type (cereal) and B-type (tuber) forms. The A-type

Table 2—Characteristics of pullulanase-debranched broadbean and pea starches[a]

Component	Broadbean starch	Pea starch
P_1		
$\overline{DP}$[b]	36.5	35.1
$\overline{DP}$ > 60	37.3	39.0
$\overline{DP}$ 45	16.4	14.6
$\overline{DP}$ 15	46.3	46.8
P_1 β_1		
Beta amylolysis limit	93.3	93.8
$\overline{DP}$ > 60	0.35	0.67

[a] Unless otherwise noted, all values are %.

[b] $\overline{DP}$ = polysaccharide in digest (glucose equivalents) / reducing capacity (glucose equivalents).

Table 3—Characteristics of beta-amylase limit-dextrins from broadbean and pea starches[a]

	Broadbean starch	Pea starch
Direct beta-amylolysis limit (β_1)[b]	66.7	67.5
β_1 P_1		
$\overline{DP}$[c]	9.7	10.2
$\overline{DP}$ 20-60	14.4 (43.3)[d]	11.1 (34)
$\overline{DP}$ 10-12	11.5 (34.5)	13.9 (42.8)
$\overline{DP}$ 2-3	7.4 (22.2)	7.5 (23.2)
β_1 P_1 β_2		
$\overline{DP}$ > 60	0.3 (0.8)	0.2 (0.5)
$\overline{DP}$ 20-60	0	0
$\overline{DP}$ 4-19	0	0
$\overline{DP}$ 2-3	33.3 (99.2)	32.3 (99.5)

[a] Unless otherwise noted, all values expressed as % of original starch.

[b] reducing capacity (maltose equivalents) / polysaccharide in digest (glucose equivalents) × 100.

[c] $\overline{DP}$ = polysaccharide in digest (glucose equivalents) / reducing capacity (glucose equivalents).

[d] Values in parentheses are % of total polysaccharide.

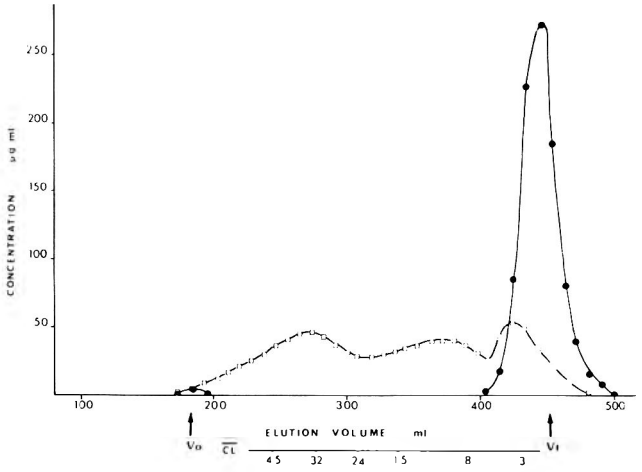


Fig. 2—Elution profiles from Sephadex G-50 of broadbean starch β-limit dextrin. Polysaccharide concentration in each fraction was determined by the amyloglucosidase-D-glucose oxidase method. Debranched beta-limit dextrin (β_1 P_1) (—□—□—□—), β_1 P_1 hydrolyzed with beta amylase (β_1 P_1 β_2) (●—●—●—●—).

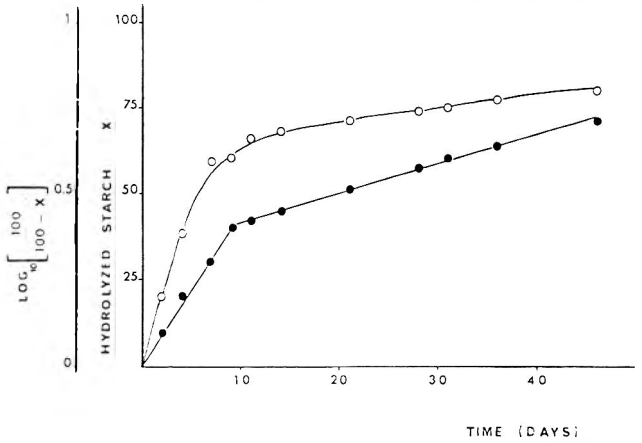


Fig. 3—Hydrolysis of broadbean starch in 2.2N HCl at 35°C. The percentage (x) (—○—○—○—) of hydrolyzed starch and the $\log_{10}$ [100/100-X] (—●—●—●—) are plotted versus time of hydrolysis.

seems to be predominant, but the presence of a peak at 5.5°C, reinforced by hydration, and of a small one at 24.0° are significant for the B-form.

After lintnerization, the X-ray patterns of the acid-resistant residues at 10.3% and 31.0% moisture content (Fig. 5.3 and 5.4) show sharper and higher peaks, indicating preferential hydrolysis of the amorphous portion. The peak characteristics of the A-form at 14.54° is more evident, but no evolution to the B-form is observed after lintnerization.

X-ray patterns of native pea starch at 15.9% and 39.6% moisture content (Fig. 6.1 and 6.2) are also characteristic of C-type starch, but with a more pronounced tendency towards the B-form, with the broad peak at 23.0°. The ratio of the peak intensities at 5.5° and 17° indicates the presence of A-type, but the general shape of spectrum is nearer the potato starch spectra.

After lintnerization, the X-ray patterns of the acid resistant residue at 15.9% and 34.5% (Fig. 6.3 and 6.4) moisture content show an increase in crystallinity without any evolution to the B-form.

Gelatinization

The temperature range (birefringence endpoint temperatures), at which the starches gelatinize, is 51-69°C for broadbean and 49-67°C for pea; at these values, 2% and 98% of the granules in the field lost birefringence. The midpoint temperatures (50% of the granules lost birefringence) are 64°C and 60.5° for broadbean and pea starches, respectively.

Differential Scanning Calorimetry (DSC) was applied to study starch gelatinization. The DSC thermogram of a pea starch-water mixture (1:2) (Fig. 7) shows an endotherm which is attributed to the gelatinization of starch granules since no starch granule can be observed after a thermal analysis. The first step is at 44°C for broadbean and 48°C for pea; the highest responses are at 65°C and 61°C for broadbean and pea starches, respectively; the conclusion is at 86°C for broadbean and 80°C for pea. The conclusion temperatures are found to be slightly dependent on the sample amount. The endotherms are broad and no shoulder could be detected. ΔH values measured are 3.8 cal g^{-1} for broadbean and 3.2 cal g^{-1} for pea.

DISCUSSION

THE AMYLOSE CONTENTS of broadbean and pea starches, determined either by an amperometric method or after chemical fractionation are very similar (32-34%) and are 5-10% higher than for normal cereal and tuber starches. Those results confirm previous work for broadbean (Greenwood and Thomson, 1962b; Lineback and Ke, 1975; Vose *et al.*, 1976) and pea (Greenwood and Thomson, 1962a; Vose, 1977; Vose *et al.*, 1976) starches, but they differ from the amylose content of 24.0% reported for broadbean starch by Naivikul and D'Appolonia (1979).

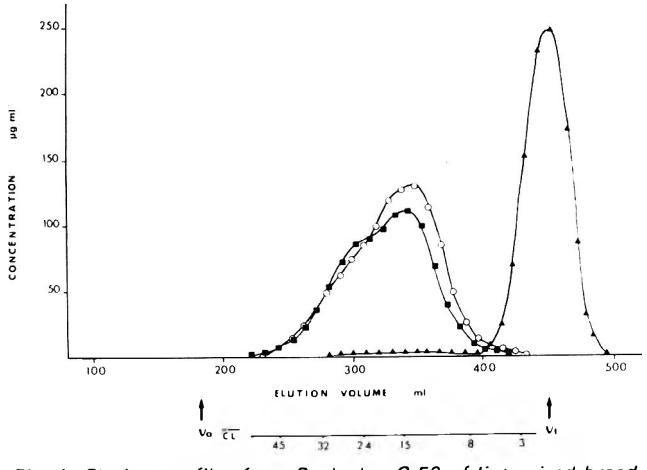


Fig. 4—Elution profiles from Sephadex G-50 of lintnerized broadbean starch. Polysaccharide concentration in each fraction was determined by the amyloglucosidase-D-glucose oxidase method. Lintnerized starch (—■—■—■—■—), debranched (P_1) (—○—○—○—○—); consecutive treatment with pullulanase and beta-amylase (P_1 β_1) (▲—▲—▲—▲—).

Debranching of legume starches yields a DP of 35-36, higher than the DP 26-28 obtained for normal cereal and tuber starches. The elution profile shows three chain populations with DP 15, 45, and higher than 60, almost completely linear corresponding to the three fractions previously observed with other starches. According to Robin *et al.* (1974, 1975), the linear DP 45 and 15 chain populations are constitutive of amylopectin. The ratio of A-chains (CL 15) to each B-chain (CL 45) (broadbean: 8.5; pea: 9.75) is higher than for potato (5.5) (Robin *et al.*, 1974) and manioc amylopectin (7.5) (Hood and Mercier, 1978), but of the same order as cereal amylopectin (8-9) (Mercier, 1973; Robin *et al.*, 1975; Lii and Lineback, 1977). These higher values indicate a greater extent of multiple branching in amylopectin. The higher amylose content of the legume starches explains the high average chain length (CL) in P_1 and the greater extent of beta-amylolysis: the experimental values for total starches agree with those calculated from the purified components.

The average chain length (CL: 9.7 and 10.2) of the debranched beta-limit dextrins are similar to those reported for potato (Gunja-Smith *et al.*, 1971), maize and waxy maize (Mercier, 1973), and manioc amylopectin (Hood and Mercier, 1978). Debranching of the beta-limit dextrin leads to chain populations of DP 2-3, 10-12 and 30-35; the DP 2-3 originate from the maltosyl and maltotriosyl stubs remaining at the 1,6-α-D glucosidic linkages after beta-amylolysis (β_1), indicating that the outer chains of the amylopectin beyond the 1,6-α-D glucosidic linkages were of DP 15. The DP values of 10-12 and 30-35 of the β_1 P_1 suggest that the inner chains of amylopectin vary from 10 to 35, representing of the CL 45 chain by beta-amylolysis. Beta-amylolysis of the debranched beta-limit dextrin is not complete for both starches and high-molecular-weight fractions remain at V_o; the same feature is observed after beta-amylolysis of the debranched starches. These residues represent less than 1% of total polysaccharide and could be attributed to slightly branched amylose. The incomplete conversion of the chemically fractionated amylose by beta-amylase into maltose (81.5%) corroborates this result. The barrier to the action of beta-amylase on amylose has been previously observed by Banks and Greenwood (1968) with wheat amylose. They attributed this to a small proportion of 1,6-α-D glucosidic linkages since pullulanase was unable to remove all the glucosidic stubs, some of them would be constituted by a single glucose residue joined by an 1,6-α-D glucosidic bond to a chain of residues linked by 1,4-α-D glucosidic linkages. On the other hand, after debranching of native starch, the high-molecular-weight fraction at V_o (37-39%) is higher than the amylose content of each starch (32-34%). This excess of material could correspond either to undebranched starch due to incomplete dispersion of the substrate or to the long C-chain of the amylopectin molecule. The first hypothesis, drawn up by Abdullah *et al.* (1966) with waxy maize, is not applicable to legume starches since debranching followed by beta-amylolysis led to residues of less than 1% of total polysaccharide (instead of 3-4% with amylopectin). Furthermore, starch solubilization by alkaline treatment, followed by neutralization, did not increase the limit of beta-amylolysis. To the contrary, the fact that the calculated CL of the amylopectins obtained from the ratio A-chain (DP 15)/B-chain (DP 45) (8.5-9.75) would be of 18-20 instead

of 23-26 after a periodate oxidation measurement of chemically fractionated amylopectins (Greenwood and Thomson, 1962a, b) demonstrates the presence of the long C-chain located under the void volume material.

The characteristics of the chemically fractionated amylose and amylopectin confirm the results of Greenwood and Thomson (1962a, b) and disagree with abnormally high value of λ_{max} (610 nm) for legume starch amylopectin-iodine complexes obtained by Biliaderis *et al.* (1979). These starch components have similar properties to those of cereal and tuber starches (Banks and Greenwood, 1975).

From our results, amylose and amylopectin of broadbean and pea starches are close to normal cereal components in terms of fine structure and properties. The main difference is due to the amylose:amylopectin ratio (1:2), which should affect their physical structure and pasting behavior.

The X-ray patterns of native broadbean and pea starches are of C-type, intermediate between the A-(cereal) and the B-type (tuber) (Sarko and Wu, 1978), with a tendency towards A-type structure for broadbean and B-type for pea. These results are in agreement with the work of Sterling (1960) and Banks and Greenwood (1975). Submitted to mild acid hydrolysis, legume starches are more resistant than waxy-maize, maize, and wheat (Robin *et al.*, 1975), rice (Maningat and Juliano, 1979) and as susceptible as potato starch (Robin *et al.*, 1974). The higher starch concentration of 10% instead of 1.5% (Robin *et al.*, 1974, 1975) during lintnerization cannot explain the higher resistance of legume starches since the hydrolysis reaction obeys first order kinetics (Robin, 1976). However, X-ray study of the lintnerized residues, showing the disappearance of the amorphous part, indicates a higher crystalline fraction (50%) than with potato (Robin *et al.*, 1974) and of the same order as cereals (Robin *et al.*, 1975). Unlike wheat starches (Hizukuri, 1961), no evolution from the A-type to the B-type spectra was observed during lintnerization; the crystallites for broadbean starch residue are still of C-type, close to A-type, whereas those of pea starch residue are C-type, close to B-type. The crystallites are composed essentially of linear chain populations of CL 15 with a shoulder, more or less important, of a singly branched material of DP 25. According to the hypothesis of Robin (1976), the ratio between DP 25 and DP 15 of the crystallites would be related to the X-ray pattern and the presence of DP 25 would imply an A-type. Indeed, the DP 25 material is more important with broadbean starch, nearer the A-type spectrum, than with pea starch.

Gelatinization was studied by two methods. The temperature ranges obtained by DSC are broader than by microscopy; they are in good agreement with previous work (Lineback and Ke, 1975; Vose, 1977; Comer and Fry, 1978; Lorenz, 1979) and call in question the results of Greenwood and Thomson (1962a) for pea starch. These values are close to those of wheat starch 53.0-58.0-63.0°C (Lii and Lineback, 1977), 56-64-82°C (Wada *et al.*, 1979) and potato starch 59-65-80°C (Wada *et al.*, 1979). The DSC parameters were chosen according to the results of Stevens and Elton (1971) and Wootton and Bamunuarachchi (1979a, b). Varying heating rates from 2-30°C min^{-1} and sample amount from 10.131 mg to 30.748 mg have no significant effect on gelatinization temperatures and heat of fusion, as noted already by Richardson (1978) and Wada *et al.*, (1979). However the conclusion temperature was

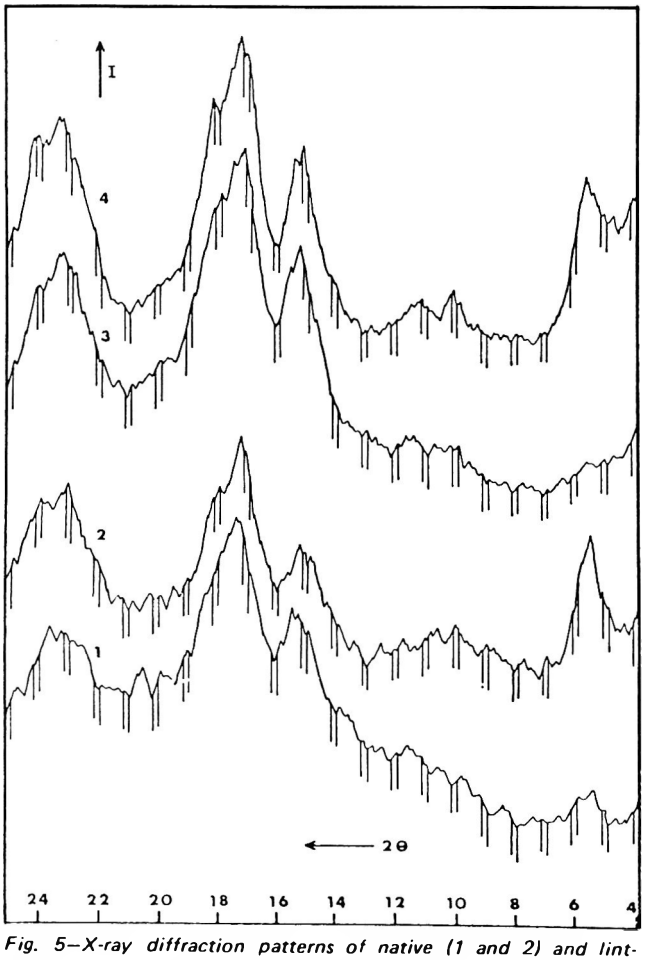


Fig. 5–X-ray diffraction patterns of native (1 and 2) and lintnerized (3 and 4) broadbean starches at different moisture content: 1, 14.0%; 2, 34.3%; 3, 10.3%; 4, 31.0%.

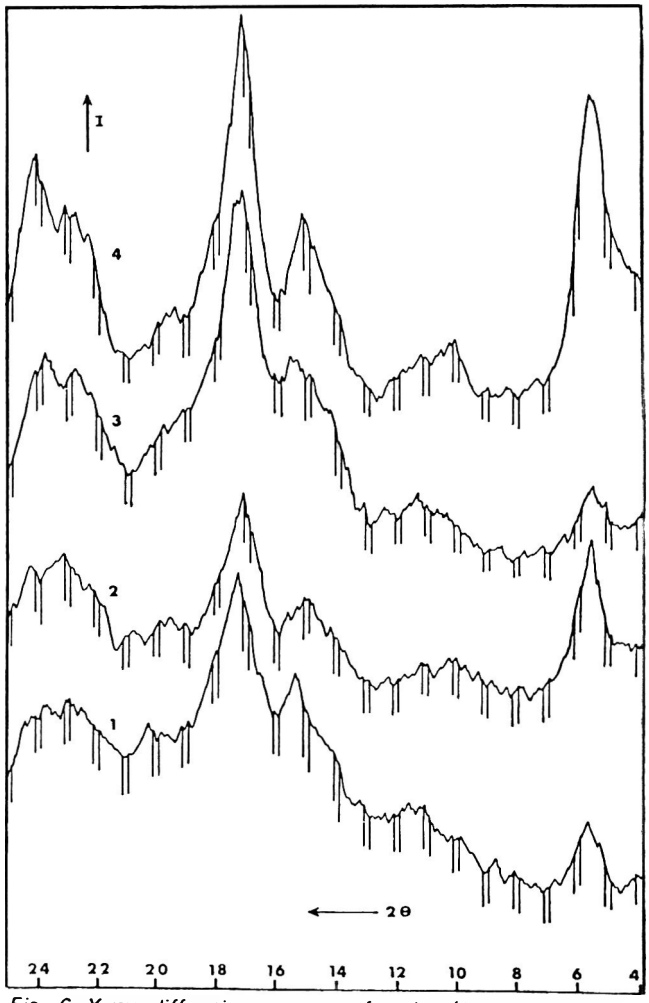


Fig. 6–X-ray diffraction patterns of native (1 and 2) and lintnerized (3 and 4) pea starches at different moisture content: 1, 15.9%; 2, 39.6%; 3, 15.9%; 4, 34.5%.

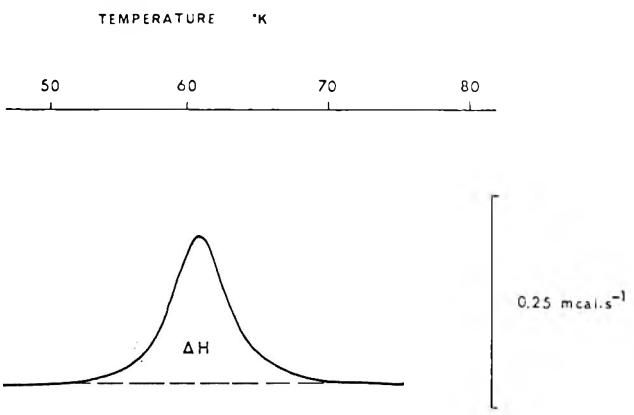


Fig. 7—DSC thermogram representative of gelatinization of pea starch:water mixture (ratio 1:2).

found to be slightly dependent on the sample amount (±3°C). The endotherm was ascribed to the melting of crystallites and weakening of hydrogen bonds, which allows one to quantify starch behavior by the heat of gelatinization. Broadbean and pea starches have intermediate heats of fusion of 3.8 and 3.2 cal g^{-1} respectively, in comparison with wheat (2.7 cal g^{-1}), maize (3.1 cal g^{-1}), potato (4.6 cal g^{-1}) and manioc (3.9 cal g^{-1}) starches, which confirm the results of Stevens and Elton (1971) for cereal and tuber starches.

This investigation demonstrates that broadbean and pea starches are similar. Besides having a higher amylose content than normal cereal and tuber starches, the fine structure and the properties of amylose and amylopectin are very close to those of cereal. However, the crystalline structure, studied by X-ray diffractometry and lintnerization, is intermediate between the cereal and tuber types.

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Recovery of Salmonellae from Foods using a Combined Enrichment Technique

W.H. SVEUM and A.A. KRAFT

ABSTRACT

The separate steps of preenrichment in lactose broth and selective enrichment in tetrathionate (TET) or selenite-cystine (SC) broths were combined into single procedures for the detection of both injured and uninjured salmonellae in foods. Following 4 hr of incubation, iodine or selenite were added to combined lactose-tetrathionate (CTET) or selenite basal broths, forming selective environments. CTET broth was as efficient as conventional TET and SC broths but the combined selenite media were not. Salmonellae were detected in foods in 30 hr using direct fluorescent antibody and combined enrichment techniques. The combined procedure resulted in savings of assay time, labor and materials.

INTRODUCTION

PROCESSED FOODS may often contain a population of stressed or sublethally injured microorganisms (Busta, 1976). A characteristic of this injured state is the inability to tolerate conditions in which normal cells would readily multiply. In a suitable recovery medium, stressed cells may rapidly (1-3 hr) repair and regain normal metabolic functions (Ray and Speck, 1973; van Schothorst, 1976; Hurst, 1977; and Speck and Ray, 1977). Detection of stressed microorganisms in semiprocessed foods has been discussed by Ray (1979). Hartman (1979) has summarized modifications of methods to detect injured coliforms and salmonellae. Subsequently, a selective enrichment step is used to favor the growth of repaired or normal salmonellae and inhibit multiplication of competing microorganisms present in food samples (Litchfield, 1973; Fagerberg and Avens, 1976).

The separate preenrichment and enrichment steps each require a 24-hr incubation period resulting in 5 days for detection of salmonellae from foods using current cultural methods recommended by the Association of Official Analytical Chemists (1975) and the U.S. Food and Drug Administration, *Bacteriological Analytical Manual* (BAM) (1978). Combining each enrichment step into a single 24-hr enrichment procedure would be most advantageous. Alford and Knight (1969) have demonstrated favorable results by delaying the addition of selenite to SC broth. Lanz and Hartman (1976) detected injured and uninjured coliforms using timed-release capsules containing brilliant green and brilliant green plus oxgall. Timed-release capsules containing selenite or iodine have been used in the isolation of salmonellae from foods (Sveum and Hartman, 1977). These methods combine a short preenrichment phase (4 hr) followed by a longer selective enrichment step and eliminate the need for transfer from a preenrichment to selective enrichment medium. The present study demonstrates the feasibility of combining preenrichment in lactose broth and selective enrichment in TET or SC broths into a single combined enrichment procedure for detection of salmonellae in foods.

Author Sveum is affiliated with Armour and Company, Research Center, 15101 N. Scottsdale Rd., Scottsdale, AZ 85260. Author Kraft is with the Dept. of Food Technology, Iowa State Univ., Ames, IA 50011. Inquiries should be directed to Dr. Kraft.

MATERIALS & METHODS

Media

The following conventional media were utilized: lactose (Lac) broth, selenite-cystine (SC) broth, tetrathionate (TET) broth, brilliant green bile 2% (BGB 2%) broth, brilliant green (BG) agar, Salmonella-Shigella (SS) agar, bismuth sulfite (BS) agar, Levine eosin methylene blue (EMB) agar, triple sugar iron (TSI) agar, lysine iron (LIA) agar, (Difco Laboratories, Detroit, Mich.) trypticase soy (TSB) broth, and trypticase soy (TSA) agar, (BBL, Cockeysville, Md). The manufacturer's directions for preparation were followed for each medium.

Four combined enrichment media were utilized, selenite one (SEL 1) broth, selenite two (SEL 2) broth, selenite three (SEL 3) broth, and combined tetrathionate (CTET) broth.

CTET broth (Sveum and Hartman, 1977) consisted of (per liter): lactose broth, 13g; sodium thiosulfate, 30 g; calcium carbonate, 10g; and bile salts, (Difco) 1g. CTET basal broth differed from TET broth due to the presence of lactose and beef extract, components of lactose broth. The CTET basal medium was adjusted to pH 6.8, heated to boiling, and 225-ml aliquots were aseptically dispensed into sterile 500 ml flasks and stored at 5°C until needed. Immediately before use 2 ml of a stock iodine solution (iodine, 6g and potassium iodide, 5g per 20 ml sterile distilled water) was added per 100 ml of CTET broth.

SEL 1 broth (Sveum and Hartman, 1977) consisted of (per liter): lactose broth, 13g; disodium phosphate, 10; and L-cystine, 0.01g. The components of SEL 2 broth were (per liter): lactose, 5g; peptone, 5g; disodium phosphate, 10g; and L-cystine 0.01g. SEL 3 broth ingredients were (per liter): lactose, 4g; tryptone, 5g; disodium phosphate, 10g; and L-cystine, 0.01g. Selenite basals were adjusted to pH 6.8, and 225 ml quantities were added to 500 ml flasks and sterilized (121°C, 15 min).

The selective agent of SC broth, sodium acid selenite, is not present in the combined selenite basals. A selective environment was created by aseptically adding 10 ml of a filter sterilized solution of 9g sodium acid selenite (BBL, Cockeysville, Md.) per 100 ml of distilled water to 225 ml of each selenite basal medium. SEL 1 and SEL 2 differed in compositon from SC broth because they contained the components of lactose broth, peptone and beef extract, or peptone without beef extract, respectively. SEL 3 and SC broths were equal in composition except for the absence of sodium acid selenite in SEL 3 broth until later addition after an incubation period.

Production of heat and freeze injured salmonellae

Cultures of heat injured salmonellae were prepared using the method of Clark and Ordal (1969) with the following modifications. Suspensions of *Salmonella typhimurium, S. anatum* and *S. heidelberg* were heat-stressed at 52°C for 30 min rather than at 48°C and the extent of injury in the heat-stressed population was determined by surface plating 0.1 ml amounts diluted in 0.1% peptone distilled water blanks on predried TSA and EMB plus 2% NaCl (EMBS) agar plates. Following 48-hr incubation at 35°C, the number of colonies on TSA represented all viable cells, injured and uninjured, while EMBS counts represented only the number of uninjured cells in the population. The number of injured cells present in a suspension was equal to the difference between the TSA and EMBS counts. The same technique was used to produce a suspension of heat injured *E. coli.*

Freeze injured salmonellae were produced utilizing techniques described by Ray et al. (1971; 1972) with the following modifications. Suspensions of salmonellae were grown and harvested as previously mentioned for the production of heat injured cells. Cells were washed once in either 100 mM sodium phosphate buffer

(pH 6.0), sterile H_2O, or Ringer's solution and resuspended in 30 ml of the appropriate freezing menstruum. The cells were freeze-injured by adding 1 ml of the suspension to 9 ml of the appropriate medium and uniformly suspending the cells in an 18 x 150 mm test tube with a Vortex mixer. Freeze-injury inducing treatments were: (i) immersion in liquid nitrogen (LN_2) for 2 min; (ii) placement in carbon dioxide snow (CO_2 snow) for 10 min; or (iii) exposure to mechanical freezing in a blast freezer at −29°C for 24 hr. Following the initial freezing treatment, the cultures were stored at −29°C.

The number of injured cells in the frozen suspensions were determined by thawing a frozen culture in a beaker of water at 5°C for 20 min. Samples were surface plated on TSA or TSA plus 0.25% sodium deoxycholate (TSAD), and Xylose Lysine Peptone (XLP) agar and XLP plus 0.25% sodium deoxycholate (XLPD). After 48-hr incubation at 35°C, colonies on TSA and XLP represented all viable cells while TSAD and XLPD counts represented the number of uninjured salmonellae.

Resuscitation of heat- and freeze-injured salmonellae

The time required for heat-injured *S. typhimurium* or *S. anatum* to undergo resuscitation was determined by inoculating lactose and CTET broths incubated at 35°C with thermally stressed cells. Samples were removed at 0, 4, 8, 12, 16 and 24 hr after inoculation and surface plated on TSA and EMBS agar plates.

The resuscitation period required by freeze-injured salmonellae to repair in lactose, CTET and SEL 1 basal broths was determined by inoculating the media with approximately 10^5 cells per ml. Samples were removed at the time of inoculation and 4 hr later and surface plated on XLP and XLPD or TSA and TSAD.

Recovery of heat-injured salmonellae and *E. coli* in conventional and combined enrichment media

Cultures of *S. typhimurium*, *S. anatum*, *S. heidelberg* and *E. coli* were heat-injured at 52°C for 30 min. An inoculum was prepared immediately by diluting the heated suspensions to obtain concentrations of 100 heat-injured organisms per ml. Five ml of the *E. coli* suspension and 1 ml of the salmonellae cultures were used as an inoculum. Recovery media and methods were: direct enrichment in SC and TET broths for 24 hr; preenrichment in lactose broth for 24 hr followed by selective enrichment in SC and TET broths for 24 hr; or enrichment in the combined enrichment media, CTET, SEL 1, SEL 2 and SEL 3 for 24 hr. All enrichments were incubated at 35°C and selective agents were added to the combined enrichment broths after 4-hr incubation. A three-tube Most Probable Number (MPN) technique was used to estimate numbers of *E. coli* (Mayou, 1976) and salmonellae (Galton et al., 1968) in the recovery media.

Comparison of conventional and combined methods

Foods naturally and artificially contaminated with salmonellae were used for comparison of the combined and conventional procedures. Foods were obtained from various retail and industrial sources. Materials examined were: frozen ground beef, frozen turkey roasts, dried beef gravy, dried vegetable beef soup and spray-dried egg albumen. Because only turkey roasts and one egg sample were found by initial testing to be naturally contaminated, all products were inoculated with known levels of salmonellae. A modification of the technique of Kafel and Bryan (1977) was used to prepare suspensions of salmonellae containing a known level of viable cells. Frozen stock cultures were thawed when needed and the contents incubated at 35°C for 20 hr. Ten-fold serial dilutions of the broth cultures were prepared in Ringer's solution. The dilutions were pour plated with TSA and counted after 24-hr incubation at 35°C. The Ringer's suspensions were stored at 5°C and showed no decrease in cell numbers during 24- or 48-hr refrigerated storage. Utilizing this procedure, foods were inoculated with a known level of salmonellae ranging from 1-2 or 10 per gram of sample.

Frozen lean ground beef was thawed overnight at 5°C, and 25-g patties were prepared the next day. Except for control samples, each patty was inoculated with *S. typhimurium*, *S. anatum* or *S. heidelberg* by "spot" inoculating different areas of the surface to obtain approximately 10 salmonellae per gram. Each patty was wrapped in aluminum foil and frozen by one of the previously described freezing techniques. The patties were then stored in plastic foam boxes at −29°C.

The patties were examined for mesophiles and psychrotrophs and the presence of salmonellae after 0 and 60 days frozen storage. Mesophilic and psychrotrophic counts were determined using the methods described in BAM. The inoculated samples were analyzed for salmonellae 24 hr after initial freezing by means of the combined enrichment methods and conventional BAM procedures for raw, unprocessed meats. Duplicate patties thawed at room temperature were placed in blender jars containing 225 ml SEL 2, SEL 3 or CTET basals or TET plus brilliant green (TETBG) or SC broth and homogenized for 2 min. Selective agents were added to the basal broths after 4-hr incubation and in all subsequent comparison studies. Each enrichment was incubated for 24 hr at 35°C prior to selective plating. Half the patties examined following 2 month's storage were incubated at 35°C and the remaining patties at 43°C. Enrichments were then plated on selective agars after 24- and 48-hr incubation.

Frozen turkey roasts were thawed overnight at 5°C and 25-g samples were analyzed for salmonellae using combined and conventional enrichment techniques. Half the samples were inoculated with 1-2 or 10 *S. typhimurium*, *S. anatum*, and *S. heidelberg* per gram. The remaining samples served as controls to test the ability of the two methods to detect natural salmonellae contaminants. Mesophiles and psychrotrophs were enumerated using previously described procedures. Enrichment broths were assayed for salmonellae after 24-hr incubation at 35°C, 24- and 48-hr incubation at 35°C or after 24- and 48-hr incubation at either 35 or 43°C.

Twenty-five gram samples of dried beef gravy, vegetable beef soup or spray-dried egg albumin were added to the combined enrichment broths and lactose broth, which were then inoculated with 1-2 *S. typhimirium* or *S. heidelberg* per gram. The number of mesophiles in each dried food was determined using BAM methods. The enrichment broths were assayed for salmonellae after 24- and 48-hr incubation at 35°C.

RESULTS AND DISCUSSION

Repair of heat injured salmonellae

Heat shocking salmonellae at 52°C produced suspensions of 99% or greater injured cells. Injury was determined by comparing the number of salmonellae on TSA plates with the number on EMBS agar, the stress medium. *E. coli* suspensions, when heated at 52°C, were more resistant to the sublethal heat treatment with only 18% of the population being injured. The repair of *S. typhimurium* in lactose broth and CTET basal broth incubated at 35°C is illustrated in Figure 1. This typical growth curve indicates the time required for resuscitation of the injured cells. *S. typhimurium* cells repaired rapidly with almost 100% of the population undergoing resuscitation within 4 hr, when differences in counts between TSA and EMBS media were only slight. Recovery from heat injury occurred in CTET broth equally as well as in lactose broth even though bile salts were present in the combined medium. Observations from similar growth curve studies also indicated that the majority of heat injured *S. anatum* cells had undergone repair after 4-6 hr incubation in lactose and CTET broths.

The effect of adding selenite to SEL 1 broth containing heat-injured salmonellae after 0, 4, and 6-hr incubation at 35°C is demonstrated in Table 1. Little difference was observed in time for repair and subsequent multiplication after addition of selenite. A 4-hr preenrichment phase was found to be sufficient particularly for *S. anatum*, and as work with mixed cultures showed, overgrowth by other organisms could occur with prolonged preenrichment.

Repair of freeze-injured salmonellae

The repair of freeze-injured salmonellae after 4-hr incubation in lactost broth and the combined enrichment media is presented in Table 2. The suspensions contained approximately 90% stressed cells. Resuscitation occurred in each repair medium; however, complete repair of the entire population did not occur in any of the enrichment broths during the 4-hr preenrichment period. Repair was more rapid

in SEL 3 and CTET broths than lactose or SEL 2 broths. This demonstrates the nonselective nature of the combined enrichment broths, similar to the conventional preenrichment medium, lactose broth. A 4-hr nonselective enrichment phase allowed repair of many injured cells.

Results from resuscitation studies using heat- and freeze-injured salmonellae illustrate the ability of stressed cells to undergo repair within 4 hr. In all subsequent studies, a preenrichment period of 4 hr was used prior to the addition of selective agents to the combined enrichment basal broths. A preenrichment phase less than 4 hr based on these studies would only allow the recovery of less than 50% of the stressed salmonellae. Any preenrichment step delayed longer than 4 hr could allow competing organisms to reach a level that may mask or inhibit the growth of any *Salmonella* present.

Growth of heat-injured salmonellae and *E. coli* in salmonellae enrichment media

The ability of heat injured salmonellae to undergo repair in various salmonellae enrichment media and multiply to measurable levels is illustrated in Table 3. Selective agents were added after 4-hr incubation. The initial concentration of the mixed population per ml was approximately 10 *E. coli* cells per *Salmonella* cell.

Multiplication of *E. coli* was significantly inhibited in TET and CTET broths. Results from this study indicate the need for a selective enrichment step in salmonellae assays. Salmonellae were greatly overgrown in lactose broth by the *E. coli*; 10^8 *E. coli* per ml and 10^2 salmonellae per ml. When aliquots of the lactose preenrichments were transferred to SC and TET broths, the salmonellae grew to greater concentrations but *E. coli* cells also multiplied to high levels. These organisms could have interfered with the isolation of salmonellae on selective agars prepared from SC and TET broths. The combined technique alleviates this problem of competitor overgrowth by utilizing only a 4-hr preenrichment step as opposed to the 24-hr period used in conventional assays. The selenite combined broths also inhibited the growth of *E. coli* more effectively than methods using SC broth. SEL 2 broth was the most effective of the combined selenite media for inhibiting the growth of *E. coli*.

There was no significant difference ($P \leq 0.01$) between methods when comparing the recovery of injured salmonellae in CTET broth to growth in TET broth with or without a lactose preenrichment step. Growth of salmonellae in SEL 2 broth was not significantly different ($P \leq 0.05$) from that obtained with direct selective enrichment in SC broth. Preenrichment in lactose broth followed by selective enrichment in SC broth produced a significantly greater population of salmonellae than enrichment in the combined selenite media. Because injured salmonellae did not grow as well in SEL 1 broth, it was eliminated from further studies.

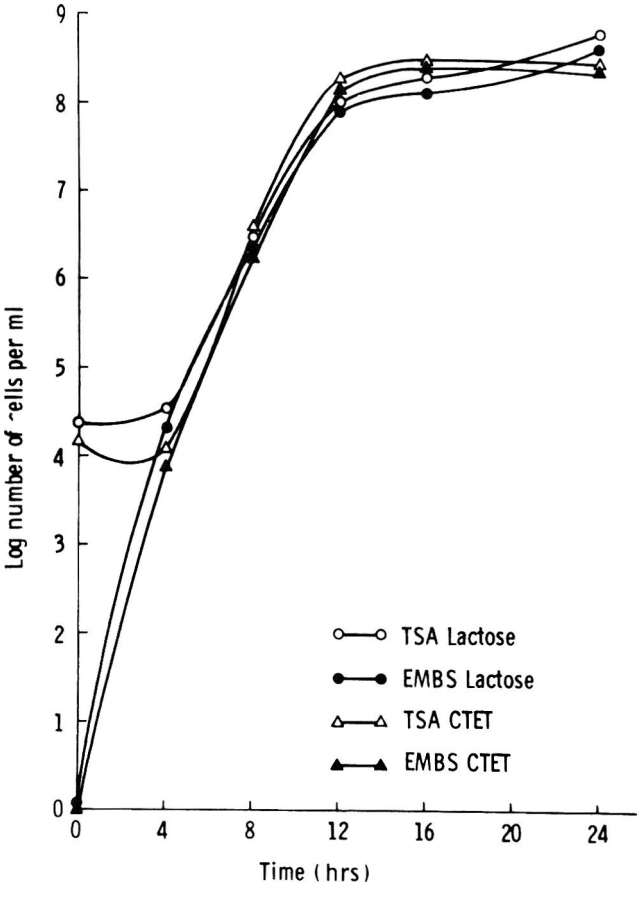


Fig. 1—Growth of heat injured S. typhimurium *in lactose broth and CTET broth.*

Table 1—Effect of adding selenite to SEL 1 broth 0, 4, and 6 hr after inoculation with heat injured salmonellae and grown for 24 hr

Time of addition	*S. anatum*	*S. typhimurium*
	No. bacteria per ml x 10^8	
0	2.80	1.40
4	1.70	1.10
6	1.90	.59
No selenite added	5.50	4.60

Comparison of combined and conventional methods

Frozen ground beef was selected as a type of food to be analyzed because competitors such as pseudomonads, coliforms and *Moraxella-Acinetobacter* were expected to be present. Unless these organisms are inhibited by the selective enrichment step, they could interfere with the detection of salmonellae in the samples of beef. Freezing the inoculated patties might also injure those salmonellae present. Temperatures recorded inside the beef patties during cryogenic freezing, $-78^\circ C$ for CO_2 snow and $-19^\circ C$ for LN_2, may have induced sublethal freeze injury. Presence of an injured population of salmonellae in the beef samples would test the ability of the combined enrichment broths to detect injured cells.

Isolation of salmonellae from patties before and after frozen storage is indicated in Table 4. Selective agents were added after 4-hr incubation in enrichment media. Before frozen storage, all samples enriched in SEL 2, CTET, TETBG and SC broths were salmonellae positive. Four negative samples were observed when SEL 3 broth was used as a recovery medium. When beef patties were analyzed after 60 days storage, the method of analysis was

Table 2—Percent of freeze injured salmonellae after 4-hr incubation at 35° C in nonselective recovery media

Organism[a]	Recovery medium[b]			
	Lac	SEL 2	SEL 3	CTET
S. heidelberg	50	65	22	33
S. typhimurium	67	29	23	24
S. anatum	65	93	61	80
Mean	61	62	35	45

[a] Injury was induced by freezing cultures suspended in phosphate buffer for 2 min in LN_2.
[b] Results are expressed as that percentage of the population that had not undergone repair after 4 hr at 35° C.

Table 3—Growth of injured salmonellae and E. coli *in various salmonellae enrichment media*[c]

Organism[a]	Enrichment medium								
	TET	CTET	SC	SEL 1	SEL 2	SEL 3	Lac	LacTET	LacSC
Salmonella	2.1×10^8	2.0×10^8	8.7×10^7	3.0×10^7	4.3×10^6	4.6×10^5	2.2×10^2	7.0×10^7	7.1×10^8
E. coli	10	2.8×10^3	8.7×10^7	8.1×10^4	1.5×10^5	1.7×10^6	3.5×10^8	7.6×10^6	9.1×10^7

[a] Counts represent the average log number of cells per ml from three repetitions with *S. typhimurium, S. anatum,* and *S. heidelberg.*

Table 4—Comparison of conventional and combined methods for the recovery of salmonellae from frozen ground beef

Storage time (Days at −29°C)	No. of samples±	Number of salmonellae positive replications[a]									
		Combined[c]						Conventional[c]			
		SEL 2		SEL 3		CTET		SC		TETBG	
		24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
0	18	18		14		18		18		18	
35°C	9	4	7	3	5	9	9	9	9	9	9
43°C	9	6	9	3	6	9	9	9	9	9	9

[a] A replication was recorded as positive if a single *Salmonella* sp. was isolated from it.
[b] All the enriched samples examined after 0 days storage were incubated at 35°C while the patties analyzed after 60 days frozen storage were incubated at either 35°C or 43°C.
[c] Conventional and combined enrichment broths were streaked onto BG, SS, and BS agar after 24-hr incubation when the patties were stored 0 days and after 24- and 48-hr incubation following 60 days storage.

modified. The same enrichment methods were used but the culture broths were streaked onto selective agars after 24-48 hr incubation at either 35°C or 43°C.

Recently, Kafel and Bryan (1977) and Gabis and Silliker (1977) have demonstrated an enhanced detection of salmonellae when 43°C was used as an incubation temperature. Increased isolations of salmonellae have also been obtained by lengthening the incubation period of selective enrichment broths from 24-48 hr (Edel and Kampelmacher, 1973, 1974; Sveum and Hartman, 1977).

After 60 days frozen storage, every sample enriched in CTET, TETBG and SC broths was salmonellae positive using the modified incubation procedures. Negative samples were observed when patties were enriched in SEL 2 and SEL 3 broths. More salmonellae isolations occurred in SEL 2 broth than SEL 3 broth at both incubation times and temperatures but the difference was not significant ($P \leq 0.05$). Detection in the combined selenite media was improved by the additional 24-hr incubation period but incubation at 43°C was not significantly advantageous.

The types of freezing treatments utilized had no significant effect ($P \leq 0.05$) on the survival of salmonellae or the total microbial population in the patties. The average number of mesophiles and psychrotrophs present after the initial freezing and storage was 6.4×10^4 and 7.2×10^4, respectively.

Frozen turkey roasts were included in the comparison study because they are often naturally contaminated with *Salmonella.* Frozen roasts are also examples of a processed meat product which would require a preenrichment step in order to enhance the detection of stressed salmonellae. Preenrichment of processed meats has been recommended by Gabis and Silliker (1974) and Edel and Kampelmacher (1973). Competitive flora including pseudomonads and *Proteus* present on the roasts, unless suppressed by a selective enrichment step, would interfere with the isolation of salmonellae. Levels of mesophiles and psychrotrophs, 9.0×10^4 and 9.4×10^4 per gram respectively, similar to those observed on the ground beef patties, were present in the turkey roasts.

Samples of the roasts were inoculated with salmonellae to compare the ability of each enrichment medium to detect a known concentration of cells. Also, control samples were analyzed by each comparison method to deter-

Table 5—Comparison of conventional and combined methods for the recovery of salmonellae from frozen turkey roasts

Experiment	Sample	No. of samples	Number of salmonellae positive samples[a]									
			Combined						Conventional			
			SEL 2		SEL 3		CTET		LacSC		LacTET	
			24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
1[b]	Control	6	–		–		2		2		2	
	Inoculated	6	4		5		6		6		6	
2[c]	Control	6	–	–	–	–	2	2	2	1	2	2
	Inoculated	6	1	2	1	2	6	5	6	6	6	6
3[d]	Control 35°C	3	–	–	–	–	1	1	–	1	–	–
	43°C	3	–	–	–	–	2	2	2	2	2	2
	Inoculated 35°C	3	–	–	–	–	3	3	2	2	3	3
	43°C	3	1	–	–	–	3	3	3	3	3	3

[a] A replication was recorded as positive if a single *Salmonella* sp. was isolated from it.
[b] Duplicate enriched control and inoculated (8-10 *Salmonella* per g) turkey roast samples were incubated at 35°C and streaked onto BG, SS, BS agar after 24-hr incubation.
[c] Duplicate enriched control and inoculated (1-2 *Salmonella* per g) turkey roast samples were incubated at 35°C and streaked onto BG, SS, BS agar after 24- and 48-hr incubation.
[d] Single enriched control and inoculated (1-2 *Salmonella* per g) turkey roast samples were incubated at 35°C and 43°C and streaked onto BG, SS, BS agar after 24- and 48-hr incubation.

Table 6—Comparison of conventional and combined methods for the recovery of salmonellae from dried beef gravy, vegetable beef soup, and spray-dried egg albumen inoculated with salmonellae

Type of food	Sample	No. of inoculated samples per recovery method[a]	Number of salmonellae positive replications[b]									
			Combined[c]						Conventional[c]			
			SEL 2		SEL 3		CTET		LacSC		LacTET	
			24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr	24 hr	48 hr
Gravy	A	2	2	2	2	2	2	2	2	1	2	2
Gravy	B	1	1	1	1	1	1	1	1	1	1	1
Soup	A	2	2	2	1	2	2	2	2	2	2	2
Soup	B	1	1	1	1	1	1	1	1	1	1	1
Egg	A	2	2	2	2	2	2	2	2	2	2	2
Egg	B	2	2	2	2	2	2	2	2	2	2	2
Egg	C	1	0	0	0	0	0	1	0	0	0	0
Total	7	11	10	10	9	10	10	11	10	9	10	10

[a] All samples were inoculated with 1-2 salmonellae per gram except spray-dried egg albumen sample C, which was naturally contaminated with salmonellae.

[b] A replication was recorded as positive if a single *Salmonella* sp. was isolated from it.

[c] Conventional and combined enrichment broths were streaked ontc BG, SS, and BS agar after 24- and 48-hr incubation at 35°C.

Table 7—Comparison of FA and conventional identification methods for the detection of salmonellae in various foods inoculated with known salmonellae

Sample	Confirmation method	Enrichment medium				
		SEL 2	SEL 3	CTET	SC	TET
Ground Beef A	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Ground Beef B	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Ground Beef C	FA	+	–	+	+	+
	Conventional	+	–	+	+	+
Turkey Roast 1	FA	+	–	+	+	–
	Conventional	+	–	+	+	+
Turkey Roast G	FA	–	–	+	+	+
	Conventional	–	–	+	+	+
Dried Gravy B	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Dried Soup B	FA	+	+	+	+	+
	Conventional	+	+	+	+	+
Dried Egg A	FA	+	+	+	+	+
	Conventional	+	+	+	+	+

mine the effectiveness of the media for isolating natural *Salmonella* contaminants.

Table 5 illustrates the results from the analysis of turkey roasts. Control samples were found to be contaminated with *S. san diego, S. st. Paul, S. heidelberg, S. anatum, S. bredeney*, and *Arizona hinshawii*. These contaminants were isolated in CTET, TET and SC broths but were not isolated from any sample enriched in SEL 2 broth or SEL 3 broth. CTET broth was as effective as the conventional enrichment media and detected natural salmonellae contaminants not isolated from SC and TET broths.

The combined selenite broths were not as effective as conventional methods used to detect salmonellae in inoculated turkey roast samples. SEL 2 and SEL 3 broths were able to detect 8-10 salmonellae per gram but were not sensitive to a low level of 1-2 salmonellae per gram. Lengthening the incubation period by 24 hr enhanced recovery in SEL 2 and SEL 3 broths slightly but not significantly in comparison to SC broth. Incubation at 43°C also did not improve the efficiency of the combined selenite techniques but it did enhance detection in CTET, TET and SC broths.

These studies with frozen turkey roasts demonstrate the reliability of CTET broth. Only one more inoculated sample was detected using the conventional tetrathionate medium than CTET broth. However, one additional control sample was salmonellae positive in CTET broth but negative in TET broth. All control samples positive in SC broth were salmonellae negative when enriched in SEL 2 and SEL 3 broths. The combined selenite media also failed to isolate salmonellae from many inoculated samples. The inefficiency of the combined selenite media is probably due to their inability to suppress the multiplication of competitors as effectively as the other enrichment media.

Isolation of salmonellae from dried foods

Dried foods were chosen because they must undergo a preenrichment phase in order to determine the presence of salmonellae sublethally injured by the drying process. Each sample was inoculated with 1-2 salmonellae per gram, except spray-dried egg sample C which was naturally contaminated. Every enrichment medium was analyzed for salmonellae after 24- and 48-hr incubation at 35°C. A *Salmonella* species was detected in dried egg sample C only by CTET broth (Table 6). CTET broth was as efficient as TET broth, which is indicated in Table 6. SEL 3 broth was equal to SC broth in efficiency, but SEL 2 broth detected one additional sample which was negative by the other selenite enrichment methods.

The results for dried foods demonstrate the reliability of CTET broth as was found for beef and turkey, but SEL 2 and SEL 3 broths were also as effective as the conventional SC medium. However, the increased sensitivity of the combined selenite broths must be interpreted with caution. Fewer mesophilic competitors per gram, 4.0×10^4 in the dried gravy mix, 6.0×10^3 in the soup, and 8.2×10^2 in the egg samples were present in the dried foods than in the meat products tested. The nature of the flora of the desiccated foods was also different because fewer gram-negative organisms were observed and more gram-positive sporeforming rods were encountered. The combined selenite media may have been more reliable with dried foods because the growth of salmonellae was not suppressed by large populations of competing gram negative bacteria.

Detection of salmonellae in the various foods analyzed in the comparison study by cultural methods and the FA technique is presented in Table 7. There were no false positive samples determined by the FA procedure, but one turkey roast sample which was culturally positive in TET broth was a false negative sample by the FA test. Only 30 hr were required to detect the presence of salmonellae in the samples with the FA technique while cultural methods required 5 days.

Fewer fluorescing cells were consistently observed on FA slides prepared from the combined selenite broths, with SEL 3 broth containing the fewest cells per positive field. Slides made from SC, TET and CTET broths generally contained similar numbers of fluorescing cells per field.

Background fluorescence was more of a problem with the ground beef and turkey samples than the dried products which did not contain as much fat as the meat samples. Results from this study indicate that salmonellae will reach levels detectable by the direct FA technique within 24 hr in the combined enrichment broths.

A Chi-square analysis of these data indicated no significant difference ($P \leq 0.01$) existed between CTET, TET and SC broths for use as salmonellae enrichment media. SC broth was significantly more effective than SEL 2 and SEL 3 broths. Although more salmonellae isolations occurred in the SEL 2 medium than the SEL 3 combined broth, the difference was not significant.

The additional 25-hr incubation period enhanced the detection of salmonellae enriched in SEL 2 and SEL 3 broths but this improvement was not statistically significant ($P \leq 0.05$). The improvement may have been due to the multiplication of the salmonellae to detectable levels after 48-hr incubation. Because competitors are not as readily inhibited in the combined selenite broths as in CTET broth, a 48-hr incubation period will enhance detection of salmonellae in foods when enriched in the combined selenite media.

The results of this study demonstrate the feasibility of combining the separate steps of preenrichment and selective enrichment used in salmonellae essays. A reduction in incubation time was obtained utilizing the combined method because only 24 hr were needed for incubation of samples containing stressed salmonellae. Forty-eight hour incubation is required when using the conventional procedures which utilize separate 24 hr nonselective and selective enrichment periods.

Reductions in materials and labor required for *Salmonella* assays are achieved utilizing the combined enrichment techniques. Transfers did not have to be made from preenrichment media to selective enrichment broths. Also, only one set of media and glassware had to be prepared and cleaned using the combined technique while conventional methods required two sets of media and glassware.

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Effect of Proteolytic Bacteria in the Natural Fermentation of Corn to Increase its Nutritive Value

PENKWAN TONGNUAL, NANCY J. NANSON, and MARION L. FIELDS

ABSTRACT

There was no significant difference in % relative nutritive value, lysine or tryptophan levels between natural lactic fermentations of Costa Rican corn meal fermented with tap water of pH 7.2 and 5.3 However, the fermented values were all significantly ($P<0.05$) higher than nonfermented values. Most of the increase in % relative nutritive value occurred in the first 48 hr. Free essential amino acids increased and the amino acid balance changed during typical fermentations. Proteolytic bacteria decreased after 3 days of the fermentation and declined to a few cells per ml by the 4th day. Proteolysis decreased by the second day of natural lactic fermentation.

INTRODUCTION

HAMAD AND FIELDS (1979) found that a natural lactic acid fermentation increased the %RNV (relative nutritive value) significantly ($P<0.05$) for wheat, barley and rice, and very significantly ($P<0.01$) for millet and maize, as measured by *Tetrahymena pyriformis*. The available lysine content, as measured by the microbiological methods of Difco (1977) increased significantly ($P<0.05$) in fermented oats, rice, millet, and maize, but the available lysine increase was highly ($P<0.01$) significant in fermented wheat. Using the same techniques, Zamora and Fields (1979) reported that the %RNV increased significantly ($P<0.05$) for the legumes, cowpeas and chickpeas. The limiting amino acids, methionine, isoleucine, and tryptophan increased significantly ($P<0.05$) for chickpeas. The methionine and isoleucine contents increased significantly ($P<0.05$) for cowpeas, but tryptophan content was not determined for this bean. In these experiments with cereals and legumes, the duration of the fermentation corresponded to the lowest pH achieved, which was considered inhibitory to food poisoning bacteria. The optimal time period to produce a pH of 3.6-4.1 was 4-6 days, depending upon the material being fermented.

In the studies of Hamad and Fields (1979), and Zamora and Fields (1979), the corn and beans were ground through a 1-mm screen in a Wiley Mill. All of the proteins were thus exposed to proteolytic activities of the flora during the fermentation, and it was this proteolysis which was thought to cause an increase in %RNV. Isolates of lactics made by Zamora (1978) and Hamad (1978) showed that only one isolate was slightly proteolytic. Thus, it was postulated that the proteolytic activities occurring in the fermentation were not due to the lactics, but to some other members of the microflora.

The question of the nature of indigenous flora on corn found at different geographic locations (for example corn grown in acidic soils) was considered as a factor which might influence the improvement of the protein quality by fermentation. Previous research (Hamad and Fields, 1979; Zamora and Fields, 1979), was done with corn grown in Missouri. Since fermentation has potential as a method for the poor to improve the protein quality of cereals and legumes in developing countries, it seemed essential to subject corn from a developing country to the same procedures that were used in Missouri. Corn from Costa Rica was obtained and fermented with the same pH (5.3) of water as occurs in San José, Costa Rica. Thus, a series of experiments were designed to focus on the improvement of protein quality by a natural flora. Emphasis was placed on proteolytic bacteria during the fermentation and their influence upon protein quality and amino acid balance.

Authors Tongnual, Nanson, and Fields are affiliated with the Dept. of Food Science & Nutrition, Univ. of Missouri-Columbia, Columbia, MO 65211.

MATERIALS AND METHODS

Preparation and fermentation of corn

Two 50-lb bags of corn, *Zea mays*, were purchased from MFA Exchange in Columbia, MO. After being passed through a #4 standard testing sieve to remove debris and broken kernels, 400g were ground through a 1 mm screen in a Wiley Mill.

Approximately 4 kg of field corn were obtained from Dr. Ronald Echandi, Univ. of Costa Rica, San Jose, Costa Rica. The corn samples were collected within a 50-mile radius of San Jose. This corn was cleaned through a sieve and ground in a Wiley mill, as stated above.

Slurries of both types of corn meal were made by adding enough tap water to give a 1:4 w/v ratio, with the exception of the 1:16 w/v ratio used in the pure culture experiment to test %RNV. The mixtures were incubated at 34°C for 4 days. The number of replications were varied with experiment. After fermentation the samples were dried at 50-55°C for 48 hr in a Freas model 835 air-circulating oven, and reground in a Wiley Mill.

Moisture and nitrogen

The moisture and nitrogen contents of the nonfermented and fermented samples were analyzed as described by AOAC (1975). The moisture content was measured by drying the sample at 130°C for 1 hr. The amount of nitrogen present was determined by using the micro-Kjeldahl method (AOAC, 1975).

Relative nutritive value (RNV)

RNV of the fermented and nonfermented corn was analyzed using *Tetrahymena pyriformis* W, (ATCC 10542), according to Stott et al. (1963). Certified casein (Fisher Scientific Co.) was used as a reference protein.

Aqueous suspensions of fat-free samples (ether extracted) were prepared to give a nitrogen content of 3 mg/4 ml, and adjusted to pH 8.2. To 4 ml of the suspensions were added solutions of minerals, nucleic acids, glucose, and vitamins in the sequence described by Stott et al. (1963). After being autoclaved, the tubes were cooled and inoculated with 3 drops of a 3-day broth culture of *T. pyriformis* W. The tubes were incubated in an inclined position, with loosened screw caps at 25°C for 4 days. After incubation, 1 ml of each culture was transferred to a test tube containing 1 ml of 36% formaldehyde preserving fluid. The Tetrahymena cells were counted in a double cell Lumicyte hemacytometer, according to Stott et al. (1963). RNV was calculated according to the formula derived by Helms and Rølle (1970) as follows:

$$RNV = \frac{\log \text{count/ml for test protein} - \log \text{ct/ml for inoculum}}{\log \text{ct/ml for casein} - \log \text{ct/ml for inoculum}} \times 100$$

Isolation and identification of proteolytic microflora

A corn meal slurry was made for Missouri corn by mixing (1:4 w/v) corn meal with sterile tap water. The slurry was fermented 48 hr at 34°C. During the fermentation, samples of the meal were taken aseptically, and plated on skim-milk agar (APHA, 1976).

A total of 20 samples were taken in three experiments. The plates were incubated at 34°C for 24-48 hr, and screened for proteolysis using a #3330 Darkfield Quebec Colony Counter, by detection of a clear zone around the proteolytic colony. The colonies surrounded by proteolysis were isolated and transferred to nutrient agar slants for later identification. The experiment was repeated three times.

Identification of the proteolytic microorganisms, isolated from fermenting corn, was determined according to *Bergey's Manual* (1974). Each of the 21 organisms isolated from Missouri corn was gram stained (APHA, 1976), and found to be a gram-negative rod. The gram negative rods were stabbed into nutrient agar tubes and the tubes were overlaid with cool, liquid agar, to a depth of 1 inch. The agar was allowed to harden, and the tubes incubated for 24 hr at 34°C. The organisms were then identified as aerobic (growth only on top of slant), and were classified in genus according to Bergey (1974).

pH and titratable acidity

Costa Rican corn meal was mixed with tap water, 1:4 w/v, and separated into 2 groups. In the first group the pH lowered from 7.2 (pH of water before addition of meal) to 6.8 (pH after addition of the meal). In the second group the pH raised from 5.3 (pH of adjusted water before addition of meal) to 6.1 (pH after addition of meal). Both groups were fermented 4 days at 34°C. Starting at time 0 and each day thereafter, a 10-ml sample was taken from each group of fermenting corn, and tested for pH and titratable acidity (T.A.). The pH was measured using a Beckman Zeromatic pH meter. The sample was titrated with NaOH and the T.A. determined by the following formula:

$$\text{T.A.} = \frac{\text{N NaOH} \times \text{ml NaOH} \times 0.09 \times 100}{\text{ml sample}}$$

Determination of total proteolytic colonies for 4 days

Missouri corn meal was mixed in a 1:4 w/v ratio with tap water, and incubated 4 days at 34°C. During the fermentation, the microflora was examined by the Gram stain. Starting at time 0 and every 4 hr thereafter, 1 ml of sample was collected aseptically from the fermenting meal, and diluted with 9 ml of 0.1% sterile peptone water to give dilutions of $1:10^2$, $1:10^3$, $1:10^4$, $1:10^5$, and $1:10^6$. Each of these dilutions was pipetted into 2 plates of skim milk agar to detect proteolytic colonies. The skim milk agar plates were overlaid with 1% sterile agar (BBL) to prevent the possible spreading of proteolytic organisms. All of the plates were allowed to harden, and were incubated 48 hr at 34°C. After incubation, the proteolytic colonies were counted with a Darkfield Quebec Colony Counter #3330, by detection of a clear zone around the colony. In addition to these determinations, the pH was measured at each of the time intervals using a Beckman Zeromatic pH meter to see if a decrease in pH might cause inhibition of the proteolytic bacteria during this time period. The experiment was repeated 3 times.

Resistance of proteolytic micoflora to varying pH

Twenty-one proteolytic microorganisms were isolated from fermenting Missouri corn meal. They were each diluted into 100 ml sterile distilled water, and thoroughly mixed. Two ml of each suspension was added to each of 6 nutrient broth tubes, adjusted with lactic acid to the following pH's: 5.3, 5.0, 4.7, 4.4, 4.1, 3.8. Uninoculated pH controls for the nutrient broth were made for each pH range, and rechecked after the autoclaving and incubation periods. Uninoculated controls at each pH range were also used as reference blanks. After inoculation, the tubes were incubated at 34°C for 4 days, and examined for growth. The experiment was run in duplicate.

Effect of pH on relative nutritive value

Costa Rican corn meal was added to each of 2 beakers of tap water, 1 preadjusted to pH 5.3 with 0.1N HCl and the other of pH 7.2, the pH of nonadjusted tap water, in a 1:4 w/v ratio. After the addition of corn meal to the water, the pH changed to a value of 6.1 and 6.8, respectively. A third sample was treated as a control and was not adjusted in pH. The 2 test slurries were fermented for 4 days at 34°C; the control was not fermented. After fermentation the slurries were dried, as above, and tested for relative nutritive value.

Relative nutritive value evaluated over 7 days

Costa Rican corn meal slurries were prepared with tap water to give a 1:4 w/v ratio. They were incubated at 34°C from 0-7 days. Each day 3 were removed from the incubator and dried at 50-55°C. The 0 day meal was not fermented, and thus served as a control. In this way, meal fermented for differing lengths of time was evaluated. After the 7-day period, the dried meals were subjected to RNV analysis.

Relative nutritive value using pure cultures

Corn meal slurries were made by mixing 1:16 w/v Missouri corn meal with tap water. The mixtures were autoclaved for 15 min at 121°C and allowed to cool. The 1:16 w/v ratio was used to avoid the production of lumpy samples which occurred after autoclaving when a 1:4 w/v ratio was used. Two of the proteolytic organisms (one loopful), isolated from Missouri corn, were added to each of 2 bottles of 100 ml sterile distilled water, and vigorously mixed. Ten ml of each suspension were added to a separate slurry and to 100 ml sterile nutrient broth, and then incubated for 4 days at 34-37°C. The nutrient broth served as a control for growth, and 100 ml uninoculated sterile nutrient broth was used as a control on sterilization. Uninoculated slurries were used as experimental controls. After fermentation, the slurries were dried at 50-55°C for 48 hr in an air-circulating oven, and reground in a Wiley Mill. The samples were then tested for moisture content and relative nutritive value as outlined above. The experiment was repeated 3 times on 3 consecutive days.

The microbiological assay method of Difco (1977) was used to determine the available lysine and tryptophan contents after a papain digestion. *Pedicoccus cerevisiae* ATCC 8042 was the test organism for the lysine assay, and *Lactobacillus plantarum*, ATCC 8014 was used in the tryptopahn assay. Reagent-grade L–(+)–lysine monohydrochloride, and DL-tryptophan (both of Eastman Kodak Co.) were used to prepare standards.

The meal used in this experiment was mixed with tap water preadjusted to either 5.3 or 7.2, as in previous experiments. Two samples of fermented and one sample of nonfermented Costa Rican corn meal, each containing 100 mg N, were predigested with 1 ml of 2% w/v papain solution for 3 hr at 56°C (Ford, 1964). Three other samples, in a second group, 2 fermented and 1 nonfermented, each containing 100 mg N, were not treated with papain. After the enzymic digestion of the first group with papain, each preparation of both groups were adjusted to pH 7.2 and diluted with distilled water to 100 ml. The samples were then shaken vigorously and 20-ml portions were diluted to 100 ml with distilled water. This dilution was added to tubes in amounts of 1, 2, 3, 4, and 5 ml. Five milliliters of the amino acid assay medium (Difco Lab., Detroit, MI) were then added to each assay tube, and all the tubes were autoclaved for 10 min at 121°C. After cooling, each tube was inoculated with one drop of diluted suspension of test culture, and the tubes were incubated at 35-37°C for 72 hr. The acidity of each culture was titrated with 0.1N NaOH to pH 6.8. The standard curves for lysine and tryptophan were constructed following the Difco Technical Information (1977). The amino acid content of each sample was calculated based on the respective standard amino acid curve. 'Free' available lysine and tryptophan were determined without papain digestion; whereas, total available values were determined after papain digestion. The difference between the digested and nondigested values was the bound value. The bound and free values added together were the total available values.

Analysis of amino acid profile (Total amino acid values)

The amino acid profile of the control and fermented Missouri corn meal was analyzed by the method of Benson and Patterson (1971). The samples were hydrolyzed with 6N HCl for 22 hr at 110°C. Prior to hydrolysis, the samples were oxidized by performic acid for the determination of cystine as cystic acid. The amino acid analyses were performed by automated, cation-exchange chromatography (Benson and Patterson, 1971). Each of the samples was analyzed with and without HCl hydrolysis. In this research, these amino acids are the total, absolute amino acid values.

Analysis of free amino acids

Water extracts of nonacid hydrolyzed corn meals were examined as above. The values obtained by this procedure are free amino acids.

Measuring protein hydrolysis using a modified formol titration

A modified formol titration (Harrow et al., 1955) was used to determine the degree of protein hydrolysis of lactic and pure culture fermentations and thus give a measure of the amount of free amino acids produced during the 4-day fermentation.

Five slurries of corn meal in tap water (1:4 w/v) were prepared

for a natural lactic fermentation, and 4 of them were incubated at 37°C for 1-4 days. The nonincubated slurry served as the zero time control and was immediately tested. A 10-ml aliquot was pipetted from the control slurry into each of 2 beakers, and adjusted to pH 8.0 using a Beckman Zeromatic pH meter. A 15-ml portion of formalin was added to each beaker and thoroughly mixed. The sample was then titrated back to pH 8.0 using 0.02N NaOH. The milligrams of amino acid nitrogen were calculated from milliliters of base used by the formula: 0.28 mg amino acid nitrogen=1 ml of 0.02N NaOH. The fermented slurries were removed 1 day at a time and analyzed in the same manner as was the control slurry.

The slurries and organisms used in this experiment for pure culture fermentations were prepared in the same way as in the previous experiment on RNV using pure cultures; however, before inoculation of the slurries the 2 bacterial suspensions used were each standardized to 87%T at 540 nm using a Bausch and Lomb Spectronic 20 spectrophotometer. This was done to insure a constant inoculum between the slurries. The remainder of the experiment was performed as above.

Statistical analyses

Data were analyzed by analysis of variance (Snedecor and Cochran, 1967) and by Duncan's new multiple range test (Duncan, 1955).

RESULTS

pH and titratable acidity

Data in Table 1 show that Costa Rican corn meal mixed with either tap water of pH 7.2 (pH of Missouri tap water) or adjusted tap water of pH 5.3 (pH of Costa Rican water) had the same pattern in terms of pH and titratable acidity. The corn meal with its initial water adjusted to pH 5.3 decreased in pH and increased in titratable acidity more than did the corn meal with the original water pH of 7.2.

Determination and identification of total proteolytic bacteria for 4 days

Plate counts of Missouri corn using milk agar increased from a log count of 2.3 to 8.3 in 24 hr. The count stayed at a log count of 8.2 at 48 hr but then dropped to a log count of 4.7 in 72 hr. At 96 hr (4 days) the log count was 0 (Fig. 1).

The proteolytic bacteria were predominately aerobic, gram negative rods which are characteristically members of the family Pseudomonadaceae. Approximately 21 proteolytic colonies were isolated from the fermentations.

Table 1—Effect of the initial pH of water before fermentation on pH and titratable acidity of Costa Rican corn meal during fermentation (N = 14).

Days of fermentation	pH Tap water[a]	pH adjusted water[b]	Titratable acidity Tap water	Titratable acidity Adjusted water
0	6.7	6.1	0.03	0.04
1	5.0	4.8	0.29	0.27
2	4.4	3.8	0.49	0.67
3	4.3	3.8	0.56	0.80
4	4.1	3.7	0.61	0.88

[a] Columbia, Missouri, water

[b] pH was adjusted with 0.1N HCl to simulate water at San Jose, Costa Rica.

Resistance of proteolytic microflora to varying pH

All of the proteolytic bacteria isolated from a natural fermentation of Missouri corn meal, (and inoculated into nutrient broth over a pH range 5.3-3.8) were inhibited at pH 4.7 and lower.

Effect of pH on RNV

When Costa Rican corn meal was fermented using water of pH 7.2 and 5.3, the %RNVs were 82.8 and 83.3, respectively, which were both significantly ($P<0.05$) higher than the nonfermented control with a RNV of 76.8%.

RNV evaluated over 7 days

Since Costa Rican corn reacted similarly regardless of the starting pH and since the pH and titratable acidity values were similar to previous fermentations of Missouri corn, data from the fermentations of both Costa Rican and Missouri corn are combined to show trends (Fig. 1).

The %RNV of fermented Costa Rican Corn meal increased significantly ($P<0.05$) during the first 4 days from 77% in the nonfermented corn to 85.8% by the 4th day in the fermented corn. Days 5, 6 and 7 (not shown in Fig. 1) were 86, 87, and 86%, respectively. During the first 5 days the %RNV increased 9%.

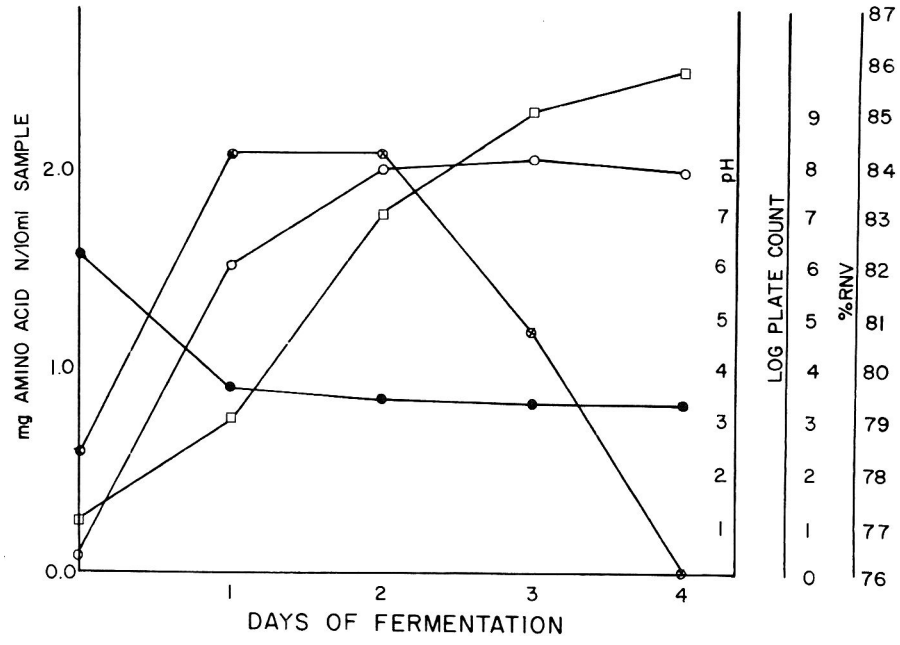


Fig. 1 – Mg amino acid nitrogen, pH, log plate count and %RNV over a 4-day fermentation: x —— x Log plate count; • —— • pH; ○ —— ○ Formol titration; □ —— □ %RNV.

RNV using pure cultures

Two of the most proteolytic isolates (as judged from the amount of proteolysis on skim milk agar), from a natural corn meal fermentation (Missouri Corn) were used to determine their influence on %RNV in a pure culture system. Pseudomonad No. 1 produced no increase at all over its control (73.5% vs. 73.7%). Although pseudomonad No. 2 produced an increase over its control (80.1% vs 74.6%), the difference was not statistically significant.

Determination of available lysine and tryptophan by microbiological analyses

The effect of fermentation on Costa Rican corn meal is given in Table 2. Since some samples were not digested with papain, the analyses of these samples reflect "free" lysine and tryptophan values. Those samples digested with papain before analysis are the total available lysine and tryptophan values. The difference between the total available and free is the bound value.

The available "free" lysine contents of the slurries using tap water of pH 7.2 and 5.3, were 97.5 mg/100g and 92.6 mg/100g, respectively. When compared to the nonfermented control value of 29.9 mg/100g, the fermented samples (regardless of the initial pH) increased significantly ($P<0.05$).

Most of the available lysine was in the "free" state after fermentation (87% and 85%); whereas, only 60% of the available lysine in the nonfermented sample was "free." Forty percent of the available lysine in the nonfermented sample was in the bound form.

The same trend that occurred with lysine also occurred with tryptophan. Most of the available tryptophan was in the free form in the fermented samples (69% and 75%), and most of the available tryptophan was in the bound form in the nonfermented samples (71%).

Analysis of amino acid profile

Both the data in Table 2 and Table 3 show that lysine as free lysine occurred in nonfermented Missouri and Costa Rican corn meal before fermentation.

Essential amino acids in Missouri corn meal in the free form are presented in Table 3. The data show that all of the amino acids increased in the free form during the first 3 days of the fermentation but decreased slightly on the 4th day.

By dividing the amount of lysine into the quantities of the other amino acids, ratios were developed. Any change in the balance (the ratio of one amino acid to another) will be indicated by the ratio values. Data in Table 3 show that the ratio did change during the fermentation when the "free" amino acids were compared.

The ratios for nonfermented corn and casein are given also. The casein ratios are more desirable than those of the nonfermented corn. The ratios for days 2 and 3 are closer to the ratios of casein than the other days.

The maximum amount of amino acids released (%) due to the proteolysis in 3 days is shown in Table 3. For the essential amino acids, the highest values were 30.0% for lysine and 20.0% for methionine. The release of nonessential amino acids is shown in Table 4.

Measuring protein hydrolysis using a modified formol titration

Another measure of protein hydrolysis is the formol titration technique. The milligrams of amino acid N increased from 0.45 to 1.49 mg per 10 ml sample at the first day of natural lactic fermentation. Proteolysis slowed at the second day of the fermentation of Missouri corn meal and formed a plateau on the third and fourth days (2.02 and 1.92 mg amino acid per 10 ml sample) (Fig. 1).

When a pure culture fermentation was measured by formol titration, the mg amino acid N increased from 0.81 mg per 10-ml sample on day 0 (control sample) to 3.22 mg on day 3 to 6.44 mg on day 5 of fermentation. The amino acid values did not level off as they did in natural lactic fermentation.

DISCUSSION

THE DATA which are a combination of reactions using Missouri and Costa Rican corn meal (Fig. 1) suggest that during natural lactic fermentation, as the lactics reduced the pH and increased the titratable acidity (Table 1), the proteolytic bacteria declined and were almost completely destroyed by the fourth day of the fermentation. Inoculation of proteolytic bacteria into nutrient broth adjusted to various pH units also showed that the proteolytic bacteria were sensitive to pH. The formol titration data for natural lactic fermentations and pH data, above, suggest that not only were the proteolytic bacteria killed due to the effect of pH and titratable acidity, but their enzymes became inactive at the more acidic pH. If their enzymes had not been inactivated, the free amino acid values of the formol titration would have kept increasing, which they did not.

The data presented herein suggest that water with a pH of 5.3 did not prevent the proteolytic bacteria from hydrolyzing the proteins and allowing an increase in available amino acids (lysine and tryptophan). Presumably the in-

Table 2—Available lysine and tryptophan of fermented and nonfermented Costa Rican corn meal using tap water adjusted to pH 7.2 or 5.3

Treatment	Lysine (mg/100g)					Tryptophan (mg/100g)				
	% Free[b]	Free	Total available	Bound[c]	% Bound	Free[b]	% Free	Total available	Bound[c]	% Bound
Fermented Costa Rican corn meal using tap water of pH 7.2, resulting in a final pH of 6.8	97.6a	87	111.6a	14.1	13	12.7a	69	18.4a	5.7	31
Fermented Costa Rican corn meal using tap water adjusted to pH 5.3, resulting in a final pH of 6.1	92.6a	85	109.2a	16.6	15	14.7a	75	19.7a	5.0	25
Nonfermented Costa Rican corn meal using tap water of pH 7.2	29.9a	60	50.1b	20.2	40	1.0b	29	3.4b	2.4	71

[a] Corn was fermented for 4 days at 34°C. Each value represents an average of 14 replications. Values with different letters are significantly different ($P<0.05$) when compared vertically in the table.

[b] Free means that sample was assayed without papain digestion.
[c] Bound means that total available (with papain digestion-free).

Table 3—Essential amino acids, mg/100g of corn meal and ratios of lysine to other essential amino acids in Missouri grown corn

Essential amino acids	Free amino acids after days of fermentation[a]					Total amino acids in corn meal[b] (nonfermented)	Total amino acids in casein[c]	Maximum amount release (%) due to proteolysis in 3 days[d]
	0	1	2	3	4			
Lysine	10	50	56	69	43	230	1077	30.0
Leucine	18	49	58	110	99	1040	1262	10.6
Isoleucine	4	8	8	19	16	290	718	6.6
Phenylalanine	12	28	36	59	53	410	695	14.4
Methionine	2	9	13	26	20	130	370	20.0
Valine	10	27	32	49	46	400	894	12.3
Threonine	5	7	5	13	11	280	618	4.6

	Free amino acids					Total amino acids in nonfermented corn meal	Total amino acids in casein
	Lysine						
	0	1	2	3	4	Lysine	Lysine
Leucine	1.80	0.98	1.04	1.59	2.30	4.52	1.17
Isoleucine	0.40	0.10	0.14	0.28	0.37	1.26	0.67
Phenylalanine	1.20	0.55	0.64	0.86	1.23	1.78	0.65
Methionine	0.20	0.18	0.23	0.38	0.47	0.57	0.34
Valine	1.00	0.54	0.57	0.71	1.07	1.74	0.83
Threonine	0.50	0.14	0.09	0.19	0.26	1.22	0.57

[a] Water extracted with column chromatography — free available amino acids.
[b] Acid hydrolysis followed by column chromatography — total absolute amino acids.
[c] Composition of casein according to FAO (1970).
[d] Free amino acid values on day 3 divided by total amino acids in nonfermented corn meal.

crease in available lysine and tryptophan was responsible for the increase in %RNV with water at pH 5.3 and 7.2, and the acidic pH of 5.3 did not inhibit an increase in %RNV.

From Figure 1, it can be seen that the %RNV increased the most during the first 2 days of fermentation (77-83%) and slightly increased from the second to the fourth day (83-85.5%). The fifth, sixth and seventh days were not shown since the %RNV varied only by 1% (86%, 87%, and 86%), respectively. Thus, most of the biochemical activities which help to cause an increase in %RNV during natural lactic fermentations seem to be happening in the first 48 hr.

The %RNV did not increase significantly when pure cultures were used in the fermentation; however, the milligrams amino acid N, as seen from the formol titration, increased over a 5-day fermentation period and did not level off as in the natural lactic fermentation. This is to be expected, since in a pure culture fermentation, no lactics are present to cause the production of lactic acid and a subsequent drop in pH. The pseudomonads would not be inhibited by an acidic pH and would continue their growth and production of proteolytic enzymes. Since, the %RNV did not increase when an individual pseudomonad was used in the fermentation, it might be concluded that more than one proteolytic organism from the flora is necessary for the increase in %RNV or that other nonproteolytic organisms are needed.

Data obtained by microbiological analysis showed that an increase both in lysine and tryptophan occurred during the fermentation. The increase in lysine was also confirmed by the increase in "free" lysine, as determined by chemical analysis. This increase occurred when using tap water at either pH 7.0 or pH 5.3 for fermentation, and indicated that fermentations carried out in Costa Rica, using water of pH 5.3, would probably increase these limiting amino acids.

When ratios were developed for the free essential amino acids in Table 3, it can be seen that in most cases, the balance approached that for total casein amino acids by the second to third day. This is also the time when %RNV and mg amino acid nitrogen begin to level off, and the plate count for proteolytic organisms begins to decrease.

Table 4—Maximum amount of amino acids released due to proteolytic activity (3 days) mg/100g of nonessential amino acids in Missouri grown corn

Amino acid	Total	Released	% of Total released
Asparatic acid	630	33	5.2
Serine	350	37	10.5
Glutamic acid	1590	102	6.4
Proline	773	125	16.2
Glycine	320	19	5.9
Alanine	620	81	13.1
Cystine	110	3	2.7
Tyrosine	260	46	17.7
Histidine	240	38	15.8
Arginine	330	34	10.3

The ratios for total amino acids in nonfermented corn meal were not as close to the casein ratio as were the free amino acid values. Thus, acid hydrolysis of nonfermented corn meal would not improve the amino acid balance as compared to the balance of amino acids in casein.

The data reported herein confirm previous observations of Hamad and Fields (1979) and Zamora and Fields (1979) that a natural fermentation increases lysine and methionine limiting amino acids in other cereals and legumes. Since lysine and methionine are limiting in corn, one would expect that their maximum release due to proteolysis might be less than the more abundant amino acids. This was not the case; however, since lysine (30% release) and methionine (20%) release had the highest values. It would seem that a biochemical mechanism such as transamination might be operating during fermentation to explain the presence of high levels of free limiting amino acids.

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Functional Properties of a Food Colorant Prepared from Red Cabbage

G. M. SAPERS, I. TAFFER, and L. R. ROSS

ABSTRACT

Spectral, colorant, and stability properties of colorants based on red cabbage, grape, cranberry, beet, and Red No. 40 were compared in buffer and in a simulated beverage. At pH 3, red cabbage imparted a red color similar to that of beet juice and less orange than that of the other colorants. Increasing the pH of red cabbage extracts to 4 produced a bathochromic shift and decreased color strength. Tristimulus parameters displayed maxima when L was varied. Red cabbage pigments were more stable during heating and storage than were the other colorants tested. pH had little effect on color stability; exposure to light greatly increased color loss during storage.

INTRODUCTION

CONSIDERABLE INTEREST has been shown in the utilization of unconventional colorants as replacements for synthetic red food dyes since the banning of FD&C Red Nos. 2 and 4 and the threatened loss of Red No. 40. Natural colorants have not yet proven satisfactory as substitutes for the synthetic dyes because of their high cost, limited supply, and technical limitations (Riboh, 1977).

Shewfelt and Ahmed (1977, 1978) proposed that red cabbage (*Brassica oleracea*) be used as a colorant for dry soft drink mixes. They demonstrated that pigment preparations obtained from this plant imparted a color superior to that obtained with Red No. 40. Color stability was satisfactory both in the dry state and in reconstituted beverage mixes during refrigerated storage. Preliminary studies conducted in our laboratory with carbonated beverages containing red cabbage pigments also suggested a high degree of color stability.

The pigments of red cabbage consist primarily of cyanidin 3-sophoroside-5-glucoside and cyanidin 3-sophoroside-5-glucoside acylated with sinapic, ferulic, p-coumaric, and malonic acids (Tanchev and Timberlake, 1969; Hrazdina et al., 1977). Van Buren et al., (1968) reported that acylated anthocyanins were more stable to light than were nonacylated anthocyanins.

The objectives of our research were twofold: (1) to characterize the spectral and colorant properties of red cabbage pigments at pH values between 3 and 4 and compare them to other natural colorants; and (2) to determine the stability of red cabbage pigments in aqueous solution as a function of pH, light, and temperature.

EXPERIMENTAL

Colorant preparation

Fresh red cabbage samples representing ten different cultivars (Debut, Hybrid Red YR150, Red Storage 4004, Mammoth Red Rock, Meteor, Red Danish, Red Drumhead, Red Head Hybrid, Resistant Red Acre, and Ruby Ball) were obtained from Fordhook Farms, the experiment station of the W. Atlee Burpee Co. in Doylestown, Pa. Cabbages were quartered, cored, and blended with 1% HCl in MeOH (1:1 w/v) in a 1 gal Waring Blendor. The resulting homogenates were held overnight at room temperature and then filtered, first through cheesecloth and then through Whatman No. 1 filter paper in a centrifugal filtration device. Filtrates were refrigerated and then further clarified by the addition of 1% Celite Analytical Filter-Aid (Johns-Manville Products Corp., Lompoc, Cal.) followed by filtration through Whatman No. 2 paper with suction. The clarified filtrates were concentrated ten to twentyfold with a Thomas Magne-Flash Evaporator Model 38 (Arthur H. Thomas Co., Philadelphia, Pa.) operated at a water bath temperature of 35-40°C and a vacuum of 0.03 kg/cm² (29 in Hg). Foaming was controlled by the application of Dow Corning Antifoam A Spray (Dow Corning Corp., Midland, Mich.) Pigment concentrates were frozen and stored at −18°C until required.

Samples of other natural and synthetic colorants were obtained from commercial sources so that their functional properties could be compared with those of red cabbage pigments. These samples included a grape concentrate, cranberry concentrate, beet juice concentrate, and FD&C Red No. 40.

Measurement of spectral and colorant properties

Aqueous pigment solutions were prepared by diluting concentrates with pH 3, 3.5 or 4 McIlvaine's buffer (Hodgman, 1954) and then readjusting to the original pH value of the buffer by adding 10% NaOH to compensate for the high acidity of the pigment preparations. The pigment solutions were diluted further with buffer so that absorbance and tristimulus coordinate measurements would fall within a useful range. Additional dilutions were made at pH 1 and 4.5, following the procedure of Fuleki and Francis (1968), so that values of their Simplified Degradation Index could be calculated for the anthocyanin-based colorants. A Perkin-Elmer Model 552 UV-VIS Double Beam Spectrophotometer (Perkin-Elmer Corp., Norwalk, Conn.) was used to measure λ_{max} and corresponding absorbance values for visible absorption bands. A Gardner XL-23 Tristimulus Colorimeter (Gardner Laboratory, Inc., Bethesda, Md.), operated in the transmission mode, was used to obtain values of the tristimulus coordinates L, a_L, and b_L for a series of colorant dilutions ranging in absorbance at λ_{max} between 0.4 and 3.0. Tristimulus measurements were made in a clear glass optical cell, 57.1 mm in diameter (ID) and 40 mm in height, filled to a depth of 20 mm with a 50-ml sample.

To determine the pH sensitivity of anthocyanin-based colorants, absorbance values for pH 3, 3.5, and 4 solutions were expressed as a percentage of the absorbance obtained at pH 1. To compare the color strength of different colorants, the quantity of product (or raw material, in the case of red cabbage) required to produce an absorbance at λ_{max} of 1.0 in 1L of pH 3.0 buffer was estimated from the spectral data and appropriate dilution factors. Values of the hue angle θ ($\tan^{-1} b_L/a_L$) and saturation index $(a_L^2 + b_L^2)^{1/2}$ were computed from the tristimulus coordinates. Tristimulus parameters of colorants samples were compared at different degrees of lightness, including L-values corresponding to $A\lambda_{max} = 1.0$ and L = 50. In addition, since the tristimulus parameters are known to vary with L and may, in some cases, display a maximum (VanBuren et al., 1974), values of the hue angle were plotted against L for each colorant sample so that L-values corresponding to maxima could be determined.

Evaluation of heat stability

The heat stability of red cabbage pigments and other natural colorants was determined by immersing duplicate loosely capped 125 x 16 mm screw cap test tubes containing 12-14 ml of colorant solution in a boiling water bath for as long as 5 hr (depending on pigment losses) followed by cooling in an ice bath for 3 min. Color-

At the time the work reported in this paper was done, all authors were with the USDA-SEA-AR Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118. Author Taffer was a student trainee in the Cooperative Education Program at ERRC. Author Ross is now retired.

ant solutions to be heated were prepared with pH 3 or 3.5 McIlvaine's buffer, adjusted in concentration so that the initial absorbance at λ_{max} was between 1.0 and 1.5. In addition, a solution of red cabbage colorant (Meteor cultivar) was prepared by diluting the pigment concentrate with a simulated beverage containing 10% sucrose and 0.3% citric acid monohydrate, adjusted to pH 3 ot 3.5 by titration with 10% NaOH solution. Following heating and cooling, the sample absorbance was measured at λ_{max} with a Bausch and Lomb Spectronic 88 Spectrophotometer.

Evaluation of storage stability

The storage stability of colorants prepared from red cabbage and cranberry concentrates was determined in air at 25°C by a procedure used previously for beet pigments (Sapers and Hornstein, 1979). Colorant concentrates were boiled 2 min to inactivate enzymes, cooled, clarified by the addition of 1% Celite followed by filtration through Whatman No. 2 paper with suction, diluted with pH 3 or 3.5 McIlvaine's buffer to a concentration yielding an absorbance at λ_{max} of 1.2-1.5, readjusted to the original pH of the buffer with 10% NaOH, sterilized by membrane filtration and dispensed aseptically into sterile tubes for storage. Stability tests were also conducted with the simulated beverage described previously instead of buffer; samples were adjusted to pH 3 and 3.5. Sample tubes were stored under fluorescent light (90-100 ft.-c) or in darkness for as long as 9 months.

Following storage, measurements of absorbance at λ_{max} and the tristimulus coordinates L, a_L, and b_L were made, as described previously.

Table 1—Spectral properties of colorants prepared from red cabbage extracts, grape concentrate, and cranberry concentrate

Colorant	pH	λ_{max}	% of absorbance at pH 1.0	Degradation index[b]
Red cabbage extract[a]	3.0	524	43.1	1.17
	3.5	528	29.6	–
	4.0	531	19.0	–
Grape concentrate	3.0	518	51.5	1.16
	3.5	519	32.0	–
	4.0	520	18.9	–
Cranberry concentrate	3.0	523	78.5	1.70
	3.5	526	64.5	–
	4.0	530	52.4	–

[a] Meteor cultivar
[b] Simplified Degradation Index, as defined by Fuleki and Francis (1968).

RESULTS & DISCUSSION

Spectral properties

To be suitable as replacements for synthetic food dyes, natural pigments should be capable of imparting the desired color over a useful pH range. The spectral and colorant properties of extracts of nine red cabbage cultivars, other natural colorants, and Red No. 40 were compared at pH values between 3 and 4, a pH range which encompasses many artificially colored food and beverage products. Visible absorption maxima (λ_{max}) for aqueous anthocyanin solutions vary with pH. All red cabbage cultivars studied were similar with respect to pH response and the general appearance of spectra. Data for Meteor cabbage, a more highly pigmented but otherwise similar cultivar, are given in Table 1. At pH 3, λ_{max} values for solutions of red cabbage pigments were between 519 and 526 nm, depending on the cultivar. Absorption maxima shited to 524-529 nm at pH 3.5 and to 526-534 nm at pH 4. Values of λ_{max} were higher for red cabbage than for grape at the same pH or for Red No. 40 (496 nm), which is not pH-sensitive. Absorption maxima were similar for red cabbage and cranberry colorants at the same pH. Values of λ_{max} for red cabbage solutions at pH 3-4 were lower than λ_{max} for beet juice concentrate (532 nm), which is not pH-dependent.

Increasing the pH of solutions containing red cabbage pigments not only produced a bathochromic shift but also substantially lowered their color strength, as measured by the absorbance at λ_{max}. Similar changes occurred with solutions of grape concentrate. The smaller pH effect seen with cranberry probably reflects the substantial contribution of degradation products to the sample absorbance at λ_{max}. This is consistent with the higher Degradation Index obtained with cranberry concentrate in comparison to that for red cabbage and grape. The pH sensitivity of these colorants within a relatively narrow pH range represents a serious limitation to their utilization.

Colorants prepared from the different red cabbage cultivars varied in color strength over a twofold range. These differences may be related to cultivar, maturity, extraction efficiency, or other factors. We have estimated from the spectral data that the quantity of colorant obtained from 26-63g of red cabbage, depending on the sample, would produce an absorbance at λ_{max} of 1.0 in 1L of pH 3 buffer solution; this absorbance also could be obtained with 23 mg of Red No. 40, 3 ml of beet juice concentrate, 6 ml of

Table 2—Colorant properties of red cabbage extracts and other colorants

		Tristimulus value								
		Constant absorbance ($A\lambda_{max}$ = 1.0)					Constant L value (L = 50)			
Colorant	pH	L	a_L	b_L	θ	Sat'n index	a_L	b_L	θ	Sat'n index
Cabbage ext.[a]	3.0	50.5	75.4	6.4	4.9	75.7	78.3	8.9	6.5	78.9
	3.5	45.2	75.4	3.8	2.9	75.6	74.5	1.3	1.0	74.5
	4.0	39.7	72.6	−2.6	357.9	72.6	65.0	−7.5	353.4	65.5
Grape conc[b]	3.0	49.0	67.4	20.4	16.8	70.4	66.6	19.8	16.5	69.5
	3.5	42.8	65.7	19.0	16.1	68.4	61.0	16.3	15.0	63.2
	4.0	35.3	61.2	16.0	14.7	63.3	51.8	13.2	14.3	53.5
Cranberry conc[b]	3.0	39.7	66.9	19.1	15.9	69.5	59.1	16.8	15.8	61.4
	3.5	35.3	64.0	17.0	14.9	66.3	52.9	14.7	15.5	54.9
	4.0	32.2	61.8	15.1	13.7	63.7	47.8	12.8	15.0	49.5
Beet conc[b]	3.0	42.7	80.3	9.5	6.8	80.7	73.9	1.2	0.9	74.0
	5.0	43.3	80.5	14.0	9.9	81.7	74.0	7.5	5.8	74.4
Red No. 40[b]	3.0	61.4	65.4	29.5	24.1	71.7	77.8	33.1	23.0	84.6
	4.0	61.4	65.4	29.5	24.1	71.7	77.4	33.1	23.2	84.2

[a] Meteor variety
[b] Commercial colorant

grape concentrate, or 35 ml of cranberry concentrate. The pigment contents of these products were not estimated since data on the proportions of individual anthocyanins and their extinction coefficients were not available for the red cabbage extracts. The relatively low color strength obtained with cranberry concentrate may be a consequence of pigment loss during processing or storage, as indicated by the higher Degradation Index of this colorant.

Colorant properties

Comparisons of the colorant properties of red cabbage pigments, other natural colorants, and Red No. 40 were based on tristimulus measurements made on solutions at pH 3-4, diluted to provide a wide range of color strengths (Table 2). Values of the tristimulus coordinates L, a_L, and b_L, as well as the hue angle θ and saturation index were similar for red cabbage colorants prepared from seven different cultivars at each of the pH's tested. Increasing the pH of cabbage pigment solutions, adjusted in concentration to $A\lambda_{max}$ = 1.0, tended to make them darker (lower L) and more blue (lower b_L). Solutions adjusted in concentration to constant lightness (L = 50) became less red (lower a_L) with increasing pH. In both cases, the change in hue angle θ with increasing pH was indicative of a color change from orange-red at the lower pH to violet-red at the higher pH.

Solutions of red cabbage pigments and other colorants differed in lightness when compared at $A\lambda_{max}$ = 1.0 (Table 2). Red cabbage produced darker solutions (lower L) than did Red No. 40, but was similar in lightness to grape and was lighter (higher L) than were solutions of cranberry and beet concentrates. Red cabbage pigments produced a less orange shade of red (lower hue angle θ) than did the other colorants, except for beet pigment which was similar. This is a reflection of slightly higher a_L values and much lower b_L values for red cabbage and beet pigments. The saturation index, an indication of color purity, generally paralleled a_L.

pH-Related color differences appeared to be greater with red cabbage pigments than with the other colorants in this study. This is evident in the spread of b_L and θ values at pH 3-4. The color and tristimulus coordinates of Red No. 40 solutions were not affected by pH; a small increase in b and hue angle occurred when the pH of beet juice was increased from 3 to 5.

With each of the colorants compared in this study, the chromaticity parameters (a, b, θ, and the saturation index) generally increased to a maximum value and then decreased as the pigment concentration was increased. L-values corresponding to these maxima (L_m) are given in Table 3. Differences in L_m between red cabbage cultivars were small. Maximum values of a and the saturation index occurred at similar L-values with red cabbage, grape, and beet colorants; with cranberry, these maxima appeared at lower L-values. Maxima for b and θ occurred at lower L-values with red cabbage extracts than with the other colorants. Chromaticity parameter maxima were not observed with cranberry concentrate (θ at pH 3.5 and 4), beet juice concentrate (b and θ), and Red No. 40 (a and the saturation index). With the anthocyanin-based colorants, increasing the pH shifted the maxima to lower L-values.

Van Buren et al. (1974) suggested that an anthocyanin solution observed in a container such as a wine glass, for which the light path varies over a wide range, would exhibit a variety of hues according to the θ vs L relationship. However, Eagerman et al. (1973) reported that the appearance of such solutions did not agree with the predicted hue at lower L-values; i.e., on dilution, colorant solutions might shift in hue with increasing L but would not return towards the original hue of the concentrated solution when L_m was exceeded. They attributed the discrepancy to an inherent weakness in the equations relating chromaticity parameters to the X, Y, Z color scale. This error could affect the accuracy of color measurements made on dark-colored, highly concentrated pigment solutions. In our study, however, all tristimulus data for red cabbage extracts and beet juice concentrates reported herein corresponded to L-values greater than L_m. Values of b and θ for pH 4 grape concentrate and for cranberry concentrate at $A\lambda_{max}$ = 1.0 (Table 2) did correspond to L-values near the maximum and consequently would be subject to error. Johnson et al. (1976) obtained relatively poor correlations between Gardner b or hue values and visual rankings or the anthocyanin concentration of cranberry juice cocktail samples, although they stated that their samples were not dark enough to be sub-

Table 3—Chromaticity parameter maxima with red cabbage extracts and other colorants

Colorant	pH	L-value at maximum			
		a	b	θ	Sat'n index
Cabbage ext.[a]	3.0	41-44	34-38	33-37	39-41
	3.5	38-41	28-33	27-32	37-39
	4.0	32-33	22-26	20-25	30-33
Grape conc[b]	3.0	39	41	41	39
	3.5	36	37	39	36
	4.0	32	35	38	32
Cranberry conc[b]	3.0	35	38	43	35
	3.5	29	35	>58[c]	31
	4.0	27	33	>56[c]	28
Beet conc[b]	3.0	39	<32[c]	<32[c]	37
	5.0	39	<34[c]	<34[c]	37
Red No. 40[b]	3.0	<49[c]	53	57	<49[c]
	4.0	<49[c]	53	58	<49[c]

[a] Range for 7 cultivars
[b] Commercial colorant
[c] No maximum observed with dilutions tested

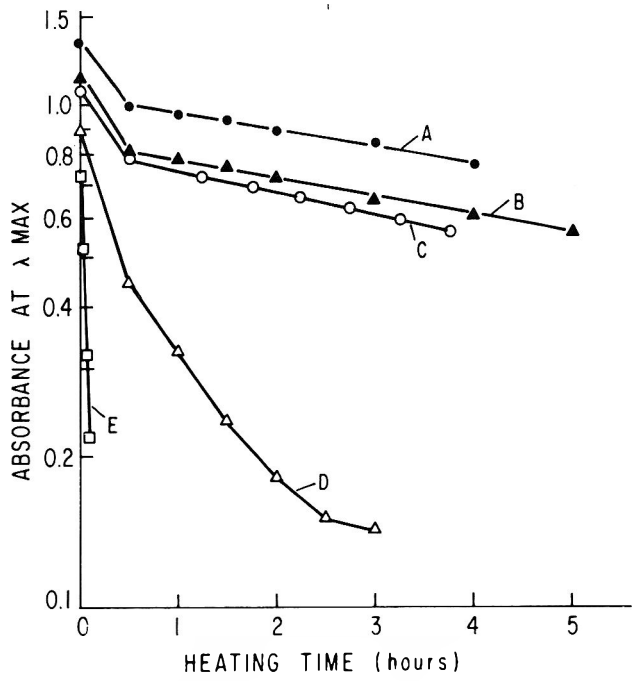


Fig. 1—Effects of heating at 100°C on the absorbance of pH 3 buffer solutions of colorants: (A) Meteor cabbage; (B) Resistant Red Acre cabbage; (C) Red Head cabbage; (D) cranberry concentrate; and (E) red beet.

ject to the L_m error.

Heat stability of red cabbage pigments

Measurements of the change in absorbance at λ_{max} of colorants prepared from three red cabbage cultivars (Red Head, Resistant Red Acre, and Meteor), cranberry concentrate, and red beet (Detroit Dark Red cultivar; data taken from Sapers and Hornstein, 1979), diluted with pH 3 buffer and heated at 100°C, are compared in Figure 1. Semi-log plots of the red cabbage stability data show an initial decrease in absorbance during the first 30 min of heating, followed by a more gradual decrease which appears to be linear. Pigment extracts obtained from the three red cabbage cultivars yielded similar stability curves and displayed a much greater degree of heat stability than did the pH 3 solutions of cranberry concentrate or red beet juice. Differences in stability between anthocyanin-based colorants may be related to their degree of acylation, the acylated red cabbage anthocyanins (Hrazdina et al., 1977) being more heat stable than the nonacylated cranberry anthocyanins (Zapsalis and Francis, 1965). Van Buren et al. (1968) reported that wines containing acylated pigments retained their color more completely during heating (6 days at 50°C) than did wines containing nonacylated anthocyanins.

The heat stability of a colorant prepared from Meteor cabbage was determined at pH 3 and 3.5 buffer and in a simulated beverage containing sucrose and citric acid at concentrations similar to those used in such products as carbonated beverages, fruit drinks, and frozen fruit pops. Semi-log plots of the absorbance vs heating time data gave similar curves with buffer and the simulated beverage at both pH values (Fig. 2). Other workers have reported that the heat stability of anthocyanins is independent of pH in the absence of air and is greater at lower pH values in the presence of air (Markakis, 1974). We suggest that our system approached the former condition as a consequence of the venting of dissolved O_2 and headspace air from the loosely capped sample tubes during the first few minutes of heating.

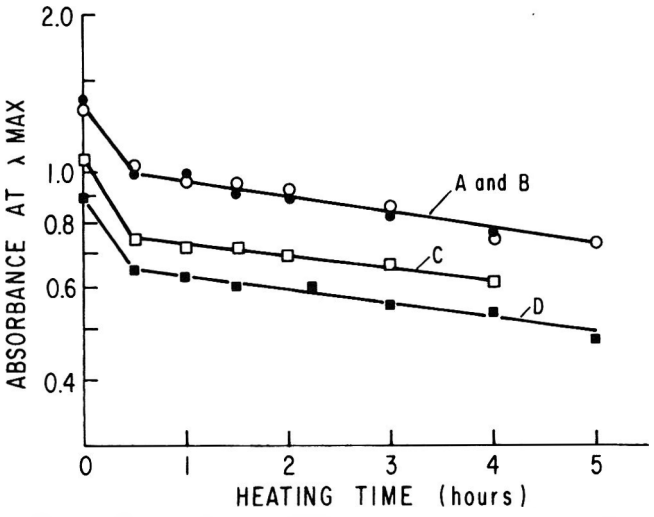


Fig. 2—Effects of heating at 100°C on the absorbance of solutions of Meteor cabbage colorant: (A) in pH 3 buffer; (B) in pH 3 simulated beverage; (C) in pH 3.5 simulated beverage; and (D) in pH 3.5 buffer.

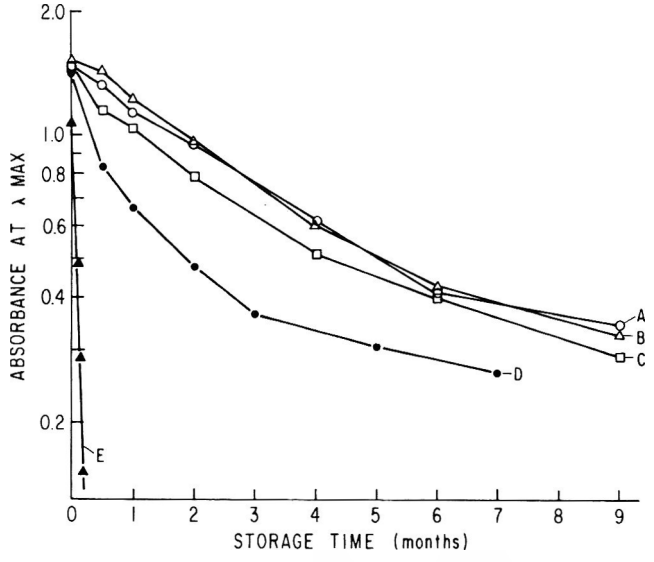


Fig. 3—Effects of storage in light at 25°C on the absorbance of pH 3 buffer solutions of colorants: (A) Meteor cabbage; (B) Red Head cabbage; (C) Resistant Red Acre cabbage; (D) cranberry concentrate; and (E) red beet.

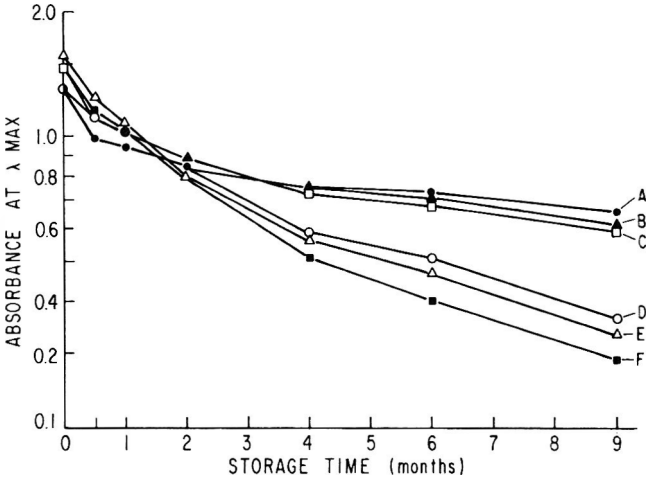


Fig. 4—Effects of storage at 25°C on the absorbance of colorant solutions prepared from Resistant Red Acre cabbage: (A) pH 3 simulated beverage, darkness; (B) pH 3.5 buffer, darkness; (C) pH 3 buffer, darkness; (D) pH 3 simulated beverage, light; (E) pH 3.5 buffer, light; (F) pH 3 buffer, light.

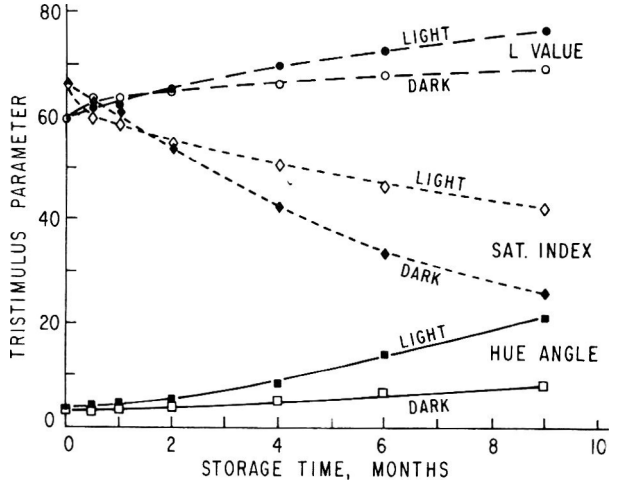


Fig. 5—Effects of storage at 25°C in light and in darkness on tristimulus parameters of Resistant Red Acre cabbage colorant in simulated beverage at pH 3.

Storage stability of red cabbage pigments

During storage at 25°C in air, the color of aqueous solutions of red cabbage pigments gradually faded and became more orange. Changes in the absorbance at λ_{max} of pH 3 solutions of colorants prepared from red cabbage (Resistant Red Acre, Red Head, and Meteor cultivars), cranberry concentrate, and red beet (Detroit Dark Red cultivar; data taker. from Sapers and Hornstein, 1979) during storage in light at 25°C are shown in Fig. 3. The three red cabbage colorants responded similarly during storage, undergoing a progressive decrease in $A\lambda_{max}$. Solutions containing red cabbage colorant were more stable during storage than were solutions containing cranberry colorant. This may be due to the presence of acylated anthocyanins in the former, as discussed previously. Both red cabbage and cranberry colorants were much more stable during storage than was the colorant prepared from beef juice which had been evaluated under similar conditions in a previous study.

A smaller decrease in $A\lambda_{max}$ occurred when solutions of red cabbage colorant (Resistant Red Acre cultivar) were stored in darkness rather than in light (Fig. 4). This effect overshadowed relatively small differences between samples representing the two pH values and the simulated beverage formulation. Absorbance changes were consistently smaller in the simulated beverage than in buffer at both pH values and with all cultivars, in samples stored in the dark as well as in light. The basis of this protective effect is not known.

Visual changes in solutions of red cabbage colorant during storage can be represented by the L value, hue angle and saturation index, determined by tristimulus colorimetry (Fig. 1). Increasing L values indicate the extent of color fading during storage; changes were relatively small in samples stored in the dark. Increasing values of the hue angle in samples exposed to light indicate a shift in color from a red to a more orange-red shade. This may be due to the occurrence of browning reactions in parallel with anthocyanin degradation during storage. The decrease in saturation index, an indication of color purity or vividness (Van Buren et al., 1974), reflects the loss in redness due to anthocyanin degradation. Similar changes occurred at each of the pH values studied and in buffer as well as in the simulated beverage. These results demonstrate the high degree of color stability that can be attained with red cabbage colorant when light is excluded.

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Physicochemical Changes in Potato Granules During Storage

B. OORAIKUL and K.H. MOLEDINA

ABSTRACT

Physicochemical properties of freeze-thaw (F-T) and add-back (A-B) potato granules, such as swelling power (SP), water-holding capacity (WHC), degree of starch retrogradation, moisture change, and rate of rehydration were monitored over about 100 wk of storage. SP of both types of granules decreased to minima after 40 wk then increased on further storage, while WHC showed an opposite trend, reaching maxima after 52 wk. Degree of starch retrogradation in the granules increased to maxima after 40 wk then declined. Moisture content of the granules also increased slightly during the first 33 wk then decreased on further storage. These changes caused the reduction in the rehydration rate of the granules from 8.3 x 10^{-2} sec^{-1} to 5.5 x 10^{-2} sec^{-1} (F-T) and 5.2 x 10^{-2} sec^{-1} (A-B) after 36 wk of storage, after which the rate again increased. Addition of Ca^{++} to the granules during precooking and cooling steps of the process appeared to cause similar changes.

INTRODUCTION

A MAJOR PROBLEM in fabricating French fries and similar products from potato granules is the very rapid rehydration rate of the granules, preventing the formation of a uniform dough. The dough so made usually has wet and dry spots resulting in poor handling and extrusion properties, making a product of poor eating quality. Ooraikul (1977a) experienced greatly reduced rehydration rate if the granules were stored for about 6 months. He attributed the reduction to the molecular rearrangement and probable interaction of starch, protein, sugar and pectin during storage. However, lengthy storage of granules would be impractical on an industrial scale, and old granules normally suffer from rancidity problems.

Jadhav *et al.* (1976) attempted to correct the situation by increasing the quantity of water for reconstitution to allow a more uniform distribution of water in the dough. The method appeared to succeed on a lab scale. However, the same improvement was not attained on a pilot scale production as the dough became too moist and weak and either stuck together or disintegrated on frying.

It was felt that the nature of physicochemical changes in potato granules during storage should be further elucidated so that a more practical technique could be developed to modify the rehydration rate of the granules. In this study the changes in degree of starch retrogradation, water-holding capacity (WHC), swelling power (SP), moisture content and rehydration rate of potato granules during storage were investigated.

MATERIALS & METHODS

FREEZE-THAW (F-T) potato granules were produced from cultivar Netted Gem potatoes grown in Southern Alberta with the F-T process described by Ooraikul (1977b). The process involves peeling, slicing, steam cooking, mashing, freezing, thawing, predrying, granulation, drying and sifting. Its major difference from the commercial add-back (A-B) process is that it does not require recycling of dry granules. Ca-added granules were produced according to Haydar *et al.* (1980), where the potatoes were first precooked and cooled in water containing 100 ppm Ca^{++} before being steam cooked.

Add-back (A-B) granules were obtained from a local manufacturer. The process involves peeling, slicing, precooking, cooling, steam cooking, mash-mixing with about two parts of recycled dry granules, conditioning, fluff-mixing, air-lift drying, fluidized bed drying and cooling, and sifting.

The granule samples of about 200g each were vacuum packed in moisture impervious polyethylene bags and stored in ambient atmosphere (about 25°C and 35% R.H.) until use.

Swelling power (SP)

The method described by Potter (1954) with minor modifications was used to determine SP values of potato granules. Samples of 2.5g each of granules were placed in 50 ml graduated centrifuge tubes and mixed with sufficient distilled water at 25°C to make 25 ml of homogeneous slurry. The tubes were stoppered and agitated for 1 hr with a wrist-action shaker placed in a constant temperature (25.0 ± 1.0°C) chamber, then centrifuged for 15 min at 1,000 x G. The supernatant was swiftly decanted, then 10 ml of distilled water was carefully pipetted into the tubes and the total volume determined. The volume of the swollen material, designated as SP, was calculated from (total volume −10) x 4. The SP values, expressed on the basis of 10g dry solids, were the average of four determinations.

Water-holding capacity (WHC)

The method of Medcalf and Giles (1965) as modified by Morrow and Lorenz (1974) was used to determine % WHC of potato granules. A sample of 0.5g granules was added to 10 ml distilled water in a tared 15 ml tapered centrifuge tube. The tube was stoppered and agitated for 1 hr with a wrist-action shaker placed in a constant temperature (25.0 ± 0.1°C) chamber, then centrifuged for 30 min at 2,000 x G. The supernatant was carefully decanted and excess water wiped dry with paper tissue. The tube was weighed to determine % WHC, expressed as weight of water retained per 100g dry matter. The results reported were the average of four determinations.

Degree of retrogradation

Granule sample of 2g and 25 ml distilled water were thoroughly blended for 30 min in a tissue disintegrator (Buhler Tissue Disintegrator, Edward Buhler & Co., Tubinge, W. Germany) containing ≅ 25 glass beads (6 mm diam). The apparatus was kept cool with a water jacket at 18 ± 1°C. The homogenate was transferred into a 250 ml volumetric flask, made up to the mark with distilled water then magnetically stirred at ambient temperature for 1 hr. About 10 ml of the mixture was centrifuged for 30 min at 2,500 x G. The supernatant contained soluble starch, 2.5 ml of which was mixed with 7.5 ml distilled water, vortexed for 15 sec, heated in a 100°C water-bath for 10 min then cooled to room temperature. The cooled supernatant was mixed with 0.2 ml standard iodine solution (0.02N KI_3), vortexed for 15 sec and allowed to stand 10 min for color development before reading an absorbance at 605 nm. The soluble starch, expressed as absorbance units per g dry matter (blue value index [BVI]), was an average of four determinations. The amount of soluble starch indicates the degree of retrogradation (Potter, 1954). However, the absolute degree of retrogradation could not be obtained in this experiment since the soluble starch was measured in terms of BVI, not absolute quantity. Hence, for the purpose of comparison a relative degree of retrogradation, expressed as % retrogradation (relative), was calculated using the following formula:

$$\frac{A - B}{A} \times 100$$

Authors Ooraikul and Moledina are with the Dept. of Food Science, Univ. of Alberta, Edmonton, Alberta, Canada T6G 2N2.

where: A = BVI of freshly mashed potatoes with no surfactant added and B = BVI of the sample.

Moisture content

Moisture content of the granules was determined at regular intervals over the storage period of 103 wk. About 10g of granules were dried until constant weight was reached (≅ 48 hr) in a vacuum oven (76 mm Hg) at 70°C. The % moisture, reported on wet basis, was an average of three determinations.

Rehydration Rate

An extensive search for a suitable technique to measure the rate of rehydration of potato granules, including the trials with Brookfield viscosimeter, Haake Rotovisco, and a modified Mixograph was unsuccessful. None of the available techniques and instruments were amenable to measuring the time, in seconds, in which a quantity of granules would fully absorb a quantity of water. Thus, a simple method based on the reconstitution procedure adopted for institutional production of extruded French fries (Tamura and Packer, 1976) was developed. The method is as follows. A 30-g sample of granules was gravitationally fed through an 8.5 cm glass funnel with its opening adjusted, by means of a flexible tube and a clamp, to allow all granules to pass through in 12 sec. The granules dropped into a beaker of 9 cm diameter containing 63 ml of tap water at 18 ± 1°C, vigorously stirred over a magnetic stirrer (Bell-Stir Heavy Duty Magnetic Stirrer, Bellco Glass Inc., Vineland, N.J.). The magnetic rod was 1 x 7.5 cm in size, and the stirrer speed was set at 4.5 on the speed dial. The time in sec was taken from the first contact of granules with water till the magnet stopped moving due to the "setting" of the slurry into a firm dough. The relative rehydration rate is the reciprocal of time (sec^{-1}), reported as an average of three determinations.

RESULTS & DISCUSSION

Swelling power (SP)

The SP for both types of granules decreased from about 37 ml/10g dry matter to a minimum of 27 ml/10g (A-B) and 31 ml/10 g (F-T) after 40 wk of storage, then increased on further storage (Fig. 1). Physicochemical changes in granules constituents during the 40 wk of storage caused the insoluble residue to form a more dense pellet in the centrifuge tubes when SP was determined.

Potter (1954) measured SP of potato starch gels of varying starch contents, and of potato granules of varying moisture contents stored for different periods of time at different temperatures. His results showed that SP of starch gels was lower the longer they were stored at 25°C. That of the "moist-mix," taken from the mash-mixing stage of the A-B process, stored for up to 96 hr at 25°C, decreased the fastest when its moisture was between 36-43%. He stated that the swollen material consists of granule sacks enveloping gelatinized starch, some of which was dissolved. If it was allowed to cool and age the free starch as well as the rest of the starch within the swollen granules becomes associated resulting in lower SP, and less starch will diffuse into solution. Thus, SP could also be used as an indication of degree of retrogradation of starch.

Results from the present study collaborate that of Potter's (1954) quite well. They indicated that aging for up to 40 wk steadily decreased the ability of the granules to swell, allowing them to be uniformly mixed with water during the preparation of the dough for extruded French fries.

Water-holding capacity (WHC)

WHC of F-T granules increased gradually during the first 20 wk of storage, then sharply from about 350 to a maximum of 478g water per 100g dry matter at 52 wk (Fig. 1). That of A–B granules, on the other hand, increased rapidly from 350 to 440 within 4 wk of storage, then gradually to a maximum of 530g per 100g dry matter at 49 wk. Further storage resulted in the decline of WHC in both types of granules. The opposite trend of change of WHC from SP indicated that it was a measurement of mainly "free" water trapped inside and between granules.

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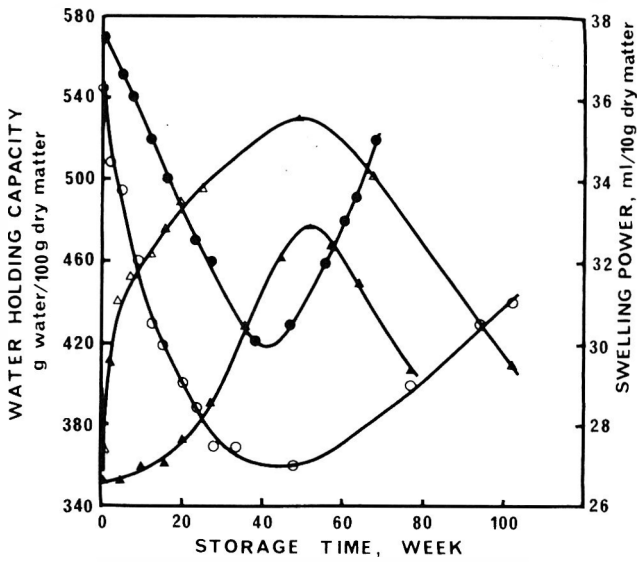


Fig. 1–Changes in water-holding capacity (WHC) and swelling power (SP) of potato granules during storage: – ● – ● – Swelling power (F-T granules); – ○ – ○ – Swelling power (A-B granules); – ▲ – ▲ – Water Holding Capacity (F-T granules); – Δ – Δ – Water Holding Capacity (A-B granules).

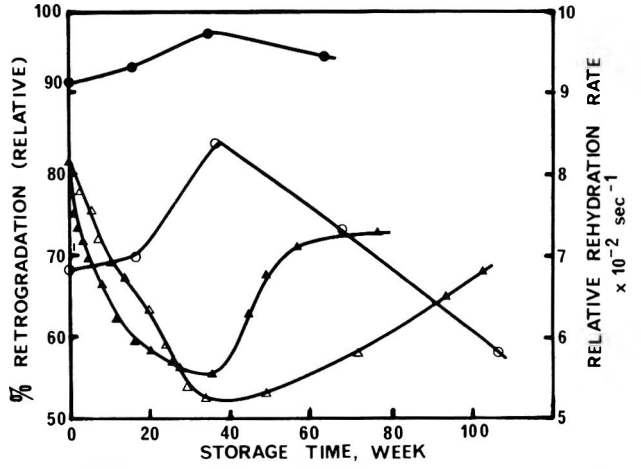


Fig. 2–Changes in % retrogradation of starch and relative rehydration rate of potato granules during storage: – ● – ● – % retrogradation in F-T granules; – ○ – ○ – % retrogradation in A-B granules; – ▲ – ▲ – Rehydration rate of F-T granules; – Δ – Δ – Rehydration rate of A-B granules.

*Table 1–Changes in moisture content of A-B and F-T granules during storage**

Storage period (wk)	Moisture content, %	
	A-B Granules	F-T Granules
0	7.05 ± 0.05[a]	6.32 ± 0.04[a]
16	7.64 ± 0.05[b]	6.55 ± 0.03[b]
33	7.83 ± 0.03[c]	6.55 ± 0.03[c]
77	–	5.57 ± 0.01[d]
103	7.16 ± 0.05[a]	

* Values followed by different letters are statistically different from one another (p = 0.01).

Degree of retrogradation

Starch, a major component of the granules, retrograded gradually during 30-40 wk of storage. Relative retrogradation increased from 91 to 97% (F-T) and 68 to 83% (A-B) during this period, then declined on further storage (Fig. 2).

Potter (1954) reported that moisture content plays an important role in retrogradation. He found no measurable change in the solubility of starch at moisture content below 15%. Hellman *et al.* (1954) also demonstrated interdependence of starch retrogradation and moisture. French (1950), however, suggested that starch retrogradation can take place even in the solid state. When the solubility of starch was used as a measure of its retrogradation, the results of this study showed that starch could retrograde even at a low moisture content of about 7% in potato granules.

Moisture content

Moisture content of potato granules, stored in sealed, moisture impervious polyethylene bags, appeared to increase during the initial 33 wk of storage, from 7.05 to 7.83% (A-B) and 6.32 to 6.73% (F-T), then decreased (Table 1). The slight increase in moisture might be due to physicochemical changes, such as starch retrogradation, which caused part of the molecularly "bound" water to be released. The decrease in moisture on further storage followed the decrease in the degree of retrogradation. No fully satisfactory explanation to this occurrence could be offered at this juncture, though it may be suggested that the moisture might be reabsorbed by starch molecules in a manner similar to the refreshening of stale bread by heating. However, the exact mechanism might differ.

Rehydration rate

The relative rehydration rate of A-B and F-T granules decreased from 8.3×10^{-2} sec^{-1} to a minimum of 5.2×10^{-2} (A-B) and 5.5×10^{-2} (F-T) after about 36 wk of storage, then increased (Fig. 2). Experience showed that 6 months old granules with a rehydration rate of about 5.7×10^{-2} sec^{-1}, formed a dough of satisfactory handling and extrusion property for extruded French fry manufacture. However, such granules presented a rancidity problem.

Changes in the rate of rehydration were the reverse of those of starch retrogradation. Retrograded starch loses its swelling power and water absorptive capacity, causing the decrease in rehydration rate of the potato granules. The lower the rate of rehydration, the more desirable are the granules for the manufacture of extruded French fries and similar products. Doughs made from such granules, with proper binding agents, had a slightly moist appearance and extruded easily to form a firm but smooth, continuous strip which retained its coherence on frying.

Thus, the changes in physicochemical properties of potato granules appeared to be closely interrelated. Results from this study indicated that among the properties studied, changes in starch during storage were of primary importance in affecting other properties of the granules. For example, molecular alignment of starch during retrogradation might have caused the release of part of its "bound" water, as detected in the slight increase in moisture content of the granules during storage. The retrograded starch lost its solubility, resulting in a decrease in the SP of the granules. The alignment of the starch molecules might also have created minute voids in the solid matrix which would trap a quantity of water on reconstitution, contributing to the increase in WHC. The loss of solubility and SP of starch, therefore, caused the reduction in rehydration rate, allowing the granules to absorb cold water more slowly and uniformly.

The change in the degree of retrogradation, itself, is of prime interest. Potato starch consists of 17-21% amylose (Chung, 1979), part of which may leach out, during processing, to the cell wall or even out of the cell through primary wall pits (Reeve, 1954). Hence, the major fraction of starch remaining inside the granules is amylopectin. Retrogradation is a phenomenon associated mainly with amylose, where linear chains align themselves to form crystalloid regions (Hodge and Osman, 1976). These regions become hydrophobic as "bound" water is replaced by intermolecular bonds of carbon-bound hydroxyl groups. It is possible that amylose, deposited on the surface of the granules during processing, formed crystalloid regions on the cell walls during storage, thus inhibiting easy passage of water into the granules during reconstitution and contributing to the decrease in rehydration rate.

Amylopectin is less involved in retrogradation, as bonding among the branches of its molecules is much less extensive than those of amylose. For that reason the retrogradation of amylopectin was found to be reversible by mechanisms such as heating, as in the refreshening of stale bread (French, 1950; Hellman *et al.*, 1954). This may partially account for the reverse trend of starch retrogradation in potato granules, on prolonged storage. Retrogradation of amylose, on the other hand, is irreversible (Hodge and Osman, 1976).

Fedec *et al.* (1977) observed KCl crystals on the surface of fresh potato granules. These crystals disappeared on prolonged storage. It is possible that the salt crystals absorbed moisture which had been released from starch on retrogradation, dissolved and rediffused into the granules. K^+ might then bind with orthophosphate groups of amylose. Basing their study on potato starch depleted of cations, Haydar *et al.* (1980) found that K^+ had much less effect on suppression of SP and solubility of the starch than Ca^{++} or Mg^{++}. It is possible, therefore, that the reintroduction of K^+ into the "native" starch molecules resulted in the increase in its SP and solubility and in the slight reduction of moisture content experienced in potato granules on prolonged storage.

Hence, to achieve a reduction in the rehydration rate of potato granules, modification of physicochemical properties of the starch fraction is of utmost importance. This may be accomplished by aging, which is undesirable due to flavor problems, or by addition to starch modifying agents,

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*Table 2–Swelling power (SP), water-holding capacity (WHC), starch solubility (as BVI), and relative rehydration rate of fresh, aged and Ca-added potato granules**

Granules	SP ml/10g dry matter	WHC g water/100g dry matter	BVI	Relative rehydration rate, $\times 10^{-2}$ sec^{-1}
1. Normal F-T granules, fresh	37.5 ± 2.1[a]	350 ± 14[a]	46.1 ± 9.2[a]	8.3 ± 0.6[a]
2. F-T granules, 6 mo old	31.3 ± 0.6[b]	405 ± 20[b]	15.6 ± 1.1[b]	5.7 ± 0.2[b]
3. F-T granules, precooked and cooled in water containing 100 ppm Ca^{++}	36.0 ± 1.5[a]	401 ± 23[b]	3.4 ± 0.7[c]	5.9 ± 0.3[b]

* Values followed by different letters are statistically different from one another ($p = 0.05$).

Properties of Free Radicals in Protein and Amino Acid Pyrolysates

SADAO UCHIYAMA and MITSURU UCHIYAMA

ABSTRACT

The ESR intensities (g=2.0042 ~ 2.0053) of free radicals in L-lysine and casein pyrolysates increased with increase of the heating time. In L-lysine pyrolysate (200°C, 20 min), the fraction insoluble in organic solvents and water accounted for 48.1% of the ESR intensity of the whole pyrolysate; the component responsible appeared to be a product of condensation polymerization, but the elemental analysis data indicated a smaller value for nitrogen than that expected for polylysine. The pyrolysis of casein brought about denatured products insoluble in water. The free radicals formed were stable for a week in distilled water and were unaffected by digestion with pepsin or pancreatin (37°C for 3 hr).

INTRODUCTION

THERE HAVE BEEN recent reports that mutagenic substances are formed in pyrolysates of amino acids, proteins and foods (Kosuge et al., 1978; Uyeta et al., 1978). Kosuge and co-workers reported that basic fractions from pyrolysates of tryptophan, phenylalanine, glutamic acid, serine, lysine, and creatine showed more potent mutagenicities in Ames's test (Ames et al., 1975) than those of other amino acids. Among them, the active principles in the pyrolysate of tryptophan were identified as two kinds of β-carboline derivatives.

Uyeta et al. (1978) also reported that there was a correlation between the mutagenic activities of pyrolysates and the protein contents in food. The appearance of mutagenicity in pyrolysates of proteinous foods poses a new problem in the field of food safety.

The authors have studied energy-requiring reactions of foods, such as pyrolysis or γ-irradiation, physico-chemically by electron spin resonance (ESR) spectrometry (Uchiyama and Uchiyama 1979). Heating and γ-irradiation of Kamaboko, a Japanese staple food (a kind of fish cake), caused the formation of free radicals (g=2.0030 ~ 2.0049). The ESR intensities of the free radicals increased with increasing temperature or dose of γ-rays and the radicals were mainly present in the protein fraction of Kamaboko.

Subsequent studies on free radical formation in amino acid pyrolysates revealed particularly high levels of pyrolysates of L-lysine, L-tryptophan, and L-phenylalanine, with L-lysine showing the highest level. Some amino acids whose pyrolysates showed mutagenicity produced high levels of free radicals, but it was not clear whether there was a good correlation between the mutagenicity and the free radical formation.

On the other hand, Renner et al. (1973) studied the mutagenicity of γ-irradiated milk powder (Renner et al., 1973), and found that γ-irradiated milk powder containing a high concentration of free radicals did not exhibit any toxicity toward rats. No detailed studies on the properties of the free radicals were undertaken. Szent-Gyorgyi proposed a link between cancer and free radicals, especially free radicals in protein (Szent-Gyorgyi, 1975). Imoto also supported the existence of a relationship between carcinogenesis and free radicals in his reviews (Imoto, 1976, 1979).

In this work, therefore, we carried out basic research on the properties of free radicals in L-lysine and protein pyrolysates. The structural elucidation of the pyrolysates was attempted, and the stability of the free radicals was investigated to determine their interaction with other components in food, i.e., the probability of radical transfer. The influence of digestive action in the human body on the free radicals is discussed.

Authors Sadao Uchiyama and Mitsuru Uchiyama are with the National Institute of Hygienic Sciences, 1-18, Kamiyoga 1-chome, Setagaya-ku, Tokyo, 158, Japan.

EXPERIMENTAL

Fractionation of L-lysine pyrolysate

L-lysine (Wako Pure Chemical Industries Ltd.) was heated in an open beaker at 200°C for 20 min on a hot plate. The pyrolysate (100 mg) was extracted with 5 ml of chloroform by shaking for 10 min (chloroform extract, Fraction 1) and then extracted with 5 ml of methanol by shaking for 20 min (methanol extract, Fraction 2). The residue was extracted with 5 ml of hot water (80°C) for 40 min (aqueous extract, Fraction 3), leaving a final residue of 5 mg (Fraction 4). The sample solutions of Fraction 1, 2, and 3 were concentrated to 0.5 ml and each then subjected to ESR measurement. Fraction 4 was dried over anhydrous Na_2SO_4 and then subjected to ESR and IR measurements (IR: Jasco DS-403G diffraction grating infrared spectrophotometer; KBr discs).

Determination of L-lysine in acid hydrolysate of Fraction 4

Fraction 4 was washed again with hot distilled water (10 ml) at 80°C on a water bath and the absence of L-lysine in the washing water was confirmed. After 20% HCl hydrolysis for 24 hr, determination of L-lysine in the hydrolysate was conducted with a Hitachi KLA-313 amino acid analyzer, using Hitachi resin 2611.

Gel permeation chromatography (GPC) of heated casein

Casein (Miles laboratories, Inc.) was heated in a beaker at 150°C for 2 hr in an electric drying machine. As a first experiment, the heated casein was dissolved in distilled water at a concentration of 0.5%. The denatured fraction insoluble in water was eliminated by centrifugation at 1000 rpm for 20 min. The water-soluble fraction was injected into a column and subjected to high speed gel permeation chromatography (GPC). Secondly the heated casein was completely dissolved in 4 ml of 0.05N NaOH by standing at 80°C for 30 min. After cooling, the solution was neutralized to pH 7.0 with 10% phosphoric acid and adjusted to 5 ml total volume by addition of distilled water, followed by GPC as above.

The machine used was a Hitachi 634 A liquid chromatograph under the following conditions; gel, Shodex OH Pak B-804 (polyester resin); column, 8 x 500 mm, steel; column temperature, 40°C; eluent, 0.01M NaCl; pressure, 10.0 kg/cm^2; flow rate, 1.0 ml/min; detector, UV 254 nm, 0.16 ~ 0.32 AUFS.

Stability of free radicals stored in aqueous solution

Casein (50 mg) heated at 150°C for 2 hr was added to 0.5 ml of distilled water in a quartz tube for ESR measurement and left to stand at 5°C for a week. Each sample was then frozen at −196°C under liquid nitrogen and subjected to ESR spectrometry.

Digestion of radical-rich protein

Casein (10g) heated at 150°C for 3 hr was dissolved in 0.5N NaOH at 80°C for 40 min, then the solution was filtered through a glass filter. The filtrate was adjusted to pH 4.0 by addition of

1N HCl so that a precipitate was obtained. The precipitate, consisting of water-soluble and free radical-rich protein, was separated by centrifugation at 1500 rpm, then washed with distilled water and dried over P_2O_5 under reduced pressure. The yield of the radical-rich protein was 33.3%.

Two ml of a solution containing 25 mg per ml of the prepared protein in 1/15M Na_2HPO_4 was added to 10 mg of pepsin or pancreatin dissolved in 7 ml of 0.05 HCl or 1/15M Na_2HPO_4, respectively. Both enzymes were purchased from Wako Pure Chemical Industries Ltd. The pepsin powder was diluted with lactose to an extent such that it could digest 100 times its weight of denatured egg white. The mixtures were incubated at 37°C for 3 hr. Each incubated solution was neutralized to pH 7.0 and subjected to GPC. A solution prepared in the same manner was concentrated to 0.5 ml by lyophilization, and the concentrated solution was subjected to ESR measurement at −196°C.

ESR measurement

The ESR spectra were measured with a JEOL JES-FE3X ESR spectrometer after lyophilizing a solid sample at room temperature or after freezing a liquid sample at −196° under liquid nitrogen. The operation conditions were as follows. Temperature, room temperature of −196°C; field, 3356 ± 100 G or 3356 ± 50 G; sweep time, 4 min; mode, 6.3 GHz; amplitude, 4 x 100 ~ 5 x 1000; response, 1.0; g value standard, tetracyanoquinodimethane, 2.0026; radical intensity correction, bivalent manganese (MnO); spin number standard, 2,2',6,6'-tetramethyl-4-hydroxylpiperdine-1-oxl.

RESULTS & DISCUSSION

Pyrolysate of L-lysine

The formation of free radicals upon pyrolysis of amino acids was reported in the previous paper (Uchiyama and Uchiyama, 1979). It was clear that the highest level of free radicals was obtained with L-lysine among the amino acids tested. The distribution of free radicals in L-lysine pyrolysate was therefore examined and their nature was investigated in the present work.

L-lysine was heated at 200°C for 20 min in air. The brownish pyrolysate was fractionated into four portions by extracting sequentially with chloroform, methanol and water. The chloroform extract (Fraction 1) contained no radicals, while the methanol extract (Fraction 2) contained 8.7 ± 0.1% of the total free radicals of the initial L-lysine pyrolysate, and the water extract (Fraction 3) contained 15.8 ± 1.6%. On the other hand, the residue insoluble in water (Fraction 4) contained the bulk of the free radicals: 48.0 ± 5.9% (n=3).

This final residue showed an ESR signal at g = 2.0042, and its infrared (IR) spectrum showed an amide absorption at 1680 cm^{-1} in contrast to intact L-lysine (Fig. 1). The free radicals were stable on standing, and even in water. This indicates that radical-rich products are formed by dehydro-condensation of L-lysine upon heating; the free radicals may be accumulated at the same time. The structure of the principal product was therefore assumed to be polylysine.

Elemental analysis of Fraction 4, however, showed that the nitrogen value of the fraction was smaller than the theoretical values for L-lysine and polylysine (condensation number: 100), as shown in Table 1. After washing Fraction 4 again with distilled water to ensure that it contained no free L-lysine (no L-lysine was found in the washing water), Fraction 4 was hydrolyzed with 20% hydrochloric acid. L-lysine was obtained in 9.8% yield, as determined with an amino acid analyzer. The same value was obtained on hydrolysis for 24 hr and also for 48 hr. This suggests that this radical-rich product might be formed not only by dehydro-condensation but also an amino group-releasing reaction (for instance, loss of the ε-amino group of L-lysine) and oxidation. Thus, various free radicals, including carbon and peroxide radicals, etc., may be produced. These free radicals might be stabilized in the complex products in Fraction 4.

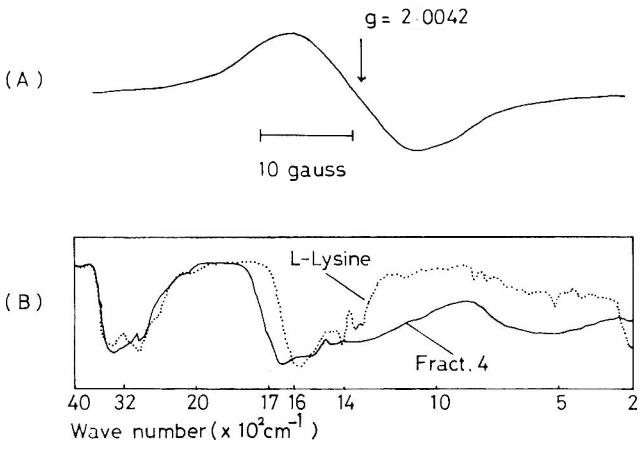


Fig. 1—ESR (A) and Infrared (B) spectra of Fraction 4 of the L-lysine pyrolysate.

Table 1—Elemental analysis of Fraction 4 of the L-lysine pyrolysate

Element[a]	Found	L-lysine calculated (%)	Polylysine calculated[b] (%)
C	56.82 ± 0.83	48.29	56.15
H	6.92 ± 0.16	9.65	9.44
N	14.30 ± 0.38	19.16	21.83

[a] Mean ± SD (n=3)
[b] Theoretical value for a condensation number of 100.

Pyrolysate of protein

The formation of free radicals on pyrolysis of proteins was investigated using cow's milk casein; this does not contain protein-bound manganese and ferric ions, which interfere with free radical measurement by ESR. As shown in Figure 2, the signal at g = 2.0053 increased as the heating time was increased. This free radical may be produced by oxidation and amino group-releasing reaction in the same way as in the case of L-lysine and, in fact, may include a substantial fraction of peroxide radicals. Next, the relation between the denaturation of protein and the heating time was examined. The casein samples heated at 150°C were

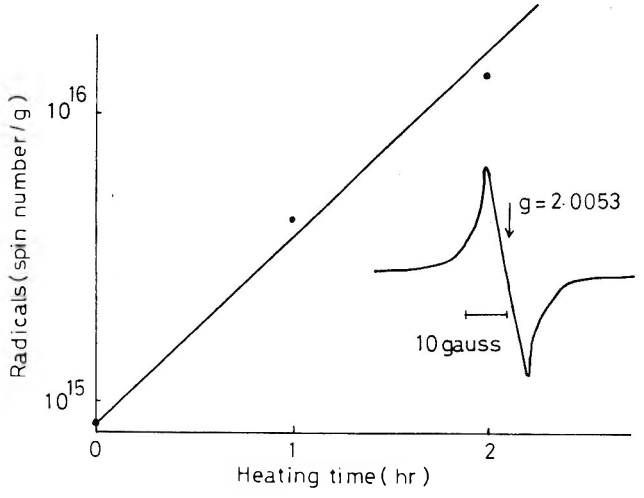


Fig. 2—Relationship between the amount of free radicals in heated casein and the heating time at 150°C, and ESR spectrum of free radicals in heated casein.

dissolved in distilled water and the insoluble material was eliminated by centrifugation. The water-soluble fractions were gel-permeation chromatographed on a high speed liquid chromatograph using a polyester resin column. The main peak of intact casein (retention time: 13.0 min) decreased with increase of the heating time, as shown in Figure 3. This indicates that denaturation of casein occurred progressively.

Next, in order to investigate possible structural change, the heated casein was dissolved completely in 0.05N NaOH and the hydrolysates were gel-permeation chromatographed in the manner described above. Figure 4 showed that the prolonged heating of casein led to more bulky hydrolysates. Since no dissociation agent was used in the procedure, it is difficult to define whether the increase of the hydrolysate's bulk is done to condensation polymerization or association and, therefore, it is difficult to discuss real change of molecular weight.

However, the above two experiments indicate that casein is denatured by heating, its bulk increases, and free radicals are simultaneously produced and accumulated in the pyrolysates. The authors assume that a great probability of condensation polymerization would be present in the procedures. These results are closely consistent with those for the L-lysine pyrolysate.

Stability of free radicals in protein

Casein heated at 150°C for 2 hr was stored in distilled water for a week and then subjected to ESR measurement after freezing at −196°C. As shown in Figure 5, the signal intensity after a week was the same as that measured immediately after adding the heated casein to distilled water. It appears that the free radicals produced in the protein are stable in the same way as the free radicals in heated Kamaboko, reported in the previous paper (Uchiyama and Uchiyama, 1979), and that the probability of radical transfer is very small, at least in an aqueous system.

Effect of digestion on the free radicals in protein

The change of the free radicals by digestion was examined to evaluate the reactivity of the radicals and other compounds in digestive organs. Radical-rich, water-soluble protein was prepared by 0.5N NaOH hydrolysis of casein heated at 150°C for 3 hr. Prolonged heating was carried out to obtain a larger amount of free radicals, and alkaline hydrolysis at high concentration yielded a larger amount of water-soluble product from the heated casein. The prepared protein was digested in a system containing pepsin or pancreatin at high concentration, incubating at 37°C for 3 hr. Figure 6 shows the results of GPC of the digested mixture. The main peak (a) coincided with that of egg albumin, having a molecular weight of 45,000. On digestion with pepsin, the main peak (a) decreased slightly, but peak (b) decreased markedly. On digestion with pancreatin, the main peak (a) decreased slightly and new lower molecular peaks (d) and (e) showed up in the pattern. Although peak (b) increased as compared with that in the prepared protein, this can be attributed to pancreatin, the peak of which is very close to peak (b).

The radical intensities in the digested mixture were not affected by the action of these enzymes as compared with the control, as shown in Figure 7. Even though a measure-

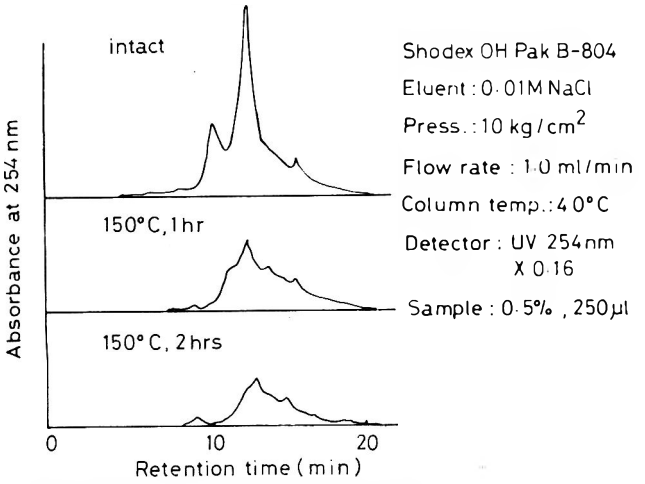


Fig. 3—High-speed gel permeation chromatography of the water-soluble fraction of casein heated at 150°C.

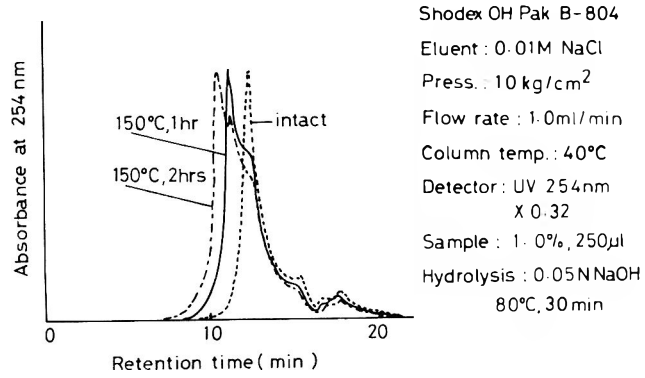


Fig. 4—High-speed gel permeation chromatography of the 0.05N NaOH alkaline hydrolysate of heated casein.

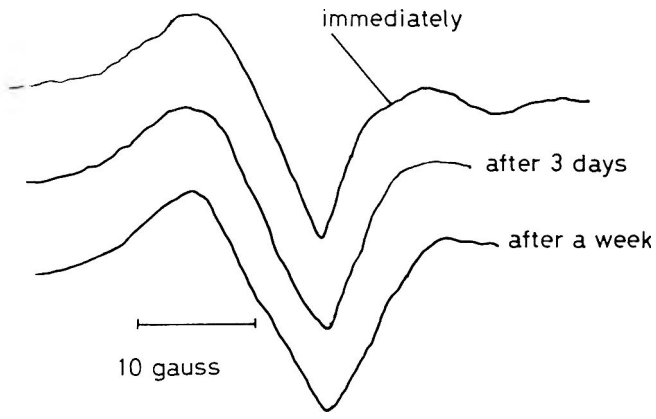


Fig. 5—ESR spectra of free radicals in heated casein stored in distilled water at 5°C. (ESR measurement was conducted after freezing the samples at −196°C).

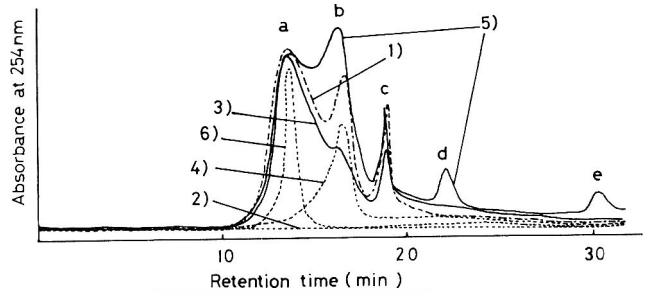


Fig. 6—High-speed gel permeation chromatography of mixtures digested with pepsin and pancreatin: (1), control; (2), pepsin; (3), pepsin digestion mixture; (4), pancreatin; (5) pancreatin digestion mixture; (6), egg albumin. (The measurement conditions for GPC were the same as in Fig. 4.)

ment of the real change in molecular weight was not carried out, the prepared protein was decomposed to some extent, as shown in Figure 6. Consequently, there may be a specific resistant structure carrying the free radicals in the heated protein, which is not readily destroyed by the digestive action.

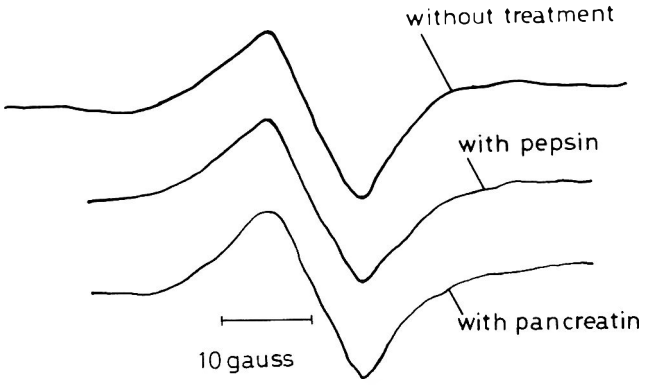


Fig. 7—ESR spectra of free radicals in heated casein digested with pepsin and pancreatin at 37°C for 3 hr. (ESR measurement was conducted after freezing the samples at −196°C.)

In conclusion, it appears that the free radicals in protein and L-lysine pyrolysates may be stable in water and even in digestive juice. Since many foods may contain substantial amounts of free radicals, as reported by Kohno (1978), further studies are required to assess their significance for food safety.

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such as Ca^{++}, during processing.

When Ca-added potato granules were analyzed for their physicochemical properties, it was found (Table 2) that their WHC was comparable to that of 6 mo old granules (difference is not significant at p = 0.05) while their starch solubility index (BVI) was even lower than that of 6 mo old granules (significant at p = 0.05). However, their SP remained inexplicably high at 36.0 ml/10g which is not significantly different (p = 0.05) from that of fresh F-T granules without added Ca. Nevertheless, relative rehydration rate of the Ca-added granules was similar to that of the 6 mo old granules (not significant at p = 0.05) at 5.9×10^{-2} sec^{-1}. The granules were found to form satisfactory dough for the manufacture of extruded French fries.

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Effect of Nisin on the Outgrowth of *Clostridium botulinum* Spores

VIRGINIA N. SCOTT and STEVE L. TAYLOR

ABSTRACT

Nisin, an antibiotic produced by certain strains of *Streptococcus lactis*, is effective in preventing the outgrowth of *Clostridium botulinum* spores. Type A *C. botulinum* spores were the most resistant to the inhibitory action of nisin requiring 1000-2000 I.U. of nisin/ml for a 50% inhibition of outgrowth on TPYG agar plates. Type E spores were more sensitive requiring only 50-100 I.U./ml for 50% inhibition of outgrowth on TPYG agar plates. Type B spores displayed an intermediate level of sensitivity requiring 500-1000 I.U. of nisin/ml for 50% inhibition of outgrowth on TPYG agar plates. Similar levels of nisin were necessary to prevent spore outgrowth in TPYG broth and BHI broth over a 7-day incubation period. With prolonged incubation periods of up to 65 days in TPYG broth, spore outgrowth was observed sporadically at higher nisin levels with the type A and B spores which may indicate some decomposition of nisin with storage. Nisin levels of 5000 I.U./ml for the type A spores and 2000 I.U./ml for the type B spores and the Minnesota E spores were insufficient to prevent spore outgrowth by *C. botulinum* in cooked meat medium. For the Beluga E spores, a nisin level of 2000 I.U./ml was necessary to prevent spore outgrowth in cooked meat medium. The need for higher levels of nisin in cooked medium to prevent spore outgrowth may be due to the binding of the nisin by meat particles.

INTRODUCTION

NISIN, an antibiotic produced by certain strains of *Streptococcus lactis*, consists of several closely related polypeptides. Since it is a product of *S. lactis*, nisin is a normal constituent of certain foods, particularly cheeses. As an antibiotic, nisin is particularly effective in preventing the outgrowth of spores (Lipinska, 1977). Nisin also induces the lysis of vegetative cells, but its effects on spores are much more profound than its effects on vegetative cells.

Nisin is used as a food additive, primarily to control spore outgrowth, in a number of countries although not in the United States. Nisin has been added to cheese to prevent the outgrowth of clostridial spores and the resultant problems associated with clostridial gas production (Fowler, 1979; Tramer, 1966). Nisin is frequently used as an adjunct to thermal processing. Since nisin is effective in preventing the outgrowth of the thermophilic sporeformers, the use of nisin in heat-processed foods allows a decrease in the heat process requirements and increases the shelf life of the product (El-Sadek et al., 1976; Heinemann et al., 1965). A variety of other uses for nisin in foods have been found, and these additional uses have been reviewed elsewhere (Fowler, 1979; Lipinska, 1977; Tramer, 1966).

One further use of nisin that should be investigated is the possible use of nisin in cured meats to control the outgrowth of *C. botulinum*. Although nisin is effective in preventing the outgrowth of spores from many different bacterial species (Campbell and Sniff, 1959; Denny et al., 1961; Jarvis, 1967; Mahmoud et al., 1976a; O'Brien et al., 1956), only scattered reports on the effectiveness of nisin against the spores of *C. botulinum* exist (Anderson et al., 1953; Denny et al., 1961; O'Brien et al., 1956). The study reported herein was conducted to obtain data on the effectiveness of nisin in preventing the outgrowth of *C. botulinum* spores of types A, B, and E.

Author Taylor is with the Food Research Institute, Dept. of Food Microbiology & Toxicology and the Dept. of Food Science, Univ. of Wisconsin, Madison, WI 53706. Author Scott, formerly affiliated with the Food Research Institute, Dept. of Food Microbiology & Toxicology, Univ. of Wisconsin-Madison, is currently with the National Food Processors Association, 1133 20th St. N.W., Washington, D.C. 20036.

MATERIALS & METHODS

Nisin

Partially purified nisin (25 million I.U./g) and Nisaplin (1 million I.U./g) were obtained from Aplin and Barrett Ltd. (Beaminster, Dorset, England). Nisaplin is a commercial nisin concentrate composed of 2.5% nisin in denatured milk solids and sodium chloride (Fowler, 1979). Stock solutions of purified nisin containing 100,000 I.U./ml or 20,000 I.U./ml in 0.02N HCl were prepared weekly. The pH was adjusted to 2 with concentrated HCl and the solutions were autoclaved (15 min at 121°C) and stored at 4°C. The actual nisin levels in these autoclaved stock solutions were confirmed with the plate diffusion assay employing *Micrococcus flavus* (Tramer and Fowler, 1964). Since nisin is stable to autoclaving at pH 2 (Tramer, 1966), no decreases in the nisin levels were found following this treatment.

Spores

Frozen spore suspensions of *C. botulinum* strains 69A (5×10^8 spores/ml), 56A (1×10^8 spores/ml), 113B (1.1×10^8 spore/ml and 213B (1.3×10^8 spores/ml) were obtained from Dr. Nobumasa Tanaka (Food Research Institute, Madison, Wisc.). These spore suspensions had been prepared by the method of Christiansen et al. (1974). The harvested spores had been washed, sonicated to destroy vegetative cells, washed to remove debris, and frozen until thawing for enumeration and use in the described experiments (Tanaka et al., 1980).

Spore suspensions of two type E strains, Minnesota E and Beluga E, were prepared from lyophilized cultures by transferring the lyophilized cultures into cooked meat medium and incubating them for 4 days at 30°C and 3 additional days at room temperature. A small aliquot of the cooked meat medium cultures was then inoculated into 10 ml of modified TPG medium. The inoculated cultures were heat-shocked at 60°C for 10 min and incubated at 30°C for 2 days. At this stage, microscopic examination revealed approximately 70% sporulation for Minnesota E strain and 90% sporulation for Beluga E strain. The TPG cultures were then heat-shocked again at 60°C for 10 min and a 1 ml inoculum was dispensed into bottles containing 150 ml of modified TPG medium. The cultures were incubated at 30°C for 2 days. Sporulation checks under the phase contrast microscope revealed adequate percentages of free spores (70-80%). The cultures were centrifuged at 6,000 rpm for 10 min, washed with sterile cold distilled water, and centrifuged again at 12,000 rpm for 10 min. The washing procedure was repeated five times. The final pellets were resuspended in water and frozen. Thawed spore suspensions were subjected to sonication (30 sec at a time for a maximum total sonication time of 5 min in an ice bath using a Bronwill Biosonik IV sonicator at the high setting) to insure the absence of vegetative cells. Microscopic observations were made to determine the efficacy of sonication for destroying vegetative cells. Prior to use or enumeration, the spore suspensions were heat-shocked which provided additional insurance against the presence of vegetative cells. The sonicated spores were washed seven times with sterile cold distilled water, the pellets were resuspended to 10 ml, and the spore suspensions were stored frozen in 2 ml

aliquots. A portion of the frozen spore suspension was thawed and diluted for enumeration of the spores. The Minnesota E spore suspension had 2.2×10^8 spores/ml; the Beluga E spore suspension had 1.6×10^9 spores/ml.

Spore counts for all strains were performed by the five-tube Most Probable Number (MPN) method in TPYG medium (FDA, 1976). Spores were heat-shocked at 80°C for 10 min for the type A and B spores and 60°C for 10 min for the type E spores before enumeration. The MPN procedure was compared to a serial dilution plating method for assessing the number of spores in a spore suspension of strain 56A. The plating procedure utilized TPYG agar. The MPN procedure estimated 1.3×10^8 spores/ml in the suspension of 56A spores, while the plate procedure estimated 7.5×10^7 spores/ml in the same suspension. While these counts are roughly comparable, the MPN method was preferred, due to the difficulty in obtaining reproducible and accurate plate counts because of spreading colonies on the agar surface.

The estimated spore loads used in the inhibition experiments are indicated in Table 1.

Media

TPYG medium consisted of 5% trypticase peptone, 0.5% Bacto-peptone, 2% yeast extract, 0.4% glucose, and 0.1% sodium thioglycollate. TPYG agar consisted of TPYG medium plus 1.5% agar. The TPYG agar was also supplemented with 0.1% soluble starch. Modified TPG medium contained 5% trypticase peptone, 0.5% Bacto-peptone, 0.4% glucose and 0.05% cysteine-HCl. Brain heart infusion broth (BHI) was obtained from Difco Laboratories (Detroit, MI) and supplemented with 0.1% sodium thioglycollate. The pH was adjusted to 7 prior to autoclaving. (Cooked meat medium (CMM, Difco Laboratories) was prepared by using one capful of dehydrated meat in a 16 x 125 mm screw-cap test tube. The meat was rehydrated with 10 ml of 0.3% glucose-0.2% soluble starch adjusted to pH 7.

Inhibition of spores by nisin in agar plates

Spores of *C. botulinum* were diluted in 0.1% peptone-water and heat-shocked at 80°C for 10 min for types A and B and 60°C for 10 min for type E. A 0.1 ml aliquot (approximately 10^2 or 10^3 spores) was then spread on a TPYG agar plate containing one of several concentrations of nisin. The sterile nisin solution was incorporated directly into the molten agar before solidification. For strains 56A, 69A, 113B, and 213B, the nisin concentrations used in these experiments were 0, 500, 1,000, 1,500, 2,000, 2,500, and 5,000 I.U./ml. Levels above 5,000 I.U./ml exceeded the solubility limits for nisin in TPYG broth and thus were avoided in both the solid and liquid media experiments. For strains Minnesota E and Beluga E, the nisin concentrations used in these experiments were 0, 50, 500, 1,000, and 1,500 I.U./ml. Duplicate plates were prepared for each set of conditions. The plates were incubated anaerobically at 35°C (30°C for type E) for 48, 96, or 168 hr. Following incubation, the colonies appearing on the TPYG agar plates were counted. The effectiveness of nisin was measured by determining the level of nisin necessary to cause a 50% decrease in the number of colonies appearing on the TPYG agar by comparison to control plates which had no nisin.

Inhibition of spores by nisin in liquid medium

Spore suspensions of ths six strains of *C. botulinum* were diluted to suitable concentrations in 0.1% peptone-water. The diluted suspensions were heat-shocked as described above. The heat-shocked suspensions were inoculated into TPYG broth containing nisin. The final spore levels in the inoculated broth were approximately 10^2, 10^3, and 10^4 spores/ml of TPYG broth. Duplicate TPYG tubes were inoculated for each set of conditions. Tubes without nisin served as controls. The nisin levels used for type A strains were 0, 1,000, 1,500, 2,000, 2,500, and 5,000 I.U./ml; for type B strains were 0, 200, 500, 1,000, 1,500, and 2,000 I.U./ml; and for type E strains were 0, 50, 100, 500, 1,000 and 1,500 I.U./ml. Growth was monitored by visual observation for turbidity. Occasionally, tubes displaying growth were checked for toxin production with the standard mouse bioassay (FDA, 1976). These observations were made daily for a 7-day incubation period at 35°C (30°C for type E). Further incubation and occasional observations for growth were made for a total of 65 days. These experiments were repeated at least twice for each strain. In the liquid medium experiments, the effective nisin concentrations were determined by the amount necessary to completely inhibit outgrowth (as opposed to the 50% inhibition endpoint used in the agar plate experiments). Since duplicate tubes were inoculated for each set of conditions, complete inhibition of outgrowth is defined as inhibition of outgrowth in both tubes of the duplicate pair.

Experiments using cooked meat medium or brain heart infusion broth instead of TPYG were incubated for 7 days or until growth occurred.

RESULTS

Preparation of nisin solutions

Some initial problems were encountered with the solubility of nisin. At the outset, Nisaplin was used. Since Nisaplin contains appreciable quantities of dried milk solids which would have an unknown effect on planned experiments, attempts were made to extract the nisin with 0.02N HCl. Although nisin has antibiotic properties, we still felt that it would be desirable to sterilize the extracted nisin either by autoclaving or filter sterilization. Initially, filter sterilization was attempted and considerable problems were encountered at the final filtration, due to the presence of dissolved milk solids in the acid extract. Initial experiments with these filtered extracts also indicated that *C. botulinum* type A strains were relatively resistant to nisin. Consequently, we faced several problems: a lack of solubility and a need for stock solutions containing as much as 100,000 I.U./ml. To determine the best approach to nisin preparation, the plate diffusion assay using *Micrococcus luteus* (*M. flavus*) (Tramer and Fowler, 1964) was employed. A straight-line response is produced when the logs of nisin concentrations between 0.5 and 10 units are plotted against the diameters of the zones of inhibition. Dilutions of an unsterilized nisin solution containing 2,500 I.U./ml were used to obtain a standard plot. The zones of inhibition obtained with the unsterilized, stock solutions of nisin in this study were very similar to those obtained by Tramer and Fowler (1964) which confirms that the basic test materials, nisin (25 million I.U./G) and Nisaplin (1 million I.U./g), contained the indicated amounts of nisin. Filter sterilized Nisaplin (100,000 I.U./ml) and autoclaved Nisaplin (10,000 I.U./ml) deviated substantially from the standard plot. Both autoclaved and filter sterilized purified nisin (100,000 I.U./ml) were similar to the standard, as was filter-sterilized Nisaplin containing 10,000 I.U./ml.

Experiments comparing autoclaved nisin, filter sterilized nisin, and unsterilized nisin using strain 56A and TPYG broth resulted in comparable levels of inhibition. However Nisaplin (both unsterilized and filter sterilized) was unable to inhibit outgrowth of spores, even at the highest concen-

Table 1—Estimated spore loads

Spore level	*Clostridium botulinum* strains					
	69A	56A	113B	213B	Beluga E	Minnesota E
10^4	10,000[a]	10,000	11,000	13,000	16,000	11,000
10^3	1,000	1,000	1,100	1,300	1,600	1,100
10^2	100	100	110	130	160	110

[a] Spore counts were determined by the most probable number method.

tration tested (5,000 I.U./ml).

As a result of these preliminary studies, all further nisin stock solutions were prepared from the purified nisin and were autoclaved (15 min at 121°C) at pH 2 to insure sterility. This approach eliminated the problems encountered by attempting to extract and sterilize nisin from Nisaplin. Data from the *M. flavus* plate diffusion assay indicated that nisin stock solutions prepared in this manner had consistent nisin activity levels with virtually no loss of nisin during the sterilization step.

Inhibition of spores by nisin in agar

Table 2 shows the nisin levels required for 50% inhibition of spore outgrowth on TPYG agar. Type A strains were the most resistant to nisin, requiring 1,000-2,000 I.U./ml for 50% inhibition of outgrowth. The type B strains showed intermediate resistance with 500-1000 I.U. of nicin/ml required for 50% inhibition. The type E strains were fairly sensitive to nisin with 50-100 I.U./ml effecting 50% inhibition. No differences were encountered as a function of the length of incubation of the plates. Data from 48 hr, 96 hr, and 168 hr incubation times yielded essentially identical estimates of 50% inhibition levels.

Inhibition of spores by nisin in liquid media

Figure 1 shows the nisin levels required to inhibit the outgrowth of spores in TPYG broth with spore loads of 10^2, 10^3, and 10^4 spores/ml. The type A strains were again the most resistant to nisin with the type B strains having intermediate resistance and the type E strains being fairly sensitive to nisin inhibition. Duplicated experiments showed excellent reproducibility in some cases and only fair reproducibility for other strains. Substantial differences in nisin sensitivity as a function of spore load were observed only with the type E strains. Occasional toxin tests confirmed that the observed growth was produced by the expected type of *C. botulinum*.

The levels of inhibition indicated in Figure 1 were determined after 7 days of incubation. However, after prolonged incubations of greater than 25 days, sporadic, growth was observed in TPYG broth, especially with spores from type A and B strains. In the most extreme case, growth was observed after 63 days of incubation at 35°C for strain 213B inoculated with 13,000 spores/ml into TPYG containing 5,000 I.U. of nisin/ml. The growth after prolonged incubations was somewhat haphazard, with respect to nisin levels.

The nisin levels required for inhibition of spore outgrowth in BHI broth and CMM are indicated in Table 3. The nisin levels required for inhibition of spores in BHI broth do not differ significantly from those in TPYG broth. In cooked meat medium growth occurred at all nisin levels used except at the 2000 I.U./ml level for strain Beluga E spores at the 10^3 spores/ml level.

Table 2—Nisin levels required for 50% inhibition of spore outgrowth on TPYG agar[a]

Spores/Plate	Nisin Levels (I.U./ml)	
	Strain 56A	
10^3	2000	1000
10^2	1500	1000
	Strain 69A	
10^3	2000	1500
10^2	1000	1500
	Strain 113B	
10^3	1000	500
10^2	500	500
	Strain 213B	
10^3	500	1000
10^2	500	500
	Strain Minnesota E	
10^3	50	–[b]
10^2	75	100
	Strain Beluga E	
10^3	50	–
10^2	50	100

[a] The multiple columns represent the results of distinct trials.
[b] Not determined

DISCUSSION

NISIN is effective in preventing the outgrowth of *Clostridium botulinum* spores in liquid media and on agar plate surfaces. Type E spores were the most sensitive to the effect of nisin. Type A spores were the most resistant to nisin and type B spores displayed an intermediate sensitivity to nisin. The nisin concentrations required to inhibit the outgrowth of the spores ranged from 50-1,000 I.U./ml for type E spores (dependent on the spore load) to 500 to greater than 2,500 I.U./ml for type A and B spores. The inhibition of the outgrowth of type A and B spores was much less dependent on the spore load than was inhibition of type E spore outgrowth.

These observations of nisin effectiveness confirm previous reports that nisin is capable of inhibiting the outgrowth of *C. botulinum* spores. The original report by Anderson et al. (1953) showed that 800 I.U. of nisin/ml would allow

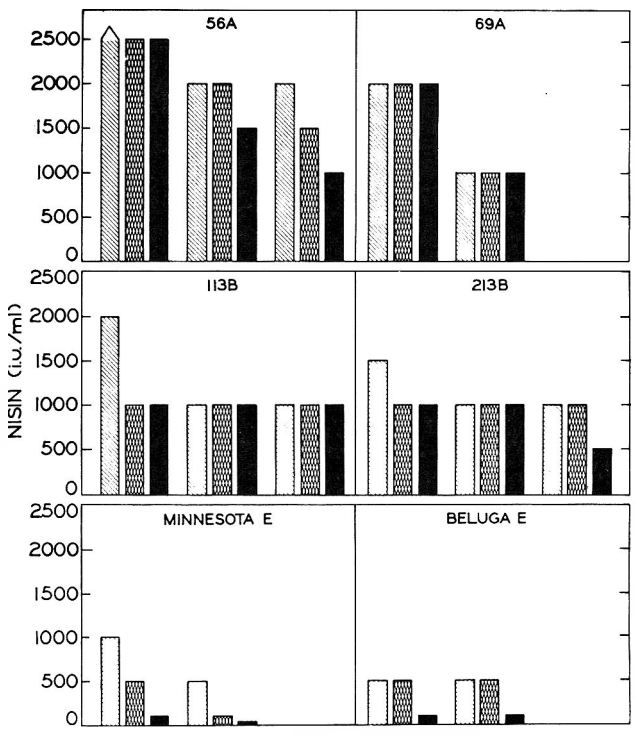


Fig. 1—Inhibition of spore outgrowth by nisin in TPYG broth. Bars indicate the nisin level required to inhibit outgrowth in both tubes of a duplicate set inoculated at each particular spore load. Spore loads are as follows: cross hatched bars, 10^4 spores/ml; diamond hatched bars, 10^3 spores/ml; solid bars, 10^2 spores/ml. Bars with arrow tips indicate that the highest nisin level shown was insufficient to prevent outgrowth of spores in both tubes. The multiple sets of bars for a strain indicate distinct experiments. Levels of inhibition were determined after 7 days incubation.

only very limited outgrowth of *C. botulinum* strain 62A spores on an agar surface. A later report by O'Brien et al. (1956) indicated that the D values for spores in pea puree could be decreased to 58% of control for strain 62A and to 22% of control for strain 25B in the presence of 560 I.U. of nisin/ml. Denny et al. (1961) showed that 4,000 I.U. of nisin/ml were required to eliminate outgrowth of strains 62A and 213B when 20,000 spores/ml were inoculated into a tryptone beef extract medium.

Nisin is much more effective against spores of certain species than it is against spores of other species. *C. botulinum* type A spores which require 1,000 to greater than 2,500 I.U. of nisin/ml to inhibit outgrowth are among the most resistant spores. For comparative purposes, Jarvis (1967) has demonstrated that spores of certain strains of *Bacillus stearothermophilus* are sensitive to less than 2 I.U. of nisin/ml and that outgrowth of spores of other *Bacillus* species is inhibited by 3-13 I.U. of nisin/ml. Some bacilli seem to be more resistant to nisin with *B. cereus* requiring 75-100 I.U./ml (Gupta et al., 1972; Jarvis, 1967), *B. megaterium* requiring 25-100 I.U./ml (Gupta et al., 1972; Jarvis, 1967), and *B. polymyxa* requiring 50 I.U./ml (Jarvis, 1967) to inhibit spore outgrowth. Clostridia seem to be more resistant to nisin. Denny et al. (1961) were unable to inhibit the outgrowth of *C. thermosaccharolyticum* spores with 4,000 I.U. of nisin/ml. *C. perfringens* spores would not outgrow in the presence of 1,000 I.U./ml of nisin (Mahmoud et al., 1976b).

However, considerable variability in nisin sensitivity may even be found within a given species. While Jarvis (1967) reports that *B. stearothermophilus* spores are inhibited by less than 2 I.U. of nisin/ml, Denny et al. (1961) reported that 1,000 I.U. of nisin/ml was required to inhibit spore outgrowth by a particular strain of *B. stearothermophilus.* Since their strain was isolated from thermally processed food, it may be a relatively heat resistant strain. Heat resistance may parallel nisin resistance but this requires further proof. Alternatively, the key comparative factor between these two studies may have been spore load since Denny et al. (1961) used a high spore load of 4,200 spores/ml and Jarvis (1967) does not report the spore level used in his study. Other factors may also affect the nisin sensitivity of spores. Heinemann et al. (1965) showed that heat-damaged spores were much more sensitive to the inhibitory effects of nisin.

Table 3—Nisin levels required for inhibition of spore outgrowth in BHI[a] broth and CMM[b]

Spore levels	Nisin levels (I.U./ml)[c] BHI	CMM
	Strain 56A	
10^4	>5000	>5000
10^3	5000	>5000
10^2	2000	>5000
	Strain 69A	
10^4	5000	>5000
10^3	5000	>5000
10^2	2000	>5000
	Strain 113B	
10^4	2000	>2000
10^3	1500	>2000
10^2	500	>2000
	Strain 213B	
10^4	2000	>2000
10^3	1000	>2000
10^2	500	>2000
	Strain Minnesota E	
10^4	500	>2000
10^3	100	>2000
10^2	100	>2000
	Strain Beluga E	
10^4	100	>2000
10^3	500	2000
10^2	<50	>2000

[a] Brain heart infusion broth
[b] Cooked meat medium
[c] The figures represent the nisin levels required to inhibit outgrowth in both tubes of a duplicate set for each spore level.

Our experiments suggest that nisin is approximately equivalent to nitrite in controlling the outgrowth of *C. botulinum* spores. A nisin level of 2,000 I.U./ml corresponds to 50 ppm. Such levels of nisin were effective in preventing the outgrowth of *C. botulinum* spores under most conditions in these studies and compare favorably with the currently used nitrate levels in bacon of 120 ppm. Of course, these studies emphasized nisin effectiveness in a TPYG medium. Nisin must be shown to be equivalently active in cured meat systems before such comparisons are valid.

Several observations from this study suggest that much higher nisin concentrations may be required to effectively inhibit the outgrowth of *C. botulinum* in cured meats. First, nisin was much less effective in cooked meat medium than in either TPYG broth or BHI broth (Table 3). The probable explanation for this observation is that nisin binds to the meat particles, and its effectiveness is thereby limited. However, recent evidence acquired by Rayman et al. (1980) shows that nisin is effective at levels of 3000-4000 I.U./g (75-100 ppm) in preventing the outgrowth of heat-damaged *Clostridium sporogenes* spores in a meat slurry system. The possible binding of nisin to proteins could also explain some of the difficulties encountered in extracting nisin from Nisaplin. If nisin binds to the milk proteins in the Nispalin formulation, then incomplete extraction might result. This phenomenon could also explain the outgrowth of *C. botulinum* strain 56A in TPYG broth containing high levels of nisin as Nisaplin. When high levels of Nisaplin were added to TPYG medium, a precipitation of milk solids was observed to occur during incubation. If nisin binds to the milk solids, the precipitation would effectively lower the nisin concentration of the medium and thereby allow spore outgrowth.

The second factor, which may increase the nisin requirements in cured meat systems, is the observation of increased spore outgrowth on prolonged storage. The most likely explanation for this observation is that nisin slowly decomposes in TPYG broth on storage at 35°C. Since nisin is reportedly sporostatic rather than sporicidal (El-Sadek et al., 1976; Mahmoud et al., 1976a; Tramer, 1966), outgrowth might be expected once the nisin concentration drops below the minimal effective levels. The relatively high resistance of type A and B spores to nisin likely plays an important role in this phenomenon also. The instability of nisin in foods on prolonged storage has been noted previously (Tramer, 1966). Any decreased effectiveness of nisin on prolonged storage may impose some practical limitations toward its use in inhibiting *C. botulinum* outgrowth in foods. However, the decomposition of nisin has been slow enough in other food systems that it has not seriously impaired the utilization of nisin (Campbell et al., 1959; Heinemann, 1965; Tramer, 1966).

Numerous additional factors have been identified which may have an impact on the effectiveness of nisin in preventing the outgrowth of *C. botulinum* spores. These factors include temperature of heat-shocking, pH, temperature of incubation, and spore load. The following paper presents data from a detailed investigation of the effects of heat-shocking temperature, pH, and spore load on the effectiveness of nisin in preventing the outgrowth of *C. botulinum* spores in TPYG broth. Further studies aimed at defining the effect of incubation temperature are currently in progress.

—Continued on page 126

Temperature, pH, and Spore Load Effects on the Ability of Nisin to Prevent the Outgrowth of *Clostridium botulinum* Spores

VIRGINIA N. SCOTT and STEVE L. TAYLOR

ABSTRACT

The effectiveness of nisin in preventing the outgrowth of spores of *Clostridium botulinum* types A, B, and E in TPYG broth was profoundly affected by pH, temperature of heat-shocking, length of the heat-shocking period, and spore load. Nisin was considerably more effective at pH 6 than at either pH 7 or pH 8 in limiting the outgrowth of all six tested strains. Heat-damaged spores were also more sensitive to nisin. Both higher heat-shocking temperatures in the range 20-30°C higher than the optimal heat-shocking temperatures for the particular strain and longer heat-shocking periods served to lower the levels of nisin required to inhibit spore outgrowth. Nisin was more effective against spore loads of 10^2 spores/ml than higher spore loads of 10^3 or 10^4 spores/ml. With all of these variables taken into consideration, the order of sensitivity of the spores of the various strains of *C. botulinum* was strain 56A < strain 69A < strain 113 B = strain 213 B < strain Beluga E < strain Minnesota E.

INTRODUCTION

IN THE PRECEDING REPORT, Scott and Taylor (1981) demonstrated that nisin is effective in preventing the outgrowth of *Clostridium botulinum* spores for up to 60 days in bacterial media. Type E spores were the most sensitive *C. botulinum* spores to the inhibitory effect of nisin with the order of sensitivity to nisin being type E > type B > type A. The inhibitory levels of nisin were 500 to 2,500 I.U./ml for the type A and B spores and 50-1,000 I.U./ml for the type E spores with the variability dependent on experimental conditions, spore loads, and other factors. The levels correspond to 12.5-62.5 ppm for the type A and B spores and 1.25-25 ppm for the type E spores. The inhibitory levels of nisin for type A and B spores are comparable with previously reported data (Anderson et al., 1953; Denny et al., 1961). Previous studies on type E spores are not available.

The inhibitory levels to nisin defined in the preceding study are equivalent to the levels of nitrite currently used in cured meat products. However, several factors require further investigation before such comparisons are valid. The previous study did not fully address such important variables as pH, heat-shocking temperature, incubation temperature, the effectiveness of nisin in meat-containing systems, and the storage stability of nisin.

Since cured meats generally have a pH of approximately pH 6, the effectiveness of nisin at pHs lower than pH 7 should be assessed. Nisin is know to have increased solubility and stability in more acidic environments (Lipinska, 1977). Duncan and Foster (1968) showed nitrite was markedly more effective against putrefactive anaerobe 3679h at pH 6 than at pH 7. The influence of pH on the effectiveness of nisin in inhibiting the outgrowth of *C. botulinum* spores is unknown.

The temperature of heat-shocking may be another important variable in assessing the effectiveness of nisin. Heinemann et al. (1965) showed that heat-damaged spores were much more susceptible to the inhibitory effects of nisin.

The study reported herein was conducted to obtain detailed information on the combined effects of pH, heat-shocking temperature, and spore loads on the effectiveness of nisin in preventing the outgrowth of *C. botulinum* spores.

Author Taylor is with the Food Research Institute, Dept. of Food Microbiology & Toxicology and the Dept. of Food Science, Univ. of Wisconsin, Madison, WI 53706. Author Scott, formerly affiliated with the Food Research Institute, Dept. of Food Microbiology & Toxicology, Univ. of Wisconsin-Madison is currently with the National Food Processors Association, 1133 20th St. N.W., Washington, D.C. 20036.

MATERIALS & METHODS

Nisin

Nisin was prepared according to Scott and Taylor (1981). A stock solution containing 100,000 I.U. nisin/ml was used for type A strains, a solution containing 20,000 I.U./ml was used for type B strains and solutions containing 5,000 and 500 I.U./ml were used for type E strains.

Nisin levels used in the heat and pH experiments were as follows: for type A strains, 0, 200, 500, 1,000, 1,500, and 2,000 I.U./ml; for type B strains, 0, 100, 200, 500, 1,000, and 1,500 I.U./ml; for type E strains, 0, 5, 10, 50, 100, and 500 I.U./ml.

Spores and media

Spore suspensions of *Clostridium botulinum* were those used by Scott and Taylor (1981). TPYG broth was prepared as indicated by Scott and Taylor (1981). In experiments requiring a particular pH concentrated HCl or 10N NaOH was used to adjust the pH of the TPYG broth.

Heat and pH experiments

TPYG broth was adjusted to the required pH (ususually 6, 7 or 8) and distributed in 16 mm screw-capped test tubes. Nisin was added after autoclaved media had cooled. Spores were added just prior to heating; spore levels were those used by Scott and Taylor (1981). Duplicate tubes for each spore level and nisin concentration were used. Tubes were heated for 5 min in a water bath (60°C, 70°C, 80°C, 90°C) or an oil bath (100°C, 110°C,) and cooled in cold water (for the 60°C temperature treatment or an ice bath for the higher temperature treatments). All experiments were repeated with the addition of nisin after the heat-shocking process. Tubes were incubated 7 days at 35°C (30°C for type E strains).

Time-temperature experiments

Nisin levels were the same as used above. Spores at a level of 10^3/ml were added just prior to heating of TPYG broth (approx. pH 7.0) containing nisin. Tubes were placed in an oil bath for 5, 10, 15 or 20 minutes. Control tubes were placed directly in an ice bath. Heated tubes were cooled in an ice bath. Types A and B were heated at 100°C and type E at 90°C.

RESULTS

Heat and pH experiments

Figure 1 demonstrates the combined effects of pH, heat-shocking temperature, and spore load on the ability of nisin to prevent the outgrowth of *C. botulinum* strain 56A spores. At pH 7 and pH 8, strain 56A spores displayed considerable resistance to nisin until the heat-shocking tem-

perature was raised to 110°C. Below 110°C at pH 8, 2000 I.U. of nisin/ml were insufficient to inhibit outgrowth of strain 56A spores. At 110°C and pH 8, a definite effect of spore load was observed. At 10^2 spores/ml, the heat shocking temperature of 110°C alone at pH 8 was sufficient to completely prevent outgrowth. With 10^4 spores/ml at the heat-shocking temperature of 110°C and pH 8, even the highest nisin level of 2,000 I.U./ml failed to impede spore outgrowth by strain 56A. At pH 7, some nisin inhibition of outgrowth was noted at lower heat-shocking temperatures for the 10^2 spores/ml level. The 110°C heat-shocking treatment was more effective at pH 7 than at pH 8. Nisin was considerably more effective in preventing the outgrowth of strain 56A spores at pH 6 than at the higher pHs. Even at the optimal heat-shocking temperature of 80°C, only 1,000-1,500 I.U. of nisin/ml were required to inhibit spore outgrowth depending upon the spore load. More nisin was needed to inhibit outgrowth at the optimal heat-shocking temperatures than at either lower or higher heat-shocking temperatures. At the lower heat-shocking temperatures of 60°C and 70°C, lower numbers of heat-activated spores may be present, thereby lowering the required nisin concentrations. At pH 6, heat-shocking at 100°C in the absence of nisin was sufficient to inhibit spore outgrowth at spore loads of 10^2 and 10^3 spores/ml. At pH 6 and a heat-shocking temperature of 90°C, a definite spore load effect was observed.

The effects of pH, heat-shocking temperature, and spore load on the ability of nisin to prevent the outgrowth of *C. botulinum* strain 69A spores in TPYG broth are shown in Figure 2. In general, nisin is more effective against strain 69A spores than strain 56A spores. At pH 8, strain 69A spore outgrowth was not inhibited by 2,000 I.U. of nisin/ml until the heat-shocking temperature reached 100°C. At pH 8 and a heat-shocking temperature of 100°C, nisin becomes increasingly effective with decreasing spore loads. The required inhibitory concentration of nisin at pH 8 is still lower at heat-shocking temperatures of 110°C with 200 I.U./ml effective against a spore load of 10^2 spores/ml. The inhibitory effectiveness of nisin increased dramatically at lower pHs. At pH 7, with heat-shocking temperatures of 100°C and 110°C nisin levels of 500 I.U./ml or less were effective. As with the strain 56A spores, outgrowth of the strain 69A spores was affected most dramatically by nisin at pH 6. Even at a spore load of 10^4 spores/ml, only 1000 I.U. of nisin/ml or less were necessary to inhibit the outgrowth of strain 69A regardless of the heat-shocking temperature. Heat-shocking in the absence of nisin was sufficient to prevent outgrowth at pH 6 for all spore levels at 100°C and for the lower two spore levels at 90°C.

Spores of strain 113B are more sensitive to the inhibitory actions of nisin than spores of either of the tested type A strains (Fig. 3). As with the type A spores, nisin was most effective at pH 6 and at low spore levels. At pH 8, 1,500 I.U./ml or more of nisin were required to inhibit outgrowth at heat-shocking temperatures up to 90°C for the higher spore loads of 10^3 and 10^4 spores/ml. Nisin was somewhat more effective against the 10^2 spores/ml level. At the higher heat-shocking temperatures, lower nisin levels were effective in preventing outgrowth. With strain 113B, only minimal differences in nisin effectiveness were observed between pH 7 and pH 8. At pH 7 and high heat-shocking temperatures, as low as 100 I.U. of nisin/ml were effective in preventing outgrowth in some cases. At lower heat-shocking temperatures, the effects paralleled those observed at pH 8 with definite evidence being provided of an increased effectiveness of nisin at lower spore loads. At pH 6, nisin was considerably more potent at all of the tested heat-

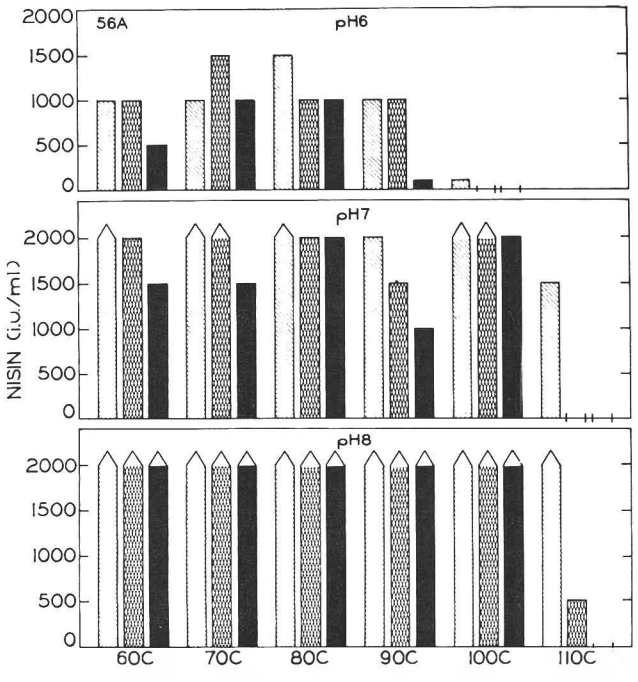


Fig. 1—Inhibition of outgrowth of strain 56A spores by nisin in TPYG broth as a function of heat-shocking temperature, pH, and spore load. Bars indicate the nisin levels required to inhibit outgrowth in both tubes at a particular spore load. Spore loads are as follows: cross hatched bars, 10^4 spores/ml; diamond hatched bars, 10^3 spores/ml; solid bars, 10^2 spores/ml. Bars with arrow tips indicate the highest nisin level shown was insufficient to prevent outgrowth of spores in both tubes. Vertical dashes at the baseline indicate no growth in the nisin-free control tubes at the indicated heat-shocking temperature and spore load.

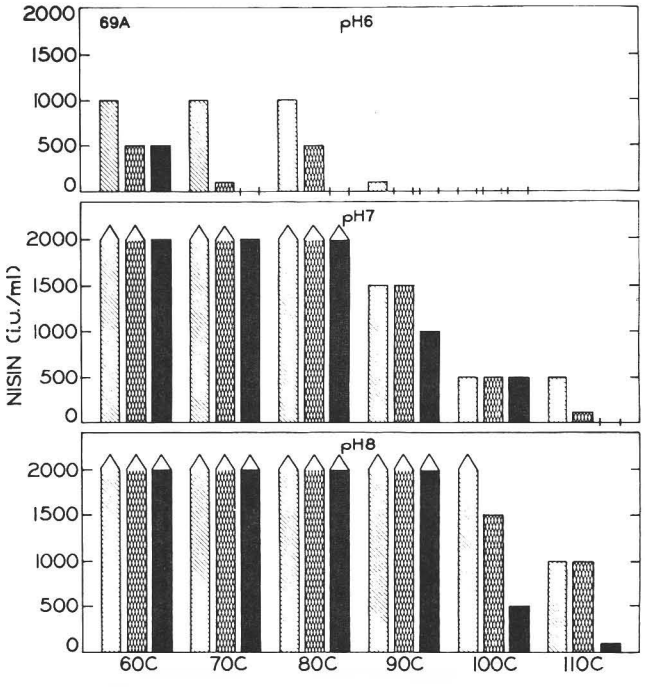


Fig. 2—Inhibition of outgrowth of strain 69A spores by nisin in TPYG broth as a function of heat-shocking temperature, pH, and spore load. Bars indicate the nisin levels required to inhibit outgrowth in both tubes at a particular spore load. Spore loads are as follows: cross hatched bars, 10^4 spores/ml; diamond hatched bars, 10^3 spores/ml; solid bars, 10^2 spores/ml. Bars with arrow tips indicate that the highest nisin level shown was insufficient to prevent outgrowth of spores in both tubes. Vertical dashes at the baseline indicate no growth in the nisin-free control tubes at the indicated heat-shocking temperature and spore load.

shocking temperatures and spore loads. Even at the optimal heat-shocking temperature of 80°C, 100-1,000 I.U. of nisin/ml were sufficient to prevent outgrowth at all spore levels. With the higher heat-shocking temperature of 100°C, nisin was virtually unnecessary since the pH effect alone prevented outgrowth in the absence of nisin at the lower spore loads. Nisin appears to be more effective at heat-shocking temperatures below 80°C but as with strain 56A that phenomenon may be due to the presence of lower numbers of heat-activated spores.

The effect of nisin on strain 213B spores is nearly identical to that observed with strain 113B spores (Fig. 4). At pH 8, more than 1,500 I.U. of nisin/ml were necessary to prevent outgrowth at heat-shocking temperatures up to 100°C for the 10^4 spores/ml level. Lesser amounts of nisin were effective against the lower spore loads. At pH 8 with a heat-shocking temperature of 110°C, 200 I.U. of nisin/ml were sufficient to eliminate outgrowth at all spore levels. The differences in nisin effectiveness between pH 7 and pH 8 were minimal. However, as already seen with the other spore strains, a dramatic increase in nisin effectiveness was observed at pH 6. For example, only 100 I.U. of nisin/ml were needed to prevent outgrowth at pH 6 after heat-shocking 10^4 spores/ml at 100°C. Even at the optimal heat-shocking temperature of 80°C, only 1,000-1,500 I.U. of nisin/ml were sufficient to inhibit outgrowth. Again, nisin appeared to be more effective at lower than optimal heat-shocking temperatures.

The combined effects of pH, heat-shocking temperature, and spore load on the effectiveness of nisin in preventing the outgrowth of *C. botulinum* strain Minnesota E spores are shown in Figure 5. As expected, nisin was more effective in preventing outgrowth of Minnesota E spores than it was in preventing outgrowth by the type A and B spores under all tested conditions. At pH 8, 500 I.U. of nisin/ml were effective in preventing outgrowth at all spore levels when the optimal heat-shocking temperature of 60°C was employed. With the heat-shocking temperatures of 90°C and 100°C at pH 8, rather low levels of nisin were sufficient to prevent outgrowth. For example, at 90°C and pH 8, only 5 I.U. of nisin/ml were necessary to prevent outgrowth. At pH 7, nisin seems to be somewhat less effective. However, at the higher heat-shocking temperatures of 90°C and 100°C, quite low levels of nisin were again sufficient to block outgrowth. As shown with the other strains, nisin was most effective in preventing the outgrowth of Minnesota E spores at pH 6. Even at the optimal heat-shocking temperature of 60°C, only 5 I.U. of nisin/ml were required to inhibit outgrowth at the spore level of 10^2 spores/ml. A distinct spore load effect was noted at pH 6 and heat-shocking temperatures of 60°C and 70°C. Very low levels of nisin were needed to prevent outgrowth at pH 6 when higher heat-shocking temperatures of 80°C or above were used.

The effect of nisin on the outgrowth of strain Beluga E spores under various pH, heat-shocking temperatures, and spore load conditions is shown in Figure 6. Nisin is somewhat less effective against Beluga E spores than Minnesota E spores, but the type E strains are definitely more susceptible to inhibition by nisin than the type A and B strains. At pH 8, 500 I.U. of nisin/ml were required to inhibit outgrowth at the optimal heat-shocking temperature of 60°C for all spore levels. However, at higher heat-shocking temperatures of 80°C or greater at pH 8, rather low nisin levels were sufficient to block outgrowth. Nisin was somewhat less effective at pH 7. At both pH 7 and pH 8, the spore load effect was haphazard. At pH 7, heat-shocking temperatures in excess of 80°C were needed to dramatically lower the inhibitory nisin level from 500 I.U./ml. Again, nisin was most effective at pH 6. A definite spore load effect was noted at pH 6 for the heat-shocking temperatures of 60°C, 70°C, and 80°C. A nisin level of 50 I.U./ml was effective against spore loads of 10^2 spores/ml with heat-shocking treatments of 70°C and 80°C. At pH 6 and

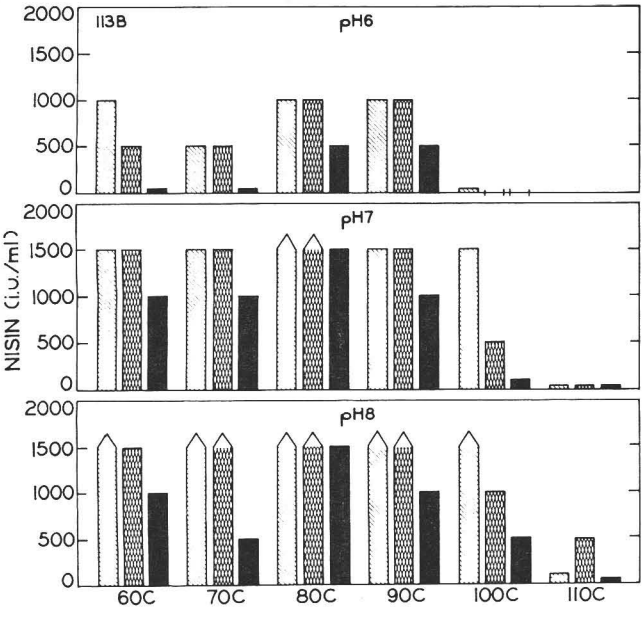


Fig. 3—Inhibition of outgrowth of strain 113B spores by nisin in TPYG broth as a function of heat-shocking temperature, pH, and spore load. Bars indicate the nisin levels required to inhibit outgrowth in both tubes at a particular spore load. Spore loads are as follows: cross hatched bars, 10^4 spores/ml; diamond hatched bars, 10^3 spores/ml; solid bars, 10^2 spores/ml. Bars with arrow tips indicate that the highest nisin level shown was insufficient to prevent outgrowth of spores in both tubes. Vertical dashes at the baseline indicate no growth in the nisin-free control tubes at the indicated heat-shocking temperature and spore load.

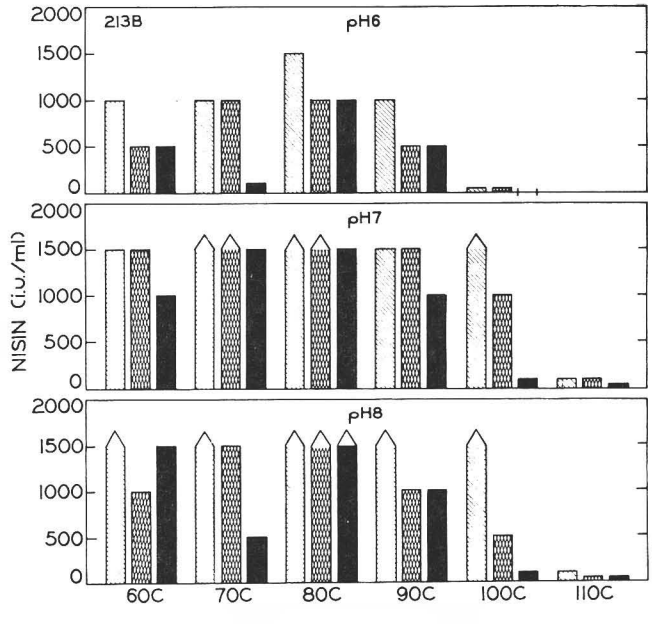


Fig. 4—Inhibition of outgrowth of strain 213B spores by nisin in TPYG broth as a function of heat-shocking temperature, pH, and spore load. Bars indicate the nisin levels required to inhibit outgrowth in both tubes at a particular spore load. Spore loads are as follows: cross hatched bars, 10^4 spores/ml; diamond hatched bars, 10^3 spores/ml; solid bars, 10^2 spores/ml. Bars with arrow tips indicate that the highest nisin level shown was insufficient to prevent outgrowth of spores in both tubes. Vertical dashes at the baseline indicate no growth in the nisin-free control tubes at the indicated heat-shocking temperature and spore load.

90°C, the pH effect alone was sufficient to inhibit outgrowth in the absence of nisin.

It should be noted that, while it cannot be shown on Figures 1-6, nisin delayed outgrowth in many cases even though outgrowth was eventually observed during the 7-day incubation period. The delay of outgrowth in the presence of nisin was also observed in the time-temperature experiments (Tables 1-3) which will be discussed below. Also, no consistent effect was noticed due to the addition of nisin after the heat-shocking treatment rather than before heat-shocking.

Time-temperature experiments

The results of the time-temperature experiments are portrayed in Tables 1-3. Increasing the length of time of heat-shocking at 100°C, decreased the nisin level required for effective inhibition and delayed spore outgrowth for the type A spores (Table 1). Strain 56A spores, the most resistant to nisin in all experiments, were markedly more sensitive to nisin when the heat-shocking time at 100°C was increased to 20 min. With 20 min of heating, outgrowth was observed in only one tube at nisin concentrations of 500 I.U./ml or above. A similar effect was noted for the strain 69A spores, although outgrowth was virtually eliminated at nisin levels of 200 I.U./ml or greater by heating for 10 min at 100°C.

Similarly for the type B strains, increasing the length of time of heat-shocking at 100°C decreased the nisin level required to prevent spore outgrowth (Table 2). With both strain 113B and strain 213B, 15 min of heating at 100°C lowered the nisin requirement for prevention of outgrowth to 100 I.U./ml. Delays in spore outgrowth as a function of length of heat-shocking were slight by comparison to the effects seen with the type A spores.

With the type E strains, the heat effect alone was sufficient to prevent outgrowth in most cases (Table 3). In the absence of nisin, heating at 90°C for 5 min or longer resulted in only sporadic incidents of outgrowth. These effects contrast with the effects portrayed in Figures 5

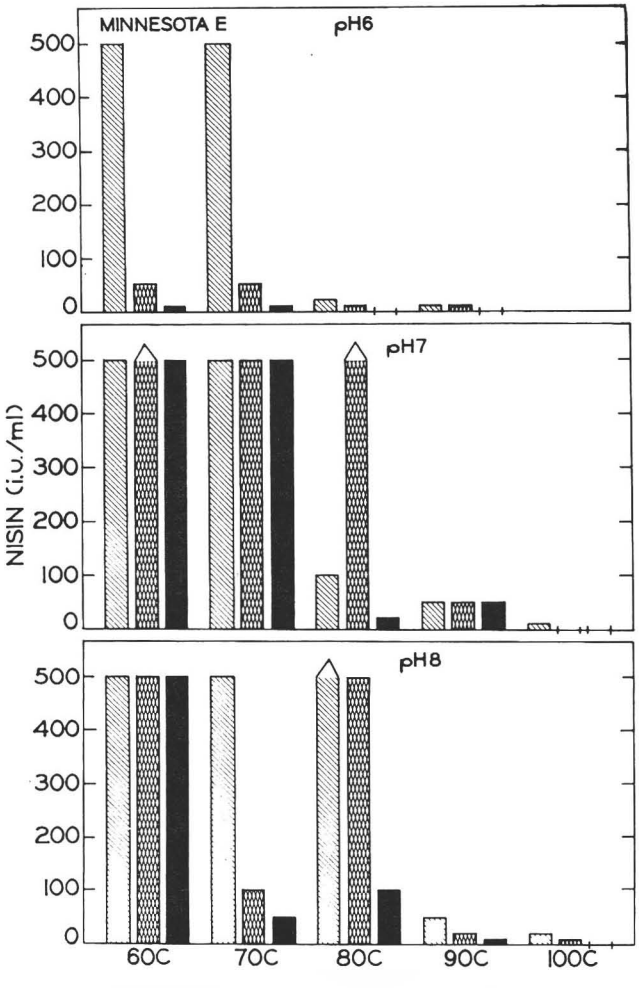


Fig. 5—Inhibition of outgrowth of strain Minnesota E spores by nisin in TPYG broth as a function of heat-shocking temperature, pH, and spore load. Bars indicate the nisin levels required to inhibit outgrowth in both tubes at a particular spore load. Spore loads are as follows: cross hatched bars, 10^4 spores/ml; diamond hatched bars, 10^3 spores/ml; solid bars, 10^2 spores/ml. Bars with arrow tips indicate that the highest nisin level shown was insufficient to prevent outgrowth of spores in both tubes. Vertical dashes at the baseline indicate no growth in the nisin-free control tubes at the indicated heat-shocking temperature and spore load.

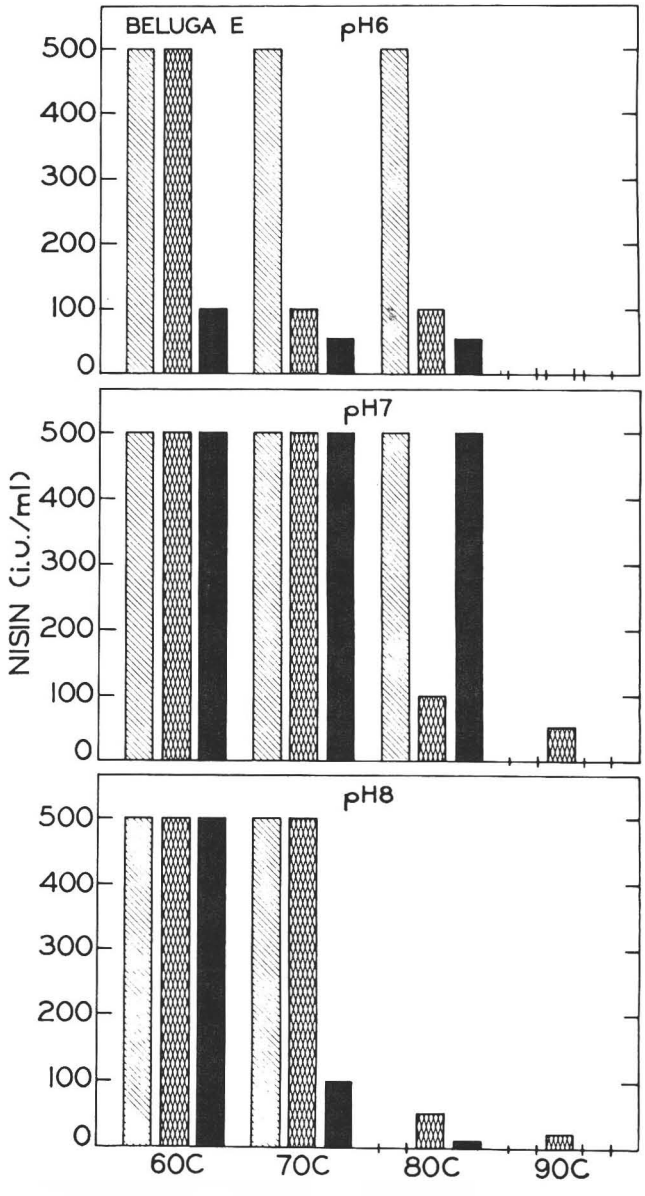


Fig. 6—Inhibition of outgrowth of strain Beluga E spores by nisin in TPYG broth as a function of heat-shocking temperature, pH, and spore load. Bars indicate the nisin levels required to inhibit outgrowth in both tubes at a particular spore load. Spore loads are as follows: cross hatched bars, 10^4 spores/ml; diamond hatched bars, 10^3 spores/ml; solid bars, 10^2 spores/ml. Vertical dashes at the baseline indicate no growth in the nisin-free control tubes at the indicated heat-shocking temperature and spore load.

and 6. In the heat and pH experiments at pH 7, a heat-shocking temperature of 90°C for 5 min, and a spore load of 10^3 spores/ml, 50 I.U. of nisin/ml were necessary to prevent outgrowth for both type E strains. Such variations in nisin sensitivity from experiment to experiment have been observed on several occasions for unknown reasons.

DISCUSSION

TEMPERATURE of heat-shocking, length of the heating period, pH, and spore load were all shown to be important variables that had an impact on the effectiveness of nisin in preventing the outgrowth of *C. botulinum* spores. As noted in the previous study (Scott and Taylor, 1981), type A spores were the most resistant to nisin under all tested conditions with type E spores being the most sensitive and type B spores displaying an intermediate level of sensitivity. After consideration of the effects of heat-shocking temperature, length of the heating period, pH, and spore load, strain 56A spores were the most resistant to the inhibitory effects of nisin. The order of sensitivity appears to be strain 56A < strain 69A < strain 113B = strain 213B < strain Beluga E < strain Minnesota E, although the order changes under various specific conditions.

For spores of all six tested strains of *C. botulinum*, nisin was more effective in preventing outgrowth at pH 6 than in less acid environments (Figs. 1-6). The mechanism of this pH effect is unknown but likely involves several factors. Of course, *C. botulinum* spores become less likely to germinate and outgrow as the pH decreases. Nisin may magnify this effect. For example, the stability of nisin in solution is dependent on the pH of the solution with nisin being more stable at acid pHs (Lipinska, 1977; Schaller, 1960). The stability of nisin may not be as important in short-term, 7-day incubation experiments as in long-term incubation studies. However, nisin is much more stable to heating and autoclaving in acid environments (Tamer, 1966). Nisin is completely stable to autoclaving at pH 2 but loses 40% of its activity on autoclaving at pH 5 and loses over 90% of its activity on autoclaving at pH 6.8 (Tramer, 1966). In our studies, nisin was added after autoclaving the media. However, the nisin was added prior to the heat-shocking step. If heat-shocking adversely affected the nisin activity, more nisin would be expected in TPYG broth, pH 6, than in the same broth at pH 7 or pH 8. However, the heat-shocking treatment probably did not affect the nisin activity, since no consistent difference was noticed in the heat and pH experiments that could be ascribed to the addition of nisin after the heat-shocking treatment rather than before the heat-shocking treatment.

Heat also had a profound impact on the effectiveness of nisin. In all cases, nisin was more effective in inhibiting spore outgrowth following heating at the higher heat-shocking temperatures (Fig. 1-6). In addition, heating at a specified high heat-shocking temperature for progressively longer periods of time showed that longer heating periods decreased the required inhibitory concentration of nisin even further (Tables 1-3). Consequently, nisin appears to be more effective against heat-damaged spores of *C. botulinum*. This finding extends the previous observation by Heinemann et al. (1965) that nisin was more effective against heat-damaged spores of *Bacillus stearothermophilus, B. coagulans, Clostridium thermosaccharolyticum*, and putrefactive anaerobe, PA3679.

Spore load was another important factor in determining the effectiveness of nisin in preventing spore outgrowth by *C. botulinum*. As expected, nisin was more effective in preventing outgrowth at spore loads of 10^2 spores/ml than at spore loads of 10^3 or 10^4 spores/ml (Fig. 1-6). In our initial studies, spore load was shown to be an important factor only with the type E strains (Scott and Taylor, 1981). However, in these studies, spore load was an important factor in determining the effectiveness of nisin against type A and B spores also. Fowler (1979) indicated that spore load was one of the key determining factors in controlling the amount of nisin necessary to prevent outgrowth in foods.

Under appropriate conditions of heating, pH, and spore

Table 1—Time-temperature experiments with spores of type A C. botulinum

Length of heating	Nisin level in medium[a] (I.U./ml)					
(min) at 100°C	0	200	500	1000	1500	1000
56A						
0	2[b]	2	2	2	3	5
	2	2	2	2	3	5
5	2	2	2	3	5	5
	2	2	2	5	5	5
10	2	2	3	3	—[c]	5
	2	3	3	5	—	7
15	2	3	5	5	—	—
	2	5	5	—	—	—
20	2	2	—	7	—	—
	3	7	—	—	—	—
69A						
0	2	2	2	4	4	4
	2	2	4	5	—	5
5	2	2	6	6	—	—
	2	5	6	—	—	—
10	4	—	6	—	—	—
	4	—	—	—	—	—
15	2	—	—	—	—	—
	4	—	—	—	—	—
20	4	—	7	—	—	—
	4	—	—	—	—	—

[a] TPYG broth
[b] Numbers designate the number of days of incubation at 35°C before growth occurred in these tubes.
[c] Dashes indicate that no growth had occurred in these tubes at the completion of the experiment (7 days of incubation).

Table 2—Time-temperature experiments with spores of type B C. botulinum

Length of heating	Nisin level in medium[a] (I.U./ml)						
(min) at 100°C	0	100	200	500	1000	1500	2000
113B							
0	3[b]	3	3	3	4	6	ND[d]
	3	3	3	3	5	6	ND
5	3	3	3	—[c]	—	—	ND
	3	3	—	—	—	—	ND
10	3	3	—	—	—	—	ND
	3	—	—	—	—	—	ND
15	3	—	—	—	—	—	ND
	3	—	—	—	—	—	ND
20	3	—	—	—	—	—	ND
213B							
0	1	2	2	2	4	ND	7
	1	2	2	2	5	ND	7
5	1	2	2	—	—	ND	—
	1	2	2	—	—	ND	—
10	1	2	—	—	—	ND	—
	1	—	—	—	—	ND	—
15	1	—	—	—	—	ND	—
	1	—	—	—	—	ND	—
20	2	—	—	—	—	ND	—
	2	—	—	—	—	ND	—

[a] TPYG broth
[b] Numbers designate the number of days of incubation at 35°C before growth occurred in these tubes.
[c] Dashes indicate that no growth had occurred in these tubes at the completion of the experiment (7 days of incubation).
[d] Not done

load, the amount of nisin required for inhibition of outgrowth can be lowered considerably by comparison to the amounts found necessary in our initial studies (Scott and Taylor, 1981). Since the pH of cured meats is near pH 6 and nisin is more effective at pH 6, an analysis of the effective concentrations of nisin at pH 6 should be enlightening. At optimal heat-shocking temperatures (80°C for type A and B spores and 60°C for type E spores), the effective nisin levels at pH 6 with a spore load of 10^3 spores/ml were 1,000 I.U./ml (25 ppm) for strain 56A, 500 I.U./ml (12.5 ppm) for strain 69A, 1,000 I.U./ml (25 ppm) for strain 113B, 1,000 I.U./ml (25 ppm) for strain 213B, 50 I.U./ml (1.25 ppm) for strain Minnesota E, and 500 I.U./ml (12.5 ppm) for strain Beluga E (Fig. 1-6). The sensitivity to nisin can be increased by as much as several orders of magnitude by subjecting the spores to a heat-damaging treatment. In many cases, these effective nisin concentrations are dramatically lower than the levels found necessary in previous studies with *C. botulinum* (Anderson, 1953; Denny et al., 1961; Scott and Taylor, 1981).

Table 3—Time-temperature experiments with spores of type E C. botulinum

Length of heating (min) at 90°C	Nisin level in medium[a] (I.U. /ml)					
	0	5	10	50	100	500
Minnesota E						
0	1[b]	1	1	1	1	–[c]
	1	1	1	1	1	–
5	–	–	–	–	–	–
	–	–	–	–	–	–
10	3	–	–	–	–	–
	–	–	–	–	–	–
15	–	–	–	–	–	–
	–	–	–	–	–	–
20	3	–	–	–	–	–
	–	–	–	–	–	–
Beluga E						
0	1	1	1	1	1	–
	1	1	1	1	1	–
5	4	–	–	–	–	–
	–	–	–	–	–	–
10	–	–	–	–	–	–
	–	–	–	–	–	–
15	–	–	–	–	3	–
	–	–	–	–	–	–
20	3	–	–	–	–	–
	–	–	–	–	–	–

[a] TPYG broth
[b] Numbers designate the number of days of incubation at 30°C before growth occurred in these tubes.
[c] Dashes indicate that no growth had occurred in these tubes at the completion of the experiment (7 days of incubation).

The effective levels of nisin at pH 6 in TPYG broth compare very favorably with currently used levels of nitrite in cured meats. However, additional factors must be taken into consideration before such comparisons become valid. Ultimately, the effectiveness of nisin in preventing spore outgrowth in meat systems must be assessed.

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Relationship between In Vitro Digestibility of Casein and its Content of Lysinoalanine and D-Amino Acids

MENDEL FRIEDMAN, JAMES C. ZAHNLEY, and PATRICIA M. MASTERS

ABSTRACT

Alkali and heat induce changes in proteins. These include racemization of L- to D-amino acids and crosslinking via lysinoalanine. Since high-quality proteins are presumably well-digested, possible effects of D-amino acid and lysinoalanine contents on food casein digestibility was examined. The following variables, which were expected to influence all three parameters, were evaluated: (a) pH (8-14); (b) temperature (25-75°C); and (c) time of treatment (10 min to 24 hr). An approximately inverse relationship was observed between D-amino acid and lysinoalanine content and the extent of proteolysis. Such changes may lower the quality and safety of foods.

INTRODUCTION

THE NUTRITIONAL QUALITY of any protein depends on its amino acid composition, digestibility, and physiological utilization of released amino acids. Since an amino acid must be liberated from a protein by digestion before nutritional assimilation can take place, it is important to establish the factors that affect protein peptide bond cleavage and nutritional quality.

Physiological availability of amino acids varies and depends on protein source, processing treatment, and on the diet and state of health of the consumer.

Food proteins are sometimes treated with heat and alkali during commercial and home processing. Many commercial foods contain proteins, such as sodium caseinate, textured soy protein, and corn flour, which have been previously exposed to alkali and/or heat. Alkali induces a number of changes in protein. These include formation of crosslinked amino acids such as lysinoalanine (LAL) and racemization of amino acid residues (Friedman, 1977a; 1978; Masters and Friedman, 1979; 1980; Fig. 1). It is, therefore, of interest to establish the factors which lead to alkali-induced chemical and nutritional changes in food proteins.

In this paper, we evaluate the relationship between in vitro susceptibilities of alkali-treated casein preparations to tryptic and chymotryptic digestion and their contents of LAL, D-aspartic acid, and D-phenylalanine.

EXPERIMENTAL

Alkali treatment

The following is a typical procedure. 1% solutions of commercial casein (International Casein Corp.) in 0.05M borate buffer were placed in glass-stoppered Erlenmeyer flasks and incubated at 65°C in a water bath. After the appropriate time interval, the samples were dialyzed against 0.01N acetic acid and lyophilized. Initial and final pH values did not differ significantly.

Amino acid analysis

A weighed sample (5 mg) of casein was hydrolyzed in 10 cc of 6N HCl in a commercial hydrolysis tube. The tube was evacuated, placed in an acetone-dry ice bath, evacuated and refilled with nitrogen twice before being placed in an oven at 110°C for 24 hr. The cooled hydrolysate was filtered through a sintered disc funnel, evaporated to dryness at 40°C with the aid of an aspirator, and the residue was twice suspended in water and evaporated to dryness. Amino acid analysis of an aliquot of the soluble hydrolysate was carried out on a Durrum Amino Acid Analyzer, Model D-500 under the following conditions: single column ion-exchange chromatography method; Resin, Durrum DC-4A; buffer pH, 3.25, 4.25, 7.90; photometer 440 nm, column, 1.75 mm X 48 cm; analysis time, 105 min. Norleucine was used as an internal standard. The accuracy of the analyses is estimated to be ± 1-2% (Friedman et al., 1979).

In this system LAL elutes as a well-resolved peak between PHE and HIS. For example, the following elution times (in min) were observed for selected amino acids in casein treated at pH 10: PHE, 55.70; LAL, 61.60; HIS, 62.53; ARG, 88.05.

The color constant (1.26 relative to leucine) and elution time for LAL was determined with an authentic sample purchased from Miles Laboratories.

Enantiomeric analyses

Approximately 30 mg of protein were hydrolyzed in 12 ml double-distilled 6M HCl for 24 hr at 96°C. The HCl solution was then brought to dryness at 50°C under partial vacuum in a rotatory evaporator. The residue was redissolved in double-distilled water and desalted on Dowex 50W-X8 (100-200 mesh) resin that has been cleaned with NaOH and protonated with double-distilled HCl. Amino acids were eluted from the column with 1.5M NH_4OH prepared by bubbling NH_3 through double-distilled water. The effluent was again evaporated to dryness. Aspartic acid was separated by chromatography on a calibrated column of BioRad AG1-X8, 100-200 mesh, anion exchange resin. The resin was regenerated with 4 column volumes of 1M sodium acetate followed by 2 column volumes of 1M acetic acid. Half of the sample was applied to the column, elution was carried out with 1M acetic acid. The aspartic acid fraction was evaporated to dryness. The L-leucyl-DL-aspartic acid dipeptides were synthesized by the procedure of Manning and Moore (1968). The D/L aspartic acid ratio was determined with a Beckman Model 118 Automatic Amino Acid Analyzer.

The D/L ratios for phenylalanine in the remaining half of the desalted amino acid fraction were obtained by gas chromatography (Hoopes et al., 1978). The N-trifluoracetyl-L-prolyl-DL-amino acid esters were synthesized, then separated on a Hewlett-Packard Model 5711A Gas Chromatograph with flame ionization detector and a 12-ft column of Chromasorb W-AW-DMCS solid support coated with 8% SP 2250.

The following modification was used to measure D-aspartic acid content of commercial foods. 300 mg of the food sample suspended in 350 ml 6N HCl was refluxed for 24 hr. The HCl was evaporated on an aspirator and the residue was suspended in 20 ml of water. The suspension was centrifuged, the supernatant liquid was desalted, and its D-aspartic acid content was determined as described above. The accuracy of the method is estimated to be ± 3%.

The results are discussed mainly in terms of LAL/LYS + LAL (calculated from mole % values) and D/L ratios rather than in terms of mole or weight percent for each amino acid. The use of ratios to indicate trends in transformation of lysine to lysinoalanine and an L- to a D-amino acid is generally more accurate because it avoids errors occurring in all operations during amino acid analysis. However, mole % and wt % values for LAL shown in Tables 1-4 indicate analogous trends.

Proteolytic digestion assays

Casein samples were dissolved or dispersed (5 mg/ml) in 0.05M

Authors Friedman and Zahnley are with the USDA-SEA Western Regional Research Center, Berkeley, CA 94710. Author Masters is with the Scripps Institution of Oceanography, Univ. of California at San Diego, La Jolla, CA 92093.

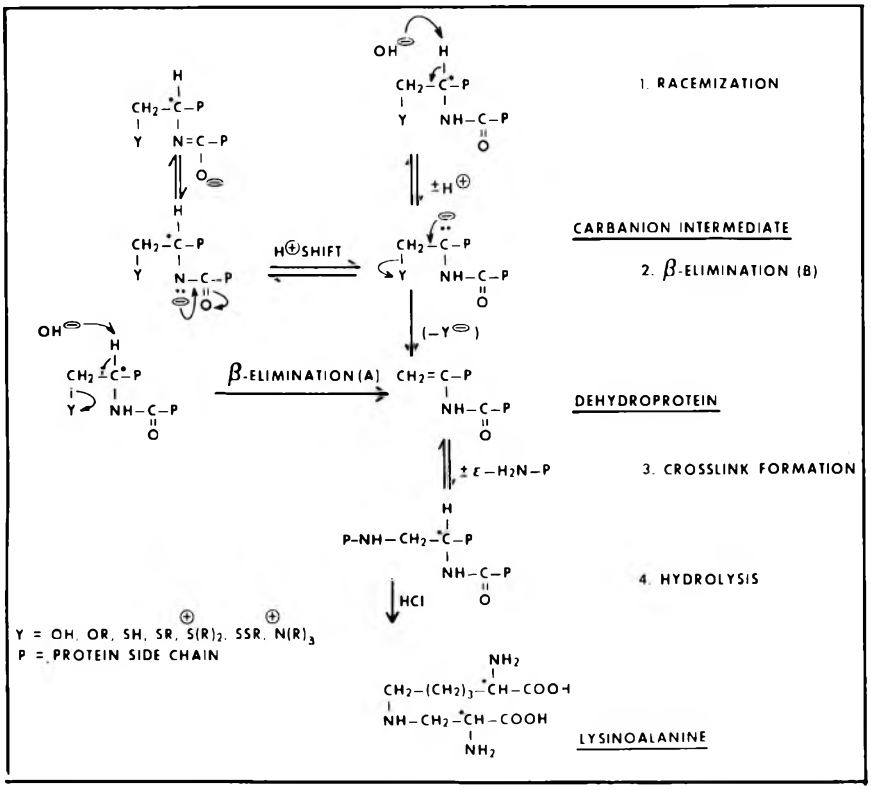


Fig. 1—Possible mechanisms for alkali-induced lysinoalanine formation and amino acid racemization in a protein via a common carbanion intermediate. Note that two β-elimination pathways are possible: (a) a concerted, one-step process (A) forming the dehydroprotein directly; and (b) a two-step process (B) via a carbanion intermediate. The carbanion, which has lost the original asymmetry, can recombine with a proton to regenerate the original amino acid residue which is now racemic. Proton transfer may take place from the environment of the carbanion or from adjacent NH groups, as illustrated on the left-hand part of the figure.

KCl with stirring and adjusted to pH 7.5. To determine digestibility by trypsin or chymotrypsin, aliquots (10 mg of protein) were pipetted into plastic titration vessels (Radiometer V 524) at 37°C, and diluted to 4.0 ml. Samples were incubated under N_2 to minimize CO_2 absorption. In a typical run, the substrate was brought to pH 7.5 and 37°C, and the baseline rate of uptake of titrant (0.02N NaOH) was determined. Then, 25 μl of bovine trypsin or α-chymotrypsin (~8 mg/ml) were added. (Trypsin was dissolved in 2 mM HCl-0.02M $CaCl_2$, chymotrypsin in 2 mM HCl. Both were crystallized preparations obtained from Worthington.) Net base uptake was determined from the graphic plot on the recorder after 20 min, since reaction was usually essentially complete at this time. Since cleavage of each peptide bond liberates 1 mole of carboxyl groups, the results permitted calculation of the number of peptide bonds hydrolyzed per average molecular weight of casein.

The following average values demonstrate the reproducibility of the enzyme digestion. The number of bonds cleaved by trypsin with the standard deviation given by three replicates was found to be 5.83 ± 0.20 per molecule (an average molecular weight of 25,000 was assumed; Brunner, 1977) for commercial casein; 5.32 ± 0.22 for casein treated at 25°C under conditions shown in Table 3; 5.17 ± 0.19 for casein treated at 35°C; and 1.61 ± 0.27 for casein treated at 75°C. Similarly, the average number of bonds in untrated casein cleaved by chymotrypsin in four determinations was 5.85 ± 0.39.

RESULTS & DISCUSSION

PROCESSED FOODS containing alkali-treated casein are expected to contain significant amounts of racemized amino acids. Determining D-amino acid contents of commercially available foods would provide an estimate of the extent of this problem. Results listed in Table 1 show that the D-aspartic acid content of five commercial food products ranged from 9% relative to the L-form for textured soy protein, to 17% for a nondairy creamer. The high content of D-aspartic acid in corn chips could be due to the use of heat and/or alkali during the preparation of the corn flour product.

Since high-quality proteins are presumably well digested (Ford, 1975; Stahmann and Woldegiorgis, 1975; Marshall et al., 1979), it is of general interest to establish the relationships between digestibilities of food proteins and processing conditions. These studies were carried out with casein as a model protein. The lysinoalanine content of casein is used as a measure of crosslinking (Friedman, 1978) and the D/L ratios of aspartic acid and phenylalanine, which were previously shown to racemize at fast rates (Masters and Friedman, 1979; 1980), as indices of the extent of racemization. The following variables which were expected to influence lysinoalanine formation, racemization, and, therefore, the digestibility of casein were evaluated: (a) pH; (b) temperature; and (c) time of treatment.

Effect of pH

Formation of LAL starts around pH 9 and increases rapidly with pH to 35% at pH 12.5 (Fig. 2). The extent of racemization parallels the extent of LAL formation up to pH 12.5 but continues to rise with increasing basicity. In contrast, the susceptibility of the treated casein samples to tryptic cleavage decreases with increasing pH. The number of bonds cleaved by trypsin range from about 6 per mole for casein subjected to pH 8 for 3 hr at 65°C to about 2 bonds for casein treated under analogous conditions at pH 12.5. Figure 2 shows the approximately inverse relationship between casein cleavage by trypsin and its LAL or D-ASP and D-PHE content.

The described pH effect on LAL formation may be understood in terms of a two-step postulated mechanism (Fig. 1). First, hydroxide ion-catalyzed β-elimination reactions of serine, threonine, and cystine give rise to a dehydroalanine intermediate. Since such elimination reactions are second-order reactions that depend directly on the concentration of both hydroxide ion and susceptible amino acid, the extent of lysinoalanine formation should vary directly with hydroxide ion concentration. Results in Table 2 show that this is indeed the case within a broad range. The dehydroalanine residue, which contains a conjugated carbon-carbon double bond, then reacts with the ε-amino group of lysine to form a lysinoalanine crosslink. This step is governed not only by the number of available amino groups but also by the location of the dehydroalanine and amino group

potential partners in the protein chain. Each protein, therefore, may have a limited fraction of potential sites for forming crosslinked residues. The number of such sites is presumably dictated by the protein's size, composition, conformation, chain mobility, steric factors, and extent of ionization of reactive amino (or other) nucleophilic centers.

A postulated mechanism of concomitant racemization via a carbanion intermediate is also shown in Figure 1. The carbanion, which has lost the original asymmetry, can recombine with a proton to form a racemic amino acid residue. Formation of the carbanion is the rate-limiting step in the racemization reaction. Increasing basicity facilitates the abstraction of the α-proton. Previous studies (Masters and Friedman, 1979; 1980) have shown racemization rates to be pseudo-first-order with respect to hydroxide-ion concentration. Thus, both lysinoalanine formation and racemization proceed rapidly at high pH.

Trypsin catalyzes the hydrolysis of peptide bonds derived from carboxyl groups of arginine and lysine residues (Keil, 1971). Alkali transforms lysine residues to lysinoalanine side chains and simultaneously degrades arginine residues (Friedman, 1978). The decreased susceptibility of alkali-treated proteins, such as casein, to trypsin digestion (Table 2) could, therefore, partly arise from loss of susceptible sites for enzyme cleavage. In addition, examination of complete amino acid profiles (not shown) reveals that about one-fifth of the arginine side chains were in fact degraded at the higher pH and temperature values used in this study.

Chymotrypsin catalyzes the hydrolysis of peptide bonds of proteins adjacent to carboxyl groups of aromatic amino acids (phenylalanine, tyrosine, tryptophan and to a lesser extent leucine, isoleucine, and methionine residues; Hess, 1971). The resistance of alkali-treated casein to hydrolysis by chymotrypsin (Table 3) may be due to racemization of certain aromatic acid side chains. Under the cited conditions, about 22% of phenylalanine, and 29% of aspartic acid residues were racemized (Table 2).

Table 1—D-Aspartic acid content in commercial food products

Product	D/L ASP	D-ASP[a] / D + L-ASP
Texturized soy protein	0.095	0.09
Baby formula (soy protein)	0.108	0.10
Simulated bacon (soy protein)	0.143	0.13
Corn chips	0.164	0.14
Dairy creamer (sodium caseinate)	0.208	0.17

[a] Relative ratios of aspartic acid as D- and L-enantiomers

Table 2—Effect of pH on lysinoalanine formation, aspartic acid and phenylalanine racemization, and tryptic digestion of casein (Conditions: 1% casein; 65° C; 3 hr)

pH[a]	LAL (g/100g)	LAL / LYS + LAL	D/L ASP	D/L PHE	No. peptide bonds cleaved by trypsin/ mole casein[b]
Comlcase n	—	—	0.022	0.03	5.83 ± 0.20
8.0	0.0	0.0	0.034	0.05	6.06
9.0	0.37	0.019	0.041	0.03	6.09
10.0	0.61	0.043	0.063	0.04	5.05
11.0	1.47	0.100	0.159	0.04	5.05
12.0	2.98	0.213	0.319	0.09	3.91
12.5[c]	5.29	0.345	0.397	0.29	2.66
13.9[d]	5.34	0.365	0.943	0.71	2.21

[a] pH measured at room temperature
[b] Based on an average molecular weight of 25,000
[c] pH of 1% casein solution in 0.1N NaOH
[d] pH of 1% casein solution in 1.0N NaOH

Effect of temperature

The dependence of lysinoalanine formation, racemization, and hydrolytic susceptibility of casein treated in 0.1N NaOH, as a function of temperature, are shown in Table 3 and Figure 3. It is striking that both LAL and D-ASP and D-PHE acid formation appear to be a nearly linear function of temperature, and that the extent of peptide bond hydrolysis by trypsin appears to follow an inverse relationship with temperature. These results suggest that, as in the case of pH, one or both changes in casein may be responsible for the decreased digestibilities.

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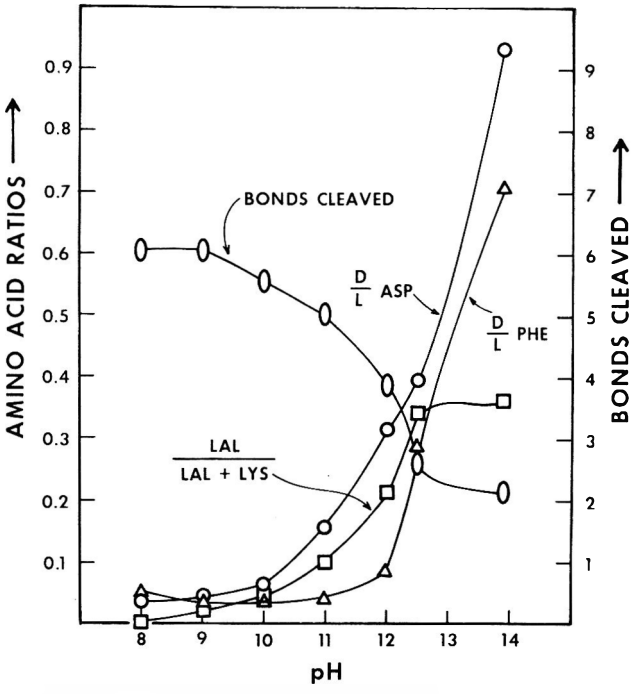


Fig. 2—Effect of pH on the relationship between the extent of hydrolysis of peptide bonds by trypsin in casein and its content of lysinoalanine, D-aspartic acid, and D-phenylalanine. (Conditions: 1% casein; 65° C; 3 hr.)

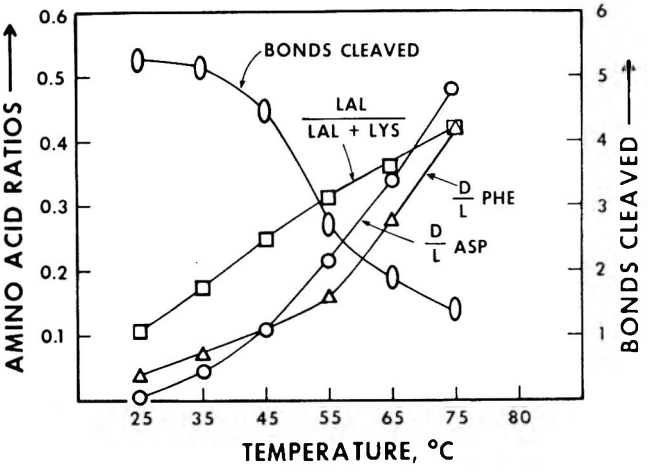


Fig. 3—Relationship between the extent of peptide bond cleavage in casein by trypsin and levels of lysinoalanine, D-aspartic acid, and D-phenylalanine as a function of temperature of treatment. (Conditions: 1% casein; 0.1N NaOH (pH ~ 12.5); 3 hr.)

Table 3—Effect of temperature on lysinoalanine formation, aspartic acid and phenylalanine racemization, and tryptic and chymotryptic digestion of alkali-treated casein (Conditions: 1% casein; 0.1N NaOH; 3 hr)

Temp °C	LYS Mole %	LYS Wt. % (g/100g)	LAL Mole %	LAL Wt % (g/100g)	LAL / LYS+LAL	D/L ASP	D/L PHE	No. peptide bonds cleaved per mole casein: Trypsin	No. peptide bonds cleaved per mole casein: Chymotrypsin
Coml casein[a]	7.01; 7.17	6.91; 6.78	–	–	–	0.022	0.03	5.83 ± 0.20[c]	5.85 ± 0.39[d]
25	7.31	7.08[b]	6.99; 7.07	0.0; 0.0	0.00; 0.0	0.0; 0.0[b]	0.108	0.04	5.07; 5.45	4.10
35	6.42; 6.68	6.35; 7.15	0.30; 0.35	0.62; 0.78	0.045; 0.049		0.174	0.07	4.98; 5.35	
45	6.08; 6.30	5.80; 5.85	0.75; 0.81	1.51; 1.57	0.110; 0.114		0.244	0.11	4.47; 4.47	3.00
55	5.51; 5.75	5.52; 5.51	1.51; 1.66	3.62; 3.63	0.218; 0.216		0.311	0.16	2.44; 2.93	3.10
65	4.47; 4.51	3.97; 4.51	2;31; 2.40	4.26; 4.71	0.339; 0.350		0.356	0.28	1.70; 2.10	
75	3.96; 4.12	3.73; 2.99	3.08; 3.18	6.08; 6.77	0.402; 0.437		0.486	0.42	1.35; 1.50	1.90

[a] Average molecular weight of 25,000
[b] Values obtained with samples treated separately with base as described in the experimental section
[c] Average of three separate determinations
[d] Average of four separate determinations

Table 4—Effect of time of treatment on lysinoalanine formation, aspartic acid, and phenylalanine racemization, and susceptibility to trypsin digestion of alkali-treated casein (Conditions: 1% casein; 0.1N NaOH; 65° C)

Time hr	LAL Mole %	LAL Wt. %	LAL / LYS + LAL	D/L ASP	D/L PHE	No. peptide bonds cleaved per mole casein[a,b]: Trypsin	Chymotrypsin[b]
Coml casein	–	–	0.022	0.03	5.83	5.85	
1/6	0.19	0.48	0.028	0.153	0.04	4.54	3.46
1/3	0.38	0.88	0.055	0.255	0.11	3.62	3.32
2/3	0.74	2.02	0.103	0.303	0.09	3.26	2.82
1.0	1.29	2.79	0.143	0.333	0.19	2.43	2.51
2.0	2.04	4.05	0.293	0.366			
3.0	2.70	4.48	0.354	0.387	0.28		
4.0	2.75	4.92	0.390	0.415			
5.0	2.69	5.22	0.408	0.463	0.34		
6.0	3.07	6.39	0.440				
7.0	3.11	6.30	0.446	0.510	0.41		
8.0	3.26	6.81	0.468	0.517	0.42		
24.0	3.20	5.98	0.455	0.733	0.57		

[a] Based on an average molecular weight of 25,000
[b] Average from two separately treated samples

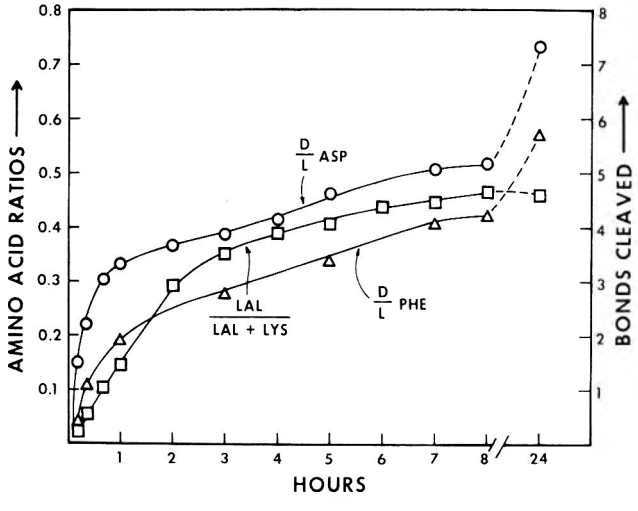


Fig. 4—Comparison of rates of formation of lysinoalanine, D-aspartic acid, and D-phenylalanine as a function of time. (Conditions: 1% casein, 0.1N NaOH (pH ~12.5), 65° C.)

Effect of time

Both LAL formation and racemization of aspartic acid and phenylalanine in casein increase with treatment time (Table 4, Fig. 4). The three curves appear to be approximately parallel. Note initial rapid rates up to 1-3 hr, followed by slow rates up to about 24 hr. Rapid initial rates could reflect a more ready formation of the carbanion intermediate during the early stage of the protein denaturation. As the native conformation is altered, different sets of amino acid residues are exposed to alkali. An alternate possibility is that the primary structure of the protein dictates the exposure of different sets of amino acid residues to alkali. Similar factors may govern the time-dependence of lysinoalanine formation. Results in Table 4 indicate that adverse effects of alkali on digestibility of casein occur quite early during the treatment.

Nutritional implications

pH and temperature and to a much lesser extent length of exposure affect LAL formation and racemization. Both processes may be responsible for the observed increased resistance of the treated casein to digestion by trypsin and chymotrypsin. Crosslinking of proteins via LAL formation would be expected to slow but not completely prevent hydrolysis by enzymes. In contrast, L-D, D-L, and D-D peptide bonds introduced during the racemization process would resist attack by proteolytic enzymes which function best with L-L bonds. They may, in fact, be competitive inhibitors of the cleavage of L-L peptide bonds (Hess, 1971; Keil, 1971; Schechter and Berger, 1966). Even if protein-bound D-amino acids could be liberated in the digestive tract they might not be transported into the blood stream and utilized for growth (Berg, 1959; Baker, 1979; Stegink, 1977). L-Amino acids are invariably taken up faster than the D-enantiomers in the intestine (Finch and Hird, 1960; Gibson and Wiseman, 1951) and kidney (Rosenhagen and Segal, 1974). If absorbed, D-amino acids can be utilized by undergoing reversible transamination pathways. D-Amino acid oxidases may catalyze oxidative deamination to α-keto acids, which can be specifically reaminated to the natural L-forms (Meister, 1965). This enzyme system should permit human utilization of D-amino acids for growth and maintenance.

Of the D-isomers of the eight essential amino acids, however, only D-phenylalanine and D-methionine were found to maintain human nitrogen equilibrium in early studies (Rose, 1949). More recently, Kies et al. (1975), Zezulka and Calloway (1976), and Printen et al. (1979), presented evidence that D-methionine is, in fact, poorly utilized by humans. When mixtures of D-amino acids are fed to rats, the oxidase system can be overloaded so that the D-enan-

tiomers of essential amino acids cannot be transaminated in sufficient quantity to support growth (Wretlind, 1952). In fact, excretion studies indicate that D-isomers are less readily reabsorbed through the glomeruli of the kidneys (Stegink, 1977).

We have shown that commercial foods contain varying amounts of D-aspartic acid. As a single index of racemization, we have used aspartic acid because of its analytical precision and because it undergoes racemization more rapidly than the other amino acid residues measured. Also, racemization rates of individual amino acids vary among proteins, but in any given protein the relative rates are similar so that one D/L measurement can serve as an index of racemization (Masters and Friedman, 1979).

Lysinoalanine (Gould and McGregor, 1977; Woodard et al., 1975) and D-serine (Ganote et al., 1974) can induce nephrotoxic changes in animals under some conditions. Recently, peptide-bound serine has been shown to racemize as fast as or faster than bound aspartic acid (Smith and Silva de Sol, 1980). Because the commercial foods analyzed in this study contain D-aspartic acid, it is now probable that they also contain similar amounts of D-serine. The long-term consequences of human and animal consumption of processed proteins are not known. Systematic surveys of commercial foods for D-amino acid compositions are needed to establish the importance of this problem to human nutrition and food safety. In addition, because utilization of protein-bound D-amino acids depends on the ability of an organism first, to liberate the amino acid from an L-D, D-L, or D-D peptide bond in a food protein and, second, to transform the D- to an L-form, further studies are desirable to define metabolic and nutritional effects of D-amino acids in animals and humans.

Finally, Erbersdobler (1977) and Erbersdobler et al. (1978) observed that heat-damaged casein which contained Maillard products such as fructosyl-lysine but no lysinoalanine was associated with (histo) pathological changes in rat kidneys such as enlarged epithelial cells similar to those observed with lysinoalanine (Woodard et al., 1975), D-serine (Wachstein, 1947; 1952; Ganote et al., 1974), and DL-2,3-diaminopropionic acid (Kaltenbach et al., 1979). Racemization undoubtedly also takes place under the experimental conditions used by Ebersdobler. In fact, the presence of glucose may increase the racemization rate of aspartic acid two- to threefold (Zumberge, 1979). These results suggest that the (cyto) toxicity observed by Erbersdobler may be due to D-enantiomers of amino acids, as well as to Maillard products. A cell could respond similarly to structural (stereochemical) features common to one or more of the four possible diastereoisomers of lysinoalanine ($R\tilde{N}HCH_2CH(NH_2)COOH$), D-serine ($HOCH_2CH(NH_2)$-$COOH$), or D-diaminopropionic acid ($NH_2CH_2CH(NH_2)$-$COOH$) (*cf.* Tas and Kleipool, 1976; Friedman, 1977[b]). Moreover, lysinoalanine could be a precursor for the last two compounds, since its hydrolytic cleavage at the starred nitrogen atom could form either racemic serine or 2,3-diaminopropionic acid.

In conclusion, this and related studies suggest that racemization of amino acids and concurrent formation of lysinoalanine in food proteins can impair the nutritional quality and safety of foods by: (a) decreasing the amount of essential L-amino acids; (b) decreasing digestibility and biolavailability of proteins; and, (c) forming toxic products.

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Measurement of Available Lysine Using the Guanidination Reaction

JOSEPH A. MAGA

ABSTRACT

Available lysine with its free ϵ-amino group can be chemically converted to homoarginine by means of a guanidination reaction using methylisourea (MIU). Various factors that can affect this reaction were investigated. For milk protein optimum conditions were found to be pH 10.8 for 96 hr at 20°C with a MIU concentration of 0.5M. Other protein sources had a different optimum pH for the guandination reaction. Addition of various carbohydrate sources and amounts did not interfere with the quantitation of available lysine.

INTRODUCTION

PROTEIN SOURCES that are heat treated or stored for long periods of time in the presence of reducing sugars can lose their nutritional value due to Maillard reactions which involve the interaction of reducing sugars with the ϵ-amino group of lysine. This reacted lysine is not susceptible to enzymatic attack and thus becomes nutritionally unavailable. Therefore, the measurement of lysine in certain processed foods results in a value for total lysine which may not be appropriate for estimating nutritional contributions from lysine.

During the past two decades numerous biological, biochemical, and chemical techniques, comparisons and reviews have been reported in an effort to more accurately quantitate these two important forms of lysine. Several of the most popular include the methodologies of Carpenter (1960), Carpenter and Booth (1973), Hurrell and Carpenter (1974, 1977) and Hurrell et al. (1979).

In addition to the above, the guanidination reaction, which was originally proposed by Mauron and Bujard (1963), has also found limited application. The compound o-methylisourea (MIU) can react in an alkaline medium with the free ϵ-amino group of lysine resulting in the formation of the guanidine residue of homoarginine. Since homoarginine is stable to acid hydrolysis (Nair et al., 1978), its quantitation by conventional chromatographic or colorimetric means can serve as an accurate and specific measurement of available or reactive lysine. Researchers (Finot and Mauron, 1972; Hurrell and Carpenter, 1974; Creamer et al., 1976; Burvall et al., 1978; Nair et al., 1978) have reported that the guanidination method gives an accurate measurement of available lysine in products that have undergone both minor and excessive browning. This is not the case for other measurement techniques. However, its apparent limitations include the facts that although not a complicated procedure, a great deal of time is required, due to a several day incubation period, and also, the reaction is pH dependent with protein source. As suggested by Hurrell and Carpenter (1974), modifications in the procedure, which have never been published in detail, could perhaps minimize these limitations.

Thus, the objectives of this study were to investigate, using a model system approach, those variables which could influence the accuracy of the guanidination reaction and apply this information to the analysis of various protein sources.

Author Maga is affiliated with the Dept. of Food Science & Nutrition, Colorado State Univ., Fort Collins, CO 80523.

MATERIALS & METHODS

Guanidination

The procedure as suggested by Mauron and Bujard (1963) was followed except for the variables of incubation time and temperature, pH, and MIU concentration which were evaluated in the current study. Different pH values have been reported in the literature, including 10.3 (Mauron and Bujard, 1963), 10.5 (Finot and Mauron, 1972; Burvall et al., 1978) and 10.8 (Hurrell and Carpenter, 1974; Creamer et al., 1976). Likewise, various incubation times, ranging from 3-7 days and temperatures (4°C-room temperature) have been utilized. Another important factor to consider is MIU concentration.

Therefore, the first phase of this study was designed to investigate these variables. Incubation times used ranged from 12-144 hr with sampling occurring at 12-hr intervals. MIU concentration was varied from 0.1-0.6M. Guanidination temperatures of 10, 20, 25, and 30°C were investigated as a possible means of shortening the reaction time knowing that the higher temperatures could perhaps result in artifact formation.

Another variable investigated was the addition of various types and amounts of carbohydrate materials, including glucose, sucrose, and potato starch. This was an attempt to evaluate their potential to interfere with the quantitation of available lysine. All of the above variables were evaluated using 200 ml portions in test tubes containing freshly prepared 5% by weight lysine solution. Readjustment of pH during incubation was done at 12-hr intervals with 0.1 NaOH and temperature was controlled by means of a thermostatically controlled water bath. All chemicals and reagents used are commonly commercially available and were of analytical grade.

The second phase of this study involved the determination of the optimum pH to maximize the guanidination reaction of various common protein sources. In their original communication, Mauron and Bujard (1963) indicated that various proteins had different homoarginine conversion rates as influenced by pH. The 8 protein sources evaluated in this study included raw whole milk, raw whole egg and unprocessed cottonseed, wheat, rice, soy, peanut and corn. The grains and legumes were frozen and ground to pass through a 50 mesh screen just before analysis. 20 ml of 20% suspensions in water on an as-is weight basis of each protein source was incubated in the pH range 9.0-11.0 at each 0.1 unit using NaOH. Thus, each protein was subjected to incubation at 21 different pH's. pH readjustment during incubation was done at 12-hr intervals as described above. As an aid in quantitation, a known quantity of butyl sterate was added as an internal standard to each sample before incubation (Nair et al., 1978).

Protein isolation and hydrolysis

After guanidination the amino acids (residual lysine and homoarginine in the model systems) and/or protein were precipitated with trichloracetic acid (final concentration of 12.5%) and washed with ethanol-diethyl ether (1/1) and finally with diethylether as described by Mauron and Bujard (1963). The resulting precipitate was hydrolyzed with 6N HCl at 110°C for 20 hr.

At this stage in sample preparation Nair et al., (1978) reported on an extensive and lengthy ion exchange column cleanup whereas Creamer et al., (1976) did not use a cleanup step. Preliminary experiments in the current study indicated that cleanup of the hydrolysate was not necessary to obtain quantitative results. The hydrolysate was then carefully evaporated to dryness.

Sample derivatization and gas chromatographic analysis

Samples were esterified and derivatized with trifluroacetic anhydride as described by Kaiser et al. (1974). A glass column (1.6M x 4 mm i.d.) packed with 2.0% w/w OV 17 and 1.0% w/w OV 210 on 100/120 mesh Gas Chrom Q was used for the gas chromatographic separation of the derivatives. A Hewlett-Packard Model 402 gas chromatograph equipped with a Model 3370B integrator operating at an isothermal oven temperature of 185°C with an argon carrier gas flow rate of 28 ml/min was employed. The peak area ratio of lysine to homoarginine relative to the internal standard peak area was used to measure the conversion rates of lysine to homoarginine as influenced by the variables investigated. Elution of all peaks of interest occurred in approximately 7 min.

RESULTS & DISCUSSION

Guanidination time

The time required to maximize the guanidination reaction (conversion of available lysine to homoarginine) can be seen from the data presented in Table 1. Most would consider a conversion rate in excess of 99% as being adequate in a quantitative analysis and thus, under the conditions employed, 96 hr (4 days) was required.

The time required to obtain quantitative results using the guanidination reaction has been cited as an obvious disadvantage of the method. It is granted that the method is not suitable for the rapid screening of samples but it should also be considered that the time factor represents little work for the investigator. Samples are incubated at room temperature and the only attention required during the 4-day period is pH adjustment. In practice pH would drop 0.1-0.2 units over 24 hr. It can be suggested that if working with the same types of samples, incubation time can be shortened to approximately 1 day if an appropriate guanidination conversion rate factor has been calculated. The data in Table 1 indicate that under the conditions employed, conversion of available lysine to homoarginine was essentially complete after 4 days of incubation, with no noted improvement in conversion associated with longer incubation times.

MIU concentration

The influence of MIU concentration on the conversion of available lysine to homoarginine during guanidination is seen in Table 2. Apparently, a concentration of at least 05.M is required for maximum conversion, with the use of lower levels resulting in incomplete conversion.

Guanidination temperature

As would be expected, the guanidination reaction is temperature dependent (Table 3). Maximum conversion to homoarginine at 10°C required 132 hr and fell to a low of 60 hr at 30°C. However, in actual practice the 30° temperature of incubation is not recommended due to the potential for nonenzymatic browning to occur. At refrigerated temperatures the reaction proceeds quite slowly. Thus, the compromise would be close to room temperature incubation.

Potential microbial problems associated with the room temperature storage of liquid samples can be minimized by the addition of thymol as a preservative (Hurrell and Carpenter, 1974). However, at such an alkaline pH, microbial problems should be negligible.

Influence of added carbohydrates

A major limitation of the commonly used Carpenter (1960) method for the chemical assessment of available lysine is the problem that the presence of reducing sugars can react with lysine and its derivatives during subsequent hydrolysis (Finot and Mauron, 1972). Since most food sources have some degree of reducing sugars present, care should be taken in the interpretation of quantitative avail-

Table 1—Influence of guanidination time on rate of homoarginine formation[a]

Time (hr)	% Lysine converted to homoarginine
12	14.3 (± 1.9)
24	62.5 (± 1.5)
36	70.4 (± 1.1)
48	90.1 (± 0.6)
60	90.4 (± 0.5)
72	93.4 (± 0.3)
84	96.0 (± 0.3)
96	99.7 (± 0.1)
108	99.8 (± 0.2)
120	99.8 (± 0.2)
132	99.7 (± 0.1)
144	99.8 (± 0.2)

[a] 20°C incubation, 5% lysine solution, pH 10.5, 0.5M MIU, average of 10 individually reacted sampled.

Table 2—Influence of MIU Concentration on rate of homoarginine formation[a]

MIU conc (M)	% Lysine converted to homoarginene
0.1	19.2 (± 2.1)
0.2	40.3 (± 1.7)
0.3	60.9 (± 1.6)
0.4	79.8 (± 0.8)
0.5	99.8 (± 0.2)
0.6	99.7 (± 0.1)

[a] 20°C for 96 hr of incubation, 5% lysine solution, pH 10.5, average of 10 individually reacted samples.

Table 3—Influence of guanidination temperature on rate of homoarginine formation[a]

Temp (°C)	Time (hr)	% Lysine converted to homoarginine
10	120	96.2 (± 0.3)
10	132	99.9 (± 0.1)
20	84	95.1 (± 0.4)
20	96	99.7 (± 0.1)
25	72	96.0 (± 0.4)
25	84	99.7 (± 0.1)
30	48	97.9 (± 0.3)
30	60	99.7 (± 0.1)

[a] 5% lysine solution, pH 10.5, 0.5M MIU, average of 10 individually reacted samples.

Table 4—Optimum guanidiantion pH for various protein sources[a]

Protein source	pH
Whole milk	10.8
Cottonseed	10.6
Wheat	10.5
Rice	10.4
Soy	10.3
Peanut	10.1
Corn	10.0
Whole egg	9.4

[a] 20% suspensions in water, 20°C for 96 hr of incubation, 0.5M MIU, average of 10 individually reacted samples. All products were in the raw state.

able lysine data. However, the guanidination technique apparently is not influenced by carbohydrate type and amount since additions of 5 and 20% of glucose, sucrose and potato starch resulted in lysine to homoarginine rates in excess of 99.6%.

Protein source guanidination pH

Mauron and Bujard (1963) reported that optimum guanidination pH can vary with protein source; however, no specific data relative to this observation were reported. Also, it is important to note that with food protein sources conversion of availble lysine to homoarginine is apparently only 80-90% complete (Mauron and Bujard, 1963; Hurrell and Carpenter, 1974; Nair et al. 1978) but is very consistent for each protein source dependent on the conditions of guanidination. Probably this is a function of protein structure. Thus, conversion factors are required to obtain quantitative data. In certain instances, this can present problems, especially in a mixed protein food of unknown protein content or ratios.

As seen in Table 4, all protein sources evaluated, except whole egg, had a maximum guanidination pH above 10.0 with whole milk having the highest at pH 10.8. Optimum pH was considered to be the pH where homoarginine yield was the highest. Thus, a mixed protein food that may contain egg and other protein sources, maximization of guanidination pH may be difficult to achieve. However, in single protein source foods the pH of guanidination can be a significant factor in attempting to quantitate available lysine.

Two theoretical potential limitations of the guanidination reaction as performed in this study are acknowledged, but other research would indicate that these problems are of little overall significance.

The first area involves the potential for lysinoalanine formation under alkaline conditions. Raymond (1980) has reported that temperature is an important aspect in its formation and only in food systems that have been exposed to elevated temperature was lysinoalanine detected. In contrast, the current study was conducted at room temperature. Also, Bohak (1964) had suggested that lysinoalanine formation primarily occurs within a protein structure and recently, Hayashi and Kameda (1980) have demonstrated that the addition of lysine derivatives to an alkaline/thermal model system did not increase the amount of lysinoalanine formed.

The other question involves potential reaction of MIU with the α-amino group as well as the ϵ-amino group. Apparently, the reaction is quite specific for only the ϵ-amino group since early researches (Hughes et al., 1949; Roche et al., 1954) could not demonstrate α-amino group alteration after guanidination.

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Reference to a company and/or product named by the Department is only for purposes of information and does not imply approval or recommendation of the product to the exclusion of others which may also be suitable.

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Characterization of a Heat-Resistant Strain of *Bacillus coagulans* Isolated from Cream Style Canned Corn

S. FEIG and A. K. STERSKY

ABSTRACT

A highly heat resistant strain of *Bacillus coagulans* was isolated from canned cream style corn. Spores of this organism grown at 45°C on Mn^{++} supplemented nutrient agar had the following heat resistance characteristics: in phosphate buffer, D_{100} = 86.00, D_{105} = 27.5, D_{110} = 6.1 min and z = 8.6C; in cream style corn extract, D_{110} = 9 min. For spores grown at 50 and 55°C, the D_{110} values in buffer were 2.5 and 1.8 min, respectively. The *B. coagulans* isolate matched the description in *Bergey's Manual of Determinative Bacteriology*, except for its higher heat resistance. Two additional *Bacillus* strains were intermediate between *B. coagulans* and *B. stearothermophilus*.

INTRODUCTION

THE NORMAL heat resistance of *Bacillus coagulans* is relatively low, with D_{110} ranging from 0.2-0.65 min (Amaha and Ordal, 1957; Anderson et al., 1949; El-Bisi and Ordal, 1956a; 1956b; Frank, 1955; Frank and Campbell, 1957; Knock et al., 1959; York et al., 1975; Youland and Stumbo, 1953) as compared to about 2.5 min for *C. botulinum* (Williams, 1969). Thus, the detection of *B. coagulans* in low acid canned foods is usually considered as an indication that the food has not been heated sufficiently to destroy *C. botulinum*. Only one relatively heat resistant *B. coagulans* isolate (from canned spinach) with a D_{110} value of 2.4 min, has been reported (Put and Wybinga, 1963).

B. coagulans and *B. stearothermophilus* are the two most common causes of flat sour spoilage. However, the high heat resistance (D_{110} = 55 min in our laboratory) of *B. stearothermophilus* would not cause concern with respect to underprocessing for *C. botulinum*, should it be found in spoiled low acid canned food.

This report characterizes a heat-resistant *B. coagulans* strain isolated from flat sour cans of cream style corn. The study also includes a comparison of differential characteristics of this isolate with those of two other isolates: strain 320, considered by Murrell et al. (1950) as *B. coagulans*, with a reported D_{110} value of 60 min and a very similar strain (1518-A) with a D_{110} value of 50 min (Denny, 1975).

MATERIALS & METHODS

Identification of the corn isolate

The characteristics of strain D1 were determined according to methods outlined in *The Genus Bacillus* (Gordon et al., 1973). *Bergey's Manual for Determinative Bacteriology* (Buchanan and Gibbons, 1974) was consulted for the species description of *B. coagulans* and *B. stearothermophilus*.

Source of other (control) Bacillus strains

Sp 33, isolated from spinach (H.M.C. Put, Carl C. Conway Laboratory, The Netherlands); ATCC 8038 and ATCC 12980 (American Type Culture Collection); 320, isolated from cabbage (W.G. Murrell, Commonwealth Scientific & Industrial Reaseach Organization (CSIRO), Australia); 1518-A, isolated from corn (C. Denny, National Food Processors Association, U.S.A.).

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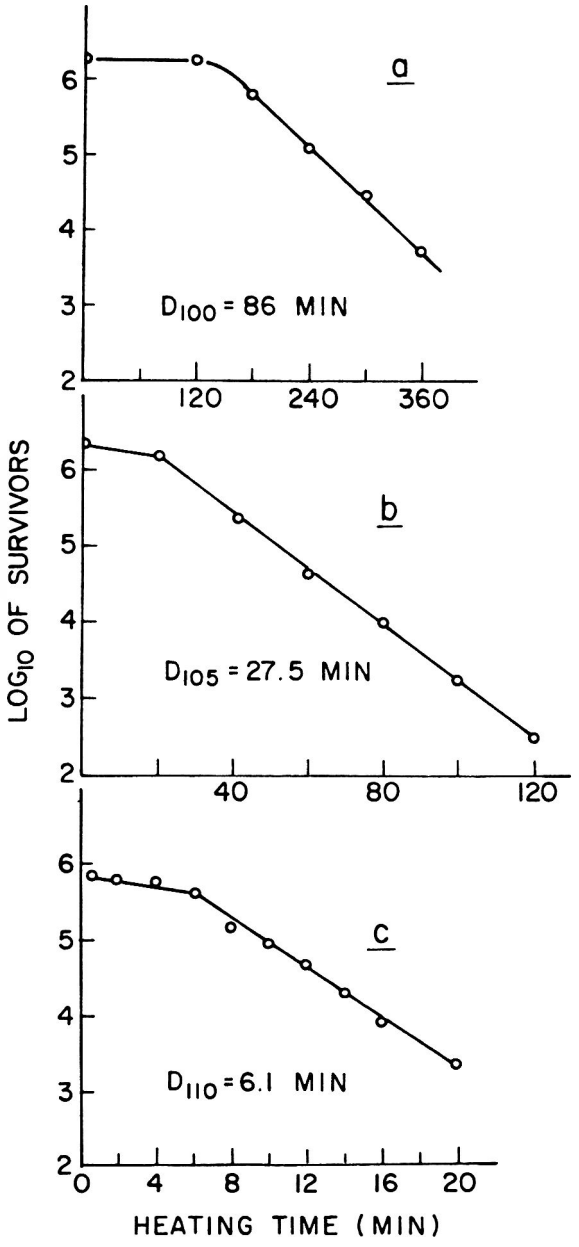


Fig. 1—Thermal resistance of spores of strain D1 produced on nutrient agar + 50 ppm Mn^{++} at 45°C heated in 1/40M Na phosphate (PO_4) buffer pH 6.8: (a) heated at 100°C; (b) heated at 105°C; (c) heated at 110°C.

Author Stersky is with the Food Directorate, Bureau of Microbial Hazards, Health Protection Branch, Health and Welfare Canada, Tunney's Pasture, Ottawa, K1A 0L2 Canada. Author Feig's present affiliation and address is Kupat Cholim Projinin, c/o Izhaki, Jerusalem, Israel.

Table 1—Differential characteristics of B. coagulans *and* B. stearothermophilus *and of some strians isolated from canned foods*

	B. stearo-thermophilus[a]	ATCC 12980[b]	320[b]	1518-A[b]	B. coagulans[a]	D1[b]	ATCC 8038[b]	SP 33[b]
Growth at 65°C	+	+	+	+	–	–	–	–
Growth at 55°C	+	+	+	+	+	+	+	+
Growth at 30°C	– slow	–	+ slow	–	+	+	+	+
Growth at 0.02% Azide	–	–	–	–	+	+	+	+
Sabouraud Dextrose Agar	–	–	+	+	+	+	+	+
Catalase	+ or –	+	+	+	+	+	+	+
Acetoin production	–	–	–	–	+	+	+	+
D^b110°C		55	32	24		6.1	0.5	1.9

[a] Bergey's Manual for Determinative Bacteriology
[b] Data obtained in our laboratory

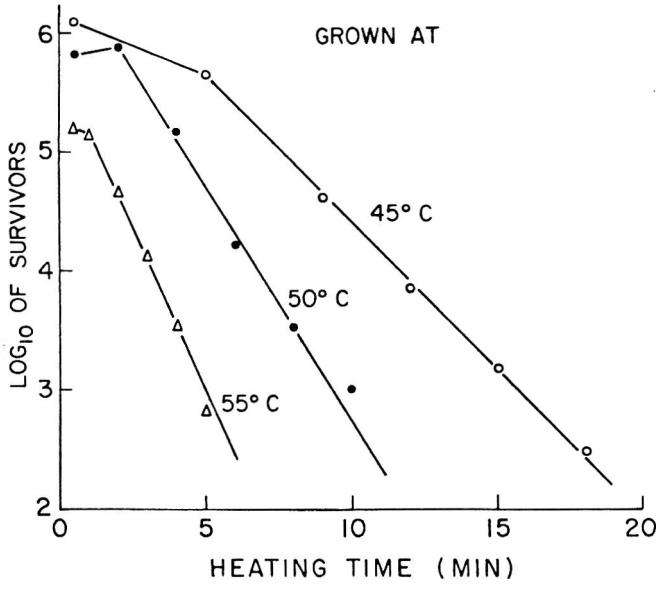


Fig. 2—Thermal resistance of spores of Strain D1 heated at 110°C in 1/40M PO_4 buffer, pH 6.8. Spores produced on nutrient agar + 50 ppm Mn^{++}: ○ – ○ Spores produced at 45°C, D_{110} = 4 min; ● – ● Spores produced at 50°C, D_{110} = 2.5 min; Δ – Δ Spores produced at 55°C, D_{110} = 1.8 min.

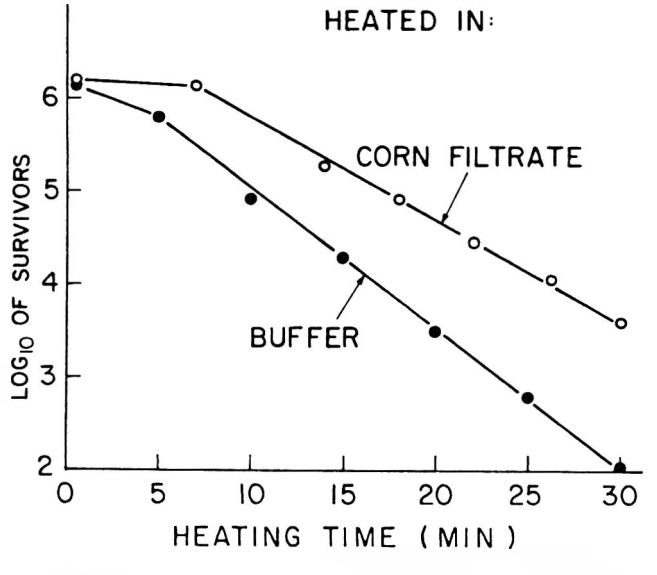


Fig. 3—Thermal resistance of spores of strain D1 heated at 110°C. Spores produced as in Fig. 1: ○ – ○ Heated in filtrate from cream style corn, D_{110} = 9 min; ● – ● Heated in 1/40M PO_4 buffer, D_{110} = 6.6 min.

Thermal resistance studies

Spore production. Heat-resistant spores were produced on nutrient agar, supplemented with 50 ppm of $MnSO_4$ and incubated at 45°C for 3 days. The culture was scraped off and suspended in 1/40M Na phosphate (PO_4) buffer at pH 6.8. The suspension was centrifuged and the sediment was resuspended in fresh buffer.

Heating. The heat resistance was determined by a modified (Hauschild and Hilsheimer, 1977) tube method of Kooiman and Geers (1975), except that the spores were heated in the above pH 6.8 buffer. In one experiment, the heating medium was a cheesecloth filtrate of commercial cream style corn (pH 6.4).

Enumeration of Survivors. The heated and cooled spore suspensions were diluted in the pH 6.8 buffer, and 0.1-ml volumes of the dilutions were spread, in duplicate, on plate count agar (Difco) containing 0.1% soluble starch. The plates were incubated at 55°C and the colonies counted after 2 and 4 days.

RESULTS & DISCUSSIONS

Identification of organisms

Table 1 shows that all the pertinent characteristics of our corn isolate D1, and of strains ATCC 8038 and Sp. 33 are identical to those of *B. coagulans.*

Strains 320 and 1518-A resemble *B. coagulans* in their growth on Sabouraud dextrose agar but are consistent with *B. stearothermophilus* in their temperature growth range, susceptibility to azide and lack of acetoin production. We found D_{110} values of 32 and 24 min for strains 320 and 1518-A, respectively, and 55 min for *B. stearothermophilus* ATCC 12980.

Heat resistance of D1

The thermal resistance curves of strain D1 heated at 100, 105, and 110°C are shown in Figure 1. The D values obtained from these curves were 86, 27.5 and 6.1 min, respectively. The corresponding linear death time curve (not shown) had a z-value of 8.6°C. The D_{110} value of 6.1 min for Strain D1 is 2.5 times higher than value reported for strain Sp 33 (Put and Wybinga, 1963) and about 10 times higher than the previously reported values (0.2-0.65 min) for other strains. The initial lag in the heat resistance curves has been noted and discussed by others (Amaha and Ordal, 1957; El-Bisi and Ordal, 1956a; Yokoya and York, 1965).

The D values obtained were greatly influenced by various factors. For spores of strain D1 grown on nutrient agar without Mn^{++} the D_{110} value was only 1.3 min. Amaha and Ordal (1957) reported a similar effect of Mn^{++} on the heat resistance of strain ATCC 8038 spores. Spores produced at elevated temperatures had a lower heat resistance (Fig. 2). In contrast, El-Bisi, and Ordal (1956a) and Lechowich and Ordal (1962) repored increased heat resistance as the temperature for growth and sporulation was increased from 30 to 52°C.

The effect of the heating menstruum on the heat resist-

ance of *B. coagulans* has been reported by several authors (Amaha et al., 1956; Anderson, et al., 1949; El-Bisi and Ordal, 1958; Frank, 1955; Put and Wybinga, 1963; Yokoya and York, 1965). When strain D1 was heated in a filtrate from cream style corn (pH 6.4), a D_{110} of 9 min was obtained as compared to 6.6 min for the same spore preparation in PO_4 buffer pH 6.8 (Fig. 3).

The recovery medium has also been shown to affect the D-values obtained (Murrel et al., 1950; Schmidt, 1955; Yokoya and York, 1965). We have noted that plate count agar (Difco) with 0.1% soluble starch gave higher recoveries than thermoacidurans agar or heart infusion agar.

Our conditions for determining D-values of *B. coagulans*, strain D1, differed in several aspects from those reported in the literature. Therefore, spores of strains D1, ATCC 8038 and Sp 33 were all produced and treated as described above. The D_{110} values for the three strains were 7.2 min, 0.5 min, and 1.9 min, respectively (Fig. 4). Thus, in spite of differences in methodology, these values are in fair agreement with the published D_{110} values of 0.2-0.65 min for ATCC 8038 and 2.4 min for Sp 33.

In view of the above, one may conclude: (a) that detection of *B. coagulans* in canned low acid food may, on occasion, be indicative of underprocessing for *C. botulinum* to a lesser degree than previously assumed; (b) growth on Sabouraud agar should be interpreted with caution when differentiating *B. stearothermophilus* from *B. coagulans*; and (c) the widely held concept that spores produced at higher temperatures are more heat resistant may not always pertain.

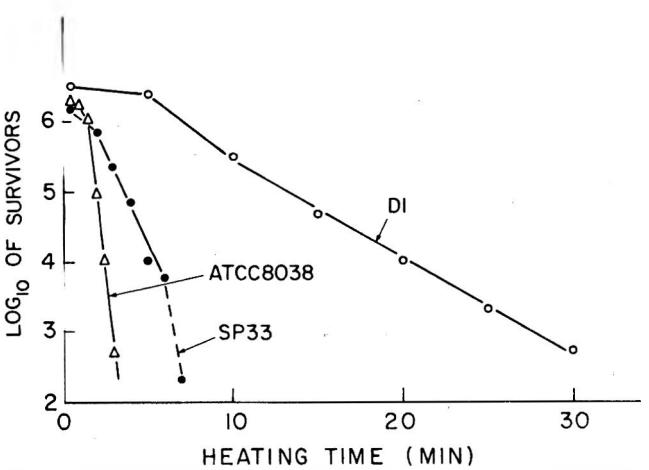


Fig. 4—Thermal resistance of spores of strains of B. coagulans *produced on nutrient agar + 50 ppm Mn^{++} and incubated at 45°C, heated at 110°C 1/40M PO_4 buffer pH 6.8: ○ – ○ Strain D1, D_{110} = 7.2 min; ● – ● Sp 33, D_{110} = 1.9 min; Δ – Δ ATCC 8038, D_{110} = 0.5 min.*

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Comparison of Growth Response by Chemostat Cultured and Batch Cultured *Clostridium perfringens* Cells in Various Food Substrates

STEVEN B. GOLDNER and MYRON SOLBERG

ABSTRACT

A constant source of exponential phase cells of *Clostridium perfringens* ATCC 3624 could reduce the time required to carry out the protein quality evaluation test of Solberg et al. (1979) to 4 hr. *Clostridium perfringens* ATCC 3624 was grown in an anaerobic chemostat using the chemically defined medium, R&S, with glucose as the growth limiting nutrient. The dilution rate was set at 0.06 hr^{-1}, the pH at 7.2, and the temperature at 43°C. In the transition from a batch culture to a continuous culture an initial oscillatory cell density response was observed. In the steady state, which was continued for as long as 50 days, the cells were typical Gram positive rods, occurring singly or in chains as long as 15 rods, which were occasionally without septa. The fermentative and biochemical responses of the cells did not change. No sporulation occurred when the cells were growing in the chemostat, but spores were observed in the glucose free culture effluent after incubation at 37°C for 24 hr. When cells produced in the chemostat, were cultured in complex media they demonstrated a growth response similar to cells which had been grown in a batch culture. In defined medium the generation time for the chemostat cultured cells was decreased approximately 17%. The chemostat cultured cells can be used as inoculum for the *C. perfringens* protein quality assay.

INTRODUCTION

MICROBIAL ASSAYS for evaluation of protein quality have been described by Fernell and Rosen (1956), Ford (1960) and Solberg et al. (1979). These studies used the protozoan flagellate *Tetrahymena pyriformis*, the bacterium *Streptococcus zymogenes* and the bacterium *Clostridium perfringens*, respectively. The *Clostridium* assay described by Solberg et al. (1979) requires an inoculation approximately 9-10 hr prior to the starting time of the experiment. There is a need to eliminate such an inoculation if the protein quality evaluation is to be completed from start to finish, within a single working day. A continuous culture of exponential phase cells would provide the means for achieving such an objective.

Chemostats have been used to grow microorganisms for long periods of time (Herbert et al., 1956; Rosenberger and Elsden, 1960; Zacharias and Bjorklund, 1968; Gill et al., 1977). The essential features of this method are that microbial growth occurs at a constant rate and in a constant environment. Herbert et al. (1956) found that the concentration of *Enterobacter cloacae* organisms could be maintained indefinitely in continuous culture if the flow rate remained unaltered. No organisms differing from the original strain in microscopic morphology, colonial morphology, biochemical reactions or growth characteristics were observed during long periods of continuous growth in a chemostat. Finn and Norwey (1958) found that cells of *Clostridium saccharoacetobutylicum* were less likely to degenerate in continuous cultures when kept in the logarithmic phase. Stability depended upon the maintenance of an environment where substances which could cause a change in properties were kept at a minimal concentration. Displacement of the original strain by a mutant strain could happen if the new strain possessed a selective advantage such as a faster growth rate (Novick, 1958). Novick and Szilard (1950) found that mutants would accumulate if there were no selective pressure for or against the mutation. Rosenberger and Elsden (1960) cultivated *Streptococcus faecalis* for 23 days in an anaerobic chemostat using a chemically defined medium and found that once steady state growth was achieved the cell density remained practically constant. Findley (1974) also observed that the cell density remained constant when *Clostridium perfringens* NCDC 2617 was cultivated for 19 days in a glucose limited chemostat using a fluid thioglycollate medium as the growth supporting substrate. Holstrom (1968) found that a chemostat culture of a β-hemolytic *Streptococcus* was stable for periods up to at least 20 days. Zacharias and Bjorklund (1968) observed no deterioration in the toxin producing capacity of a chemostat culture of *Clostridium tetani* which was maintained as long as 65 days. Chou (1971) used a tryptone salts medium for continuous growth and alpha toxin production of *C. perfringens* S-107. During steady state growth there was no change in the biochemical and fermentative activity of the culture. Gill et al. (1977) grew *Candida* 107 in a chemostat for periods up to 21 wk and observed that the fatty acid composition remained almost unchanged.

This study was carried out to characterize the continuous culture growth of *C. perfringens* ATCC 3624 in a chemically defined medium and to compare the growth response of chemostat cultured cells with batch cultured cells in a variety of substrates.

MATERIALS & METHODS

Microorganism

The microorganism used was *C. perfringens* ATCC 3624 which was obtained from the Food Science Dept. at Rutgers Univ.

Maintenance of stock culture

Stock cultures were maintained in Cooked Meat Medium (CMM) (Difco) at 4°C. Every 3-4 wk the culture was transferred by passing it twice through fresh fluid thioglycollate medium (Difco) at 37°C for 24 hr. Cultures were then inoculated into freshly prepared CMM, incubated at 37°C for 24 hr and then stored at 4°C.

Chemostat construction and operation

The laboratory chemostat used in this study was adapted from the original model of Novick and Szilard (1950). The modified chemostat is diagramatically shown in Figure 1. The modifications permit anaerobic conditions to be maintained and allow for internal sterilization after the medium and glucose reservoirs were connected to the chemostat and also after the reservoir was changed.

Inoculation was through the septum which covered the side arm orifice of the 300 ml culture vessel. The culture volume was maintained at a constant 200 ml.

The assembled chemostat was disconnected at the polypropylene tube connectors forming 3 assemblies which were autoclaved for 25 min at 121°C. Prior to the sterilization the 9.5-L medium reservoir was filled with 6L of glucose-free R&S medium (Riha and Solberg, 1971), the 250 ml water trap was filled with 50 ml of water, the 1-L sterile water vessels were filled with 400 ml of distilled deionized water, and the 250-ml glucose reservoir was filled with 100 ml of 50% glucose solution (w/w). Special precautions were required after reassembly of the chemostat and after each re-

Authors Goldner and Solberg are with the Dept. of Food Science, Rutgers–The State Univ., Cook College; New Brunswick, NJ 08903.

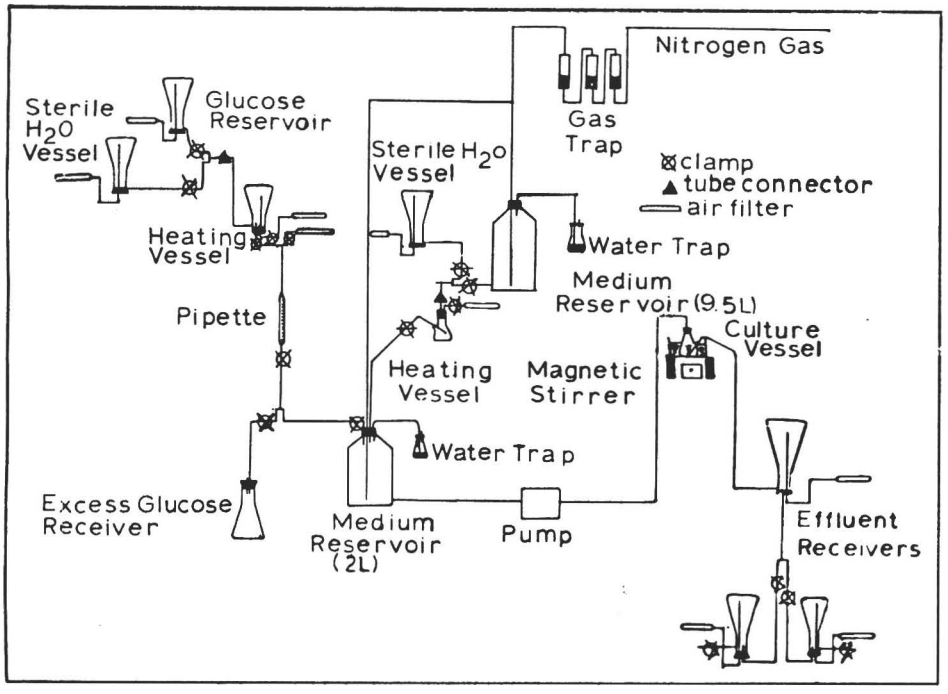


Fig. 1—Schematic representation of an anaerobic chemostat with provisions for internal thermal treatment to ensure system sterility during long term operation.

placement of the 9.5-L medium reservoir or the glucose reservoir to assure continued sterility. This was accomplished by using an internal heat treatment. The reservoir inflow lines were flushed with 200 ml of sterile water from the sterile water vessels to wash any potential contaminant into the heating vessels, which were in an upright position. The 200 ml of water in each heating vessel were then completely evaporated using an electric hot plate. The steam was released through an air filter. The flushing and evaporating procedure was then repeated. The air filter and the sterile water line valves were then closed and the heating vessel was inverted.

The 2-L medium reservoir was filled with R&S medium. Glucose, at a concentration of 2.5g/L was the growth limiting nutrient. Five ml of the 50% glucose solution (w/w) were quantitatively added per liter of R&S medium. The glucose solution was transferred to the inverted heating vessel. The pipette and latex rubber tubing below the pipette were filled with the glucose solution. The appropriate amount of glucose solution was added to the 2-L medium reservoir.

Anaerobiosis in the chemostat was maintained by continually flushing the medium in both media reservoirs with high purity grade nitrogen gas (Union Carbide Corp.). Residual oxygen in the nitrogen gas was removed by a 3-tube gas trap (Umbreit and Stauffer, 1964).

The Chemostat was entirely enclosed within a 43°C incubator with the culture vessel in a water bath to minimize temperature fluctuations.

Inocula preparation

The inoculum for the continuous culture was prepared by inoculating two sterile pyrex tubes containing 20 ml of R&S medium at pH 7.0 with one-half ml of the CMM stock culture. The tubes were incubated at 37°C for 9-13 hr. Two sterile Coleman spectrophotometer tubes (25 mm x 105 mm) containing 18 ml of R&S medium at 46°C were each inoculated with 2 ml of the 9-13 hr culture. Growth was monitored spectrophotometrically with a Coleman Junior Spectrophotometer set at 600 nm. At an absorbance of 0.30-0.35, a 15-ml aliquot of the culture was inoculated into the culture vessel using sterile syringes. Immediately after the inoculation, the peristaltic pump was turned on at a dilution rate of 0.06 hr^{-1}, which was maintained constant unless stated otherwise.

Batch culture cells for food substrate inoculation were prepared according to the procedure described by Solberg et al. (1979) except that the final inoculum was adjusted to A_{600} = 1.5.

Subcultured chemostat cells for food substrate inoculation were prepared by inoculating five sterile Coleman cuvettes, each containing 18 ml of R&S medium (at 46°C), with 2 ml of the chemostat culture. Growth was monitored spectrophotometrically (A_{600}). Readings were taken at 20-min intervals until an absorbance of 0.6–0.7 was reached. The cells were then washed and resuspended to A_{600} = 1.5. One-half ml of this culture was inoculated into the appropriate culture tubes.

Directly used chemostat cells for food substrate inoculation were prepared by increasing the flow rate of medium into the chemostat to 5 ml/min and collecting from the culture vessel. The sample was washed and the cell concentration was adjusted to A_{600} = 1.5. One-half ml of this culture was inoculated into the appropriate culture tubes.

Composition of test growth media

Four test growth media were used. These included the chemically defined R&S medium and three types of protein-containing media in which beef stew, chicken a la king, and breaded fish served as the protein source in R&S base (R&S medium without any amino acids). The test foods and their preparation were described by Solberg et al. (1979) and by Blaschek and Solberg (1978). The amount of each test food added to 20 ml R&S base provided a medium with 0.26%N (0.93g chicken a la king, 1.16g beef stew and 0.93g of the breaded fish). The pH was adjusted to 7.0 and one drop of antifoam (Dow Corning) was added to each tube to reduce foaming during N_2 flushing. The tubes were flushed with streaming O_2 – free N_2 for 20 min prior to the start of the experiment. One-half ml of each inoculum type was inoculated into five pyrex culture tubes containing R&S medium and five pyrex culture tubes containing a test food. One pyrex culture tube containing R&S medium was inoculated with sterile R&S base and was used as a control.

Continuous culture examination

Culture purity was examined by checking for characteristic biochemical and fermentative responses, colonial morphology and microscopic morphology. The tests carried out included Gram stain, plate counts in Tryptose Sulfite Cycloserine (TSC) agar (Harmon et al., 1971) without cycloserine and API -20A Anerobic Diagnostic tests (Analytab Products, Inc., Plainview, N.Y.) which consist of 21 biochemical and fermentative tests. Nitrate motility agar was used to check for nitrate reducing response and motility (FDA, 1972).

Plate counts

Viable cell density was measured by serial dilutions which were made in 0.1% peptone water blanks. Pour plates in duplicate were made using TSC agar. The plates were incubated anaerobically at 37°C for 18-24 hr in the GASPAK anaerobic system (Becton, Dickinson & Co.). Colony forming units were counted using a Quebec colony counter.

Glucose analysis

Glucose concentration was measured by the modified glucose oxidase-chromagen method described by Lin et al. (1976).

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Data analysis

Culture development was followed by measuring gas evolution manometrically (Curran et al., 1981). Growth curves were obtained by plotting gas evolution in manometer units vs time on semilogarithmic paper. Linear regression was used to determine the slope of the gas production curves during the exponential phase. Generation times were calculated from the slopes using the formula G = log 2/slope. Analysis of variance was utilized for evaluation of the dependent variable, generation time.

RESULTS AND DISCUSSION

STEADY STATE GROWTH was established at a dilution rate of 0.06 hr^{-1} which was at an absorbance of 0.60 (Fig. 2) in the glucose limited anaerobic chemostat. The culturable vegetative cell count in the continuous culture effluent remained in the range 3.7×10^8 to 5.3×10^8 cells/ml. *C. perfringens* ATCC 3624 was cultivated for as long as 50 days using the chemically defined R&S medium, without degeneration in any of the biochemical and fermentative responses measured. Tests conducted every 5 days during the study always showed fermentation of glucose, lactose, saccharose, maltose, trehalose and mannose; proteolysis of gelatin and reduction of nitrate to nitrite. The other tests were always negative.

The cells of *C. perfringens* ATCC 3624 grown in continuous culture and examined by phase contrast microscopy possessed characteristic morphology. The organisms were 3-5μ long, nonmotile, Gram positive rods occurring singly or in chains of 4-6 rods which were occasionally without septa. Chains of up to 15 cells were also present but constituted a small fraction of the population.

No spores were observed in the culture vessel. Findley (1974) also found that no sporulation occurred in the culture vessel with a continuous culture of *C. perfringens* NCDC 2617.

In the transition from a batch culture to a continuous culture a transient oscillation in the cell density was observed (Fig. 3). An increase in the absorbance from 0.7-0.8 occurred in the first 8-12 hr. This was followed by a decline in the cell density. The minimum absorbance was reached within the first 30 hr. A second variable oscillation of reduced amplitude, occurred over the next 40-50 hr after which steady state growth was established (Fig. 3). There have been no reports of any sustained oscillations of cell numbers where the growth parameters were kept constant. However, there have been reports of transient oscillations (Finn and Wilson, 1954; Gilley and Bungey, 1967; Dean and Rogers, 1967). Chou (1971) using a tryptone salts medium and Findley (1974) using fluid thioglycollate medium did not report transient oscillations in continuous cultures with *C. perfringens* strains S-107 and NCDC 2617, respectively.

Occasionally a black precipitate appeared in the culture vessel during steady state growth. The culture effluent appeared to contain H_2S. The black precipitate would disappear gradually when operation of the chemostat continued. There were no deleterious effects on the culture as observed by the absence of changes in any of the characteristic biochemical and fermentative responses measured or in the microscopic and colonial morphology of the cells. The cell count in the culture effluent remained in the range 3.7×10^8 to 5.3×10^8 cells/ml. There was no consistent correlation with changing the medium in the reservoir and the appearance of the black precipitate. When the cells were growing at a dilution rate of 0.06 hr^{-1}, increasing the dilution rate to 1.5 hr^{-1} for 5-10 min did not consistently stop the formation of the black precipitate.

Wood and Trim (1942) found that *C. perfringens* could metabolize cystine and produce H_2S. Fuchs and Bonde (1952) observed that H_2S could be produced by growing a culture of *C. perfringens* in a medium containing thiosulphate, cysteine, cystine, and glutathione but not methionine.

H_2S can be detected by adding ferrous sulphate to the medium which forms a black iron sulphide (Salle, 1973). Sulphur was present in the R&S medium as methionine, cystine, $FeSO_4$, $MgSO_4$ and $ZnSO_4$. The black precipitate was still observed after $FeSO_4$, $MgSO_4$ and $ZnSO_4$ were replaced with $FeCl_2$, $MgCl_2$ and $ZnCl_2$. The black precipitate could have formed as a result of H_2S being formed from cystine and subsequently reacting with Fe^{++} ions present in the medium.

The culture effluent was continually collected. When a 5-ml sample was incubated at 37° C for 24 hr and then examined by phase constrast microscopy, refractile spores were observed. Approximately 20% of the cells had sporulated. Half of the spores were in the sporangia and the rest were free spores. Glucose was not detected in 0.5 ml samples of the culture effluent using the Glucostat Test which was sensitive to 10^{-5}g of glucose. The conditions in the

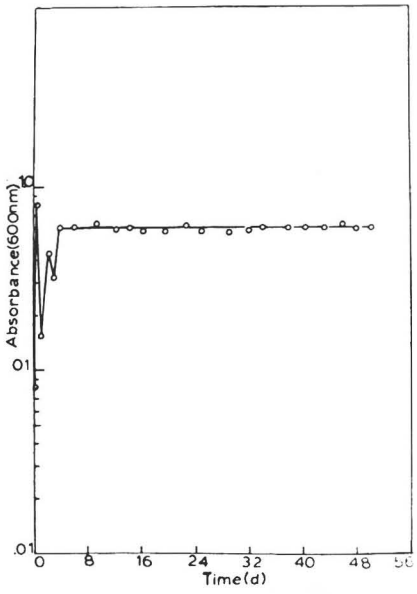


Fig. 2—Establishment and maintenance of steady state growth of Clostridium perfringens *in a chemostat at a dilution rate of 0.06 hr^{-1} at 43° C in a defined medium.*

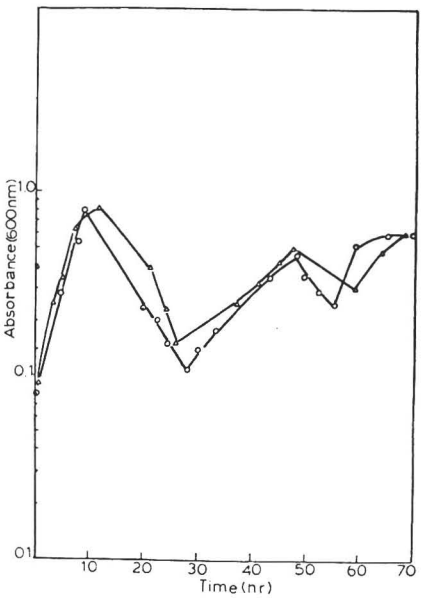


Fig. 3—A transient oscillation in cell density of Clostridium perfringens *during the first 70 hr of cultivation in a chemostat.* ○—○ *Run 1;* △ — △ *Run 2.*

culture effluent appear to be conducive for sporulation of *C. perfringens* ATCC 3624.

The growth response of the three inocula types of *Clostridium perfringens* ATCC 3624 were compared in batch culture using R&S medium, as well as beef stew, chicken a la king, and breaded fish as protein sources in R&S base. In R&S medium the mean generation time for the batch cultured cells was 22.7 min and for the directly used chemostat cultured cells 18.5 min with a standard error of ± 0.33 min. In a second study the mean generation time for the batch cultured cells was 24.2 min, for the subcultured chemostat cells 21.4 min, and for the directly used chemostat cultured cells 20.4 min with a standard error of ± 0.47 min. The mean generation time of the batch cultured cells and the chemostat cultured cells differed by 4.2 min in the first study and by 2.8 and 3.8 min in the second study. Table 1 shows that the directly used chemostat cultured cells and the subcultured chemostat cells consistently grew faster than the batch cultured cells in R&S medium. An analysis of variance confirmed that there was no difference in the growth response between the subcultured chemostat cells and the directly used chemostat cultured cells but that there was a significant difference in the growth response between the batch cultured cells and the chemostat cultured cells in R&S medium. Chemostat cells cultivated at 46°C, when subsequently grown in batch culture using R&S medium at 46°C had mean generation times in two experiments of 16.0 min and 19.0 min with a standard error of ± 0.60 min. The Student-Newman Keuls multiple range test confirmed there was no difference in the generation times between chemostat cells cultivated at 43°C and 46°C when grown in batch culture using R&S medium at 46°C.

The growth response of the batch cultured cells, subcultured chemostat cells and directly used chemostat cultured cells in the media containing the three test foods are summarized in Table 2. An analysis of variance confirmed that there was no difference in the generation time between the three inoculum types in any of the test foods. The results also showed that *C. perfringens* ATCC 3624 grew better in beef stew than in chicken a la king or breaded fish which is in agreement with the results reported by Solberg et al. (1979) for the same foods. The growth response of *C. perfringens* ATCC 3624 in each food was supported at a unique maximum rate. In addition, the culture age of the chemostat cells had no effect on the growth response in the three test food substrates (Table 3) or in R&S medium (Table 1).

The difference in the growth response of the chemostat cultured cells and the batch cultured cells, all of which were compared in batch culture using R&S medium could be due to changes brought about by the continuous cultivation. In a long term chemostat culture there is selection pressure as a result of continuously washing cells out of the culture vessel. The constant selective pressure discriminates against cells with generation times greater than the dilution rate (Powell, 1956; Beran, 1966). The slower growing cells should be maintained at a fixed concentration or eliminated entirely. It is unlikely that in every experiment a spontaneous mutation would occur and would produce a cell which when subsequently batch cultured would give a reproducible decrease in the mean generation time (Kubitschek 1970; Herbert et al., 1956). Herbert et al., (1956) studying the chemostat culture of *Enterobacter cloacae*, never observed mutant organisms with faster growth rates but observed that the chemostat culture of *E. cloacae* grew faster relative to a batch culture of *E. cloacae*. Dalton and Postgate (1969) found that faster growing variants were selected for high dilution rates in a chemostat culture of *Azotobacter chroococcum*. The observed difference in the growth response between the batch cultured cells and the chemostat cultured cells in R&S medium was probably due to the selection for faster growing variants in the chemostat.

The chemostat cells, when subsequently grown in batch culture containing R&S medium, had a consistently shorter generation time than the batch cultured cells. In the test foods there was no difference in the growth response between the different inocula types. Bacteria respond to an alteration in the growth medium by changing their chemical composition which is reflected by a change in the growth rates (Herbert, 1961). Table 2 shows that *C. perfringens* ATCC 3624 had generation times varying from 15-28 min

Table 1—Relative generation time[a] *of* Clostridium perfringens *cultivated at 43°C in a chemostat containing R&S medium and subsequently grown at 46°C in batch culture containing R&S medium*

	Relative generation time Chemostat cells		
		Direct[d]	
Days[b]	Subcultured[c]	Run 1	Run 2
4	–	70.1	–
5	–	74.6	–
6	–	87.2	–
7	87.7	80.0	83.5
11	–	97.5	–
12	–	76.5	–
14	88.9	–	99.2
17	–	87.3	–
20	79.1	–	93.8
25	71.1	–	65.0
29	91.3	–	88.9
35	103.6	–	70.7
44	85.3	–	78.5
46	91.2	–	91.9

[a] Relative generation time = (Generation time for chemostat grown cells ÷ generation time for batch culture cells) x 100.
[b] Days = length of time that the chemostat cultivated cells have been in steady state growth.
[c] Subcultured = Inoculum type which was harvested from chemostat and reinoculated into a batch culture using R&S medium. Growth was monitored spectrophometrically until an A_{600} 0.6-0.7 was reached. The cells were then washed and resuspended to A_{600} = 1.5. One-half ml of this culture was inoculated into the appropriate tubes.
[d] Direct = Inoculum type which was harvested from chemostat, washed, and cell concentration adjusted to A_{600} = 1.5. One-half ml of this culture was inoculated into the appropriate tubes.

Table 2—Generation time of Clostridium perfringens *harvested from a chemostat and subsequently grown in batch cultures at 46°C in medium containing R&S base and various nitrogen sources*

	Generation time (min)		
		Chemostat cells	
Nitrogen source	Batch culture	Subcultured	Direct
Fish	26.9	27.5	25.2
Beef Stew	15.9	15.4	15.5
Chicken a la king	18.2	19.2	17.8

Table 3—Effect of chemostat culture age on generation time of Clostridium perfringens *grown at 46°C in batch culture medium containing R&S base and various nitrogen sources*

Fish		Beef Stew		Chicken A La King	
Age of culture (days)	Generation time (min)	Age of culture (days)	Generation time (min)	Age of culture (days)	Generation time (min)
7	28.6	14	17.5	18	22.2
20	26.3	25	13.5	29	16.1
35	25.8	44	15.5	46	17.3

depending on the growth medium. Blaschek and Solberg (1978) found that the extracellular proteolytic activity and growth rate of *C. perfringens* varied as a function of medium and strain. Schaechter et al. (1958) found that *S. typhimurium* had generation times varying from 22-97 min depending on which medium was used for cultivation.

A lag phase was observed when the chemostat cells and the batch cultured cells were inoculated into a test food. During the lag phase, the cells were adapting to the new growth medium. In the chemostat, the cells were selected for their greater ability to grow in R&S medium. Once the cells were harvested from the chemostat and inoculated into a medium other than R&S medium, the selected variants no longer maintained any growth advantage. The chemostat cultured cells and the batch cultured cells adapted to the test foods in a similar manner which was reflected in their growth responses (Table 2).

The directly used chemostat cultured cells can replace the batch cultured cells as an inoculum source in the microbial assay evaluating protein quality which was developed by Solberg et al. (1979). Modifications in the RSR values of Solberg et al. (1979) will result from the faster growing controls in R&S medium but the relative responses will be comparable. The use of the chemostat cultured cells reduced the time required to conduct of the *C. perfringens* protein quality assay to approximately 4 hr.

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Regulation of Aflatoxin Biosynthesis: Characterization of Glucose as an Apparent Inducer of Aflatoxin Production

A. ABDOLLAHI and R.L. BUCHANAN

ABSTRACT

Aflatoxins were not produced when *A. parasiticus* was cultured on medium containing peptone plus mineral salts. Transferring the microorganism to a glucose-containing medium resulted in aflatoxin production. Initiation of aflatoxin synthesis could be blocked by treatment with cycloheximide or actinomycin D. It appears that glucose or a product of its metabolism, acting at the transcriptional level induces one or more of the enzymes responsible for aflatoxin synthesis.

INTRODUCTION

AFLATOXINS are secondary metabolites (Detroy and Hesseltine, 1970) synthesized by *Aspergillus flavus* and *Aspergillus parasiticus* via the condensation of two carbon units (Biollaz et al., 1970; Hsieh et al., 1975). Various investigators have reported that the production of aflatoxins is influenced by both the identity (Mateles and Adye, 1965; Davis and Diener, 1968) and concentration (Mateles and Adye, 1965; Davis et al., 1966, 1967; Hsieh and Mateles, 1971, Shih and Marth, 1974a, 1974b, Applebaum and Buchanan, 1979) of available carbon sources. Mateles and Adye (1965) reported that the type of carbohydrate employed as the sole carbon source of a synthetic medium strongly influenced the accumulation of aflatoxin. They found that sucrose, glucose, and fructose yielded the greatest aflatoxin production, while lactose, maltose, xylose, sorbose, glycerol, and glutamate supported growth but little aflatoxin accumulation. Starch. mannitol, sorbitol, and galactose, yielded good growth and intermediate amounts of aflatoxin. Using media composed of a carbon source plus yeast extract, Davis and Diener (1968) reported that glucose, ribose, xylose, and glycerol support abundant growth and aflatoxin production, while oleic acid, fumaric acid, shikimic acid, sodium acetate, pyruvic acid, ethanol, α-keto glutaric acid, succinic acid, and citric acid produced reduced growth and no aflatoxin accumulation. Gupta et al. (1974) found that fatty acids generally supported mold growth, but little aflatoxin production. Similarly, preliminary studies in our laboratory indicated that protein hydrolyzates as a sole carbon source supported good growth but no aflatoxin production. It would appear that maximal aflatoxin production is dependent on the availability of elevated concentrations of specific carbohydrates.

Detroy and Hesseltine (1970) demonstrated that the synthesis of aflatoxins required *de novo* protein synthesis, and suggested that this may represent the induction of the proposed aflatoxin synthetase complex (Detroy et al., 1971). The association of abundant aflatoxin production with specific carbohydrates suggested that these carbon sources may influence the induction of the aflatoxin biosynthetic pathway. The objective of the present study was to examine this hypothesis by determining the effect of glucose in a medium that does not support aflatoxin production.

Authors Abdollahi and Buchanan are with the Dept. of Nutrition & Food Sciences, Drexel Univ., Philadelphia, PA 19104.

EXPERIMENTAL

Microorganism

Aspergillus parasiticus NRRL 2999 was used throughout the study. Stock cultures were maintained on Potato Dextrose agar (Difco) slants and stored at 4°C.

Inoculum

Spores were harvested by adding 5.0 ml of sterile distilled water plus Tween 80 to a stock culture slant, and agitating gently. The spore suspension was then transferred to a sterile bottle, enumerated with hemocytometer, and diluted to contain 2×10^6 conidia/ml.

Media

The basal and replacement media employed in the present study were modifications of those described by Hsieh and Mateles (1971). The basal medium was a peptone-mineral salts medium consisting of peptone (Difco 0118), 60g; $(NH_4)_2SO_4$, 4.0g; KH_2PO_4, 10g; $MgSO_4 \cdot 7H_2O$, 2.0g; metal mix, 1.0 ml; and distilled water, 1000 ml. Replacement medium was composed of a carbon source (peptone or glucose), 60g; $(NH_4)_2SO_4$, 4.0g; KH_2PO_4, 5.0g; $MgSO_4 \cdot 7H_2O$, 0.5g; KCl, 0.5g; metal mix, 1.0 ml; and distilled water, 1000 ml. The metal mix contained (per liter) $Na_2B_4O_7 \cdot 10H_2O$, 700 mg; $Na_2MoO_4 \cdot 2H_2O$, 680 mg; $Fe_2(SO_4)_2 \cdot 6H_2O$, 10g; $CuSO_4 \cdot 4H_2O$, 300 mg; $ZnSO_4 \cdot 7H_2O$, 1.76g; and $MnSO_4$, 110 mg. The media were adjusted to pH 4.5 using 10N HCl.

Culture techniques

Flask cultures were prepared by transferring 25 ml aliquots of medium to 125 ml Erlenmeyer flasks (10 ml in 50 ml Erlenmeyer flasks were employed for the actinomycin D experimentation). The flasks were sealed with foam plugs and sterilized by autoclaving for 15 min at 15 psi. After autoclaving, cycloheximide or actinomycin D was added to the appropriate replacement medium flasks to produce a concentration of 150 μg/ml.

Growth was initiated by inoculating the peptone basal medium with 2×10^5 conidia (0.1 ml). The cultures were incubated at 28°C on a rotary shaker set for 110 rev/min. After the designated incubation period (usually 48 hr), the mycelial pellets were collected on filter paper (Whatman #1). The pellets were washed thee times with 25 ml aliquots of sterile replacement medium minus a carbon source. The pellets were then resuspended in replacement medium and incubated at 28°C on a rotary shaker (110 rev/min). After incubation (usually 24 hr), the cultures were assayed for aflatoxin production.

The effect of actinomycin D on the uptake of [^{3}H(G)] uridine was determined using a modification of the above procedure. Peptone cultures were incubated for 42 hr, harvested, washed, and resuspended in replacement medium containing glucose or glucose + actinomycin D (150 μg/ml). The cultures were then supplemented with [^{3}H(G)] uridine (New England Nuclear, Boston, Mass.) to achieve a specific activity of 0.5 μCi/ml. The cultures were incubated for 0, 3, 6, 9, and 12 hr, and harvested by centrifugation (15,000 x G). The mycelia were washed twice with distilled water, weighed, and resuspended in 0.05M phosphate buffer (pH 7.0) such that there was 100 mg mycelium/ml. The mycelia were frozen and thawed twice, and then homogenized using a Potter-Elvehjem tissue grinder. A 0.1 ml aliquot of the homogenate was transferred to scintillation vials containing 15 ml of scintillation fluid (Aquasol) and counted using a liquid scintillation detector (Model DPM-100, Beckman Instruments, Fullerton, Calif.).

Aflatoxin assays

Aflatoxins were extracted from the cultures (mycelium + medium)

according to the techniques described by Buchanan and Ayres (1976). Extracts were concentrated to a volume of 5 ml using a flash evaporator, and stored at −20°C while awaiting analysis. Aflatoxins were separated by thin-layer chromatography using Adsorbosil-1 (Applied Science Laboratories, Inc., State College, Pa.) plates developed with chloroform/acetone (9:1). Aflatoxins were quantitated fluorodensitometrically using a Turner TLC Scanner (Model 111, Turner Associates, Inc., Palo Alto, Calif.). All aflatoxin determinations were performed in duplicate.

RESULTS & DISCUSSION

CYCLOHEXIMIDE inhibits the 80S eukaryotic ribosome, blocking protein synthesis at the translational level without impairing other cellular activities (Lehninger, 1975). Detroy and Hesseltine (1970) demonstrated that the addition of cycloheximide (150 μg/ml) to exponential phase *A. parasiticus* completely blocked growth and protein synthesis, and prevented aflatoxin production. Cycloheximide was employed in the present study to determine if glucose affects the initiation of aflatoxin synthesis. *A. parasiticus* cultures were grown in basal medium (peptone or glucose as sole carbon source) for 48 hr, transferred to replacement medium containing peptone, glucose, or glucose + cycloheximide, and reincubated for 24 hr. These results are summarized in Table 1.

Aflatoxins B_1 and B_2 were detected in cultures initially grown on peptone and then transferred to glucose-containing replacement medium. Aflatoxins B_1, B_2, and G_1 were detected in cultures initially grown on glucose and transferred to glucose + cycloheximide. No aflatoxins were detected in cultures grown on peptone and transferred to either peptone or glucose + cycloheximide. In all cases, the addition of cycloheximide blocked further growth, while those cultures without the antibiotic continued to increase in mass. The data indicated that aflatoxins were not synthesized when the microorganism was cultured on peptone medium, but were produced when the cultures were transferred to glucose medium and allowed to undergo a period of *de novo* protein synthesis. This suggests that either peptone was repressing the aflatoxin biosynthetic pathway or that glucose (or a product of its metabolism) induced one or more of the enzymes necessary for aflatoxin production. The former option was eliminated by culturing the microorganism in peptone basal medium, and transferring to replacement medium containing peptone, glucose, and glucose + peptone (Table 2). Aflatoxin production was evident in the replacement cultures containing glucose or glucose + peptone indicating that the lack of aflatoxin production in the peptone cultures was not due to nitrogen catabolite repression.

The effect of actinomycin D on the apparent induction of aflatoxin production by glucose was examined by growing cultures on peptone basal medium for 42 hr, transferring the mycelia to replacement medium containing glucose or glucose + actinomycin D, and reincubating for 24 hr. These results are summarized in Table 3. Actinomycin D blocked growth and aflatoxin production, while aflatoxins B_1 and B_2 were detected in replacement cultures containing only glucose. The level of aflatoxin production (0.59 μg/mg) was approximately half that of the previous experimentation (1.35 μg/mg); however, this probably reflects the 42 hr initial incubation used in the actinomycin D study as compared to the 48 hr incubation employed with cycloheximide.

Since no literature was available on the effect of actinomycin D in *A. parasiticus*, the effect of the antibiotic on the incorporation of [^{3}H(G)]-uridine was examined to determine whether it effectively blocked transcription. These results are depicted in Figure 1. Transferring cultures to replacement medium containing only glucose resulted in increased uridine incorporation after 6 hr. This increase was not evident in the actinomycin-treated cultures, indicating an effective inhibition of transcription. These data again indicate that the transfer of the microorganism from peptone medium to glucose medium induced aflatoxin production after a period of *de novo* protein synthesis. The blockage of aflatoxin production by both cycloheximide

Table 1—de novo synthesis of aflatoxins[a]

Carbon source		Aflatoxin (μg/25 ml)				Aflatoxin/Mycelium (μg/mg)
Basal medium	Replacement medium	B_1	B_2	G_1	Total	
Peptone	Peptone	ND[c]	ND	ND	–	–
Peptone	Glucose	300	266	ND	566 ± 77[b]	1.35
Peptone	Glucose + Cycloheximide	ND	ND	ND	–	–
Glucose	Glucose + Cycloheximide	687	24	14	725 ± 21	1.87

[a] Cultures were grown in peptone or glucose basal medium for 48 hr, transferred to replacement medium containing peptone, glucose, or glucose + cycloheximide (150 μg/ml), and reincubated for 24 hr. Values are means of three replications with two determinations per replicate.
[b] Mean ± standard deviation
[c] No detectable aflatoxin

Table 2—Production of aflatoxin in glucose + peptone replacement medium[a]

Replacement medium	Aflatoxin (μg/25 ml)				
	B_1	B_2	G_1	G_2	Total
Peptone	ND[b]	ND	ND	ND	–
Glucose	155	65	22	ND	242
Glucose + Peptone	238	31	77	ND	346

[a] The cultures were grown in peptone medium with agitation for 48 hr and then transferred to glucose, peptone, and glucose - peptone replacement media. The cultures were reincubated for 24 hr and analyzed for aflatoxin. Values represent mean of two replicates with two determinations per replicate.
[b] No detectable aflatoxin

Table 3—Effect of actinomycin D on the de novo synthesis of aflatoxins by A. parasiticus[a]

Replacement medium	Aflatoxin (μg/10 ml)			Aflatoxin Mycelium (μg/mg)
	B_1	B_2	$B_1 + B_2$[b]	
Glucose	1.3	1.3	2.6 ± 2	0.59
Glucose + actinomycin D	ND[c]	ND	–	–

[a] Cultures were grown in peptone basal medium for 42 hr, transferred to replacement medium containing glucose or glucose + actinomycin D (150 μg/ml), and reincubated for 24 hr. Values are means of three replicates with two determinations per replicate.
[b] Mean ± standard deviation
[c] No detectable aflatoxin

and actinomycin D indicates that the control of the induction process is at the transcriptional level.

The inability of peptone-grown *A. parasiticus* to synthesize aflatoxins was further examined to determine if the lack of aflatoxin production may have been due to the cultures not having attained sufficient growth. Cultures were initially incubated in peptone basal medium for 48, 72, 96, and 120 hr, transferred to replacement medium containing glucose + cycloheximide, and reincubated for 24 hr. Replacement cultures containing glucose only were also employed to determine if the duration of the initial incubation influenced the initiation of aflatoxin production by glucose. These results are summarized in Table 4. No aflatoxins were detected in replacement cultures containing glucose + cycloheximide, regardless of the duration of the initial incubation period. This indicates that the lack of aflatoxin production in peptone-grown cultures was due to a lack of one or more of the enzymes needed for aflatoxin synthesis, and not due to changes in the growth pattern of the microorganism.

Aflatoxin B_1 and B_2 were detected in those replacement cultures containing only glucose. The greatest aflatoxin accumulation was associated with the cultures initially incubated in peptone basal medium for 48 hr, while much less aflatoxin was evident in the 72 and 96 hr cultures. Relatively high levels of aflatoxin were detected in the 120 hr cultures; however, it was observed that these cultures had undergone abundant conidiation prior to transfer. It is possible that the increased aflatoxin production in these cultures was due to the growth of a "second generation" of cells; however, this possibility requires additional study. Overall, the data suggest that the induction of aflatoxin synthesis by glucose (or a product of glucose metabolism) was dependent on the age of the culture. Similar findings

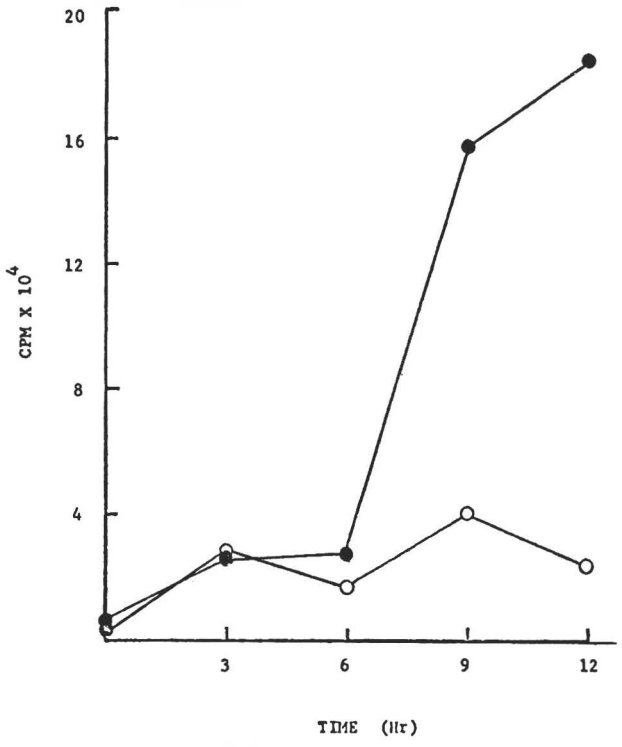


Fig. 1—Incorporation of [^{3}H(G)] uridine in A. parasiticus cultures initially incubated in peptone basal medium for 42 hr and then transferred to replacement medium containing glucose (●) and glucose + actinomycin D (150 µg/ml) (○).

Table 4—Effect of incubation time in peptone medium on aflatoxin production by A. parasiticus[a]

Incubation period (hr)	Replacement medium	Aflatoxin (µg/25 ml) B_1	B_2	$B_1 + B_2$[b]	Mycelium (mg)	Aflatoxin/Mycelium (µg/mg)
48	Glucose	122.9	41.5	165 ± 98	243	0.68
	Glucose + cycloheximide	ND[c]	ND	–	127	–
72	Glucose	9.2	1.1	10.3 ± 5	387	0.03
	Glucose + cycloheximide	ND	ND	–	335	–
96	Glucose	10.2	1.7	11.9 ± 8	443	0.03
	Glucose + cycloheximide	ND	ND	–	342	–
120	Glucose	82.5	41.6	124.1 ± 84	456	0.27
	Glucose + cycloheximide	ND	ND	–	290	–

[a] Cultures were grown in peptone basal medium for 48, 72, 96, and 120 hr, transferred to replacement medium containing glucose or glucose + + cycloheximide (150 µg/ml), and reincubated for 24 hr. Values represent means of three replicates with two determinations per replicate.
[b] Mean ± standard deviation
[c] Nc detectable aflatoxins

Table 5—Time needed for the induction of aflatoxin synthesis in A. parasiticus[a]

Time of cycloheximide addition (hr)	Aflatoxin (µg/25 ml) B_1	B_2	$B_1 + B_2$	Mycelium (mg)	Aflatoxin/Mycelium (µg/mg)
0	ND[c]	ND	–	167	–
3	ND	ND	–	108	–
6	2.1	ND	2.1 ± 0.4	132	0.02
9	7.4	0.3	7.7 ± 0.7	205	0.04
12	6.8	0.7	7.5 ± 1.5	267	0.03
24	8.8	1.3	10.1 ± 1.3	358	0.03
48	18.2	9.7	27.9 ± 7.4	360	0.08

[a] Cultures were grown in peptone basal medium for 48 hr, and then transferred to replacement medium containing glucose. Cycloheximide (150 µg/ml) was added to the replacement medium after 0, 3, 6, 9, 12, 24, and 48 hr, and the cultures were incubated for another 24 hr. Values represent the means of two replicates with two determinations per replicate.
[b] Mean ± standard deviation
[c] No detectable aflatoxin

were reported by Hsieh and Mateles (1971) who found that the accumulation of aflatoxin by resting cultures of *A. parasiticus* was greatest when the microorganism was initially incubated for 48 hr.

The results of the uridine uptake study (see Fig. 1) suggested that the initiation of *de novo* protein synthesis after trasnferring the microorganism from peptone to glucose media required approximately 6 hr. This time requirement was further characterized by determining how long the microorganism had to be exposed to glucose in order to induce aflatoxin production. This was achieved by culturing the microorganism in peptone basal medium for 48 hr, transferring to glucose-containing replacement medium, adding cycloheximide after 0, 3, 6, 9, 12, 24, and 48 hr, and continuing the incubation for another 24 hr. These results are presented in Table 5. Aflatoxins B_1 and B_2 were first detected in those cultures receiving cycloheximide after 6 hr of incubation. No aflatoxins were evident in either the 0 or 3 hr cultures. These data, along with the uridine uptake data, suggest that the induction process requires approximately 6 hr.

The pathway for aflatoxin biosynthesis remains largely unelucidated; however, Detroy et al. (1971) have estimated that the process involves over fifty individual reactions. Radiotracer studies (Hsieh and Mateles, 1970: Shih and Marth, 1974a) have demonstrated that glucose can be catabolized to acetyl-CoA, which in turn acts as the building block for the carbon skeleton of aflatoxins. Shih and Marth (1974a) and Applebaum and Buchanan (1979) reported that glucose catabolism in *A. parasiticus* in biphasic, with the sugar being used at a moderate rate during exponential growth and at an accelerated rate during aflatoxin synthesis. The results of the present study indicate that either glucose or a product of its metabolism may also have a regulatory role: acting as an inducer of one or more of the enzymes needed for aflatoxin synthesis. While confirmation of this hypothesis awaits development of techniques for measuring the activity of the enzymes of the aflatoxin biosynthetic pathway, the data in the present study are consistent with glucose acting at the transcriptional level to induce aflatoxin production.

The mechanism involved in the apparent induction of aflatoxin synthesis awaits future research. It is possible that glucose is directly inducing enzyme synthesis, or that the catabolism of the carbohydrate is altering the cell's energy status or the intracellular concentration of a key intermediate. Additional research is also needed to determine whether the induction process is specific for glucose or includes other carbohydrates capable of supporting abundant aflatoxin formation. Hopefully the observations and techniques of the present study will be useful in ultimately understanding the regulatory parameters that control the synthesis of aflatoxins and other secondary metabolites.

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Carotenoid Composition and Vitamin A Value of Fresh and Pasteurized Cashew-Apple (*Anacardium occidentale* L.) Juice

HELOISA M. CECCHI and DELIA B. RODRIGUEZ-AMAYA

ABSTRACT

The carotenoid composition of the red and yellow cashew-apples and two commercial brands of pasteurized juice were determined by column and thin-layer chromatography, visible absorption spectroscopy and specific chemical reactions. Alpha-carotene, β-carotene, ζ-carotene, *cis*-β-carotene, cryptoxanthin, aurochrome, cryptochrome, and auroxanthin were identified in all samples, with β-carotene as the predominant pigment. Color differences reflected the total amount, not the type of carotenoid present. Geographic effects on the pigment composition did not appear to be appreciable. In the processed juice, the total carotenoid content and vitamin A value were lower but the epoxide level higher. No quantitative differences were noted among lots of the same brand or between brands of pasteurized juice.

INTRODUCTION

IN SPITE OF the wide popularity of the cashew nut, knowledge and utilization of the pseudofruit called cashew-apple remain restricted to a few countries. In Brasil the whole pseudofruits, after the removal of the nuts, are disintegrated, seived, and pasteurized, thus turning what would otherwise be waste product into a nutritions, aromatic juice. The juice is highly accepted in the domestic market and even exported to a limited extent. It is an excellent source of vitamin C, the vitamin C content (average of 261 mg/100 ml) being about six times that of orange juice (Lopes, 1972; Soares and Maia, 1970). The high tannin content gives it an astringent taste which Brasilians tolerate but may not be necessarily pleasing to other people.

In Brasil, various types of the common cashew, *Anacardium occidentale* L., can be encountered and are arbitrarily grouped according to color, flavor, shape, and consistency. Based on the color of the fruits when fully ripe, the most frequently found cashew are of the yellow or red type.

Aside from the total carotenoid content, no other information about the carotenoid pigments of cashew-apple is available in the literature. In this study, the individual carotenoids responsible for the yellow or bright red color of the cashew-apple were identified and quantified. In addition, owing to the known sensitivity of carotenoids to light, heat, and oxygen, the carotenoid composition of two brands of pasteurized juice was determined to verify the effect of the processing treatment on the pigments.

Since the carotenoids are responsible not only for the color but also for the vitamin A value of fruits, we took advantage of the data already at hand and calculated the vitamin A value of cashew-apple, taking into account only the carotenoid precursors with their corresponding provitamin A activity. The value thus calculated would be more accurate than that obtained by the AOAC method (AOAC, 1975), which could include in the calculation carotenoids devoid of or possessing lower activity. For example, the vitamin A value of cling peaches determined by the AOAC method, already modified to reduce the error, was found to be about twice the value determined by a method which separated and quantified only carotenoid precursors with their respective provitamin A activity (Gebhardt et al., 1977).

Authors Cecchi and Rodriguez-Amaya are with the Faculdade de Engenharia de Alimentos e Agrícola, Universidade Estadual de Campinas, 13100 Campinas, São Paulo, Brasil.

MATERIALS & METHODS

Materials

Fruits representing the yellow and red types of *Anacardium occidentale* L. were analyzed. The fruits were collected from three states in Brasil: one lot (yellow) from Ceará (Northeast), one lot (red) from Pará (North) and two lots (red and yellow) from São Paulo (Central South). Each lot consisted of 8 to 10 fruits. Fruits from the Northern and Northeastern states were bought fresh from the farmer's market, transported by air to Campinas and analyzed immedately after arrival. The red fruits from São Paulo were gathered directly from the farm while the yellow fruits were bought from the market. For each lot, the nuts were removed, the pseudofruits homogenized in a Waring Blendor and duplicate samples of 100g each taken for analysis. Duplicate determinations were done to insure that quantitative differences were not due to experimental deviations.

Two commercial brands of juice, pasteurized and bottled in Northeastern Brasil, were also investigated. Three lots were analyzed for each brand. For each lot, two bottles were mixed and duplicate 100-g samples were taken for the carotenoid determination. Sampling was at random, the bottles being purchased from different grocery stores.

Carotenoid determination

The carotenoids were extracted with cold acetone, saponified, transferred to petroleum ether and separated on a MgO:HyfloSupercel (1:2), using a gradient of ether and acetone in petroleum ether as developing solvent. Identification was based on the visible absorption spectra, order of elution from the column, R_f values on silica gel plates developed with 3% methanol in benzene and, when necessary, specific chemical reactions. Quantitation was based on the absorbance at the maximum. Details of the extraction, separation, identification, and quantitation procedures have been described previously (Rodriguez et al., 1976).

Since the objective of this research was to identify the carotenoids which contributed to the color and vitamin A value of the juice, the colorless carotenoids phytoene and phytofluene, which are devoid of provitamin A activity, were not determined.

Calculation of the vitamin A value

The vitamin A value was calculated using the provitamin A activity of the individual active carotenoids given by Bauernfeind (1972). Thus, 1 μg of β-carotene, which has 100% activity, is equivalent to 0.5 μg of vitamin A value. One μg of α-carotene and cryptoxanthin, having 50% activity, corresponds to a 0.25 μg of vitamin A value.

RESULTS & DISCUSSION

Qualitative composition

Identical carotenoids were detected in either yellow or red cashew-apple and in the processed juice. Thus, the difference in color did not appear to be a function of the type of carotenoid present. Unlike the red-fleshed papaya and watermelon, the color of which could be directly explained by the presence of lycopene as the major pigment (Morgan, 1967; Tomes et al., 1963; Yamamoto, 1964), lycopene was

not detected in the red cashew-apple, even when the amount of sample was doubled.

The properties of the eight carotenoids identified in cashew-apple are summarized in Table 1. Six fraction were obtained from the MgO:HyfloSupercel column. Fractions 1, 2, and 3 were readily identified as α-carotene, β-carotene, and ζ-carotene, respectively, by their visible absorption spectra, affinity on the column and R_f values on the silica gel plates.

The slightly lower maxima of fraction 4 (4 nm lower than those of β-carotene) indicated that it could be a *cis* isomer or 5,6-epoxy derivative of β-carotene. The latter possibility was discounted when the yellow spot of the pigment on the silica gel plate failed to turn blue on exposure to HCl gas and the absorption spectrum remained unaltered on addition of dilute HCl to an ethanolic solution of the pigment. No hypsochromic shift in the absorbance was observed with the iodine-catalyzed isomerization test, indicating that the pigment was already in *cis* form. The properties of this fraction (absorption spectrum and polarity demonstrated on the column) resembled the *cis* neo-β-carotene reported by Gross et al. (1971) in Shamouti oranges.

The absorption spectrum of fraction 5 indicated that the basic structure was that of β-carotene. The markedly lower R_f value on the silica gel plate reflected the presence of a hydroxyl group; this was confirmed by the positive response to acetylation with acetic anhydride. The nonallylic position of the hydroxyl substituent was demonstrated by the negative reaction to methylation with acidified methanol and dehydration with acidic chloroform. Fraction 5 was thus identified as cryptoxanthin. The fraction co-chromatographed with cryptoxanthin isolated from papaya. Iodine catalyzed isomerization resulted in a hypsochromic shift, showing that the pigment was in the *trans* configuration.

Fraction 6 separated into three spots on the thin-layer plates. All three components absorbed maximally at 379, 400, and 425 nm in petroleum ether. Exposure of the chromatogram to HCl gas turned the yellow spots to blue, indicating that the pigments were epoxides. From the absorption spectrum, it could be deduced that these pigments were aurochrome and hydroxylated derivatives of aurochrome. The R_f values indicated that fraction 6-a was aurochrome, fraction 6-b cryptochrome and fraction 6-c auroxanthin. The presence and position of the hydroxyl groups were confirmed by the positive response to acetylation and negative response to methylation and dehydration.

Quantitative composition

Pronounced quantitative differences were noted between types of fresh fruit (Table 2). The red type presented a total carotenoid content (3.1-3.6 μg/g) twice as much as the yellow type (1.2-1.6 μg/g). Thus the color of the fruit was directly dependent on the total amount of carotenoids. Considering the individual pigments, the β-carotene level was about three times and the cryptoxanthin content twice as much in the red fruits as compared to the yellow fruits. The quantitative composition of fruits of the same type from different states were similar, indicating that geographical effects were not critical. This observation is being verified in a further study involving a larger number of samples from the different states.

In spite of the vivid color of the fruits, the carotenoid content is low. This is explained by the concentration of the pigments on the thin epidermal layer, the pulp being only slightly yellow in color. The whole pseudofruits were utilized in this study since we wanted to compare the fresh and processed juice. In our continuing study the pigments of the skin and the pulp are being analyzed separately.

A direct comparison of fresh and processed juice (i.e. analyzing the same lot of juice before and after processing) was not feasible at the present time. However, some observations could be drawn from the results of this study.

Considering that a mixture of fruits is generally utilized in the production of commercial juice, and taking into account the average of the two most common types of cashew (2.4 μg/g), it could be claimed that the carotenoid content diminished in the processed juice (1.9 μg/g for Brand A and 1.70 μg/g for Brand B) (Table 3). Part of the

Table 1—Carotenoids of cashew-apple[a]

Fraction	Common name	Structure	Absorption spectra in petroleum ether nm	Rf values on silica gel plates	Chemical reaction
1	α-carotene	(6'R)-β-ε-carotene	420, 443, 472	0.97	
2	β-carotene	β,β-carotene	(425), 448, 476	0.98	
3	ζ-carotene	7,8,7',8'-tetrahydro-ψ,ψ-carotene	378, 399, 424	0.96	
4	*Cis*-β-carotene	*Cis*-β,β-carotene	(421), 444, 472	0.97	– epoxide test *cis*-iodine-catalized isomerization
5	Cryptoxanthin	(3R)-β,β-caroten-3-ol	(424), 447, 474	0.52	+ acetylation – methylation – dehydration
6-a	Aurochrome	5,8,5',8'-Diepoxy-5,8,5',8'-tetrahydro-β,β-carotene	379, 400, 425	0.99	+ epoxide test
6-b	Cryptochrome	5,8,5',8'-Diepoxy-5,8,5',8'-tetrahydro-β,β-caroten-3-ol	379, 400, 425	0.52	+ epoxide test + acetylation – methylation – dehydration
6-c	Auroxanthin	5,8,5',8'-Diepoxy-5,8,5',8'-tetrahydro-β,β-caroten-3,3'-diol	379, 400, 425	0.46	+ epoxide test + acetylation – methylation – dehydration

[a] The same carotenoids were identified in the yellow and red fruits and in the processed juice.

pigment could have been lost during the sieving. However, when differences in individual carotenoids are considered, it becomes evident that some loss occurred during the heat treatment. Average concentrations of β-carotene, *cis*-β-carotene and cryptoxanthin were lower, while the levels of epoxides, known to be initial degradation products of carotenoids, were higher in the pasteurized juice.

Quantitative differences between lots of the same brand or between the two brands were insignificant (Table 3).

Table 2—Carotenoid content and vitamin A value of cashew-apple fruits

Carotenoid/Vitamin value	Lot 1[a] yellow type (μg/g)	Lot 2[a] red type (μg/g)	Lot 3[a] yellow type (μg/g)	Lot 4[a] red type (μg/g)
α-carotene	trace	0.3	0.1	trace
β-carotene	0.6	1.8	0.5	1.6
ζ-carotene	trace	trace	trace	trace
Cis-β-carotene	0.5	0.5	0.1	0.4
Cryptoxanthin	0.5	1.0	0.5	1.1
Aurochrome				
Cryptochrome	trace	trace	trace	trace
Auroxanthin				
Total	1.6	3.6	1.2	3.1
Vitamin A value	0.42	1.02	0.37	1.07

[a] Each lot consisted of 8-10 fruits: Lot 1, from Ceará; Lot 2, from Pará; Lot 3 and Lot 4 from Sao Paulo.

Table 3—Carotenoid content and vitamin A value of pasteurized cashew-apple juice

Carotenoid/Vitamin value	Brand A[a] (μg/g)	Brand B[a] (μg/g)
α-carotene	trace	trace
β-carotene	0.7 ± 0.1	0.7 ± 0.1
ζ-carotene	trace	trace
Cis-β-carotene	0.1 ± 0.1	0.1 ± 0.0
Cryptoxanthin	0.5 ± 0.0	0.5 ± 0.1
Aurochrome		
Cryptochrome	0.6 ± 0.1	0.5 ± 0.1
Auroxanthin		
Total	1.9 ± 0.1	1.7 ± 0.1
Vitamin A value	0.47	0.47

[a] Each value is the mean of three lots. Each lot was obtained by mixing two bottles of commercial pasteurized juice.

Vitamin A value

Of the carotenoids detected, only β-carotene, α-carotene, and cryptoxanthin have provitamin A activity. Converting the concentrations of these carotenoids to vitamin A value, the values for the fresh cashew-apple ranged from 37.5-107.5 μg/100g edible portion. These values are lower than that reported by INCAP (120 μg/100g edible portion) based on 8 analyses (INCAP, 1961). The type of fruit analyzed by INCAP was not specified, but even with the red fruit, which had higher vitamin A value in our work, our values are still lower. In the INCAP calculation, only β-carotene was quantified separately, its concentration being divided by two as in our work. The amount of all other carotencids grouped together was divided by four to obtain the vitamin A equivalent. The discrepancy could not be explained with certainty since the procedure used for the separation of β-carotene in the INCAP report was not described. The higher value reported by INCAP could be due to the inclusion of inactive carotenoids in the calculation and the quantitation of α-carotene with β-carotene since these two pigments could not be separated easily.

Comparing the average vitamin A value of the 2 types of fruits (0.72 μg/g) with that of the processed juice (0.47 μg/g), it could be discerned that the loss of carotenoids in the processed juice was reflected in the vitamin A value.

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Isolation, Purification and Physicochemical Characterization of Polyphenoloxidases (PPO) from a Dwarf Variety of Banana (*Musa cavendishii*, L)

MARIA A. M. GALEAZZI, VALDEMIRO C. SGARBIERI, and SPIROS M. CONSTANTINIDES

ABSTRACT

A procedure for isolating and purifying polyphenoloxidase from banana pulp is described. Maximum activity was obtained from the inner portion of the pulp by extraction with 0.2M sodium phosphate buffer, pH 7.0, containing 1% insoluble PVP and 0.25% Triton X-100. The enzyme was purified 38.8-fold after acetone precipitation, re-extraction of the precipitate with phosphate buffer containing Triton X-100, storage in the freezer for 7 days (−40°C), chromatography on Sephadex G-100 and electrophoresis on polyacrylamide gel. The purified enzyme (four isozymes) gave a polypeptide molecular weight (SDS-gel) of 31,000 ± 1,000 and molecular weight on sucrose gradient ultracentrifugation of 62,000 ± 2,000. The isoelectric point, determined by isoelectric focusing on polyacrylamide gel, was 5.2. The enzyme was stable at 55°C for 30 min; it lost more than 90% of the activity after 5 min at 85°C and was completely inactivated at 95°C for the same time. The absorption spectrum of the purified enzyme showed a maximum at 278 nm and a shoulder at 340-350 nm. This preparation, when submitted to paper chromatography revealed the presence of a fluorescent compound with R_f = 0.57, which was identical with chlorogenic acid.

INTRODUCTION

THE PRESENCE of polyphenoloxidase (PPO, o-diphenol: O_2 oxidoreductase E.C. 1.10.3.1) was demonstrated in bananas by Palmer (1963). In the same paper he also demonstrated that addition of 1% of the nonionic detergent "Cutscum" (Fisher Scientific Co.) to the extracting buffer solution increased the soluble enzyme significantly. By precipitation of the enzyme with 1.6 volumes of cold acetone and subsequent ion exchange chromatography on DEAE-cellulose, he obtained an enzyme preparation with 10-12 fold purification. Palmer and Roberts (1967) have also shown that this enzyme was inhibited by reducing and chelating agents and substrate analogues.

A tyrosine hydroxylase has been reported by Nagatsu et al. (1972) in banana pulp. This hydroxylase was activated by ascorbic acid.

Montgomery and Sgarbieri (1975), on studying the PPO of a Brazilian variety of banana of the *Musa Cavendishii* species, demonstrated the presence of 8, 9 and 10 isozymes in the interior and exterior pulp and in the peel, respectively. The isozymes were more active on o-diphenols and were inhibited by sodium diethyldithiocarbamate, cysteine, 2-mercaptoethanol, 1,2-dithiol-propane and sodium cyanide.

Griffiths (1959) demonstrated that the enzymatic darkening of banana pulp was primarily due to the oxidation of dopamine by the polyphenoloxidase.

In the present work a procedure for isolating and further purifying the PPO from banana pulp was developed. We also report some physicochemical properties of the purified enzyme.

Authors Galeazzi, Sgarbieri, and Constantinides are associated with Departamento de Planejamento Alimentar e Nutrição, UNICAMP, Nutritional Biochemistry Laboratory, Caixa Postal – 1170, 13.100 Campinas, S.P., Brasil.

EXPERIMENTAL

BANANAS (*Musa Cavendishii*, L; variety Nanica) were obtained in the local market at Campinas, State of São Paulo, Brazil in the yellow-green stage of ripening and used in the same day.

Location and extraction of the active enzyme

In order to determine the portion of the pulp most favorable for the extraction, several preliminary experiments were done with the outer and inner portions of the banana pulp. The inner part always exhibited higher enzyme activity in the extracts, on a tissue weight basis.

For the extraction, 25g of the central pulp were homogenized in a blender jar for 25 sec with 50 ml of a 0.2M sodium phosphate buffer, pH 7.0, containing 1% insoluble polyvinyl pyrrolidone (PVP) and 0.5% Triton X-100. The homogenates were centrifuged at 4°C for 15 min and 12,000 x G. The active enzyme remained in the supernatant.

Protein determination

Protein concentration in all preparations were determined by the biuret reaction according to the procedure of Weichselbaum (1946).

Measurement of enzyme activity and enzyme unit

Enzyme activity was determined by measuring the rate of increase in absorbance at 420 nm at 25°C in a model 356 double beam Perkin-Elmer spectrophotometer. The reaction mixture contained 2 ml of 0.1M catechol solution, 1 ml of distilled water and 20 µl of the enzyme solution. The reference cuvette contained only substrate solution and distilled water. The optimum pH for the activity on catechol was in the range 6.5-6.8 which coincided with the pH of the reaction mixture of catechol and water.

The straight line section of the activity curve was used to express the enzyme activity (U/min.ml). The enzyme unit of activity was defined as the unit change in absorbance under the conditions of the assay.

Effects of the extracting and dialyzing media on enzyme activity

Aliquots of 10 ml of a crude water extract and of a 0.2M, pH 7.0, sodium phosphate extract, prepared as above, were submitted to dialysis against: (a) distilled water, and (b) 0.2M phosphate buffer, pH 7.0. The dialysis was carried out at 4°C for 24 hr with four changes of the dialyzing media. The controls were maintained in test tubes at the same temperature for the same period. After the dialyzing period the enzyme activity was assayed using 0.1M catechol solution as substrate.

Purification procedure

Acetone precipitation. To the supernatant of the crude extract at 4°C two volumes of cold acetone (−13°C) were added slowly and the resulting precipitate (P) was separated by centrifugation (15 min at 12,000 x G and 5°C). The precipitate was re-extracted with 0.2M sodium phosphate buffer, pH 7.0, containing 0.25% Triton X-100 and re-centrifuged. The supernatant was frozen and kept at −40°C for at least 7 days. The insoluble material, after thawing, was removed by centrifugation at 4°C (15 min, 12,000 x G) and discarded. The soluble fraction (SF) was chromatographed on a Sephadex G-100 column.

Gel filtration chromatography on Sephadex G-100. The original acetone precipitate (P), the supernatant (S) of a reextraction of the acetone precipitate with phosphate buffer containing Triton X-100 and the soluble portion of the supernatant (SE), after storage in the freezer (−40°C) for seven days, were all submitted to chromatography on Sephadex G-100 columns (Sigma Chemical Co.). The

columns (2.5 x 40 cm) were equilibrated and subsequently eluted with 0.01M sodium phosphate buffer, pH 7.0, with a flow rate of 1.5 or 5 ml per 30 min. The collected fractions were monitored for protein by reading the absorbance at 280 nm and the fractions containing protein were assayed for enzyme activity.

Polyacrylamide gel electrophoresis. The method of Davis (1964) was used as the last step of purification as well as for characterization of the active and nonactive protein species. The pooled active fraction from Sephadex G-100 was applied in 0.9 x 9.5 cm tubes filled with gel and run for 2 hr with a current of 5 mA/tube. The region of the active bands was cut out and the enzyme eluted in test tubes with distilled water at 4°C. Protein and activity were determined in the aqueous solution after elimination of the gel.

Sedimentation coefficient and molecular weight determination

The method of Martin and Ames (1961) was used for determination of the sedimentation coefficient and calculation of molecular weight. A 5-20% sucrose gradient was employed and the enzyme lactic dehydrogenase (LDH) from Sigma Chemical Company was used as an internal marker. The gradient was centrifuged in a model L-75 Beckman ultracentrifuge with an Sw-65 rotor for 15 hr at 175,000 x G and 4°C. The molecular weight was calculated assuming a sedimentation coefficient of 7.4S and MW of 144,000 for lactic dehydrogenase.

Determination of polypeptide molecular weight

The SDS method of Weber and Osborn (1969) was used. A molecular weight versus electrophoretic mobility standard curve was constructed with serum albumin (MW 65,000), lactic dehydrogenase (sub unit MW 36,000) pepsin (MW 35,000) and lysozyme (MW 14,100).

Determination of isoelectric pH (P.I.)

The isoelectric pH was determined by isoelectric focusing on polyacrylamide gel following the technique of Wrigley (1968).

The ampholine used was pH 3.5-10.0 from LKB. The electrodes were connected to an Ortec 4100 power supply and the current was maintained at 5 mA during 4 hr.

The protein bands were revealed by Coomassie Blue R-250 (0.01%) in ethanol:acetic acid:water (7:2:11). The active bands were detected by incubating the gel with 0.08M catechol solution for 5 min by washings with a 40% solution of ethanol.

The pH gradient was determined by cutting an unstained gel into 0.5 cm fragments, elution with distilled water and reading in a pH-meter with a microelectrode.

Resistance of the enzyme to heat treatment

Aliquots of the enzyme fraction (EF) eluted from Sephadex G-100, containing 6.4 mg of protein per milliliter of a pH 7.0 phosphate buffer were incubated at 55, 65, 75, 85, and 95°C, for periods of time varying from 0-30 min. At the end of each incubation period the polyphenoloxidase activity was determined. The activity (% of original) was plotted against incubation time for the various temperatures.

Absorption spectrum of the purified preparation

The spectrum of the enzymes eluted from the polyacrylamide gel was obtained in a model 402 Perkin-Elmer spectrophotometer for the range 450-250 nm, using a concentration of 0.5 mg protein /ml.

RESULTS & DISCUSSION

Location and extraction of the PPO

The conditions for the extraction of banana PPO was established based on the following experimental facts: (1) extractions from outer pulp and from inner pulp always showed hgher enzyme activity in the inner pulp. A demonstration of the location of the active enzyme was done by cutting transversal slices of the fruit and dipping them in catechol solution. The most reactive tissue is in the central part as shown in Figure 1. Several buffers of different concentrations and pH were also tried. Sodium phosphate buffer, 0.2M pH 7.0, was found to be the best for extraction and stability of the enzyme. Sodium phosphate buffer, at pH 9.0, extracted more of the enzyme than at pH 7.0. However, the activity dropped below that of the pH 7.0 extract after standing 1 hr at 4°C. The relative stability curves for the phosphate buffer extracts at pH 7.0 and 9.0 are shown in Figure 2.

Other factors tested to improve extraction were: (a) concentration of PVP; (b) concentration of Triton X-100; and (c) centrifugal force. It was verified that 1% insoluble PVP-40T and 0.5% Triton X-100 (Sigma Chemical Company) gave the maximum enzyme extraction when centrifugation was done for 15 min at 12,000 x G.

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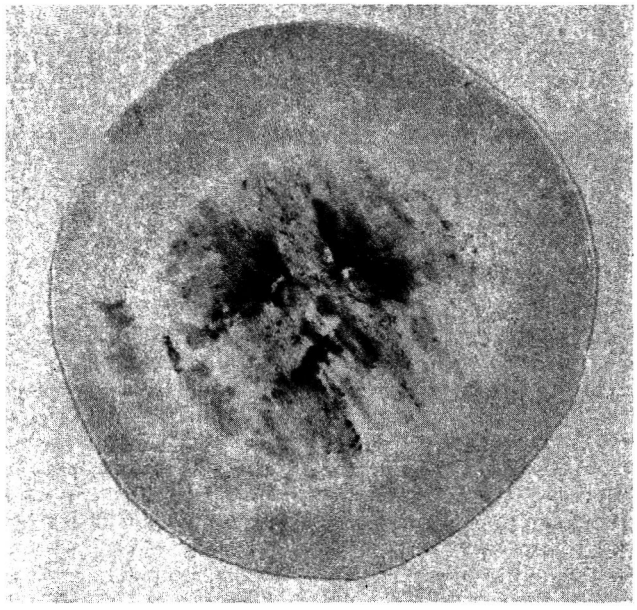


Fig. 1—Transversal section of a banana pulp in the yellow-green stage of ripening. Reaction on Whatman no. 1 filter paper soaked in 0.03M catechol solution.

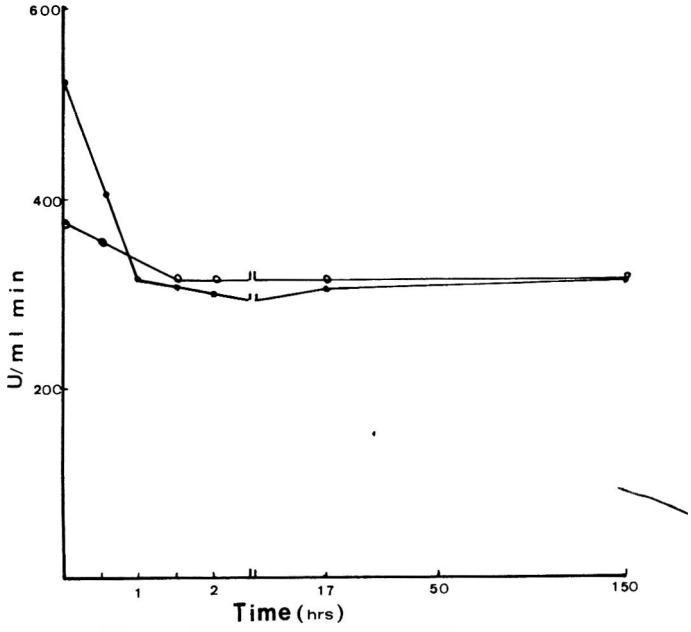


Fig. 2—Stability, at 4°C, of the polyphenol-oxidase activity in crude extracts with phosphate buffer at pH 7.0 and 9.0.

Polyvinyl pyrolidone and detergents have been used quite frequently, for improving the extraction and activity of enzymes from plant tissues (Palmer, 1963; Loomis and Bataille, 1964; Walker and Hulme, 1965).

Effect of dialysis on phosphate buffer and water extracts

It was noticed in the beginning of the purification procedure that dialysis of the extract against distilled water caused a significant loss of enzyme activity. Table 1 shows the effect of the extracting and dialysing media (24 hr, 4°C) on the enzyme activity. Data of Table 1 show a stabilizing and an activating effect of the sodium phosphate on enzyme activity. The activating effect was particularly significant in the case where the enzyme was extracted with water and than dialyzed against phosphate buffer. This effect could be due to: (a) renaturation of the enzyme in the presence of phosphate ions; (b) removal of inhibitory substances during dialysis. The fact that the enzyme activity of the control extracted and held in phosphate buffer was considerably higher than the sample extracted in phosphate buffer and dialyzed against phosphate buffer seem to indicate the possibility of removal of enzyme stabilizer by the dialysis.

Precipitation with cold acetone

Precipitation with cold acetone as a purification step for PPO had already been done by Palmer (1963) who used 1.6 volumes of acetone with respect to the extract.

In the present investigation two volumes of cold acetone (−13°C) were used. The recovery of the enzyme activity in the precipitate was greater than 90% in relation to the crude extract. However, the precipitate was partially insoluble in the phosphate buffer causing difficulty in the spectrophotometric enzyme assay and in the subsequent purification steps.

This problem was overcome by re-extracting the acetone precipitate with 0.2M sodium phosphate buffer, pH 7.0, containing 0.25% Triton X-100 and centrifuging out the insoluble particles. The recovery of activity in the supernatant was 120% which suggests an activation of the PPO by the re-extraction in the presence of Triton X-100. This recovery, above 100%, could be explained by an activation effect of the phosphate buffer, or removal of inhibiting substances.

Gel chromatography on Sephadex G-100

The elution pattern of a supernatant of the acetone precipitate, re-extracted with phosphate buffer containing Triton X-100, from a Sephadex (2 x 45 cm) column is shown in Figure 3. Most of the PPO activity appeared in the peak F_2 accounting for 63.7% of the total enzyme activity of the crude extract.

Data from a representative purification experiment are shown in Table 2.

Further increase in the activity of the acetone precipitate re-extracted with phosphate buffer containing 0.25% Triton X-100 was obtained after the solution was stored in a freezer (−40°C) for a period not less than 7 days. Under this storage condition a precipitate formed on thawing. Removal of the precipitate by centrifugation gave a more translucid preparation (SF) with a higher enzyme activity. This increase can be seen in Figure 4 which shows the change in enzyme activity on the re-extracted acetone precipitate during a 7-day storage period at 4 and −40°C. After the sixth day in the freezer there was a great increase in enzyme activity in the soluble fraction. This phenomenon was observed in several experiments. The elution pattern (Sephadex G-100 column) for the soluble material which had been stored frozen for 7 days is shown in Figure 5. In terms of enzyme activity the enzyme fraction (EF) represented 138% of the activity in the crude extract, with 18-fold purification.

The electrophoretic characterization of F_2 (Fig. 4), EF and PF (Fig. 5) eluted from Sephadex F-100 is shown in Figure 6. The preparations F_2 and EF shows the difference in purification of the enzyme peak of Fig. 4 and Fig. 5, respectively. Four active bands appeared on incubation of the gels with L-dopa, D-dopa, DL-dopa, dopamine, and catechol.

Table 1—Effect of dialyzing media (24 hr, 4°C) on PPO activity of water and sodium phosphate extracts

Extracting medium	Dialyzing medium	Enzyme activity (U/min. ml)	Change in enzyme activity (%)
Sodium phosphate buffer, 0.2M, pH 7.0	—[a]	504	0
Water	—[a]	90	0
Phosphate buffer	Phosphate buffer	360	−28.6
Water	Phosphate buffer	198	+120
Phosphate buffer	Water	144	−71.4
Water	Water	54	−40

[a]Maintained as control (not dialyzed) at 4°C for 24 hr.

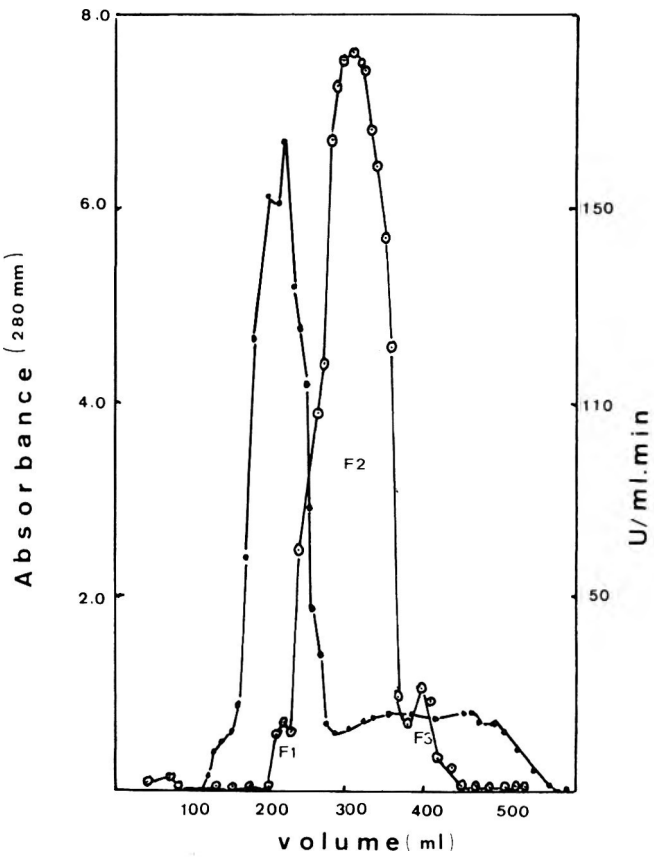


Fig. 3—Column chromatography on Sephadex G-100 of the supernatant (S) of an acetone precipitate, after re-extraction with phosphate buffer, 0.2M, pH 7.0, containing 0.25% of Triton X-100: protein ● —— ●; enzyme activity ○ —— ○.

Table 2—Purification of banana polyphenoloxidase

Purification steps	Volume (ml)	Acticity (U/min. ml)	Total units	Protein (mg/ml)	Specific activity (U/mg Protein)	Recovery (%)	Fold purification
Crude Extract	47.0	280	13,160	18.1	15.4	100.0	1.0
(P), acetone precipitate	12.2	970	11,834	28.2	34.3	89.9	2.2
(S), supernatant of the acetone precipitate re-extracted	22.5	700	15,750	9.3	75.2	119.7	4.9
Chromatography on Sephadex G-100 of (S)							
F_2	35.0	240	8,400	0.63	380.9	63.8	24.7
F_3	13.0	230	2,990	2.26	101.7	27.7	6.6

Polyacrylamide gel purification of the enzyme fraction (EF)

The pooled tubes eluted from the Sephadex column (fraction EF) were submitted to polyacrylamide gel electrophoresis as the last purification step. The zones in the gels containing the active bands were cut out and the material was eluted from the gel with distilled water. This preparation exhibited a very high specific activity (318 U/mg protein) and 38.8-fold purification as compared with the crude extract.

A representative purification experiment following all the steps described is shown in Table 3.

Sedimentation coefficient and molecular weight determination

Ultracentrifugation on sucrose gradient using LDH as a marker (S = 7.4) gave an S-value for PPO of 3.9 ± 0.1 which corresponds to a MW of 60,000 ± 2,000. On the other hand, electrophoresis on polyacrylamide gel containing sodium dodecyl sulfate (SDS gel) gave a subunit or polypeptide molecular weight in the range of 30,000 to 31,000. Similar results were obtained by Fling et al. (1963) with a crystalline and homogeneous preparation of a tyrosinase from *Neurospora*. They demonstrated that this *Neurospora* enzyme is found in two allelic forms which

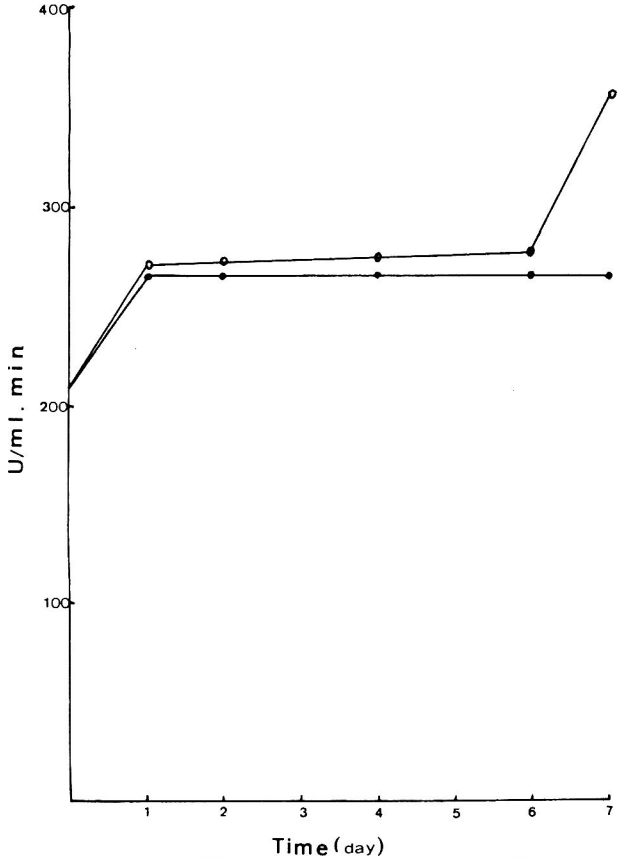


Fig. 4—Effect of storage on the polyphenoloxidase activity of the acetone precipitate re-extracted with 0.2M, pH 7.0, phosphate buffer, containing 0.25% Triton X-100; • — •, 4°C; ○ — ○, −40°C.

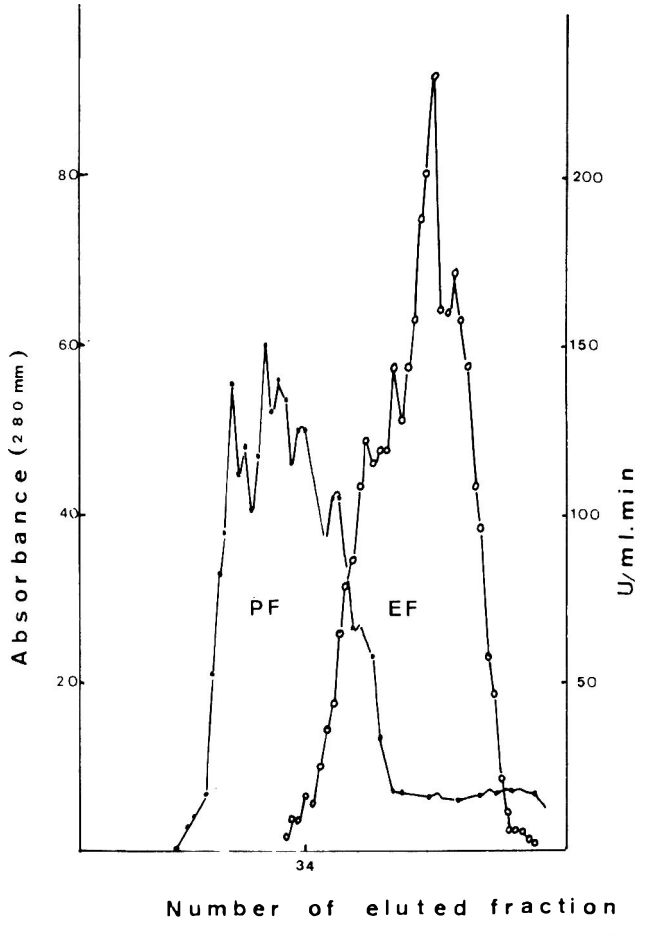


Fig. 5—Column chromatography on Sephadex G-100 of the soluble fraction (SF) originated from re-extracted acetone precipitate previously stored for 7 days at −40°C. Protein • — •; Enzyme activity ○ — ○.

Table 3—Purification of polyphenoloxidase from banana pulp

Purification steps	Volume (ml)	Acticity (U/min. ml)	Total units	Protein (mg/ml)	Specific activity (U/mg Protein)	Recovery (%)	Fold purification
Crude Extract	46.9	86.4	4,052	10.5	8.2	100.0	1.0
(SF), soluble fraction of supernatant (S) after 7-days storage in the freezer	21.0	504.0	10,584	6.8	74.1	261.2	9.0
(EF), elution of (SF) from Sephadex G-100	6.0	396.0	5,616	6.4	146.2	138.6	17.8
(EG), elution of (EF) from polyacrylamide gel	4.0	283.1	1,132	0.89	318.0	27.9	38.8

aggregate irreversibly. The molecular weight found for their purified enzyme, by sucrose gradient centrifugation, was in the range of 65,000 to 70,000.

Harel and Mayer (1963) extracted from apple cloroplasts and separated on a Sephadex G-100 column three protein fractions with catecholase activity and molecular weights of 35,000, 65,000 and 125,000.

Our results suggest that the banana polyphenoloxidases may appear in nature as dimers of slightly different peptide chains.

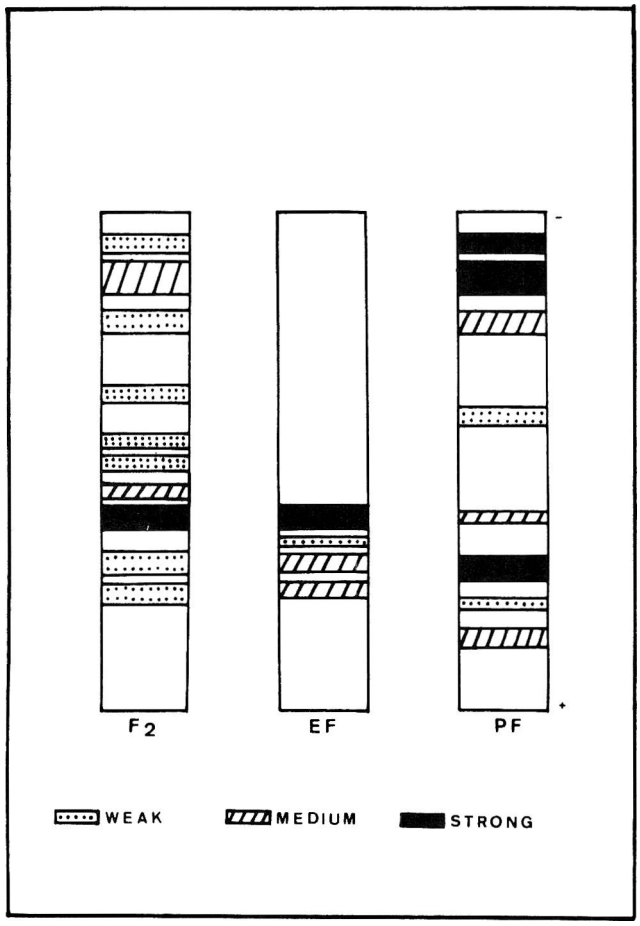


Fig. 6—Polyacrylamide gel electrophoresis of the PPO preparations eluted from Sephadex G-100 column. The chromatographed material were: F_2, enzyme fraction from Fig. 4; EF, enzyme fraction from Fig. 5; PF, protein fraction from Fig. 5.

Isoelectric pH

Isoelectric focusing of the enzyme eluted from the polyacrylamide gel was performed in polyacrylamide gel containing ampholines in the range of pH 3.5-10.0. The pH gradient of the gel after electrofocusing and the section of gel containing the active enzyme are shown in Figure 7. The most likely isoelectric pH for the enzyme is 5.2.

The data on sedimentation coefficient and isoelectric pH should be considered an average value because of the presence of isozymes. Because of the proximity of the four active bands in the polyacrylamide gel electrophoresis, detection of one single band in the SDS-gel and one single peak of activity in sucrose gradient ultracentrifugation and in isoelectric focusing, one may conclude that these isozymes are very similar with respect to molecular weight and nature and density of electric charges.

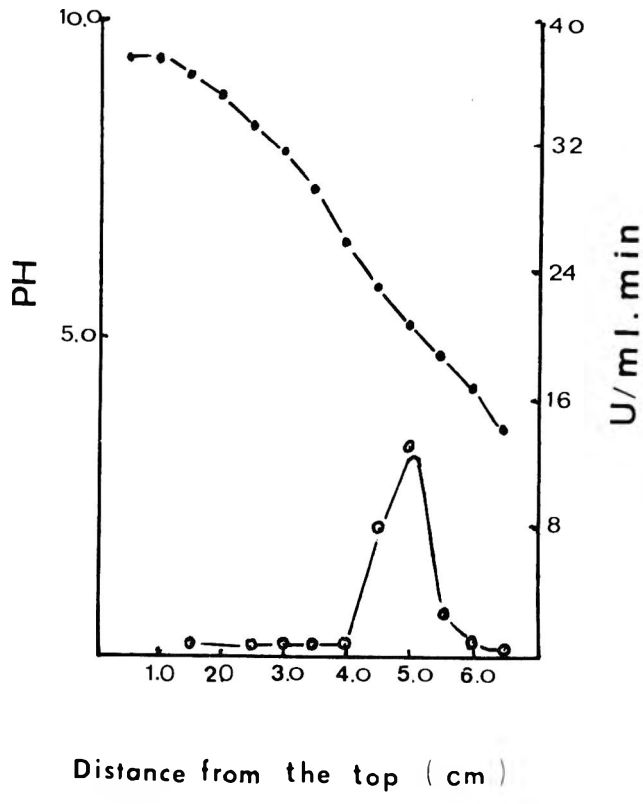


Fig. 7—Isoelectric focusing of the purified (EG) enzyme on polyacrylamide gel containing ampholines carrier pH 3.0-10.0. • — •, pH gradient; ○ — ○, enzyme activity.

Heat inactivation of the enzyme fraction (EF) isolated from the Sephadex column

The heat resistance of the purified enzyme at different temperatures was tested for various periods of time. The results are shown in Figure 8. The enzyme retained all the activity after 30 min of heating at 55°C. At 65°C the enzyme was stable up to 15 min and retained approximately 90% of the original activity after 30 min. Total activity was retained for only 5 min at 75°C and after 30 min about 40% of the activity had been destroyed. It is interesting that the activity remained constant between 10 and 15 min at 75°C, after a significant drop from 5-10 min at the same temperature. These patterns were observed in several experiments. Activity dropped to less than 10% and was completely destroyed with 5 min of heating at 85 and 95°C, respectively.

Absorption spectrum of the purified enzyme

The absorption spectrum of the purified enzyme eluted from the polyacrylamide gel had a peak of maximum absorption at 278 nm and a shoulder at 340-350 nm which is not characteristic of a protein spectrum (Fig. 9). Absorption at 350 nm was also found by Harel and Mayer (1963), Gregory and Bendall (1966), and Amorim and Josephson (1975) for apple chloroplast, potato, and coffee phenolases, respectively.

Amorin et al. (1975) demonstrated the presence of chlorogenic acid bound to coffee polyphenoloxidase.

By paper chromatography we demonstrated in our preparation the presence of a light blue fluorescent compound (370 nm) with $R_f = 0.57$ identical with that of pure chlorogenic acid.

These observations lead us to suggest that chlorogenic acid may be linked to banana polyphenoloxidase although we have no indication as to the site in the enzyme molecule or the type of linkage involved.

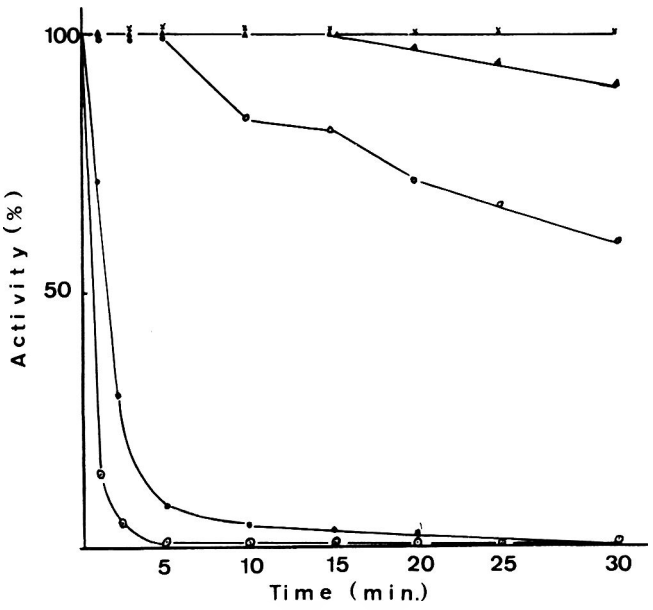


Fig. 8—Residual activity of the EF fraction after various incubation times at different temperatures. x —— x, 55°C; Δ —— Δ, 65°C; ○ —— ○, 75°C; ● —— ●, 85°C; and ○ —— ○, 95°C.

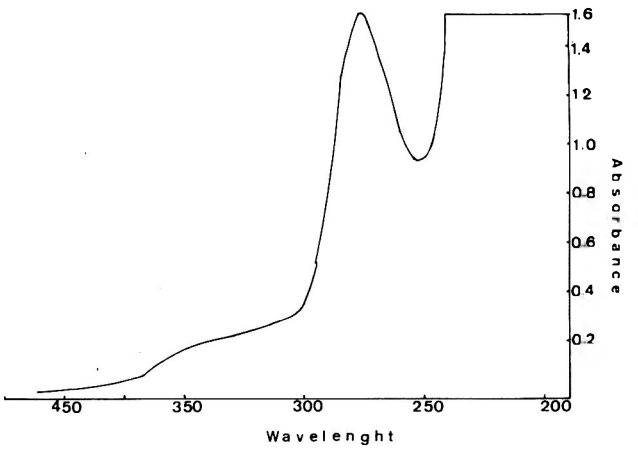


Fig. 9—Absorption spectrum of the purified (EG) enzyme from 200-450 nm.

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Determination of Alpha-Keto Acids in Banana Pulp by High Performance Liquid Chromatography

NOBUYUKI KOZUKUE

ABSTRACT

A liquid chromatographic procedure has been developed for the separation and quantification of α-keto acids. The DNPH-derivatives are separated on a column of LiChrosorb Si-60 (5 μm), eluted with petroleum ether/ethyl formate/propionic acid (65/35/5, v/v/v), and the column effluent is monitored by UV spectrophotometory at 350 nm. A total analysis of DNPH-keto acids was completed in less than 10 min. The amount of pyruvic, α-ketoglutaric, and oxaloacetic acid in ripe banana pulp was 347, 225, and 13 μg/100g fresh weight, respectively. This technique has been used effectively to analyze α-keto acids in biological samples.

INTRODUCTION

IT HAS BEEN REPORTED that α-keto acids, i.e., pyruvic, α-ketoglutaric, and oxaloacetic acid, play an important role in carbohydrates, lipids and amino acids metabolisms in biological samples. Especially, oxaloacetic acid is found to be closely related to the regulation of metabolism in plants (Hulme et al., 1964), animals (Tyler, 1960) and microorganisms (Sanwal et al., 1963).

In general, a method to which α-keto acids react with 2,4-dinitrophenylhydrazine (DNP) to form the corresponding hydrazone (DNPH) has been widely used, and the DNPH derivatives formed were separated and quantitatived by paper chromatography (Isherwood and Cruickshank, 1954; Katsuki et al., 1968) or thin-layer chromatography (Ronkainen, 1963; Berlet, 1968). However, these methods need a lot of time before a conclusion can be reached. Moreover, many problems are encountered concerning reproducibility, stability and quantification.

As for the determination of α-keto acids by high performance liquid chromatography (HPLC), Terada et al. (1977) has reported the analysis of pyruvic and α-ketoglutaric acid in serum but not that of oxaloacetic acid.

The purpose of this report is to determine the separation and quantification of α-keto acids in ripe banana pulp by using a more rapid and sensitive method than the one which has been reported as HPLC.

MATERIALS AND METHODS

Apparatus and reagent

High performance liquid chromatography (HPLC) was used in a Hitachi liquid chromatography model 635 equipped with a wavelength tunable effluent monitor and a sampling valve injector. The peaks were graphically produced by a single pen recorder (Hitachi Seisakusho, Tokyo, Japan). The chromatographic column (4.0 mm i.d. x 15 cm) was of stainless steel, packed with silica gel (LiChrosorb Si-60, particle diameter 5 μm, Merck, G.F.R.). A mobile phase (petroleum ether/ethyl formate/propionic acid, 65/35/5, v/v/v) was pumped through the column at a flow rate of 1 ml/min. The ultraviolet detector was set at 350 nm and used at a sensitivity of 0.16 absorbance units full scale. The chart speed was adjusted to 5 mm/min. Sodium pyruvate, α-ketoglutaric acid and DNP were purchased from Wako, Osaka, Japan and oxaloacetic acid from Sigma, U.S.A.

Author Kozukue is with the Dept. of Home Economics, Kenmei Junior College, 68-Honmachi, Himeji City, Hyogo Pref., Japan

Quantification

Chromatographic peak areas were calculated by the use of the electronic integrator (SiC electric company, Tokyo, Japan). The reported values were plotted against a standard calibration curve for each α-keto acid detected.

Sampling preparation

Ripe bananas were obtained from a local grocer and divided into peel and pulp.

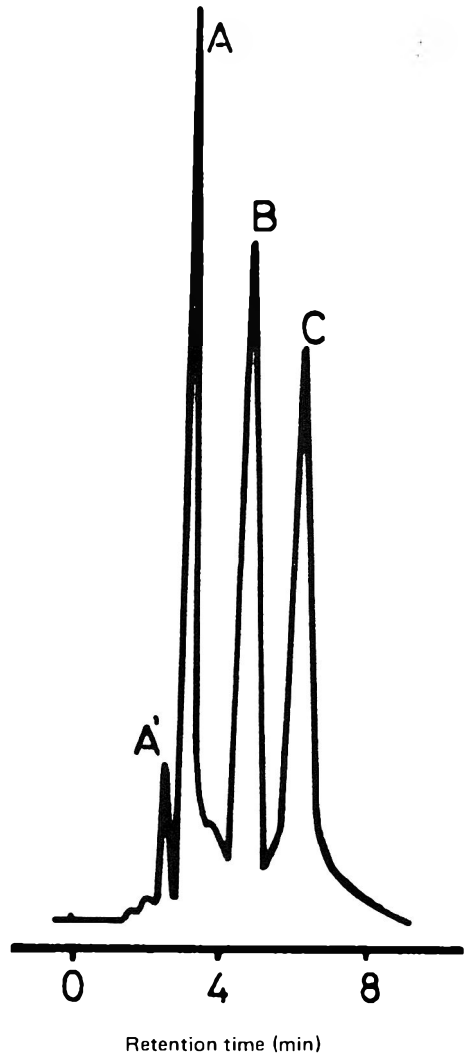


Fig. 1—Liquid chromatogram of a standard mixture of the DNPH derivatives of pyruvic acid, α-ketoglutaric acid and oxaloacetic acid. Peak: A and A'=DNPH-pyrivic acid; B=α-ketoglutaric acid; C= oxaloacetic acid. Operating conditions: Column: LiChrosob Si-60 (5 μm). Detector: UV at 350 nm and 0.16 aufs. Flow rate: 1 ml/min. Column temperature: ambient. Mobile phase: petroleum/ethyl formate/propionic acid (65/35/5, v/v/v). Chart speed: 5mm/min.

The α-keto acids were extracted by the method of Isherwood and Cruickshank (1954): 20g of pulp tissue slises put into −80°C methanol (mixture of solid CO_2 and methanol); then the acids were extracted from the tissues by 0.6M HPO_3 at 0°C. After adding DNP to the extraction, the mixture was allowed to stand for 3 hr at room temperature under darkness. Finally, the hydrazone produced was dissolved in absolute methanol of 2 ml. Then 10 μl of this solution were directly subjected to HPLC.

RESULTS & DISCUSSIONS

THE PRECISION of reproducibility was evaluated by standard pyruvic acid. Reproducibility of both peak height and peak area were good, with coefficient of variation of 1.09% and 0.38%, respectively.

The chromatographic behavior of the authentic DNPH-pyruvic, α-ketoglutaric and oxaloacetic acid was investigated on a column of LiChrosorb Si-60 with petroleum ether/ethyl formate/propionic acid (65/35/5, v/v/v) as the solvent system. Figure 1 shows the typical chromatogram obtained from a standard mixture of the three DNPH-keto acids. DNPH-pyruvic acid was separated into two isomers (A and A'), which were presumed to be syn-anti isomerism. This has already been published (Katsuki et al., 1968), together with detailed data on the effects of temperature and water content in the isomerization of both the syn-anti DNPH derivatives of pyruvic acid which have been reported by Terada et al. (1977).

The changes of the amounts of authentic three DNPH-keto acids in methanol solution stored at −20°C for 5 months's storage are represented in Table 1. Pyruvic and α-ketoglutaric acid showed a slight decrease after 5 months's storage, but oxaloacetic acid decreased significantly from 253 ng on the initial day to 186 ng after 5 months's storage and pyruvic acid, which was supposed to have been produced by nonenzymatic decarboxylation from oxaloacetic acid, increased rapidly. From the above results, it was found that DNPH-pyruvic and α-ketoglutaric acid were fairly stable under −20°C storage, but DNPH-oxaloacetic acid was extremely unstable.

The typical liquid chromatogram of DNPH-derivatives obtained from the tissues of ripe banana pulp is shown in Figure 2. Several peaks were detected. Peak A and A', B and C were presumed to be syn-anti pyruvic, α-ketoglutaric and oxaloacetic acid from the retention time of authentic keto acids.

In order to confirm the above conclusion, each component corresponding to peak A and A', B and C was collected, resubjected to HPLC and compared with the retention time or the UV spectra of standard samples in mobile phase; in addition, the R_f value of each component was also examined by thin-layer chromatography (TLC) using the three different solvents. These results are summarized in Table 2.

Table 1—Changes of the amounts of α-keto acids stored at −20°C for 5 months's storage

α-keto acid		Amounts of initial day (ng)	Amounts after 5 months's storage (ng)
Pyruvic acid[a]		368	352
α-ketoglutaric acid		586	554
	Oxaloacetic acid	253	186
Oxaloacetic acid	Pyrivic acid[a]	0	71

[a] Pyrivic acid is represented as the sum of the two isomers.

Judging from the results of the retention time, the UV spectra and the R_f value of each isolated component, peak A and A', B and C were identified as syn-anti pyruvic, α-ketoglutaric and oxaloacetic acid, respectively.

Then, on determining the amounts of the three keto acids in ripe banana pulp, pyruvic and α-ketoglutaric acid were major constituents, representing 347 and 225 μg, while the amount of oxaloacetic acid was 13 μg per 100 g fresh weight.

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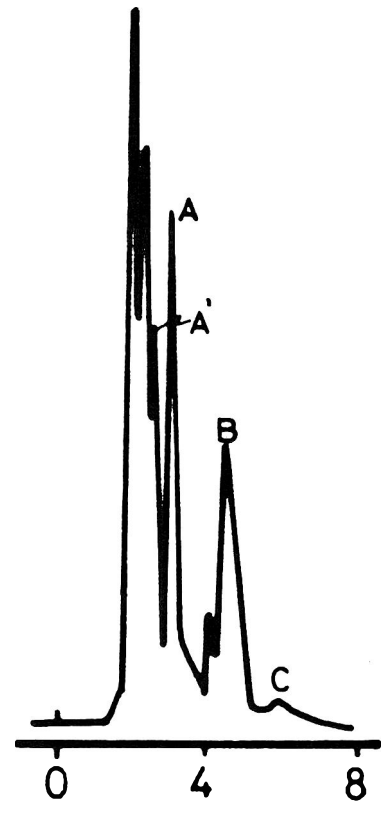


Fig. 2—Liquid chromatogram obtained from ripe banana pulp. Peak: A and A'=pyruvic acid; B=α-ketoglutaric acid; C=oxaloacetic acid. Operating conditions were as in Fig. 1.

Table 2—Retention time, maximum absorption peak and R_f value of each component of DNPH-keto acids isolated from ripe banana pulp

Peak	Retention time (min)	max (nm)	Rf value a	b	c
A'	2.44	375	0.83	0.64	0.32
A	3.35	382	0.54	0.38	0.13
B	5.80	382	0.12	0.09	0.00
C	6.21	380	0.04	0.03	0.00
Authentic					
anti-pyruvic acid	2.44	375	0.82	0.65	0.32
syn-pyruvic acid	3.34	382	0.55	0.39	0.14
α-ketoglutaric acid	5.81	382	0.12	0.09	0.00
oxaloacetic acid	6.20	380	0.04	0.03	0.00

[a] chloroform/n-butanol/acetic acid/water (100/10/10/100, v/v/v)
[b] chloroform/n-butanol/acetic acid/water (100/5/5/100, v/v/v/)
[c] isoamylalcohol/0.25N ammonia (20/1, v/v)

An Ultra-Sensitive Microbiological Method for the Semi-Quantitative Detection of Low-Level Sulfonamides

R. BOGAERTS, J. M. DE GROODT, and D. DE VOS

ABSTRACT

An improved agar-diffusion method has been developed for the detection of sulfonamides. The principle is based upon the incorporation of trimethoprim in Standard II-Nutrient Agar (pH 7.2) seeded with *Bacillus subtilis*. The sensitivity of *B. subtilis* to other antibiotics, including trimethoprim, is not influenced. This method is especially developed for the detection of sulfonamides in meat, but can be applied to pharmaceutical formulations, body fluids, etc.

INTRODUCTION

A COMMUNICATION in the 1977 Symposium of Garmisch-Partenkirchen by Gudding (1976) opened a new and useful way for the detection of sulfonamide residues in meat. His simple and brilliant idea was based upon the synergistic effect between sulfonamides and trimethoprim. By adding trimethoprim to a medium, the sensitivity of a test organism to sulfonamides can be increased 200 times. Chemical methods for the determination of sulfonamide residues in animal tissues are often time-consuming and difficult. They use a laborious extraction-method for cleanup, followed by thin-layer chromatography (TLC) or a colorimetric determination (Bratton and Marshall, 1939; Fellig and Westheimer, 1968; Tishler et al., 1968). The method which we propose is very simple although a sensitivity of 0.1-1.0 ppm can easily be achieved, without extraction or concentration procedures.

EXPERIMENTAL

Trimethoprim solution (Tr)

Dissolve 10 mg of trimethoprim in 2 ml of ethanol. Add 8 ml of distilled water. Dilute further with distilled water.

Agar-media

Standard II-Nutrient Agar (Merck) + 0.1% KH_2PO_4.
Mueller-Hinton Agar (OXOID) + 0.1% KH_2PO_4.
Antibiotic Medium no. 2 (OXOID) + 0.1% KH_2PO_4.

Escherichia coli Medium: asparagine, 1.0 g; $Na_2HPO_4 \cdot 12H_2O$, 6.3 g; Na_3 Citrate, 1.5 g; KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; glycerol (98%), 20 ml; Agar (OXOID nr. 1), 12g; and Aqua Dist., 1000 ml.

For all the media, adjust pH to 7.2 after sterilization. After inoculation, fill petri-dishes with medium to obtain a layer-thickness of 2 mm (15 ml in 9 cm petri-dishes). Inoculated petri-dishes can be stored under refrigeration (4°C) for a week.

Test organisms

Bacillus subtilis BGA or *B. subtilis* ATCC 6633. Use a spore concentration of 10^6 spores per ml medium. Incubation overnight at 30°C.

E. coli str. 28PR271 (RIV Bilthoven–The Netherlands). Use 10^5 organisms per ml medium. Incubation overnight at 37°C.

Paper disks

Schleicher & Schull nr. 2668 ϕ 6 mm.
Apply 0.01 ml of sulfonamide solution per paper disk.

All authors are affiliated with the Institute for Hygiene & Epidemiology, J. Wytsmanstr. 14, 1050 Brussels, Belgium.

RESULTS

Selection of the test organism

For years, our laboratory used an *E. coli* strain on a synthetic agar medium for sulfonamide detection. This method gave satisfactory results as far as the sensitivity was concerned. However, we found out that standardization was difficult. It was almost impossible to obtain identical results for identical assays on different days. We saw that the inoculation temperature, i.e. the temperature of the agar when organisms are added, played a major role on the sensitivity results of *E. coli* (Table 1). Therefore, we concluded that the *E. coli* method can be useful for highly-experienced and well-equipped laboratories, but in no way can it be used as a standardized routine detection method. *B. subtilis*-spores, however, give the great advantage of standardization possibilities. Moreover, this organism has already been used in the European Economic Community for the detection of antibiotics in meat. In our research we tried more particularly to modify the existing method so that an acceptable sensitivity for sulfonamides can be obtained without influencing normal antibiotic testing.

Selection of the medium

Three different media were compared in sensitivity testing of sulfonamides with *B. subtilis* as a test organism: Standard II-Nutrient Agar (Merck), Mueller-Hinton Agar (OXOID), and Antibiotic Medium No. 2 (OXOID). The sulfonamides tested were sulfadimidine, sulfamethoxazole, and sulfaquinoxaline. The results are summarized in Table 2.

It is obvious that the addition of 0.1 ppm trimethoprim to the media considerably increased the sensitivity of *B. subtilis* to the three sulfonamides. Secondly, it was proved that Standard II-Nutrient Agar was superior to Mueller-Hinton Agar, used by Gudding (1976). Besides, the sensitivity of the test is already superior to that of the *E. coli* test.

Table 1—Influence of incubating temperature on the sensitivity of E. coli[a]

	Conc in mcg/disk				
	0.125	0.250	0.500	1.00	2.00
46°C					
sulfadimidine				18.2(0.4)	20.6(0.5)
sulfamethoxazole			21.5(0.5)	26.7(0.6)	
sulfaquinoxaline	12.0(0.6)	19.0(0.4)	21.0(0.5)		
50°C					
sulfadimidine			13.4(0.4)	20.2(0.7)	24.4(0.6)
sulfamethoxazole			25.0(0.7)	28.6(0.6)	
sulfaquinoxaline	13.5(0.4)	22.6(0.4)	24.4(0.2)		

[a] ϕ inhibition zones in mm, with standard deviations, including the 6 mm disk.

Table 2—Comparison of different media in sensitivity testing of sulfonamides with B. subtilis, *with and without the addition of 0.1 ppm trimethoprim*[a]

		Standard II-Nutrient Agar		M2 Antibiotic Medium		Mueller-Hinton Agar	
	mcg/ disk	Without trimethoprim	With trimethoprim	Without trimethoprim	With trimethoprim	Without trimethoprim	With trimethoprim
Sulfadimidine	0.05	–	15.0(0.2)	–	–	–	–
	0.01	–	20.2(0.3)	–	–	–	11.0(0.3)
	0.2	–	23.7(0.3)	–	–	–	14.5(0.5)
Sulfamethoxazole	0.025	–	19.2(0.1)	–	–	–	13.6(0.6)
	0.05	–	22.6(0.2)	–	–	–	17.8(0.4)
	0.1	–	24.6(0.1)	–	–	–	22.2(0.6)
Sulfaquinoxaline	0.1	–	22.6(0.2)	–	–	–	18.6(0.2)
	0.2	–	25.9(0.4)	–	12.1(0.2)	–	22.7(0.2)
	0.4	13	28.3(0.1)	–	16.3(0.3)	–	26.9(0.4)

[a] ϕ inhibition zones in mm, with standard deviations, including the 6 mm disk.

Selection of the optimal trimethoprim concentration in the medium

We determined the most suitable trimethoprim concentration in order to develop a method with the highest possible sensitivity, without a considerable loss of precision. The results are summarized in Tables 3, 4, and 5. Using too much trimethoprim slows down the growth of the *B. subtilis* spores. Using too little reduces the sensitivity of the test. Concentrations of 0.05-0.1 mcg trimethoprim per ml medium are useful. In our laboratory the best concentration was, as an average, 0.075 ppm. Also, it gives clean, sharp inhibition zones and optimum sensitivity.

Influence of trimethoprim to antibiotic testing

The sensitivity of the test for other antibiotics (even trimethoprim itself is not affected. Adding trimethoprim to the medium is very specific for sulfonamide detection. Because of the slight reduction of *B. subtilis* growth, the inhibition zones may increase slightly from 0-8% (Table 6).

Application of the method: Detection of sulfonamides in meat

The evaluation of antibiotics in edible tissues and organs can easily be done using a sensitivity disk or swab impregnated with tissue fluid, or even using pieces of meat and putting them on two petri dishes filled with *B. subtilis* inoculated Standard II-Nutrient Agar (+ 0.1% KH_2PO_4), one with and one without trimethoprim. Any large difference between the inhibition zone sizes on the usual plate and on the Tr-plate *always* indicates the presence of sulfonamides. If inhibition zones of equal sizes are obtained in both test plates, the antibiotic residue content is prevalent.

Identification of sulfonamides in meat

Mix the meat sample with water-methanol (1:1) add a few ml aceton, centrifugate, concentrate the supernatant in a rotavapor, and identify on TLC (Siewert, 1976) and subsequent bioautography. Use cellulose TLC-plates, layer thickness 1 mm (Merck 5716), and develop with n-butanol: isopropanol:water (40:40:20).

For all sulfonamides, as a group, an Rf-value of 0.75 will be obtained, which is distinct from other current antibiotics. For autobiography, use Standard II-Nutrient Agar, with trimethoprim, as described before. Individual sulfonamides can be identified, using specific developing solvent mixtures in TLC (Cieri, 1973; Wendell and Trafton, 1975). The sensitivity of this procedure for identification of sulfonamides will be 0.1 ppm and can increase to 10 ppb.

The proposed method can also be used for autobiography of high-tension electrophoresis in agar. However, sulfonamides do not migrate as a group, but distinct products result in distinct spots. Identification will be very complicated.

Table 3—Sensitivity of sulfadimidine to B. subtilis *in Standard II-Nutrient Agar, with different trimethoprim (Tr) concentrations*[a]

	Conc of sulfadimidine (mcg/disk)			
Tr (ppm)	0.025	0.050	0.1	0.2
0.05	15.5(0.25)	22.4(0.43)	25.0(0.32)	28.3(0.13)
0.075	22.6(0.20)	27.5(1.00)	31.7(0.30)	34.0(0.26)
0.1		28.2(0.22)	30.5(0.21)	34.0(0.60)
0.2		27.0(0.66)	30.3(0.41)	33.5(0.50)

[a] ϕ inhibition zones in mm, with standard deviations, including the 6 mm disk.

Table 4—Sensitivity of sulfamethoxazole to B. subtilis *in Standard II-Nutrient Agar, with different trimethoprim (Tr) concentrations*[a]

	Conc of sulfamethoxazole (mcg/disk)		
Tr (ppm)	0.025	0.05	0.1
0.05	19.9(0.40)	25.0(0.33)	27.2(0.43)
0.075	32.4(0.50)	36.4(0.42)	38.5(0.30)
0.1	30.2(0.55)	32.6(0.33)	36.6(0.78)
0.2	29.5(0.31)	32.4(0.66)	35.9(0.72)

[a] ϕ inhibition zones in mm, with standard deviations, including the 6 mm disk.

Table 5—Sensitivity of sulfaquinoxaline to B. subtilis *in Standard II-Nutrient Agar, with different trimethoprim (Tr) concentrations*

	Conc of sulfaquinoxaline (mcg/disk)				
Tr (ppm)	0.025	0.05	0.1	0.2	0.4
0.05	21.0(0.20)	24.4(0.20)	26.8(0.38)	29.8(0.24)	32.4(0.35)
0.075	26.3(0.30)	30.4(0.51)	33.3(0.22)	36.8(0.28)	
0.1	32.3(0.76)	35.8(0.70)	37.6(0.46)		

[a] ϕ inhibition zones in mm, with standard deviations, including the 6 mm disk.

DISCUSSION

IT IS CLEAR that the incorporation of trimethoprim to a medium seeded with *B. subtilis* has a spectacular effect on the sensitivity of this test organism to sulfonamides. The sensitivity increases at least two hundred times. The use of trimethoprim has no adverse consequences in the determination of antibiotics other than sulfonamides. The only difference might be a slight increase of the inhibition zone diameter, due to slight inhibition of the growth of *B. subtilis*, and a more diffuse inhibition zone. This will exert an influence on the precision of the test. The sensitivity of this Tr-plate is ten times better than that of the *E. coli* plate.

—Continued on next page

Even so, spores are easier to handle than vegetative cells. This contributes to the simplicity of the method.

Physicochemical methods use time-consuming extraction methods to eliminate as much as possible interfering products. Final determinations always end in TLC-separation and coloration (Brandshaw, 1969; Wendell and Trafton, 1975), GLC (Daun, 1971; Fravolini and Beglionini, 1969) or coloration and spectrophotometry. The detection limits from this method vary from several mcg to 0.2-1 mcg. With the new bacterial method, 0.01-0.02 mcg of sulfonamides can be detected. Complicated extractions are not necessary, because of the high specificity of the autobiography.

Although this method can be applied to the detection of sulfonamides in pharmaceutical formulations, body fluids, etc, it was our intention to develop this principle for meat inspection. As a result, the trimethoprim (Tr)-plate will be incorporated into a four-plate antibiotic test, which will be official in the member countries of the European Economic Community (E.E.C.)

Finally, the trimethoprim (Tr)-plate alone, can be used as a simple screening test for the detection of both antibiotic and sulfonamide residues in slaughtered animals.

Table 6—Influence of different concentrations of trimethoprim in Standard II-Nutrient Agar on the sensitivity of B. subtilis *to antibiotics*[a]

	Standard II	Standard II +0.05 ppm Tr	Standard II +0.075 ppm Tr	Standard II +0.1 ppm Tr
Chloramphenicol 5 μg	25.0(0.52)	24.9(0.71)	25.7(0.52)	27.1(0.85)
Penicillin 5 μg	31.6(0.36)	32.6(0.12)	32.4(0.30)	
Erythromycin 10 μg	32.5(0.20)	34.4(0.34)	36.0(0.50)	
Tylosin 5 μg	25.9(0.57)		26.7(0.43)	
Ampicillin 2 μg	27.3(0.23)		28.5(0.33)	
CTC 5 μg	30.1(0.10)		31.2(0.27)	
TC 10 μg	33.9(0.20)	35.3(0.24)		36.7(0.84)
Neomycin 10 μg	28.0(0.26)	30.1(0.22)	29.6(0.27)	
Kanamycin 10 μg	29.3 (0.20)		29.9(0.60)	
Streptomycin 10 μf	21.2(0.17)		24.0(0.30)	

[a] ϕ inhibition zones in mm, with standard deviations, including the 6 mm disk.

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DETERMINING α-KETO ACIDS IN BANANA PULP . . . From page 157

CONCLUSION

THE IDENTIFICATION of the three keto acids in ripe banana pulp was performed in reference to standard acids. In addition, each keto acid was collected, and the retention time, the UV spectra in mobile phase or the R_f value by TLC, compared with that of standard. In conclusion, this method is an efficient technique for the separation and quantification of DNPH-keto acids.

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Beta-Galactosidase of *Streptococcus cremoris* H

S.K. JAGOTA, M.V. RAMANA RAO, and S.M. DUTTA

ABSTRACT

Beta-galactosidase (Beta-D-galactosidegalactohydrolase, EC 3.2.1.23) of *Streptococcus cremoris* H was partially purified by ammonium sulfate fractionation. Only 10% of the protein was recovered as enzyme protein and more than 50% of the enzyme in the crude extract was lost. A 2.6-fold purification only was achieved. The enzyme was most active at 65°C and recorded an optimum pH at 7.0. Km and V_{max} with ortho-nitrophenyl beta-D galactopyranoside as the substrate were recorded as 0.384 mM and 12.6 μmoles/mg protein/min. Manganese ions activated the enzyme. The enzyme was strongly inhibited by Hg^{++}, Ca^{++}, Ni^{++}, and Ag^{+} as well as parachloromercuribenzoate.

INTRODUCTION

LACTOSE utilization by organisms fermenting lactose proceeds with breakdown of lactose to glucose and galactose by the enzyme beta-galactosidase present in these organisms. The enzyme has been identified in a wide variety of microorganiams (Feniksova et al., 1968; John and Garvie, 1979; Jolly and Kosikowski, 1976; Premi et al., 1972; Ramana Rao and Dutta, 1978, 1979; Sorrensen and Crisan, 1974; Tikhomirova et al., 1974; Wierzbicki and Kosikowski, 1973). Some microorganisms (e.g. *Pseudomonas graveolens*) on the other hand, oxidize lactose to lactobionic acid (Nishizuka and Hayaishi, 1962). Vakil and Shahani (1969) reported that *S. lactis* UN utilizes lactose by hydrolytic cleavage of lactose to glucose and galactose by beta-galactosidase as well as by oxidation to lactobionic acid by lactose dehydrogenase. Yet another pathway of lactose utilization has been reported in *Staphylococcus aureus* which ferments lactose primarily by a PEP-dependent phosphotransferase system (Hengstenberg et al., 1967).

Rapid growth and lactic acid production in milk by lactic acid bacteria would suggest an active cellular beta-galactosidase system in these organisms. In fact thermophilic lactic acid bacteria have been reported to produce an active beta-galactosidase system. Mesophilic lactic acid streptococci, on the other hand, have been observed to exhibit only trace activities. Citti et al. (1965) examined 40 strains of *S. lactis* and 36 strains of *S. cremoris* for beta-galactosidase activity. All the strains of *S. cremoris* and all but one strain of *S. lactis* were observed to produce beta-galactosidase unstable to sonic vibrations. Ramana Rao and Dutta (1978) recorded the absence of beta-galactosidase activity in strains of *S. lactis*, *S. lactis* subsp. *diacetylactis* and all but one strain of *S. cremoris* examined by them. They observed a single strain of *S. cremoris* H to exhibit beta-galactosidase activity. The present report deals with the partial purification and properties of beta-galactosidase from this strain.

Author Jagota is with the Dept. of Biochemistry, Panjab Univ., Chandigarh-160014, India. Author Ramana Rao is with the Dept. of Microbiology, and Author Dutta is with the Dept. of Chemistry, National Dairy Research Institute, Karnal-132001, India.

MATERIALS & METHODS

Organism

Streptococcus cremoris H was obtained from the culture collection of dairy organisms of the National Dairy Research Institute and was maintained in sterile litmus milk. It was subcultured once a week and stored in a refrigerator at 4°C.

Preparation of cell-free extract

Cell-free extracts of the organism was prepared as described earlier by Ramana Rao and Dutta (1977) except that the organism was propagated at 30°C in reconstituted deproteinized cheese whey supplemented with peptone (1% w/v) and yeast extract (0.5% w/v).

Assay of enzyme activity

Beta-galactosidase activity of the cell-free extract of the culture was determined by incubating the cell-free extract with 0.001M ortho-nitrophenyl beta-D-galactopyranoside (ONPG; Sigma Chemical Company, U.S.A.) in phosphate buffer (0.1M; pH 7.0) for 1 min at 65°C. At the end of the incubation period the reaction was stopped by addition of 1.0M Na_2CO_3 and the color developed was measured at 420 nm (Ramana Rao and Dutta, 1977). A unit of enzyme activity was expressed as the amount of enzyme required to liberate one μmole of o-nitrophenol in 1 min under the conditions specified above.

The cell-free extract was brought to various levels of saturation using solid ammonium sulfate with constant mechanical stirring at 4°C. It was kept for 3 hr and the resulting precipitates were collected by centrifugation at 10,000 x G for 20 min at 4°C. Each precipitate was then dissolved in a minimum quantity of phosphate buffer (0.1M, pH 7.0) and analyzed for enzyme activity.

Partially purified enzyme obtained from ammonium sulfate fractionation was used for studying the properties. Each experiment was conducted in triplicate.

Protein estimation

Protein was estimated according to the method of Lowry et al. (1951).

RESULTS

Purification of the enzyme

The enzyme was subjected to purification by ammonium sulfate fractionation. Only 10% of the protein was recovered in the enzyme solution and more than half of the enzyme in the crude extract was lost. Only 2.6-fold purification at 40-50% saturation of salt was achieved by this gentle purification procedure indicating the lability of the enzyme.

Properties of the enzyme

Beta-galactosidase of *S. cremoris* H was examined for the effect of period of incubation and enzyme concentration on enzyme activity to establish the assay procedure (Fig. 1 and 2). The response was linear up to an enzyme concentration of 144 μg protein and 90 sec of incubation period, respectively. The enzyme activity was then determined at several temperatures (Fig. 3). A progressive increase in activity of the enzyme was observed up to 65°C, thereafter, it rapidly decreased. The effect of varying pH on beta-galactosidase activity of *S. cremoris* H was examined over the pH range 4.0-9.0 (Fig. 4). Optimum pH

was observed at 7.0. Lineweaver and Burk plot of enzyme with ONPG as the substrate (Fig. 5) recorded Km as 0.384 mM and V_{max} as 12.6μmoles/mg protein/min, respectively. Various metal ions were examined for their effect on enzyme activity (Table 1). Manganese ions were observed to stimulate the enzyme activity. Stimulation to a lesser degree was demonstrated also by K^+, Mg^{++}, Se^{++}, Co^{++}, and Sn^{++} at one level and by Co^{++} and K^+ at two levels. Mercury, calcium, nickel, and silver ions strongly inhibited the enzyme. The enzyme was also inhibited by parachloromercuribenzoate as well as 1,10 pehnanthroline (Table 2).

DISCUSSION

LACTOSE utilization by lactic acid bacteria has been the subject of study by several workers (Citti et al., 1965; McFeters et al., 1967; McKay et al., 1969; Premi et al., 1972; Vakil and Shahani, 1969). The capacity of these organisms for rapid growth and lactic acid production in milk suggests an active cellular beta-galactosidase (Beta-D-galactoside galactohydrolase, EC 3.2.1.23) system. Whereas thermophilic lactic acid bacteria have been reported to produce active beta-galactosidase system, mesophilic lactic acid streptococci viz. *Streptococcus lactis, S. lactis* sub sp.

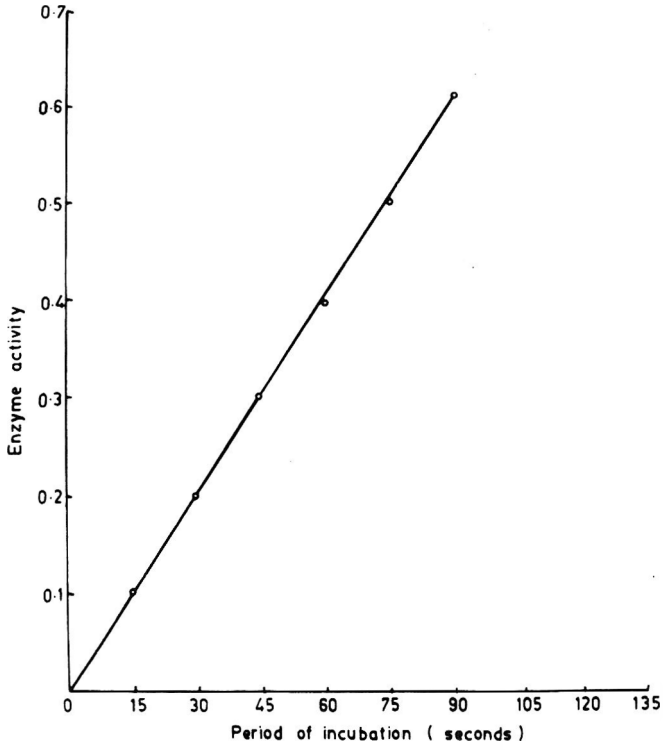


Fig. 1—Effect of period of incubation on β-Galactosidase activity.

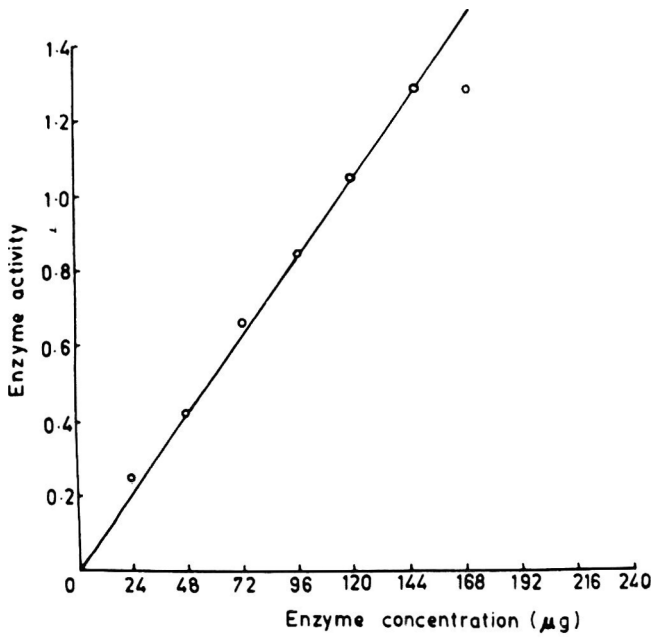


Fig. 2—Effect of enzyme concentration on its activity.

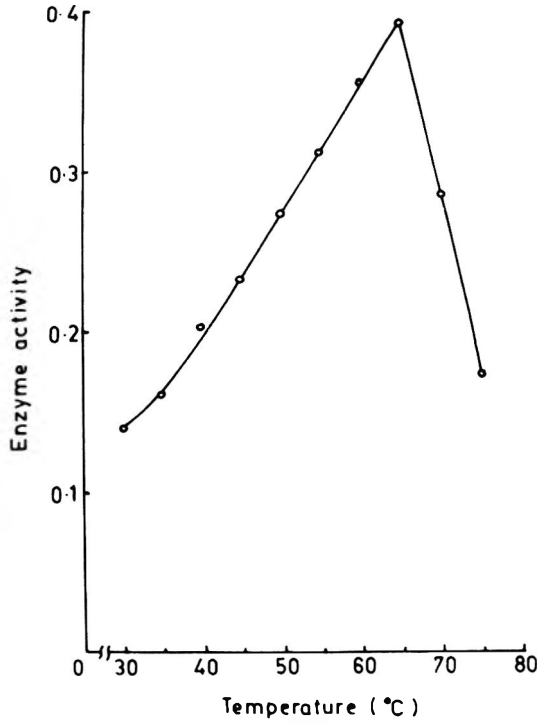


Fig. 3—Effect of temperature of incubation on β-Galactosidase activity.

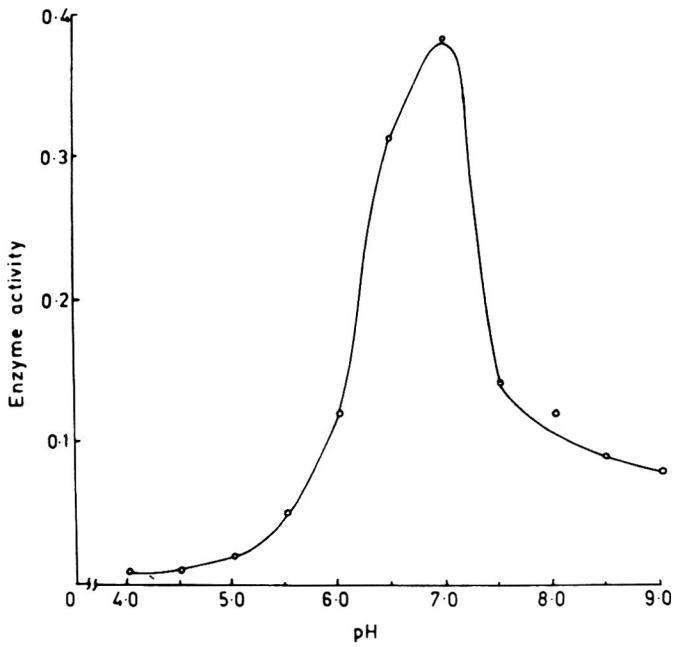


Fig. 4—Effect of pH on β-Galactosidase activity.

diacetylactis, *S. cremoris* etc. have been reported to exhibit only trace activities (Wierzbicki and Kosikowski, 1973). Citti et al. (1965) examined 40 strains of *S. lactis* and 36 strains of *S. cremoris* for beta-galactosidase activity. They observed all strains of *S. cremoris* and *S. lactis* to produce beta-galactosidase unstable to sonic vibrations or toluene treatment except a single strain of *S. lactis* 7962. Whole cell preparations of these organisms, however, were observed to exhibit low beta-galactosidase activity. Ramana Rao and Dutta (1978) screened 7 strains of *S. lactis*, 3 strains of *S. lactis* subsp. *diacetylactis* and 5 strains of *S. cremoris* for beta-galactosidase activity. Whereas no enzyme activity was detected in any strain of *S. lactis* or *S. lactis* sup sp. *diacetylactis*; one strain of *S. cremoris* H was observed to exhibit beta-galactosidase activity.

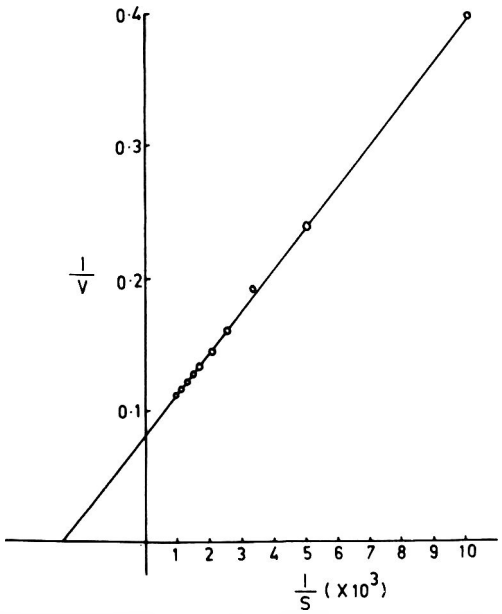


Fig. 5—Effect of substrate (ONPG) concentration on β-Galactosidase activity.

Table 1—Effect of metal ions on S. cremoris *beta-galactosidase activity*

Metal ion	Relative activity (%) at 1 x 10^{-3}M	Relative activity (%) at 1 x 10^{-4}M
Bi^{++}	90	97
K^{+}	105	103
Sr^{+++}	60	94
Fe^{+++}	94	100
Mg^{++}	103	100
Al^{+++}	84	100
Mn^{++}	122	103
Se^{++}	105	100
Na^{+}	87	89
Co^{++}	108	105
Li^{++}	78	94
Hg^{++}	0	72
Zn^{++}	56	66
Sn^{++}	105	100
Ni^{++}	37	62
Ag^{+}	50	89
Ca^{++}	41	78

Table 2—Effect of some group specific agents on S. cremoris *beta-galactosidase activity*

Chemical	Relative activity (%) at 1 x 10^{-3}M
Parachloromercuribenzoate	27
N-ethylmaleimide	69
α, α'-dipyridyl	53
Thiomersal	56
8-Hydroxyquinoline	71
1,10-phenanthroline	27

The enzyme was observed to exhibit a near neutral optimum pH as in the case of enzyme preparations from various other bacterial sources (Anema, 1964; Cohn and Monod, 1951; Goodman and Pederson, 1976; Premi et al., 1972; Ramana Rao and Dutta, 1979; Singh et al., 1979). Optimum temperature of incubation for enzyme activity observed at 65°C was much higher than the optimum temperature for growth. McFeters et al. (1967) reported an optimum temperature of 37°C for beta-galactosidase from *S. lactis* 7962. Thermophilic lactic acid bacteria have, however, been observed to record a similar higher temperature of incubation for maximum enzyme activity (Premi et al., 1972; Ramana Rao and Dutta, 1979; Wierzbicki and Kosikowski, 1973). Citti et al. (1965) observed beta-galactosidase from *S. lactis* 7962 to be unstable which could be stabilized by 0.85M ammonium sulfate. In the present study, beta-galactosidase of *S. cremoris* recorded only 2.6-fold purification by ammonium sulfate fractionation procedure and lost 57% of the activity. The enzyme was observed to be activated by Mn^{+} ions. Activation by monovalent or divalent cations has also been reported in case of beta-galactosidase from other microorganisms (Cohn and Monod, 1951; Jolly and Kosikowski, 1976; Kiyohara et al., 1976). Beta-galactosidase of *S. cremoris* was observed to be strongly inhibited by mercury and silver ions as well as parachloromercuribenzoate, indicating it belonged to the sulphydryl group of enzyme. Similar observations have been recorded in case of enzyme preparations from other organisms like *E. coli* (Wallenfels and Malhotra, 1961), *S. lactis* (McFeters et al., 1967) and *Leuconostoc cremoris* (Singh et al., 1979).

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Nutritional Evaluation of the Protein in Oilseed Products Heated with Sugars

KI SOON RHEE and KHEE CHOON RHEE

ABSTRACT

Defatted flours and protein isolates from glandless cottonseed, peanut, and soybean were complexed with glucose or sucrose in a ratio of 1:1 by weight and heated at 100°C for 0, 2, or 6 hr. Quality changes by the heat treatment were assessed by determining the extent of browning (browning index), in-vitro protein digestibility, available lysine, total amino acids, and computed protein efficiency ratio (C-PER). Whereas the sucrose-containing complexes changed very little in all the quality parameters determined, the counterparts containing glucose decreased substantially in protein quality along with the increased intensity of browning. Before heating, protein digestibility of the complex containing defatted flour of an oilseed was lower than that of the isolate counterpart. When heated, however, the isolate-glucose complexes decreased more in protein digestibility than the flour-glucose complexes. The available lysine content decreased by 76-83% for the complexes with glucose in contrast with 2-10% for those with sucrose. As to total amino acids, marked losses in arginine, lysine, tryptophan, and histidine were found in the complexes containing glucose, with the arginine loss as high as the lysine loss. Lysine-rich soy proteins lost lysine in greater percentages than lysine-poor peanut and cottonseed proteins. C-PER decreased by 63-86% in the glucose-containing complexes, with the highest decrease shown for soy flour-glucose. In-vitro protein digestibility, available lysine, and C-PER were all highly correlated ($P < 0.01$) with browning index. Available lysine was also highly correlated with C-PER.

INTRODUCTION

THE USE OF OILSEED proteins as meat extenders, dairy product substitutes, and protein supplements in cereal and snack products is expected to increase. Although oilseed protein applications in cereal and snack food products are intended mainly to increase the protein content of the products, their ultimate protein nutritive value is to be largely determined by the nature and extent of processing, and also by interactions of the supplemented proteins with other constituents in the products. Processing damage to proteins is accelerated by the presence of carbohydrates. The nonenzymatic browning reaction between proteins (amino acids) and carbohydrates (sugars) results in decreases in the availability of amino acids and digestibility of proteins, and also a decrease in the biological value of proteins beyond that accounted for by the decrease in digestibility and loss of amino acids as measured by chemical analysis (Adrian, 1974; Chichester, 1973).

In a protein, lysine is the only essential amino acid still having a free (epsilon) amino group and hence most susceptible to nonenzymatic browning. Among different sugars, pentoses are the most active in browning, followed by hexoses and disaccharides (Rubenthaler et al., 1963). Sugar reactivity also depends on the type of protein involved. The same sugar caused a different degree of lysine losses in lactalbumin versus soybean globulins (Adrian, 1974). El-Nockrashy and Frampton (1967) presented experimental data suggesting that the reduction of lysine availability in oilseed meals, such as cottonseed, peanut, and soybean meals, is a consequence of reactions between the epsilon amino groups of lysine in the proteins and the 1,2-glycosidic linkage in nonreducing sugar constituents of the seed. They showed evidence that the initial reaction is aminolysis of the 1,2-glycosidic linkage between glucose and fructose residues of nonreducing sugars (sucrose and raffinose in particular) by the epsilon amino group of lysine in the oilseed meals or flour proteins.

The protein efficiency ratio (PER) assay for protein quality evaluation, officially approved for food labeling, utilizes the rat. Because of the long 28-day assay period and its inapplicability to certain low protein or high fat foods, there has been an increasing research effort to develop rapid assays for measuring food protein quality, especially for use by the food industry in quality controls. Satterlee et al. (1977) predicted the PER for a variety of food proteins by expressing the essential amino acid (EAA) profile of each sample as a percentage of a reference casein EAA profile, after each was corrected for protein digestibility. A computed PER (C-PER) was then predicted based on the actual (rat) PER for casein. The level of protein or fat in the food tested had little effect on the C-PER assay (Hsu et al., 1978). Much more research needs to be done, however, on the applicability of the C-PER assay to foods having a wide range of protein quality and also on its sensitivity to other nonprotein food ingredients before it can be used as an effective, reliable tool to estimate food protein quality.

The present study was undertaken to assess the nutritional effect of oilseed protein-carbohydrate interactions promoted by heat treatment. Defatted flours and protein isolates from glandless cottonseed, peanut, and soybean were the oilseed protein ingredients tested, and glucose and sucrose were the carbohydrates included. In-vitro protein digestibility, amino acid profile, available lysine, and C-PER were determined to assess the nutritional quality changes caused by the interactions.

EXPERIMENTAL

Oilseed protein ingredients

Defatted soy flour (Soyafluff 200) and soy protein isolate (Cenpro M) were obtained from Central Soya. Cottonseed and peanut protein ingredients were prepared on a pilot-plant scale at the Food Protein Research & Development Center, Texas A&M Univ. Cottonseed flour was prepared from glanless cottonseeds (McNair variety) by hexane extraction and the isolate (a classical isolate without separating into storage and nonstorage protein; Martinez et al., 1970) by alkali extraction followed by isoelectric protein precipitation. Peanuts (Spanish variety) were prepressed to remove a bulk of oil before extraction with hexane and protein separation. Protein content (N x 6.25) of, and the abbreviation to be used for, each ingredient are shown in Table 1.

Preparation of protein-sugar complex

One part of a 5% suspension of an oilseed protein ingredient was mixed with one part of a 5% solution of glucose (G) or sucrose

Author K.S. Rhee is with the Dept. of Animal Science, Texas Agricultural Experiment Station, Texas A&M Univ., College Station, TX 77843. Author K.C. Rhee is with the Food Protein Research & Development Center, Texas Engineering Experiment Station, Texas A&M Univ.

(S), and the mixture was freeze-dried. The freeze-dried complex was equilibrated for 24 hr at 52% relative humidity by holding it over a saturated solution of magnesium nitrate (Rockland, 1960), tightly sealed, and heated at 100°C for 0, 2, or 6 hr. If sample analysis could not be performed immediately after the heat treatment, the samples were held at −30°C until they were analyzed.

Browning index

The extent of nonenzymatic browning of each complex was determined by the method of Fishwick and Zmarlicki (1970) with a slight modification for the digestion mixture which consisted of 0.25g sample, 14 ml distilled water, 0.6 ml 1N NaOH, 8 ml 2M Tris buffer (pH 8.0), and 2.32 g trypsin (1-100, pig pancreas, 3x crystallized pancreatin; United States Biochemical Corp., Cleveland, Ohio). The results were expressed as absorbance at 380 mμ/g complex.

Amino acid composition

Amino acids were quantitatively analyzed with a Model 120C Beckman Amino Acid Analyzer. Analyses of all amino acids, except cystine and tryptophan, were conducted on aliquots of HCl hydrolyzates of the samples as described by Spackman et al. (1958). Cystine was measured as cysteic acid as described by Moore (1963). Tryptophan was determined by the barium hydroxide hydrolysis method of Slump and Schrender (1969).

Other assays

Available lysine was determined by the sodium borohydride method of Rhee et al. (1974) and in-vitro protein digestibility by the multi-enzyme method of Hsu et al. (1977). C-PER values were computed with the EAA profile and in-vitro protein digestibility data according to the procedure described by Hsu et al. (1978).

RESULTS

Browning intensity

For all the defatted oilseed flours and isolates, browning by the heat treatment (100°C) was quite extensive in the complexes containing glucose whereas little browning was observed in those containing sucrose until they were heated for 6 hr (Table 2). Differences were found, however, within the glucose complexes; with the exception of the soy protein ingredients the isolate-glucose complex browned more than the flour-glucose complex, probably because of the higher protein content in the isolates than in the defatted flours. The browning index values of unheated complexes containing glandless cottonseed proteins, whether with glucose or sucrose, were higher than those of the peanut and soy protein counterparts (cf. the 0 hr data in Table 2), presumably due to the pigments originating from the cottonseed kernels (Blouin et al., 1979; Wan et al., 1979).

Protein digestibility

The results on in-vitro protein digestibility of heated and unheated complexes are shown in Table 3. The digestibility of the complex containing defatted flour of an oilseed was lower than that of the complex containing isolate of the oilseed. With each protein ingredient and heating time, the glucose complex had a lower protein digestibility than the sucrose complex.

When the digestibility results were evaluated in terms of changes by heating, the sucrose complexes, whether with flours or isolates, did not show appreciable changes with the exception of soy flour-sucrose heated for 6 hr. Of the glucose complexes, those with peanut flour, peanut isolate, and soy flour did not change notably (0-3% decrease) in protein digestibility after 2-hr heating but the complexes with cottonseed flour, cottonseed isolate, and soy isolate decreased by 6-7%. When heated for 6 hr, the isolate-glucose mixtures decreased in protein digestibility by 14-15% and the flour-glucose mixtures by 5-11%. Of the three flour-glucose mixtures, soy flour-glucose decreased least after 6-hr heating because its digestibility was already low even before heating.

Available lysine and total amino acids

As shown in Table 4, the glucose complexes decreased in available lysine by 76-83% (mean: 79%) after 2-hr heating whereas the sucrose complexes decreased by 2-10% (mean: 5%). Only a maximum of 38% reduction was found with

Table 1—Oilseed protein ingredients used and their protein content (N x 6.25)

Ingredient	Abbreviation	% Protein
Defatted flour		
Cottonseed, glandless	CF	60.3
Peanut	PF	63.3
Soy	SF	53.8
Isolate		
Cottonseed, glandless	CI	88.5
Peanut	PI	93.3
Soy	SI	91.5

Table 2—Browning index[a]

Protein-sugar complex	Heating time (hr) 0	2	6
Protein-glucose			
CF-G	0.9	4.0	30.5
CI-G	1.2	4.4	35.6
PF-G	0.3	1.6	18.5
PI-G	0.6	3.0	31.9
SF-G	0.4	13.2	36.8
SI-G	0.2	8.0	18.7
Protein-sucrose			
CF-S	0.9	1.1	2.3
CI-S	1.1	1.4	2.4
PF-S	0.2	0.9	1.7
PI-S	0.5	1.1	1.6
SF-S	0.4	0.8	3.6
SI-S	0.4	0.6	2.8

[a] Absorbance at 380 mμ/g complex

Table 3—In-vitro protein digestibility

Protein-sugar complex	Digestibility (%) 0	2 (hr)	6	% Decrease in digestibility after 2 (hr)	6 (hr)
Protein-glucose					
CF-G	85.2	79.8	75.6	6	11
CI-G	89.0	83.2	75.8	7	15
PF-G	84.3	82.7	77.7	2	8
PI-G	89.7	86.8	76.1	3	15
SF-G	78.0	77.8	74.2	0	5
SI-G	88.5	81.9	76.5	7	14
Protein-sucrose					
CF-S	84.1	83.4	82.9	1	1
CI-S	89.0	88.3	87.2	1	2
PF-S	86.1	84.3	83.8	2	3
PI-S	89.6	88.5	88.0	1	2
SF-S	83.4	81.4	79.2	2	5
SI-S	87.8	87.4	85.8	0	2

Table 4—Available lysine

Protein-sugar complex	Heating time (hr)	Available lysine g/16g N	% Loss after 2 hr	Loss after 6 hr
Protein-glucose				
CF-G	0	3.89	83	–
	2	0.68		
CI-G	0	2.66	79	–
	2	0.56		
PF-G	0	2.73	72	–
	2	0.76		
PI-G	0	2.57	76	–
	2	0.63		
SF-G	0	5.36	80	–
	2	1.08		
SI-G	0	5.10	83	–
	2	0.85		
Protein-sucrose				
CF-S	0	3.65		
	2	3.27	10	25
	6	2.74		
CI-S	0	2.75		
	2	2.66	3	5
	6	2.61		
PF-S	0	2.92		
	2	2.87	2	13
	6	2.53		
PI-S	0	2.68		
	2	2.63	2	22
	6	2.08		
SF-S	0	6.00		
	2	5.59	7	38
	6	3.70		
SI-S	0	4.98		
	2	4.80	4	24
	6	3.80		

Table 5—Loss of several amino acids (total amino acids)

Amino acid	Heating time (hr)	Protein-glucose complex CF-G	CI-G	PF-G	PI-G	SF-G	SI-G
		g Amino acid/16g N					
Arginine	0	11.22	10.49	12.17	11.75	6.89	7.19
	2	2.91	3.44	6.64	6.50	0.71	0.51
Lysine	0	4.16	2.83	3.18	2.65	5.80	5.58
	2	1.74	1.23	1.48	1.30	1.56	1.66
Tryptophan	0	1.29	1.07	0.89	1.03	0.81	1.17
	2	0.90	0.97	0.58	0.87	0.40	0.54
Histicine	0	2.57	2.42	2.32	2.12	2.34	2.02
	2	2.09	1.99	1.86	1.94	1.62	1.31
		% Loss after 2-hr heating					
Arginine		74	67	45	45	90	93
Lysine		58	56	54	51	73	70
Tryptophan		30	10	35	16	51	54
Histicine		18	18	20	9	31	33

the sucrose-containing complexes even when they were heated for 6 hr. In general, the complexes containing those protein ingredients with higher available lysine content before heating seemed to show a greater reduction in available lysine after heating.

Of the amino acids determined after acid hydrolysis (i.e., total amino acids), arginine, lysine, tryptophan, and histidine were lost markedly when the glucose complexes were heated for 2 hr (Table 5), but the losses were not notable for the sucrose complexes. Among the glucose complexes the percentage of total lysine loss was highest for the complexes containing soy proteins that had a higher lysine content before heating. The losses in arginine and lysine were far greater than those in the other two amino acids.

C-PER

C-PER values of the oilseed protein ingredients are shown in Table 6 along with rat-PER values reported in the literature or provided by the manufacturer of the products. C-PERs in the table are essentially those of the unheated complexes containing sucrose, not the protein ingredients themselves. C-PER of an ingredient alone, however, should not be significantly different from that of the unheated complex of the ingredient plus sucrose; C-PER change during the complexation process itself is unlikely to have occurred because the C-PERs of the sucrose-containing complexes heated at 100°C were nearly the same as those of the unheated. Some differences were shown between the C-PERs determined and the rat-PERs cited. A strict comparison, however, is difficult in this instance due to possible differences in the variety of oilseeds and product processing conditions used in different studies. The soy products were exceptional in this regard since the manufacturer of the products provided their rat-PERs. Nevertheless, the C-PERs determined and the rat-PERs cited were rather close for peanut and soy products.

The C-PER decrease in the glucose-containing complexes was 63-86%, with the highest decrease shown for soy flour-glucose (Table 7). Little or no decrease was found in the sucrose complexes.

Interrelation between quality parameters

Correlations among browning index, in-vitro protein digestibility, available lysine, and C-PER were determined with the data on unheated and 2 hr-heated complexes. The results are presented in Table 8. All of the three quality parameters were highly and negatively correlated ($P < 0.01$) with browning index, the highest correlation being shown between C-PER and browning index. Correlation between available lysine and C-PER was also very high. In-vitro protein digestibility was correlated with neither available lysine nor C-PER. The absence of significant correlation between in-vitro protein digestibility and available lysine or C-PER can be explained by the fact that available lysine and C-PER are measures of protein quality whereas in-vitro protein digestibility is a measure of only digestibility, which is in itself determined by more amino acids than just lysine and also by other factors (e.g. protease inhibitors, degree of protein denaturation, etc.).

DISCUSSION

THE OCCURRENCE of little nonenzymatic browning in the oilseed protein-sucrose complexes heated at 100°C for 2 hr, accompanied by no significant protein quality changes, suggests that a protein nutritional damage of practical importance is unlikely to occur by heat processing of oilseed protein-supplemented food products unless either they contain significant amounts of reducing sugars or suf-

ficient amounts of reducing sugars are produced through hydrolysis of complex carbohydrates by certain hydrolytic constituents (e.g. acidic ingredients) present in the products. Similarly, protein nutritional damage of the oilseed protein ingredients proper during the ingredient processing is less likely to occur because few oilseeds contain a significant amount of reducing sugars: 2.9% as glucose in whole peanuts (Tharanathan et al., 1976), virtually no reducing sugars in glandless cottonseed (Lawhon et al., 1977), and a total of less than 0.01% as arabinose and glucose in the soybean seed (Horan, 1974).

Although the amino acid profile is important in evaluating the nutritional quality of a protein, the digestibility of that protein is the primary determinant of the availability of its amino acids. Heat processing to a certain extent can improve the digestibility of proteins by destroying protease inhibitors and opening of the protein structure through denaturation. However, processing beyond this extent can cause a decrease in protein digestibility via the nonenzymatic browning reaction and thermal cross-linking (Tannenbaum, 1974). In a study by Shemer and Perkins (1974), heating a mixture of soy protein isolate and glucose caused an increased resistance to hydrolysis with pronase. Concurrently, there was a decrease in the PER value of the mixture. Hsu et al. (1977) reported that the multienzyme in-vitro method of determining protein digestibility, which was developed by them and also was used in the present study, highly correlated with the in-vivo apparent digestibility of rats and was sensitive enough to detect changes in protein digestibility which occurred during the processing of foods. In the present study, in-vitro protein digestibility as well as C-PER did decrease in the oilseed protein-glucose complexes after heating, but the decrease in C-PER was far greater than that in digestibility.

Reduction of available lysine through nonenzymatic browning and food processing has been extensively reviewed and discussed (Adrian, 1974; Bender, 1972; Carpenter and Booth, 1973; Chichester, 1973). Research findings suggest that available lysine determination of foodstuffs indicates both the occurrence and intensity of nonenzymatic browning, as demonstrated in the present study with a significant correlation between browning index and available lysine content (Table 9).

Adrian and Frangne (1969) evaluated the protein nutritive value of peanut flour-supplemented cookies to find that PER and the digestible (available) lysine content fell after baking 50 and 25%, respectively. The available lysine content, as measured in vivo and in vitro, was reported to correlate highly with PER (rat bioassay) and net protein ratio when baked and toasted breads and bread mix were evaluated (Palamidis and Markakis, 1979). The present study also showed a high correlation between the available lysine content and C-PER, suggesting that available lysine determination may be used in lieu of the more time-consuming and involving C-PER assay to evaluate protein quality changes occurring in the thermal processing of food products whose major protein component is oilseed proteins. It remains to be seen, however, whether available lysine determination can replace C-PER assay for other types of foods.

Nonenzymatic browning has been shown to cause losses in certain amino acids (total amino acids) determined after acid hydrolysis of the protein, but the kind of amino acids lost in significant amounts varied among the reports. In the present study, the interaction of defatted oilseed flours or isolates with glucose resulted in high losses in total arginine, lysine, tryptophan, and histidine, all of which are basic amino acids except tryptophan, a neutral amino acid. Badenhop and Hackler (1971), however, found no significant loss in arginine in dry roasting of whole soybeans but found a significant loss in cystine in addition to losses in lysine (total), tryptophan, and histidine. Rios Iriarte and Barnes (1966) reported a significant decrease in cystine along with decreases in arginine and lysine upon autoclaving whole soybean flour at 120°C for up to 2 hr. Autoclaving groundnuts (peanuts) at 121°C for 4 hr also destroyed much of the cystine present (Anantharaman and Carpenter, 1971). In contrast with these findings on autoclaved oilseed protein ingredients, Neucere et al. (1972) observed that, when peanut meals prepared from whole seeds were heated at 110-115°C for 1 hr, the compositions of total amino acids were not affected by heat. Their results are analogous to our finding of the sucrose-containing complexes. The extent of heat treatment used in the study by Neucere et al. (1972) as well as the 2-hr heating at 100°C used in the present study might have been insufficient to cause amino acid destruction by general oxidation.

When the losses in total lysine of the glucose complexes containing different oilseed proteins were compared, lysine in the lysine-rich oilseed proteins (soy proteins) was damaged more by the heat-promoted nonenzymatic browning than lysine in the lysine-poor oilseed proteins (peanut and cottonseed proteins). Thereby, soy proteins lost an

Table 6—Comparison of C-PERs with rat-PERs

Product	C-PER[a]	PER by rat bioassay (cited)
CF	1.9	2.4-2.5[c]
CI	1.7	2.0-2.2[c]
PF	1.8	1.5-1.8[b]
PI	1.6	
SF	2.5	2.3[d]
SI	1.9	1.6[d]

[a] Values of the unheated complexes containing sucrose
[b] FAO (1970)
[c] Martinez and Hopkins (1975)
[d] Provided by the manufacturer

Table 7—Changes in C-PER by heating

Protein-sugar complex	C-PER after heating 0 (hr)	C-PER after heating 2 (hr)	% Decrease in C-PER after 2-hr heating
Protein-glucose			
CF-G	1.9	0.7	63
CI-G	1.7	0.2	78
PF-G	1.8	0.6	67
PI-G	1.6	0.5	69
SF-G	2.2	0.3	86
SI-G	1.9	0.6	68
Protein-sucrose			
CF-S	1.9	1.8	5
CI-S	1.7	1.6	6
PF-S	1.8	1.7	6
PI-S	1.6	1.6	0
SF-S	2.5	2.4	4
SI-S	1.9	1.9	0

Table 8—Correlation coefficients between quality parameters[a]

	In-vitro protein digestibility	Available lysine	C-PER
Browning index	−0.53**	−0.58**	−0.74**
In-vitro protein digestibility		0.11	0.27
Available lysine			0.92**

[a] N=24.
**Statistically significant at $p < 0.01$.

important part of their nutritional superiority over the other oilseed proteins.

In conclusion, for the protein enrichment programs and products development effort involving oilseed protein and other protein ingredients, more emphasis should be placed on the ultimate availability of the supplemented proteins rather than merely increasing the protein content of food products.

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Casein Inhibition of *Clostridium perfringens* Growth and Exoprotease Production

JOANNE M. CURRAN, MYRON SOLBERG, HANS P. BLASCHEK, and DAVID ROSEN

ABSTRACT

Growth of *C. perfringens* strain 3624 on a defined medium containing ANRC Reference Casein as the nitrogen source was investigated. Gas production, monitored manometrically as an index of growth, indicated a depressed growth response of the organism in the casein-containing medium. Increased growth response resulting from pepsin treatment of the casein was largely due to the enzyme serving as a nitrogen source. Chelation of iron by casein was not responsible for the growth inhibition. Casein suspended in complete medium was not inhibitory to the organism. Growth studies indicated that native casein was not available as a nitrogen source to the organism. Acid hydrolyzed casein and commercial casein hydrolysate served as suitable nitrogen sources for growth of the organism, however exoprotease production was repressed in both media as well as in medium containing native casein as the sole nitrogen source. *C. perfringens* strain 3624 produced exoprotease in the completely defined medium containing synthetic amino acids, indicating the possible presence of an amino acid or a metabolite, not produced in the casein-containing medium which functioned to derepress the enzyme synthesizing mechanism.

INTRODUCTION

THE POTENTIAL VALUE of a *Clostridium perfringens* 3624 protein quality assay was reported by Solberg et al. (1979). A serious limitation of the procedure was the inability of the test organism to provide an adequate growth response when casein was provided as the sole source of nitrogen. The lack of adequate exoproteases, the presence of inhibitors, and the binding of important nutrients were suggested as causes for the restricted growth response.

Ashton et al. (1968) reported that casein inhibited the growth of *Bacillus stearothermophilus*. Studies indicated that sodium caseinate has the capacity to effect an iron deficient medium which could be relieved by adding competitive ions including Ca^{++} and Mg^{++} and the addition of H^{+} which may have increased iron solubility. Pappenheimer and Shaskan (1944) reported that iron is essential for growth, toxin production, and glucose metabolism by *C. perfringens*. Iron is also needed for activating a metallo-aldolase enzyme system (Bard and Gunsaulus, 1949). In the presence of O_2, ferrous iron is converted to ferric iron which inhibits the aldolase enzyme. This may explain the toxic effect of oxygen. Normal metabolic end products of *C. perfringens* include acetic acid, butyric acid, lactic acid, ethanol, hydrogen ions, and carbon dioxide. An iron deficient medium results in a homolactic fermentation (Pappenheimer and Shaskan, 1944). Iron is essential for the rapid growth of *C. perfringens* in chemically defined media (Boyd, 1948; Riha and Solberg, 1971).

Schroder and Busta (1973) found that *C. perfringens* S-40 growth was stimulated by the presence of beef or chicken but was inhibited by sodium caseinate. Tryptic digests of casein were stimulatory and predigestion of the caseinate increased the growth response. Jayko and Lichstein (1959) reported inhibition of gas production by *C. perfringens* BP6K in casein containing medium. Schroder and Busta (1973) reported that sodium caseinate inhibited proteinase production in *C. perfringens* cultures but the addition of 0.2% of casein hydrolysate stimulated growth. Schroder and Busta (1973) found proteins previously exposed to proteolytic bacilli were good substrates for *C. perfringens*. Blaschek and Solberg (1978) studied the exprotease activity of 16 strains of *C. perfringens* in 4 media. The results indicated that proteolytic activity was a function of medium and strain. *C. perfringens* 3624 produced measureable amounts of gas and exoprotease activity in a defined medium but did not grow well, produce gas or proteases in a casein-containing medium. An active protease system necessary for the hydrolysis of casein was reported by McKay (1978) for growth of streptococcal organisms in milk.

The purpose of this study was to investigate mechanisms which may be responsible for the growth inhibition of *C. perfringens* 3624 in a casein-containing medium.

EXPERIMENTAL

Test organism

Clostridium perfringens (ATCC 3624) was obtained from the American Type Culture Collection, Rockville, Md. The stock culture was maintained in Cooked Meat Medium (BBL) at 4°C. Every 4 wk the cultures were transferred by passing them twice through fresh Fluid Thioglycollate Medium (Difco), incubating each time at 37°C for 24 hr. Cultures were transferred to Cooked Meat Medium, incubated at 37°C for 24 hr and stored at 4°C.

Preparation of the growth media and inocula

The chemically defined medium (R&S) (Riha and Solberg, 1971) and R&S base medium which contained all of the ingredients of R&S medium with the exception of the amino acids, were prepared within 18 hr prior to use and stored at 4°C in the dark. One-half ml from warmed (37°C) stock culture tubes were inoculated into 20 ml of the R&S medium which had been passed through a micropore filter under a stream of oxygen-free nitrogen just prior to use. The R&S medium was at 37°C at the time of inoculation and was incubated in a water bath at 37°C for 9-10 hr. The inoculum was prepared by harvesting and washing cells twice with 37°C R&S base medium and resuspending the cells to an absorption (600 nm) of 1.4 in 37°C R&S base medium as described in detail by Blaschek and Solberg (1978).

No attempt was made to either reduce the contamination level or sterilize the casein and the casein derivatives which were added to R&S base medium so that heat alteration of the casein would not take place. The combined factors of high incubation temperature (45°C), anaerobiosis, and large pure culture inoculum (> 10^6/ml) tend to minimize interference from contaminating microorganisms (Solberg et al., 1979).

Enzymatic predigestion

The method of enzymatic predigestion of casein was adapted from Evancho et al. (1977). The digestion procedure was carried out in Folin-Wu NPN blood digestion tubes (Fisher Scientific Co., Fairlawn, N.J.). Each tube contained 3.5g of ANRC Reference Casein (ICN, Cleveland, Oh.). The volume of each tube was made up to 30 ml with distilled water and adjusted to pH 1.8 with 1.0N HCl. Five

Authors Solberg, Blaschek, and Rosen are with the Dept. of Food Science, Rutgers—The State Univ., Cook College, New Brunswick, NJ 08903. Author Curran, formerly with Rutgers Univ., is now affiliated with the Dept. of Food Science, Univ. of Illinois, Urbana, IL 61801.

ml of a 1% solution of enzyme were added. The enzyme solution was prepared by dissolving the 2x crystallized and lyophilized pepsin (Worthington) in distilled water and adjusting the HCl concentration to 0.05N with mixing. The enzyme solution was prepared immediately prior to use. The stoppered tubes were incubated at 55°C for 3 and 12 hr on a Roto-Torque Multipurpose Rotator Model 150V (Scientific Industries Inc., Springfield, Mass). The digest was then adjusted to pH 7.1 with 1.0N NaOH and 50 ml volume with distilled water. Five and two-tenths ml of the digestion mixture were added to 20 ml of R&S base medium.

The activity of the enzyme was assayed as described in the *Worthington Enzyme Manual* (Decker, 1977).

Acid hydrolysis

The method used for the acid hydrolysis of casein was described by Williams et al. (1949). Fifty grams of ANRC reference casein plus 250 ml of 20% HCl were refluxed for 10 hr at 100°C. The hydrolysate was vacuum evaporated at 44°C to a thick syrup and 250 ml of distilled water were added and then removed by vacuum distillation. This was repeated 3 times total. The solution was adjusted with 10.0N NaOH to pH 3.5 and diluted to 500 ml. This was stirred with 20 g of charcoal (Norite 35 x 100 Mesh, Sigma Chemical Co.) at room temperature for 30 min. The solution was adjusted with 10.0N NaOH to pH 6.0 and the charcoal treatment was repeated. The hydrolysate was membrane sterilized through a 0.45μ Micropore membrane and stored at 4°C. One ml of the final solution is equivalent to 100 mg of native casein.

Quantitative assay for available iron

Media for the iron study were prepared using the same procedure as was used for the control medium with the following exception. The iron content was adjusted by adding the appropriate amount of $FeSO_4$ to yield the calculated values of 0, 1, 2, 4, 5, 10, 20, and 30 μg/ml of iron prior to filtration. The media were membrane sterilized through a 0.45μ Micropore filter. The iron concentration in the final filtrate was measured by atomic absorption spectrophotometry (AAS) using either a Jarrel Ash Model 810 (Fisher Scientific, Fairlawn, N.J.) or a Perkin-Elmer Instrument Model 290 (Perkin-Elmer Corp., Wilton, Conn.), each equipped with an iron hollow cathode tube.

The base medium was prepared without using $FeSO_4$. A solution of $FeSO_4$ was prepared by adding 5 g/L $FeSO_4$ in 0.01N HCl and filter sterilizing. This yields a calculated value of 1800 μg/ml iron. Ten ml of this solution were added each liter of filter sterilized base medium prepared without iron thus adding 18 μg/ml iron.

ANRC reference Casein was added to the base medium, held for 4 hr at 46°C, centrifuged at 14,500 relative centrifugal force (rcf) units for 5 min and filtered through Whatman #541 filter paper. Available iron before and after the addition of casein was analyzed by AAS. A solution of $FeSO_4$ was added to the base medium. Casein was added to the base medium plus $FeSO_4$ and the above procedure was repeated.

Preparation of casein-containing growth media

Test growth medium was prepared by altering the nitrogen source added to R&S base medium as follows: (1) ANRC Reference Casein (ICN, Cleveland, Oh.) was added at a concentration of 0.26% nitrogen which is equivalent to the amino acid nitrogen in R&S medium. Percent nitrogen in the test sample was determined using a Coleman Nitrogen Analyzer (Model 29A, Coleman Ind. Inc., Maywood, Ill.). Specifically, 0.36g casein was added to 20 ml of R&S base medium. (2) Enzyme digested ANRC casein (pH 7.0) mixture containing 70 mg of casein per ml was added to R&S base medium to yield a final casein nitrogen concentration of 0.26%. The pepsin concentration in the base medium was 0.2 mg/ml. (3) Pepsin solution at pH 7.0 was added to a concentration of 0.2 mg/ml of final medium which was R&S base medium containing 0.26% nitrogen in the native casein form. (4) Acid hydrolyzed ANRC casein solution containing 100 mg/ml nitrogen was added to R&S base medium to contribute to 0.26% nitrogen. (5) Casein hydrolysate (enzymatic) (Nutritional Biochemical, Cleveland, Oh.) was added to R&S base medium to a concentration of 0.26% nitrogen. (6) ANRC Casein was added to R&S medium (containing 0.26% nitrogen to a concentration of 0.26% nitrogen. (7) Pepsin solution at pH 7.0 was added to R&S base medium such that the final concentration of pepsin was 0.2 mg/ml (≈ 0.004% nitrogen).

Conditions and measurements of growth

Colony forming units were cultured in Tryptose Sulfite Cycloserine Agar (Harmon et al., 1971) without cycloserine or egg yolk. Plates were incubated anaerobically at 37°C for 18-24 hr. Spectrophotometric culture development was monitored at 600 nm in a Coleman Jr. Spectrophotometer. Manometric culture development was carried out in the reaction vessels described by Solberg et al. (1979). Gas evolution was monitored manometrically (Blaschek and Solberg, 1978; Solberg et al., 1979). Culture vessels (in triplicate) were incubated in a 45°C water bath.

Precipitation of protein

Trichloracetic acid (TCA) was prepared in a 0.5% aqueous solution. One ml of TCA was added to 5.0 ml of culture filtrate to precipitate proteins.

Quantitative assay for extracellular protelytic activity

Following the measurement of gas production, sterile filtrates prepared from stationary phase cultures were assayed for extracellular enzyme activity using the proteolytic substrate Azocoll (50-100 mesh) (Calbiochem., San Diego, Calif.) in a colorimetric assay described by Blaschek and Solberg (1978).

Data analysis

Growth curves were obtained by plotting gas evolution in manometric units versus time on a semi log plot. Linear regression was used to determine the log slope of gas production during exponential growth. Generation times were calculated from the formula G=log 2/slope. One- and two-dimensional analysis of variance and Student Newman Keuls Multiple range tests were used to compare treatment effects (Steel and Torrie, 1960).

RESULTS & DISCUSSION

FLASK-TYPE culture vessels were used in the comparison of *C. perfringens* culture development response by counting colony forming units (cfu) and by measuring gas production in manometric units. Figure 1 shows a typical

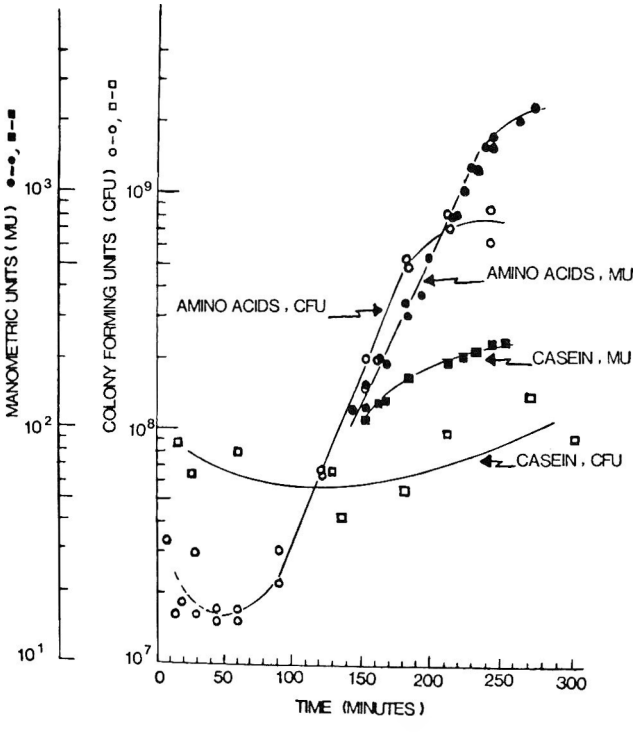


Fig. 1—Comparison of flask culture response curves for Clostridium perfringens *in R&S medium and in R&S base medium containing ANRC-casein during culture development at 45°C evaluated by counting colony forming units and by measuring gas evolution manometrically. (○ — ○ colony forming units in R&S medium; ● — ● gas in R&S medium; □ — □ colony forming units in R&S base medium containing casein; ■ — ■ gas in R&S base medium containing casein).*

growth curve in R&S medium with a generation time of approximately 19.5 min. The gas evolution curve evaluated between 10 and 100 manometer units, which is the region used when flask type culture vessels are employed, demonstrates a generation time of 22.6 min. The points plotted represent a combination of results from experiments conducted on two different days. Figure 1 also shows that in flask-type culture vessels containing R&S base medium with ANRC-casein added as the nitrogen source, there was no significant development of the culture whether evaluated by cfu or by manometer units. It is evident from Figure 1 that gas evolution continues in the exponential phase for approximately 1 hr after the cfu curve has entered the stationary phase but this does not appear to influence the slope of the curve.

When tube-type culture vessels were used, the reduced volume of medium and total number of cells necessitate the use of gas production from 1.0-10 manometer units for slope determination as may be seen in Figure 2. The flask and tube culture vessels were compared by Solberg et al. (1979) and were equivalent for generating slope data.

The demonstrated similarities of culture development response in the growth supporting R&S medium and in the growth inhibiting casein-containing medium when measured by cfu or manometer units indicate the validity of the manometric method.

Figure 2 shows a typical culture response for *C. perfringens* 3624 in R&S medium and in R&S base medium containing casein as the sole nitrogen source. Low gas production in casein containing medium was also observed for most strains of *C. perfringens* investigated by Blaschek and Solberg (1978). Jayko and Lichstein (1959) reported poor growth of *C. perfringens* in BP6K in media containing native casein. Busta and Schroder (1971) suggested that the growth inhibition of *C. perfringens* S40 by sodium caseinate may have been due to the poor solubility of casein or the presence of some inhibitory factors. The presence of peptides was important in initiating growth of *C. perfringens* S40 in sodium caseinate medium. Binding studies indicated that casein could effect an iron deficient medium. Addition of Fe^{++}, Ca^{++}, Mg^{+++} or H^{+} ions relieved the casein created inhibition (Ashton et al., 1968).

The addition of $FeSO_4$ to R&S medium, prepared without any $FeSO_4$, to provide iron additions from 0-30 μg/ml showed upon atomic absorption analysis of membrane filtered medium that the dissolved iron increased as the quantity added increased until approximately 18 μg/ml were added. At this addition level the medium contained 4.7 μg/ml which was approximately 26% of the quantity added. Figure 3 shows the levels of iron in the filtered medium compared to the iron added prior to filtration.

The generation times in Figure 3 represent the mean values obtained from several experiments. The absence of added iron always resulted in a significantly longer ($p = 0.05$) generation time than that observed when any level of iron, from 1 μg/ml to 18 μg/ml, were added. These results support the report by Shankar and Bard (1951) who found that 0.2 μg/ml of iron was required to support the growth of *C. perfringens*.

Table 1 shows that the substitution of casein for amino acids results in a binding of iron. The level of iron present is able to support the growth of *C. perfringens* albeit at a reduced rate. The addition of iron after membrane filtration of the R&S base medium increased the available iron but did not alter the generation time for microorganisms. These results indicate that the binding of iron by casein is not responsible for the decreased growth response.

Table 1 also shows that when casein was suspended in the complete R&S medium it was not inhibitory but it was stimulatory to *C. perfringens* as reflected by the decreased generation time relative to R&S medium itself. Enzyme hydrolyzed casein and acid hydrolyzed casein

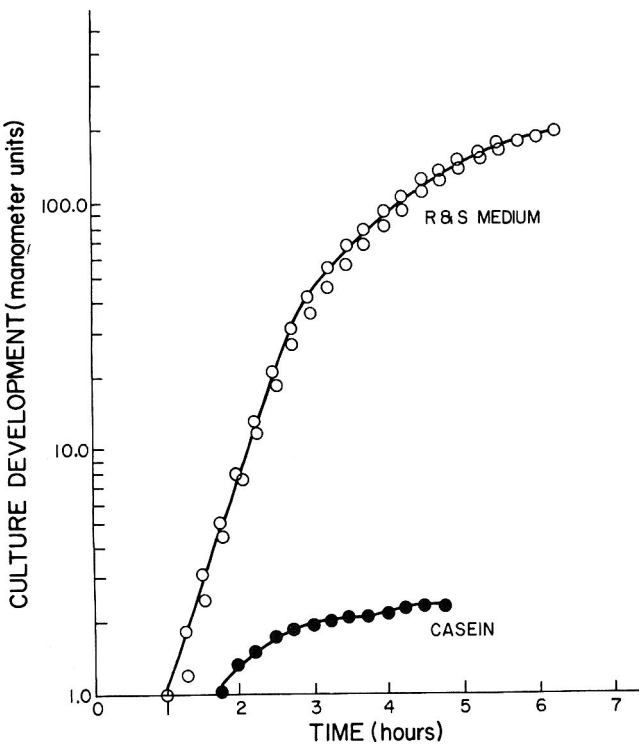


Fig. 2—Tube culture curves for Clostridium perfringens *strain 3624 in the chemically defined R&S medium and in R&S base medium containing casein as the only nitrogen source.*

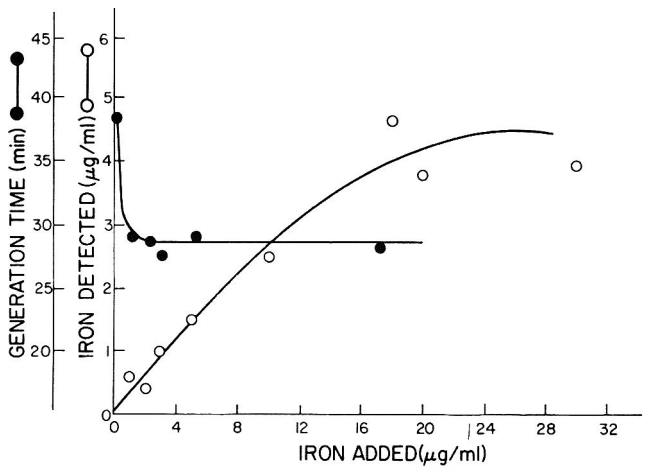


Fig. 3—Effect of iron added to R&S medium upon iron detected after membrane sterilization and upon generation time for Clostridium perfringens *strain 3624.*

supported growth of *C. perfringens* at a rate equivalent to that of R&S medium with casein added. Therefore, when casein is reduced to peptides and amino acids it is a suitable nitrogen substrate for *C. perfringens*. Based upon these data it appears that the complexity of the casein molecule is responsible for the growth inhibition which was observed.

Table 2 shows that predigesting casein enzymatically with pepsin significantly increases the growth response of *C. perfringens* in R&S base medium containing casein. Increasing the digestion time from 3 hr to 12 hr did not significantly change the generation time of *C. perfringens*. The digestion procedure added protein in the form of an enzyme into a system in which a proteolytic organism was functioning. Thus the organism may have been able to utilize the enzyme protein as a nitrogen source. Table 2 also shows that the combination of pH inactivated pepsin and native casein supported growth at a rate which was statistically equivalent to that of casein which had been pepsin digested for 3 hr or 12 hr and still contained the enzyme. The pepsin appeared to function as a growth supporting nitrogen source which when added to either native or pepsin digested casein resulted in improved growth of *C. perfringens*. The growth supported by the pepsin alone at the same concentration as found in the digested casein (0.2 μg/ml) was not significantly different from that supported by casein alone. Casein which was subjected to the treatment conditions for enzyme digestion (pH 1.8, 55°C, 12 hr) also supported growth of *C. perfringens* at an equivalent rate to native casein. It is apparent from these data that the reduced generation time observed when the combination of casein and pepsin served as the nitrogen source was due to the combined effect of the two proteins and not due to pepsin mediated hydrolysis of casein. A fully digested commercial preparation of casein hydrolysate supported significantly shorter generation times for *C. perfringens* (Table 2). These data show that the availability of nitrogen from native casein is the major growth inhibiting factor.

C. perfringens 3624 produced some exoprotease in every medium evaluated with the exception of 8 out of 14 samples containing ANRC-casein as the sole nitrogen source. The exoprotease activities are expressed as relative equivalents of trypsin in Table 3. The high levels of proteolytic activity observed in R&S medium, which contains only amino acids as the nitrogen source, indicated the possibility that a component of R&S medium may derepress an enzyme synthesizing genetic element. When casein was suspended in R&S medium it supported an apparently increased proteolytic activity, which was equivalent to that in R&S medium after normalization for growth as expressed by the ratio of RET to gas production.

Comparing the values obtained for the enzyme activity per unit of culture growth response (RET/manometer unit) showed that the proteolytic response was more a function of nitrogen source than of growth since the growth response on casein hydrolysates was equivalent to that on amino acids but the enzyme activity per unit of growth was significantly lower.

The similarity of the RET values for all nitrogen sources which did not contain amino acids indicated a minimal level of enzyme activity with measurements near the limits of instrument sensitivity. The relatively high value of RET per unit of growth presented for casein may be attributed to the combined sensitivity limits of the manometric and spectrophotometric responses. This conclusion is supported by the observed absence of measurable enzyme activity in more than 50% of the samples.

The apparent derepression of enzyme production in the R&S medium and the absence of trichloracetic acid (TCA) precipitable macromolecules in the medium, as presented in Table 3, suggested that the derepressor of exoprotease production in *C. perfringens* 3624 is a particular amino acid or group of amino acids present in the R&S medium. The rapid growth in the casein hydrolysate and casein acid hydrolysate was due to the availability of the protein for growth. The lack of TCA precipitates in the two media as presented in Table 3 indicated extensive hydrolysis of the two casein products thus making them available to the organism. The growth stimulation was not accompanied by increased enzyme production. The growth stimulation following the addition of 0.5% casein hydrolysate and casein acid hydrolysate without the production of proteolytic enzyme indicated that each hydrolysate functioned as substrate and not as derepressor of protease synthesis.

Since the acid hydrolysate and the mixed enzyme hydrolysate of casein supported growth responses of *C. perfringens* 3624 equivalent to or greater than those seen with the amino acids, and since the hydrolysates did not cause production of significant quantities of exoprotease, it seemed that an amino acid or combination of amino acids present in the R&S medium, but not present in casein hydrolysates or in R&S base medium which contains no amino acids, must be responsible for the apparent derepression which results in the exoprotease production and accumulation.

The composition of the growth medium influences enzyme formation by microorganisms (Haines, 1933; Murata et al., 1965; Blaschek and Solberg, 1978). A synthetic medium developed by Boyd et al. (1948) supported

Table 1—Effect of various nitrogen sources and the effect of iron addition before or after membrane filtration of R&S base medium on the iron detected and the effect of iron on the growth response of C. perfringens *3624 in R&S base medium containing various nitrogen sources*

Nitrogen source	Iron added (μg/ml)	Iron detected[d] (μg/ml)	Ratio detected / added	Generation[f] time (min)
Amino acids[a]	18	4.7	0.26	25.6x
Amino acids[b]	18	8.6	0.47	25.4x
Casein	18	0.2[e]	0.01	52.8y
Casein[b]	18	3.9	0.22	60.0y
Amino acids + casein	18	–[g]	–	21.3z
Casein hydrolysate[c] (enzymatic)	18	–	–	20.9z
Casein hydrolysate (acid)	18	–	–	20.0z

[a] Amino acids as nitrogen source indicate R&S Medium.
[b] $FeSO_4$ added after membrane filtration
[c] Casein hydrolysate (enzymatic); (Nutritional Biochem. Corp., Cleveland, Oh.)
[d] Determined by Atomic Absorption Spectrophotometer
[e] Calculated from standard curve extrapolation
[f] Means bearing different letters are different (P = 0.05).
[g] Not determined

Table 2—Effect of procedures for pepsin digestion of casein on the growth response of Clostridium perfringens 3624 in R&S base medium

Nitrogen source	Generation time[b] (min)
Casein	52.8x
Pepsin, (pH inactivated)	45.6x
Casein, (pH = 1.8; 55°C; 12 hr)	46.3x
Casein + pepsin, (pH inactivated)	37.2y
Digested casein (pepsin; 3 hr)	34.2y
Digested casein (pepsin; 12 hr)	29.2y
Casein hydrolysate[a]	20.9z

[a] Casein hydrolysate (enzymatic); (Nutritional Biochem., Cleveland, Oh)
[b] Means bearing different letters are different (p = 0.05).

Table 3—Effect of various nitrogen sources on growth and proteolytic activity of Clostridium perfringens *3624 in R&S base medium*

Nitrogen Source	Generation[c] time (min)	Gas produced (manometer) (units)	Proteolytic activity (RET)[d]	RET[d] / Gas produced (X 10^4)	TCA precipitate
Amino acids[a]	25.6w	106	0.536	50.4	–
Casein + amino acids[a]	22.5x	127	0.630	49.5	+
Casein + 0.5% enzyme hydrolysate[b]	27.3w	35	0.007	1.9	+
Casein + 0.5% acid hydrolysate	20.7x	75	0.007	0.9	+
Casein hydrolysate[b]	20.9x	132	0.008	0.6	–
Acid hydrolysate	20.0x	108	0.005	0.5	–
Casein	52.8y	6	0.005[e]	8.3[e]	+

[a] Amino acids as nitrogen source indicate R&S medium.
[b] Casein hydrolysate (enzymatic), (Nutritional Biochem. Corp., Cleveland, Oh.)
[c] Means with different letters are different (P = 0.05).
[d] RET = relative equivalents of trypsin.
[e] 8 of 14 samples showed a value of 0.000.

luxuriant growth of *C. perfringens* BP6K but did not support toxin synthesis. The toxins of *C. perfringens* are proteins. Many of them are also enzymes some of which are proteolytic, including kappa toxin (collagenase) and lambda toxin (proteinase) (Oakley et al., 1948). Nekvasilova et al. (1970) reported that a semi-synthetic medium, containing acid hydrolysate of casein supported growth without toxin production, whereas an enzymatic hydrolysate of casein supported growth and toxin synthesis. Fractionating the enzymatic hydrolysate of casein using column chromatography, they were able to recover a maximum toxin promoting peptide. Jayko and Lichstein (1959) and Tsukamoto et al. (1963) also reported that peptides were necessary for the production of extracellular proteins by *C. perfringens* BP6K and that the peptides could not be replaced by amino acids of the peptide in free form. Jayko and Lichstein (1959) also reported that the stimulatory effect of peptides was masked by the inhibitory effect of high concentration of free amino acids. Fry (1955) reported that the peptide bound amino acids were better utilized for transpeptidation and proteosynthesis than the same amino acids in free form. Hauschild (1965) measured the incorporation of [^{14}C] from both peptides and free amino acids. Peptide [^{14}C] was incorporated into protein 2-5 times faster than [^{14}C] from free amino acids. Pivnick et al., (1965) found that the amino acids of peptide and free amino acids entered different amino acid pools and resulted in the synthesis of different cellular and extracellular proteins.

In spite of the numerous reports concerning the stimulatory and essential role of peptides in exoprotein production, there were a few reports of a contradictory nature. Murata et al. (1965) found that the composition of the basal medium affected the growth of *C. perfringens* BP6K. They reported that a peptide free synthetic medium was able to support a high titer of toxin production provided that a high concentration of L-arginine was present. L-arginine was both growth stimulating and toxin promoting. Growth requirements were not the same as toxin production requirements. Zinc, manganese, biotin, and pyridoxamine, all components of protein synthesis, were essential factors in the production of toxin by *C. perfringens* BP6K (Murata et al. 1965). Blaschek and Solberg (1978) found the proteolytic activity was a function of both the medium and the strain. They found very low levels of activity in the spent amino acid containing synthetic R&S medium after growth of 11 of the 16 strains of *C. perfringens* tested. Strain 3624 produced exoprotease in the R&S medium. The results of Table 3 confirm the previously published data.

It appears that the data presented in Table 3 are partially contradictory to the literature supporting the need of peptides for protein or enzyme synthesis by *C. perfringens* and partially supportive of the literature which reports that the enzyme synthesis can be accomplished in a defined medium containing no peptide nitrogen. The importance of L-arginine, as described by Murata et al. (1965) may be critical. The arginine content of casein is 3.5% (0.25 g/g nitrogen) of the total amino acid content (FAO, 1970) as compared to 38% of the total amino acid content of R&S medium. The relatively low level of arginine may leave casein below the concentration required to derepress the hypothesized repressor of proteolytic enzyme synthesis. The other possible limiting amino acids of casein relative to R&S are glycine (1.8%: 3.8%) and cystein (0.3%: 3.1%) while glutamic acid, proline, and tyrosine are present in casein in significantly higher concentrations than in R&S medium: Considering the data presented in Table 3 and the assumption that a particular amino acid or group of amino acids is acting as the apparent derepressor of exoprotease synthesis, the effect of arginine alone or in combination with the other limiting amino acids may offer additional insight into the genetic regulation of exoprotease production by *C. perfringens* 3624. Studies relating to this hypothesis are in progress.

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Evaluation of the Effects of Liquid Bridges on the Bulk Properties of Model Powders

E. SCOVILLE and M. PELEG

ABSTRACT

Measured amounts of water were admixed with uniform size glass beads (107-475 micron) to form model powders with controlled interparticle liquid films. The viscosity of the films was modified by dissolving carrageenan and its surface tension by a detergent. These model powders were tested for loose bulk density, compressibility, irrecoverable work in compaction, cohesion and residual modulus after relaxation. The effect of the films on these bulk characteristics was considerable in the fine model powders but diminished with the increase of particle size. In comparison to particle size, the film viscosity (1-2000 cp) and surface tension (72 and 35 dyne cm^{-1}) had little or insignificant effect on the bulk properties. The results are explained in terms of the internal structure of the bed and their possible implications in real powders are evaluated.

INTRODUCTION

IT HAS LONG BEEN recognized that the physical and flow properties of powders are influenced by the presence of liquid interparticle films or bridges. The latter can cause spontaneous agglomeration of particles, a phenomenon that is beneficially exploited in processes like instantizing. Generally, however, the formation of liquid film at the surface of the particles, especially in soluble or partly soluble powders, may cause flow difficulties and in extreme cases severe caking problems (Burak, 1956; Pietsch, 1969; Peleg and Mannheim, 1977).

The theoretical aspects of liquid bridging in fine powders and its effect on the agglomerates strength have been thoroughly investigated (Rumpf, 1961; Derjagen, 1961; Schubert, 1975). As could be expected the factors that regulate the strength include the particle radius and the film properties, particularly surface tension.

There is less information available on the effect of liquid bridges on the *bulk* properties of powders, especially food powders. One of the difficulties in establishing such effects in real food powders is that the formation of liquid bridges, as a result of moisture condensation or melting is also accompanied by textural and geometrical changes in the particles themselves. These may include plasticizing of the solid material (Moreyra and Peleg, 1980) and partial dissolution of the particles surface. Changes in bulk behavior of real powders, therefore, are a reflection of the combined effects of a variety of possible mechanisms. Since the magnitude and relative contribution of each mechanism can vary among the different types of powders, it is interesting to establish and quantify, at least within order of magnitude, the specific role of each mechanism. In this work, an attempt to quantify the role of liquid bridges is reported and discussed.

Selection of model powders

The main problem in conducting an investigation into the specific role of interparticle films is how to avoid a simultaneous modification in the textural and surface properties of the particles themselves. The obvious solution to this problem is selection of chemically inert and mechanically hard powder. A natural choice has been glass beads of controlled size that are readily available. (The disadvantage of their use is that their solid density (2.2 g/cm^3) is bigger than any known food material).

Modification of the film could be done by dissolving a thickener (e.g. gum) to control viscosity or a detergent to reduce surface tension. The liquids could then be admixed, at known ratios, with the host powders, thus producing model powders with controlled films properties and thickness.

EXPERIMENTAL

PURIFIED glass beads of uniform size in the range 100-250 microns were used as the inert host powder. (For demonstration purposes, beads of large size, up to 4 mm in diameter, were also employed).

Control of the liquid properties was done by dissolving commercial sucrose, carrageenan and commercial laboratory detergent. The detailed composition of the liquids and their physical characteristics are given in Table 1.

Calculated amounts of the liquids were mixed with the glass beads until visual uniformity could be observed. The uniformity of the mixing was reevaluated through moisture and bulk density analysis and was found within satisfactory limits. The actual amounts added and the resulting film thicknesses are summarized in Table 2.

Determination of bulk properties

Loose bulk density was determined by pouring the powder into a cylindrical container, scraping off the excess and dividing the net weight of the powder by the volume of the container. Compressibility was determined by compression of a powder specimen by an Instron Universal testing machine. The specimen was confined in a metal cell and was covered by a specially designed lid that enables point loading during the compression. Detailed description of the cell and the procedure can be found in a recent publication by Moreyra and Peleg (1980). The force deformation curves, in the range of up to about 5 kg cm^{-2} were converted to bulk density-normal stress relationships. These were found to fit the following equation (Peleg et al., 1973):

$$\rho_B = a + b \log P \qquad (1)$$

where ρ_B is the bulk density, P the normal compressive stress, and a and b constants. The constant b representing the change in bulk density by the applied stress is defined as compressibility.

Force relaxation curves (Fig. 1c) at various strains were also re-

Table 1—Physical characteristics of the liquid films

Liquid film composition	Surface tension[a] (dyne. cm^{-1})	Apparent viscosity (CP)
Distilled water	75	1
With added detergent	35	1
1% Carrageenan solution	75	260[b]
With added detergent	40	260[b]
2% Carrageenan solution	75	2000[b]
With added detergent	50	2000[b]

[a] Measured by a Rosano Surface Tensiometer (Roller Smith Company, Newark, N.J.).
[b] Measured by a Brookfield Rotational Viscometer, Spindle No. 1 at 6 rpm.

Authors Scoville and Peleg are with the Dept. of Food Engineering, Univ. of Massachusetts, Amherst, MA 01003.

corded for about 5 min. The curves were normalized and linearized by the equation (Peleg and Moreyra, 1979):

$$\frac{Fo \cdot t}{Fo - F(t)} = k_1 + k_2 t \quad (2)$$

where Fo is the initial force, F(t) the decaying force after time t, and k_1 and k_2 constants.

Since the reciprocal of k_2 is an asymptotic value of Fo – F(t)/Fo as $t \to \infty$, it was used to calculate an asymptotic modulus (E_A) which was representative of the ability of the compressed powder to sustain unrelaxed stresses. This modulus was calculated from:

$$E_A = \frac{Fo}{A\epsilon_o}\left(1 - \frac{1}{k_2}\right) \quad (3)$$

where A is the cell cross sectional area and ϵ_o the fixed strain during the relaxation test.

Shear analysis was performed by a Jenike flow factor tester model FT-3STEH. The yield loci curves were recorded under different consolidation loads in the range of up to 1 kg·cm^{-2}. The data were used to calculate the compact cohesion and the effective angle of internal friction. The details of the experimental procedure are described in Jenike's classical work (Jenike, 1961). Schematic presentation of the method principles is given in Figure 1a.

Irrecoverable work in compresson was deermined as follows. The force deformation curves were recorded during compression-decompression tests reversing the chart movement direction during the decompression stage. The overall work is represented by the total area under the force deformation curve in compression and the irrecoverable work by the area bound by the hysteresis loop. The irreversible work is reported as percent of the total work. For graphical demonstration see Figure 1b.

Mixing uniformity and test reproducibility

The uniformity of the mixing could be controlled through the visual appearance of the beads. It was confirmed, however, by moisture and density analyses and was found to be satisfactory. The coefficient of variance (i.e. the standard deviation divided by the mean) in bulk density measurements (5 replications) was usually in the range 1-3%. For compressibility it was about 5% in the fine powders and about 10% in the coarser powders.

The procedure for cohesion measurement and calculation leaves room for experimental errors of up to 5 g · cm^{-2}. Similarly, the error range in angle of internal friction was about 1-2 degrees.

Table 2—Specific Area of the Model Powders and the Film Thickness at 1% Added Liquid

Bead diam (Micron)	Calc specivic area (cm^2 · g^{-1})	Calc film thickness (Micron)
105	260	0.19
175	156	0.32
255	107	0.47
475	57	0.87
1000	27	1.8
3000	9	5.5
4000	7	7.4

RESULTS & DISCUSSION

THE BULK DENSITY, compressibility internal friction, and cohesion of the model powders, with and without added liquid films are reported in Table 3. The percent of irrecoverable work in compression and the residual asymptotic modulus after relaxation are given in Table 4.

As could be expected, the dry powders had relatively high bulk density and low compressibility. The reason is that the dry and therefore noncohesive particles occupy most of the geometrically available space. Only few stable voids, mainly supported by frictional forces or randomly created local arches, can exist in the powder bed under such circumstances. The phenomenon is demonstrated in a somewhat exaggerated way in Figure 2a.

Since the number of such voids is very small, the powder has little compressibility as long as the applied pressure is insufficient to cause plastic deformation or considerable breakage in the particles themselves. In other words, the confined dry powders are expected to behave like solids with considerable strength under normal compressive loads. This is strengthened by the results obtained from relaxation tests; i.e., that the residual modulus after relaxation is high and that considerable part of the work invested in compression is indeed recoverable (Table 4). The irrecoverable portion is most likely due to friction since the powder returns to a free flowing state after removal of the external load. Incorporation of liquid films, has had a marked effect on the cohesiveness of the powders as can be seen in Table 3. (As could be expected, the effect on the measured angle of internal friction was fairly small in the reported experiments because glass beads have low coefficient of friction to start with.) The presence of the interparticle forces, (measured as cohesion) enabled a more open bed structure with many internal voids (Fig. 2b). These resulted in relatively low bulk density and high compressibility under small normal loads. (Carr, 1970; Peleg et al., 1973; Peleg, 1977). During the deformation of the bed, however, liquid film forms a strong binding layer (Rumpf, 1961) that results in a stable agglomerate. This was evident in the fact that all compacts, containig liquid films, remained as solid compacts even after removal of the load clearly in contrast to compacts of the noncohesive dry powders. It was also observed that practically all the work invested in the compression of the wet powder was irrecoverable (Table 4), a finding that is coherent with the explanation that the work is mainly invested in producing a stable agglomerate. Stress relaxation experiments demonstrated that the compacts could not support an appreciable amount of unrelaxed stress. This was evident from the extremely low residual

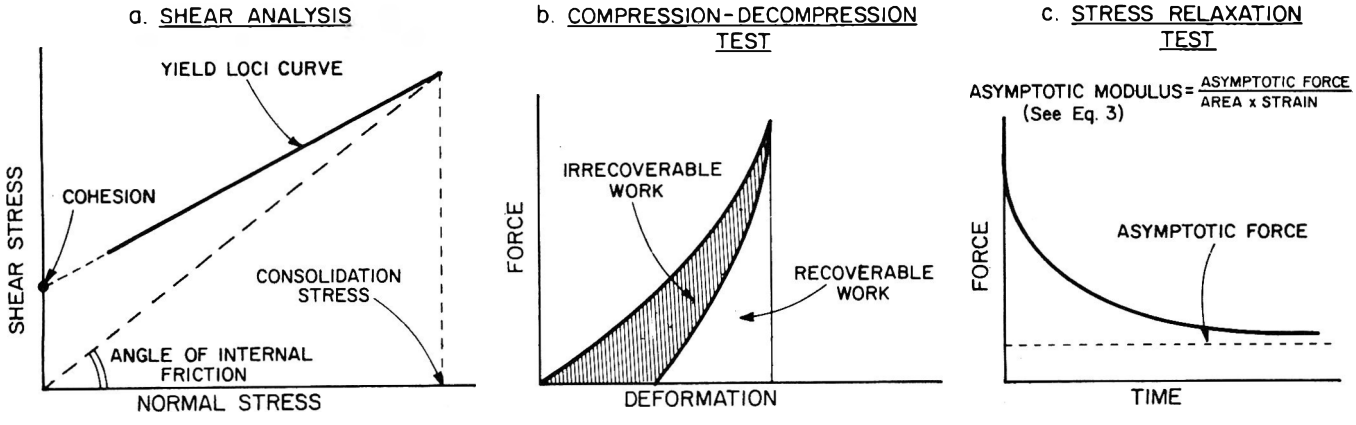


Fig. 1—Schematic presentation of some of the physical tests used for the powders characterization.

moduli (See Table 4) when compared to those of the dry powders. Although differences in density are certainly part of the explanation they do not seem to account for all the factors that are involved in this phenomenon. Apparently, much of the stress dissipated through flow and reorientation of the liquid bridges (Peleg and Moreyra, 1979) since the particles themselves were too rigid to exhibit so much stress relaxation on such a short time scale.

Effect of film properties and the bead size

Perhaps the most interesting result of this work is that neither the film viscosity in the range of up to 2000 cp nor its surface tension at 35-75 dyne · cm^{-1} had a decisive effect on the powder bulk characteristics. This was found in all the tested powders regardless of their particle size and also in sand where the shape of the particles was more irregular.

Apparently, the presence of the liquid film by itself was sufficient to cause drastic modification in the bulk properties. If there were any effects that were contributed by the specific physical characteristics of the film they must have been of minor magnitude that was obscured by the experimental errors.

It was observed, however (Table 3), that the overall effect of the liquid films on all the major bulk characteristics (i.e. density, compressibility, cohesion) diminished as the particle size increased. In this case the decrease was indeed significant and well within the accuracy of the experimental procedure.

The fact that the film thickness was different (Table 2) provides only partial explanation for this phenomenon since similar results were obtained when different amounts of film were added to the beads. A "dilution effect," i.e. that the film's own weight contributed to the decrease in bulk density must also be ruled out. Calculation showed that such effect could not induce more than 1% change in density. It should be noted that the effect of the film in the very large particles (i.e. 475-4000 micron) was almost negligible and again with respect to any type of film. (It should also be mentioned that the option of increasing the amount of added liquid to the larger particles, in order to control the film thickness, is practically limited because of drainage problems).

The most plausible explanation of this phenomenon is that one of the most dominant factors in regulating the bulk behavior is the ratio between the particle weight and the film strength. In the case of the larger and therefore heavier particles the film strength was insufficient to support a heavy structure and therefore, the bulk properties of dry and wet beds were practically the same.

In the case of the smaller beads, in contrast, the weight of the particles and the film geometry were such as to enable the latter to support a physical structure with an appredіable mechanical stability. This explanation is coherent with the differences in behavior between granular and powdered forms of materials and can be demonstrated in heavier (e.g. metal) and lighter (e.g. epoxy) beads.

Relation to food powders

Obviously, there is little similarity between glass beads and food powders. The solid density of glass (about 2.2g · cm^{-3}) is much higher than that of common food materials (up to about 1.6g · cm^{-3} for nonporous materials). The actual shape and size distribution of food powders are also far from the almost perfect state represented by the uniform beads. Despite these striking dissimilarities however, the behavior of the beads demonstrated film effects that may well be applicable to food powders especially those which are dense and relatively hard or have crystalline structure.

In such powders it is reasonable to expect that liquid films can form as a result of moisture condensation or wetting. Since penetration of liquid into the particle itself may be a slow process the major physical change (at least on a relatively short time scale) would be the presence of liquid film at the surface of the particles. (Evidence to support this existence of such possibilities has recently been reported by Peleg and Mannheim (1977).

Table 3—Effect of the presence of liquid films on the bulk properties of glass beads[a]

Diameter (micron)	Type of powder	Bulk density (g · cm^{-3})	Change in bulk density (%)	Compressibility (b in Eq. 1)	Cohesion[b] (g · cm^{-2})	Angle of internal friction[b] (Deg)
105	Dry	1.58	—	∿0	∿0	25
	Wet	1.00-1.08	37-32	0.25-0.29	13-28	18-23
175	Dry	1.72	—	∿0	∿0	23
	Wet	1.16-1.26	33-27	0.18-0.24	6-15	20-23
255	Dry	1.62	—	∿0	∿0	27
	Wet	1.31-1.40	19-14	0.07-0.12	3-12	22-26

[a] "WET" refers to the presence of liquid films whose characteristics are specified in Table 1.
[b] Determined at a consolidation stress of 260 g · cm^{-2}.

Table 4—Compressive irrecoverable work and residual (relaxed) modulus of compacted glass beads

Diameter (micron)	Film type	Irrecoverable work[a] (%)	Asymptotic modulus after relaxation (kg · cm^{-2})	Appearance after removal of load
105	dry	80	—	Free flowing
175	dry	65	60-75[(b)]	Free flowing
255	dry	65	60-75[(b)]	Free flowing
475	dry	25	45-125[(b)]	Free flowing
105-475	Any film	99	0.5-3.5[(c)]	Stable compact

[a] Maximum normal stress 1 kg · cm^{-2}.
[b] Strains of 1-2%.
[c] Strains of 35-45% for the 105 micron beads, 25-33% for the 175 and 10-18% for the 255 micron beads.

In such situations, the bulk behavior would be regulated, to a considerable extent, by the interparticle film in a similar way as in the model powders. It was demonstrated, in the latter, that a minute amount of interparticle liquid films can produce drastic changes in bulk behavior, especially in fine particle beds. These, in practical terms, are expressed in decreased flowability due to cohesion, reduced loose density, and the formation of stable compacts under pressure. It is, therefore, quite plausible that the formation of liquid films alone is sufficient to cause similar changes in food powders. Obviously, and especially in the case of porous, and highly soluble materials, the effects will even be enhanced by physical changes in the particles themselves (e.g. plasticity of the material and surface modification).

Furthermore, since the particles of most commercial food powders are both light and small, the formation or liquid film is expected to have appreciable effect on the bulk characteristics regardless of the physical properties of the film itself. It can be speculated that actual films may have even higher viscosities than tested in this work and that there might be other geometrical factors (including friction) that will also contribute to the modification of the bulk behavior. Comparison of the results of this work with previously reported data (Peleg et al., 1973, Moreyra and Peleg, 1980) especially with regard to bulk density and compressibility indicates that the surface liquid contribution is clearly the most dominant factor that outweighs the effects which are produced by textural modifications of the particles interior.

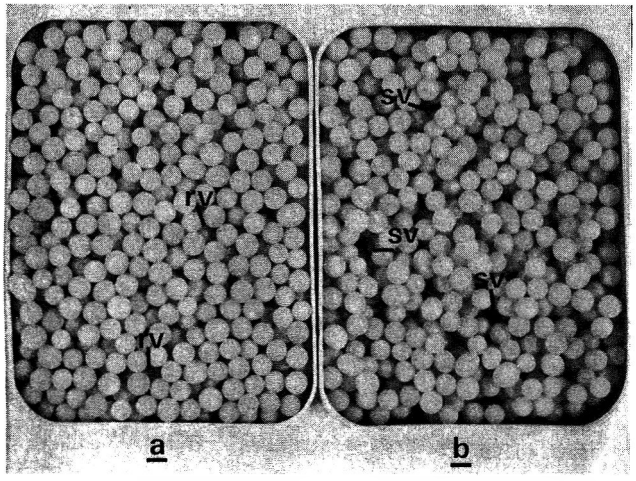


Fig. 2—The structure of dry (a) and wet (b) powders after being freely poured into a container: rv are voids created randomly and sv are considerably larger voids supported by interparticle liquid bridges. (Demonstrated with epoxy beads about 3 mm in diameter).

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Descriptive Analysis of Whiskey Sour Formulations: Magnitude Estimation Versus a 9-Point Category Scale

M. R. McDANIEL and F. M. SAWYER

ABSTRACT

The whiskey sour samples utilized in this study were specifically formulated to create differences in sensory characteristics. The effectiveness of both the magnitude estimation and 9-point category scale methods used by a trained panel were evaluated by how well these "created differences" could be perceived. No differences were found between the methods regarding scaling mix differences. However, large differences were found in other variables tested. The use of magnitude estimation resulted in far more panelist-mix interaction, while use of the category scale resulted in more variability due to panelist and replication.

INTRODUCTION

CATEGORY SCALES have long been used by psychologists to measure attitudes (Guilford, 1954). The category scale, in the form of the 9-point hedonic scale, has been a popular tool for measuring food acceptability (Peryam and Pilgrim, 1957). Other scales of varying lengths have been developed to measure intensity differences for particular characteristics of food, such as tenderness of meat, or sweetness of different sugars.

Category scales have their limitations and are subject to various biases (Stevens, 1957). For example, judges may tend to avoid using the extreme categories, thus reducing the scale's size and centering the judgments around the middle categories (Stevens and Galanter, 1957). Also, the intervals between categories may be psychologically unequal, even though the interval difference is generally given a value of "one" for analysis. The scale values have no numerical relationship. For example on a sweetness scale a value of four given a sample on one category does not mean the sample is twice as sweet as another sample given a value of two, which happens to be two categories lower on the scale.

Magnitude estimation, a ratio scaling method, has been widely used by psychologists, and it is currently growing in popularity in food research and product development. The goal of this scaling procedure is to place stimuli on a scale so that one stimulus can be rated as a specified number of times stronger or weaker than a reference stimulus. The subject is unconstrained as to the range and size of numbers used. The scale that emerges has the properties of a ratio scale of magnitude. The magnitude estimation technique also has limitations dependent on the specific procedures utilized. Panelists must be comfortable with the use of numbers. Occasionally a panelist has been shown to be constrained in his/her use of large numbers, fractions, or decimals (McDaniel, unpublished data).

Magnitude estimation has been used widely by psychophysicists for gustation and olfaction research. Some published data are available on food applications; however, little is available on direct comparisons of category scale versus magnitude estimation results. One such study on food acceptability was completed by Moskowitz and Sidel (1971).

The purpose of this study was to evaluate magnitude estimation versus category scaling as a sensory evaluation technique for scaling intensities of food quality descriptors.

Author McDaniel formerly with the Dept. of Foods & Nutrition, Univ. of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada is now located at 925 W. 500 N., West Lafayette, IN 47906; Author Sawyer is with the Dept. of Food Science & Nutrition, Massachusetts Agricultural Experiment Station, Univ. of Massachusetts, Amherst, MA 01003.

EXPERIMENTAL

Samples

The following whiskey sour mixes were prepared by Heublein, Inc., Hartford, Conn. and bottled in 24 oz bottles:

a. Standard/Formulation (as sold commercially by Heublein, Inc.) Whiskey Sour (code A) at 48° Proof.
b. Standard Formulation with no clouding agent added (code B).
c. Standard Formulation with 50% reduction in lemon flavor (code C).
d. Standard Formulation with 50% reduction in sucrose (code D).
e. Standard Formulation with 50% reduction in citric acid (code F).
g. Standard Formulation at 35° Proof (code G).

These products were developed in order to provide a relatively large number of visual, olfactory, and gustatory differences which could be scaled by the panelists.

Selection of quality descriptors

A preliminary panel of approximately 20 judges was asked to describe the appearance, aroma, taste, and mouthfeel of selected whiskey sour samples. From the list of 90 terms generated, 19 were selected by the experimenter for profile testing. Many of the original 90 terms seemed to represent the same sensory quality (e.g., sour, tart, acid). The final 19 terms were selected because they encompassed the major sensory qualities of the whiskey sours. The terms were distributed into four quality sets for testing by the selected panel (Table 1). An entire set of qualities was scaled by a panelist in one test setting. The 19 terms were selectively placed in the four sets to minimize the influence of one term on another. For example, if sweet taste were scaled directly after sour taste, a biased evaluation of sweetness might result. Although it is impossible to control completely this contrast effect when more than one quality is being scaled at one sitting, the experimenters designed the four sets to minimize, hopefully, the effect. The terms were placed within each set so that visual terms would be evaluated first, aroma second, taste third, mouthfeel and hedonic terms last.

Panel selection and training

Approximately 20 graduate students and staff members in the Food Science & Nutrition Dept. participated in a series of 20 triangle tests between the standard sample and a reformulated sample. The seven panelists best able to discriminate differences (= or > 80% correct responses) were selected for further training. The training consisted of a series of sessions where the methods of testing were discussed and reviewed. The descriptive terms to be used in the study were discussed and standards (e.g., citric acid for sourness) were evaluated so that everyone agreed on their meaning. None of the panelist had prior experience with magnitude extimations, but many had previously used category scales.

Experimental design

All samples were presented in pairs, with the standard being tested versus a reformulated sample. A set of ballots (one ballot for each quality descriptor) was presented with each pair. The sets

were described previously in Table 1. All of the ballots in the set were either magnitude estimation or category ballots. Each pair was tested twice. Pair order was randomized. Two pairs were evaluated at each testing period and there were two testing periods daily.

Sensory methods

For all product comparisons, panelists used both magnitude estimation and a nine-point category scalar technique. For the magnitude estimation procedure, each panelist evaluated the first sample (standard) and assigned it any number. The second sample was evaluated and assigned a number with respect to the number given the first sample, i.e., if sample A were assigned 10 for yellowness, and the second sample were seen as five times as yellow, then the second sample was assigned a value of 50.

All category intensity scales were nine points in length with the values 1, 3, 5, 7, and 9 corresponding to the scale points no, slight, moderate, large, and extreme, respectively. Midpoints were allowed so that any value of 1-9 could result. The judge evaluated both samples for each quality characteristic at one time, thus providing as similar a test situation as possible to the magnitude estimation procedure. The presentation of samples within the pair was randomized.

Serving conditions

All samples were served at 5°C in whiskey sour glasses coded with three digit random numbers. The testing took place in a temperature controlled sensory laboratory equipped with isolation booths illuminated with white incandescent lights.

Data analysis

The Biomed BMDO8V Analysis of Variance version of Sept. 1, 1965, Health Sciences Computing Facility, UCLA was used. The variability factors tested were mix, panelist, mix-panelist interaction, and replication. The Biomed BM02D, Correlation with Transgeneration, Health Sciences Computing Facility, UCLA, was also used. The values used in the correlations were differences between paired ratings (A vs B through G) for the category tests and ratios (A vs B through G) for the magnitude estimation tests.

All magnitude estimation data were normalized for each pair by dividing, each value by the geometric mean of the pair (McDaniel, 1974). This process was necessary because of the testing procedure selected by the experimenter. Panelists were allowed to select any number they wished to reflect the intensity of the quality being scaled.

RESULTS & DISCUSSION

APPROXIMATELY 20 consumers of whiskey sours described the appearance, aroma, taste, and mouthfeel of the standard whiskey sour sample. The resulting terms were categorized and 19 were selected and placed into sets for testing (Table 1). The taste and aroma terms selected were very similar, sweet, sour, alcohol, and citrus being common to each. Overall aroma and taste intensity terms were also included. Mouthfeel terms included those descriptors which occurred from a trigeminal or physical response rather than a chemical response. Two terms were added, pleasant and unpleasant, to obtain a hedonic reaponse from the judges.

Table 1—Nineteen descriptive profile terms distributed into four sets for use by the trained panel

Set I	Set II
cloud	yellow
citrus aroma	sour aroma
alcohol taste	nose irritation
sweet taste	citrus taste
puckery mouthful	overall pleasant
Set III	**Set IV**
sweet aroma	alcohol aroma
sour taste	aroma intensity
taste intensity	bitter taste
viscous mouthfeel	burning mouthfeel
overall unpleasant	

Intensity rating of quality descriptors

Effect of method. A total of 114 ANOVA's were completed for each method tested (Magnitude estimation and category), as each descriptive term (n=19) for each whiskey sour pairing (n=6) was analyzed separately. There was basically no difference in the judges' ability to generate differences by using either method.

Totals of incidences of significant sources of variation in the ANOVA of the magnitude and category profile data are given in Table 2. Thirteen significant differences in mixes were found using magnitude estimation as compared to 14 for the category tests. On first appraisal the number of significant differences seem small; however, an adjustment in formula of one ingredient should affect only a few of the descriptors tested for each adjustment of the formula.

Although the number of mixes significantly different was similar for both methods, the other variables tested in the ANOVA (panel, panel-mix, replication) were quite different. Panel variability in the magnitude descriptive profile was practically nonexistent, whereas in the category DIP, 83 of the 114 tests showed significant panel variability (Table 2). This large difference was due in part to the normalization procedure described earlier. Although the normalization procedure did not affect the ratios of the samples, it did drastically affect panel variability. For example, one judge's values for samples A and B (100, 10 respectively) would result in the ratio of 10/1, identical to the ratio formed by another judge's values of 1.0 and 0.1 assigned to the same samples. The normalization procedure applied to both sets of numbers results in equal values, having a geometric mean of one. The scalar values were not normalized because the judge was already working within a confined scale where the only values available were "one" through "nine". A normal distribution was assumed, and therefore an analysis of variance was used to analyze the results. A normalization procedure applied to the category values, although generally deemed unnecessary, would probably greatly diminish the occurrence of significant panel variability.

There were 48 instances of significant panel-mix interaction in the magnitude descriptive profile and only 14 instances in the category descriptive profile. Panel-mix interaction occurred when some of the panelists scored the mix in one direction and the remaining panelists scored the mix in the other direction. For example, if A and B were the samples, some of the panelists consistently scored A higher than B while other panelists consistently scored B higher than A. This could occur due to panelist confusion of the attributes being scored, but should not have been affected by scaling method.

There were 14 cases in the category descriptive profile of significant replication variability while none occurred in the magnitude method. Significant variability due to replication reflects the panelists differing use of the 9-point

Table 2—Total of incidences of significant sources of variation in the analysis of the category and magnitude descriptive profile of whiskey sours

	Sources of variation[a]			
Method	M	PM	P	R
magnitude estimation	13	48	1	0
Category	14	14	83	14

[a] M = mix; PM = panelist-mix interaction; P = panelist; R = replication.

Table 3—Significant category descriptive profile terms for whiskey sour mix comparisons and other significant ANOVA variables

Sample[a] pair	Sample adjustment	Significant descriptor	Mix mean scores			Other significant variables[b]		
			Standard	Other	Difference	P	PM	R
AB	No cloud	Cloudiness	5.5	2.1	3.4**	*		*
	No cloud	Sweet taste	3.9	3.4	0.5*			
	No cloud	Alcohol taste	4.9	5.6	−0.7*			
AC	50% less lemon	Yellow	6.2	5.4	0.8*	**	*	**
AD	50% less sugar	Yellow	6.2	5.1	1.1*	**		*
	50% less sugar	Sweet taste	4.6	2.4	2.2*	**	**	
	50% less sugar	Pleasant	4.6	2.4	2.2*	**		
AE	50% less acid	Yellow	6.0	4.8	1.2**	**	*	
	50% less acid	Pleasant	4.2	3.4	0.8*			
AF	25% dilution	Yellow	5.7	4.6	1.1*	*		
AG	35% proof	Yellow	5.8	4.6	1.2**			
	35% proof	Sweet taste	3.6	4.6	−1.0*	**	*	
	35% proof	Citrus taste	4.6	5.4	−0.8*	**	*	
	35% proof	Alcohol taste	5.9	4.4	1.5**			

[a] A = standard (48° proof) whiskey sour formula; B = no cloud; C = 50% reduction in lemon flavor; D = 50% reduction in sugar; E = 50% reduction in citric acid; F = 25% dilution of standard; G = 35% proof.
[b] P = panelist; PM = panelist-mix interaction; R = replication.
*$P < 0.05$
**$p < 0.01$

category scale from one test situation to another.

Effect of formula adjustment. Many of the significant descriptors for the mix pairs tested were significant in both magnitude and the category descriptive profile. Of the 14 category and 13 magnitude significant differences, seven were common to both. Category descriptive profile significant differences in mix are presented in Table 3. Magnitude descriptive profile significant differences in mix are presented in Table 4.

For the standard vs "no cloud" comparison, both methods provided a significant difference in cloudiness. The other descriptors whose ratings resulted in significant difference for this comparison were citrus aroma for the magnitude descriptive profile, and sweet taste and alcohol taste for the scalar descriptive profile. The presence or absence of cloud should not have affected physically but may have affected psychologically one or more of the aroma or taste characteristics of the sample. The clear sample was judged to have less citrus aroma, a less intense sweet taste, and a more intense alcohol taste. This could have been caused by one of the psychological errors common to sensory testing, the proximity error, where adjacent traits tend to be rated similarly. Because cloudiness was the first characteristic rated in a set of characteristics which included citrus aroma, sweet taste, and alcohol taste (Table 1) there may have been a carry-over effect from one scale to another. This error can be eliminated by having each characteristic evaluated at separate test session, a time consuming but effective procedure.

The sample which was adjusted to a lower lemon intensity was judged in both descriptive profiles to be less yellow than the standard. In the magnitude descriptive profile it was also judged to be less pleasant. Because yellow and pleasantness were in the same set of characteristics the proximity error could have been responsible. Another psychological error, the logical error, may have been influential here, as it results from assigning similar ratings to characteristics which appear to be related or logically associated. In the above case, less yellow may have been associated with less pleasant. Psychologically, less yellow may have meant a lower flavor or lower alcohol level, or some other characteristic change the judge might associate with less yellow to cause the sample to be scored less pleasant.

Yellow and sweet taste were significant descriptors in both descriptive profiles for the standard vs reduced sugar comparison. It is not clear why yellowness should be affected by a sugar reduction, but the reduced sugar sample was judged less yellow in both profiles. In the category descriptive profile, pleasantness was a significant characteristic. Again, this could be due to the proximity error, but results discussed earlier indicate sweetness to be of great importance to preference ratings.

Pleasantness was the common significant descriptor for the standard vs reduced citric acid sample comparison. In both profiles the standard was judged more pleasant. The standard was judged to be more yellow in the category descriptive profile, although the reason is not clear. Panelists using the magnitude descriptive profile judged the citric acid reduced sample to be more sweet, a reasonable judgment since sweetness and sourness somewhat counteract each other.

The common significant descriptor for the standard vs 25% dilution comparison was yellowness. In both profiles the standard was judged more yellow, a reasonable result since the color would be diluted along with all of the other physical characteristics. The magnitude descriptive profile also noted a significant decrease in sweet taste, sour taste, and burning mouthfeel, again a reasonable effect of dilution.

While the magnitude descriptive profile was more sensitive to the dilution effect, the category descriptive profile appeared to be more sensitive to the effect of decreasing proof. Both profiles resulted in a decreased yellowness rating for the 35° proof sample. This decreased yellowness was caused by the use of less bourbon. The panel using the category descriptive profile found the 35° proof sample to be sweeter, to have more citrus taste, and to have less alcohol taste. All of these judgments appeared to be reasonable; however, the proximity error could be an important factor as yellowness and citrus taste belong to the same set as do sweet taste and alcohol taste. Since yellowness was a common descriptor in most comparisons and citrus taste was significant only in the final comparison, the citrus taste judgment was probably valid. Sweet taste and alcohol taste were both significant in the comparison where cloudiness was the sample adjustment. Since alcohol taste was judged first in the set, sweet taste is the characteristic subject to the proximity error in this case.

The type of data generated by use of a ratio scaling technique such as magnitude estimationis of great use in intensity testing. For instance, the meaning of the ratio can be more readily explained and the ratio can be more easily used than differences in category scale points. For exam-

Table 4—Significant magnitude descriptive profile terms for whiskey sour mix comparisons and other significant ANOVA variables

Sample	Sample adjustment	Significant descriptor	Mix mean scores			Other significant variables[b]		
			Standard	Other	Ratio	P	PM	R
AB	No cloud	Cloudiness	2.58	0.53	4.87*			
	No cloud	Citrus aroma	1.38	0.82	1.68*			
AC	Lemon reduction	Yellow	1.13	0.89	1.27*		**	
	Lemon reduction	Pleasant	1.24	0.95	1.31*			
AD	Sugar reduction	Yellow	1.21	0.85	1.42*		*	
	Sugar reduction	Sweet taste	1.52	0.71	2.15*		*	
AE	Citric acid red.	Sweet taste	0.81	1.34	0.60*			
	Citric acid red.	Pleasant	1.63	0.75	2.17*			
AF	Dilution	Yellow	1.52	0.74	2.05*		**	
	Dilution	Sweet taste	1.28	0.80	1.60**		**	
	Dilution	Sout taste	1.17	0.89	1.31*			
	Dilution	Burning mouthfeel	1.39	0.74	1.87**		**	
AG1	35% proof	Yellow	1.24	0.84	1.48**			

[a] A = standard (48° proof) whiskey sour formula; B = no cloud; C = 50% reduction in lemon flavor; D = 50% reduction in sugar; E = 50% reduction in citric acid; F = 25% dilution of standard; G = 35% proof.
[b] p = panelist; PM = panelist-mix interaction; R = replication.
*$p < 0.05$
**$p < 0.01$

ple, by knowing the power function of sweetness in a product, the power function being determined by ratio scaling of sweetness over a range of sugar concentrations, one can easily adjust sweetness to the desired psychological level. If the slope of the function were 2.0, then the experimenter would know that for the addition of one unit of concentration, an increase of two units on the psychological sweetness scale would occur. Although power functions were not determined for this study, the magnitude results do take the form of a ratio of sensation. For example, the standard contains twice as much sugar as the sugar reduced sample. The sweetness ratio between the two is 2.15, or equivalent to a slope of slightly more than one (Table 4). This corresponds quite closely to the proposed slope of 1.3 for sweetness based on repeated studies of the growth of sweetness intensity (Moskowitz, 1970). The slope would be expected to differ depending upon the product's other interfering sensory stimuli. Category scores for sweetness only refer back to rather arbitrary descriptive terms. For example, from Table 3, the standard mean score was 4.6 while the sugar-reduced sample was 2.4. These numbers refer only the descriptive terms assigned to the category scale points. The 4.6 score closely corresponds to a "moderate" (scale value of 5) amount of sweetness, while 2.4 closely corresponds to a "slight" (scale value of 3) amount of sweetness. Although this information is helpful, it is difficult to relate this type of information to the psychophysics of the system.

SUMMARY

IN THE DESCRIPTIVE PROFILES, magnitude and category methods appear to have been equally sensitive to difference in product descriptive terms. The normalization procedure applied to magnitude estimation data for statistical analysis decreased panel variability. High panelist variability is frequently encountered in sensory testing, but should be minimal in trained panel work. There was a high incidence of panelist/mix interaction in the magnitude profile, which indicates that panelists were not scaling the products in the same direction. There were only a few instances of panelist/mix interaction in the category descriptive profile.

Unfortunately, the probable occurrence of psychological errors makes evaluation of both methods difficult. The proximity error and the logical error may have influenced the results. Yellow was one of the descriptive profile terms which was often a significant descriptor. In fact, the appearance terms, cloudiness and yellow, were significant 11 times out of a total of 27 significant descriptors. Appearance was evaluated first, and should not have been influenced by taste and aroma characteristics.

The hedonic term, pleasantness, was a significant descriptor four times, but three of those instances were in conjunction with a statistically significant yellow judgment where the proximity or logical error could have been influential.

Aroma terms seem to have been of little importance. In only one instance was an aroma term statistically significant (citrus aroma in magnitude descriptive profile for standard versus no cloud comparison). Sweet taste was a statistically significant descriptor six times; however, at least two of the incidences were possibly influenced by a psychological error.

CONCLUSIONS

THE WHISKEY SOUR samples utilized in this study were specifically formulated to create differences in sensory characteristics. The effectiveness of both the magnitude estimation and 9-point category scale methods used by the trained panel were to be evaluated by how well these "created differences" could be perceived. No differences were found between the methods regarding scaling mix differences. However, large differences were found in the other variables tested. The use of magnitude estimation resulted in far more panelist-mix interaction, while use of the category scale resulted in more variability due to panelist and replication. Since the same panel used each method, differences should be due either to the ability of the panel to use each method or to the statistical manipulation of the data, or a combination of these factors.

The reason for the significant panelist and replication variability from the category profile is fairly straight forward. Individual panelists use different portions of the scale and this results in significant panelist variability. The significant replication effects showed that the panel as a whole used a different portion of the scale during the first replication as compared to the second. This points out very clearly that indivdual product means from category scales out of context of the entire text, are of limited usefulness.

Although the number of significant mix differences was nearly equal between the two methods, the usefullness of the sample mean data differs. The category scale mean values represent descriptive terms which are useful in obtaining a generaly feeling for the degree of sweetness, but

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Preference Testing of Whiskey Sour Formulations: Magnitude Estimation Versus the 9-Point Hedonic

M. R. McDANIEL and F. M. SAWYER

ABSTRACT

Consumer preferences for different whiskey sour formulations were determined by a consumer laboratory panel and by a home panel using two different sensory techniques: the magnitude estimation procedure and the nine-point hedonic method. Use of the magnitude estimation testing procedure resulted in more statistically significant differences in preference in both the home panel and the consumer laboratory panel. Many more significant differences in preference were expressed by the home panel than by the consumer lab panel. The standard whiskey sour formula scored very well in the preference tests in comparison to the formula adjusted samples. In only two of the comparisons were the adjusted samples preferred over the standard, the 25% dilution of the standard and the 35° proof samples.

INTRODUCTION

SENSORY EVALUATION of food acceptability in the food industry has centered around category scaling methods since the development of the 9-point hedonic scale at the Quartermaster Food and Container Institute in 1957 (Peryam and Pilgrim, 1957). This method has been widely used because the analysis is straight forward and the task is easily understood by panelists. However, the method has several limitations, as it is subject to several systematic biases of human judges. The intervals between neighboring categories may be psychologically unequal, and judges may tend to lump their judgements into the center of the scale.

A number of researchers have used magnitude estimation successfully to scale preferences. Magnitude estimation is a method used to estimate the intensity of characteristics where the judge is free to use any range and size of numbers to describe a sensation. The original goal of the scaling procedure was to be able to report that one stimulus was a specified number of times stronger or weaker than a standard stimulus.

This same system has been applied to the scaling of hedonics since all of the sensory qualities possess hedonic as well as intensity characteristics. See Pfaffmann et al. (1971) for a short review on hedonics of taste psychophysics.

Moskowitz and Sidel (1971) conducted a series of nine experiments to compare magnitude (ratio) and hedonic 9-point (category) scales of food acceptance. They concluded that both evaluation procedures to be equally sensitive to differences in food acceptability, but each procedure provided additional information as well. Magnitude scales quantified the ratios of food acceptability among different items, and the hedonic scale provided numerical and verbal categories of acceptance. However, Moskowitz and Sidel did not discuss the fact that the method of magnitude estimation resulted in a larger number of significant differences in preference than did the hedonic scale for most products tested. Because of this suggestion of differences in sensitivity between the two methods, further preference testing with the two methods is merited.

This study was designed to compare magnitude estimation versus category scaling of preference over two distinct types of preference panels, a consumer laboratory panel vs a consumer home panel, thus allowing evaluation of both method and panel effectiveness.

Author McDaniel formerly with the Dept. of Foods & Nutrition, Univ. of Manitoba, Winnipeg, Manitoba R3T 2N2, Canada is now located at 925 W. 500 N., West Lafayette, IN 47906. Author Sawyer is with the Dept. of Food Science & Nutrition, Massachusetts Agricultural Experiment Station, Univ. of Massachusetts, Amherst, MA 01003.

EXPERIMENTAL

Selection of panelists

Two panels were used in this study: a consumer laboratory panel and a consumer home panel. The consumer laboratory panel consisted of anyone who liked and was at least an occasional consumer of whiskey sours and was available in the laboratory on the day of testing. The majority of the panelists were either students or staff members from the Dept. of Food Science & Nutrition. Most of the panelists were male and ranged in age from 20-30 years.

The number of panelists ranged from 11-24. Although this panel is small, its use was to enable the authors to evaluate the ability of such a small panel to reflect consumer preference. Many companies in the food industry regularly run in-house preference tests of this size.

Fifty volunteers who liked and were at least occasional consumers of whiskey sours participated in the home panel. Forty-seven of the 50 volunteers completed the study. The 47-member panel consisted of 18 males and 29 females. The average age of panelists was approximately 35 years. Results of a questionnaire concerning information on the whiskey sour consumption habits of the home panelists were summarized by McDaniel (1974).

Methods of sensory testing

The 9-point hedonic scalar technique was utilized in both the consumer laboratory panel and the home panel. The scale ranged from like extremely (9) to neither like nor dislike (5) to dislike extremely (1).

Magnitude estimation was also used by the consumer laboratory panel and the home panel to assess preference. Sample were presented in pairs, the standard vs a reformulated sample. The reformulated sample was assigned a number with respect to the number given the standard sample. If the second sample were 1½ times as well liked as the standard, it was given a number 1½ times as large as the number assigned to the standard. If it were ¼ aas well liked, it was given a number ¼ as large.

Samples

Samples used in the study are identified in Table 1. Formulation details are described in a previous paper (McDaniel and Sawyer, 1981). They consisted of a standard 48° proof whiskey sour formulation and six different formula modifications. Samples for distribution to the home panel were bottled in 2-oz, clear glass bottles with plastic screw caps.

Serving procedure and test design

Consumer laboratory panel. The standard whiskey sour sample was tested against each of the other six samples, twice for each preference method, scalar (9-point Hedonic) and magnitude estimation, for a total of 24 days of testing. Because of the intensity of the sample's flavor impact, only two samples could be tested at one sitting. The paired comparison method was selected in order to have the standard formulation rated in each comparison. The 24 tests were run in random order. Daily sample order presentation was

randomized for hedonic test, while in the magnitude estimation test the standard was always presented first.

All samples for the consumer lab panel were served at 5°C. Samples were poured directly from the refrigerator into 1-oz whiskey sour glasses. The two samples were coded with a three-digit random number, placed randomly on the tray with a preference ballot and a glass of distilled water. All panelists were instructed as to the nature of the test at their first test session. The methods and ballots were reviewed until the experimenter felt the panelist had full understanding of the procedure. Each panelist was interested in the test and was willing to participate. Panelists were seated in fully lighted isolation booths. The panel evaluated the samples between 3:00-4:00 p.m.

Home panel. The home panel completed the same series of tests as the consumer laboratory panel, the standard vs each of the other samples twice for each preference method for a total of 24 days of testing. Daily sample order presentation was the same as in the consumer laboratory panel.

A set of samples was prepared for each of the 50 panelists. Because it was undesirable to have all panelists testing the 24 comparisons in the same order, ten different orders were used. They were prepared by randomly drawing the 24 comparisons until ten orders were completed. The design provided five replications for each order. All of the 48 samples were bottled, coded, and given to each individual panelist with 24 ballots. Each panelist was given precise written and verbal instructions regarding the test methods and the test procedures as well as a short questionnaire concerning whiskey sour consumption habits.

The home panel was instructed to serve the samples in a fashion normal to them. They were to evaluate samples at a convenient time and as close as possible to the time when a whiskey sour would normally be consumed at home. They were instructed to label two identical glasses with the corresponding 3-digit random numbers on the sample bottles for that day. The samples could be served with the normal condiments that particular person would use to prepare a whiskey sour at home, for example, a maraschino cherry. At the end of the 24-day session, the ballots were collected and the judges were questioned regarding any difficulties encountered during the test period. None of the judges reported irregularities in the prescribed regimen but a few reported a certain amount of disinterest toward the end.

Data analysis

Analysis of variance. The Biomed BMDO8V Analysis of Variance version of Sept. 1, 1965 Health Sciences Computing Facility, UCLA, was used. The analysis gave four measures of variability for the home panel: sample formulation between panelist, within panelist, and sample panelist interaction. The factors for the consumer lab panel were mix and panelist. The magnitude estimation values underwent a geometric mean normalization across each panelist prior to analysis (McDaniel, 1974).

Correlation. The Biomed BM02D, Correlation with Transgeneration, Health Sciences Computing Facility, UCLA, was used. The values used in the correlation were differences between paired ratings for the scalar tests and ratios for the magnitude estimation tests.

RESULTS & DISCUSSION

Magnitude estimation vs the 9-point hedonic in food preference testing

The sensitivity of magnitude estimation vs the 9-point hedonic scalar technique in food preference testing was evaluated by comparing results from both methods over two whiskey sour preference panels studied. The sensitivity of each preference method was evaluated by observing the number of significant differences between samples each method allowed. The number of significant differences in preference indicated which method provided greater ease of discrimination.

Results of the consumer laboratory panel are presented in Table 1. A total of 24 panels were run, 12 by each method. Of the 12 tests where preferences were judged using magnitude estimation, four resulted in a significant preference. The ratio A/O (Standard/Other sample) is given for each magnitude estimation comparison because of the ratio properties of the magnitude estimation scale. As an example, for the second comparison of sample A and sample D (AD^2), the judges liked sample A 2.82 times as much as sample D. The difference A – O is presented for each hedonic comparison. For example, a difference of 2.1 scale points resulted from the first hedonic comparison of samples A and D (AD^1). Of the 12 tests where the 9-point hedonic method was used, only two significant differences in preference resulted. Although the magnitude estimation procedure resulted in more statistically significant preferences and, therefore, might be considered more sensitive, neither method resulted in both tests of the same pair of products showing a significant preference.

Factors which were probably responsible for results of this type are variation in panel size, where some days as few as 11 people were available for testing, and variation in panel composition from day to day, a problem frequently encountered when judges are not readily available.

Results of the home panel are presented in Table 2. The home panel was planned so that every judge performed each evaluation twice. The results of the two evaluations were averaged and an analysis of variance was performed on the resulting data. Of the six magnitude estimation comparisons, five significant preferences resulted. Three of the six 9-point hedonic comparisons were significant. The magnitude estimation procedure appears to have been more sensitive to differences in preference for the home panel.

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Table 1—Consumer laboratory panel average preference scores of whiskey sour mix variations tested by use of magnitude estimation and the 9-point hedonic scale

Pair tested[a]	Magnitude estimation Standard (A) (mean)	Other (O) (mean)	A/O (ratio)	n	Significance Level	Hedonic[b] Standard (A) (mean)	Other (O) (mean)	A-O	n	Significance Level
AB^1	1.51	1.50	1.00	14	–	5.3	5.2	0.1	19	–
AB^2	1.05	1.01	1.04	11	–	4.4	4.5	–0.1	18	–
AC^1	1.22	0.91	1.34	24	0.01	6.4	5.6	0.8	19	–
AC^2	1.18	1.02	1.11	11	–	4.5	5.0	–0.5	18	–
AD^1	2.49	0.87	2.86	23	–	6.2	4.0	2.1	22	0.01
AD^2	1.80	0.64	2.82	17	0.01	4.4	3.8	0.6	16	–
AE^1	1.75	1.05	1.63	16	–	5.8	5.7	0.1	22	–
AE^2	1.41	0.88	1.60	16	0.01	5.4	5.0	0.4	14	–
AF^1	0.83	1.91	0.43	23	0.05	5.5	5.4	0.1	20	–
AF^2	1.19	1.42	0.83	21	–	5.7	6.1	–0.4	19	–
AG^1	1.10	1.28	0.85	20	–	4.7	6.2	–1.5	19	0.05
AG^2	0.87	2.30	0.38	16	–	4.9	5.7	–0.8	21	–

[a] A = standard (48° proof) whiskey sour formula; B = no cloud; C = 50% reduction in lemon flavor; D = 50% reduction in sugar; E = 50% reduction in citric acid; F = 25% dilution of standard; G = 35° proof.
[b] 9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely.

The analysis of variance used to analyze the home panel data also allowed a test of panel variability, within-panelist variability, and mix-panel interaction. More instances of statistically significant panel variability were found using the 9-point hedonic method (Table 2). This result could be due to the normalization procedure applied to the magnitude estimation data prior to analysis.

Significant panel variability is a common finding in scalar tests because panelists use different parts of the scale. In the magnitude estimation analysis, every occurrence of significant panel variability was accompanied by significant mix-panel variability. The significant mix-panel interaction indicated that the panelists were rating the mixes in a different manner. Some preferred one, some the other, a common occurrence in preference testing. In only one case (hedonic AF comparison) was there a statistically significant within panelist variability (variation within a panelist from one replication to another). This suggests that the panelists responded differently to the sample over the two presentations.

Comparison of preference panels

It must be understood that a comparison of the two panels is justified only within the context of this particular test. The study was not designed to study in-lab vs in-home testing, but to study the difference between results from laboratory preference tests run under less than perfect conditions, i.e., varying numbers of judges, vs a more well-balanced home panel where numbers were static but also were experimentor control was minimized. Many food companies rely on in-house testing for "direction," and often use small numbers of biased observations, however questionable the practice.

It is obvious from the results that more significantly different preferences were expressed by the home panel [66% (8/12) for the home panel vs 25% (6/24) significant differences in preference for the consumer laboratory panel]. The following points probably contributed to this result.

1. **Motivation.** All 50 home panelists were eager to participate in the 24-day study. Consumer laboratory panel members were often distracted from their work in order to participate.

2. **Information.** The home panel was better informed on the scope of the project. For instance, they knew the panel lasted for 24 consecutive days and that it was necessary not only to complete the series but to maintain the schedule prescribed for them.

3. **Experienced consumers.** Although the panelists on both the consumer laboratory panel and the home panel were consumers of whiskey sours and alcoholic beverages in general, the home panel consisted of more experienced consumers. Many young students participated in the consumer laboratory panel. Most young students drink beer, rather than mixed drinks. Some were foreign students, and came from countries where whiskey sours were not commonly served and the drink was relatively new to them. The home panelists were generally older and were more familiar with whiskey sours.

4. **Test situation.** Normally it is assumed that most successful preference tests are carried out in a normal consuming atmosphere. The home panelists prepared the whiskey sours in the manner to which they were accustomed. The samples were served in familiar glasses and were probably consumed in a more comfortable and relaxed atmosphere.

5. **Time of day.** Although the consumer laboratory panel was conducted in the late afternoon, most of the panelists had not completed their day's work by panel time. The home panel tested the samples on their own schedule.

6. The larger "n" for the home panel may have been a factor.

Results from this study differ from those of Moskowitz and Sidel (1971). They concluded that the magnitude estimation and 9-point hedonic techniques were equally sensitive. They conducted only nine experiments while results from this study were generated from many more tests.

Magnitude estimation results are in the form of a ratio between two samples. The numerical value of one sample only has meaning when it is in a ratio with the sample to which it was compared. In the 9-point hedonic tests, each sample is assigned a value which corresponds to a scale point. The average hedonic score of a sample is influenced by the other samples tested at the same time. For example, scores of the standard sample in the consumer laboratory panel study (Table 1) ranged from a low of 4.4 (AB^2) to a high of 6.4 (AC^1). In fact, variation in standard score within the same comparisons sample AC, ranged from 4.5 (AC^2) to 6.4 (AC^1). Therefore, hedonic terms associated with scale points can be very misleading. Still it does give one some idea of how acceptable a sample is by its relative placement on the scale.

It would be unfair to judge the hedonic test by the consumer laboratory panel results because that panel was not as sensitive as the home panel. Hedonic values for the standard sample from the home panel (Table 2) were very similar ranging from 5.1 (AG) to 5.7 (AD). The comparisons which generated these values demonstrate one of the biases which influence the hedonic test. A superior sample tends to make an inferior sample appear to be more inferior than it actually is, and an inferior sample tends to make a superior sample appear more superior than it is. Sample D received the lowest of all hedonic scores while sample A served at the same time received the highest score. In the comparison AG, G received the highest hedonic score of all formula adjusted samples, while at the same time the standard A sample received the lowest A score.

When magnitude estimation was used, no category scale value resulted because no structured scale was used (however, comparisons of scale values with magnitude estimation values are possible (Moskowitz, 1977). Therefore, comparing values of the standard (A) resulting from the magnitude home panel study is meaningless exercise. Each value of A has meaning only when compared to the corresponding test sample. And this comparison must be accomplished by forming a ratio between the two scores. One might ask what the ratio means. In preference testing, the obvious need is to determine what ratio is necessary to influence acceptability, use, or purchasing practice. The ratio alone does not give enough information, just as the hedonic averages do not give enough information. The ratio may differ depending on the number of panelists and type of product being tested.

Effect of whiskey sour formulae adjustments on preference ratings

The whiskey sour formula was originally adjusted for two purposes. The main purpose was to produce large enough differences to allow testing of the two sensory methods by both a descriptive intensity profile (McDaniel and Sawyer, 1980) and by preference panels. It was also possible to evaluate the acceptability of the standard whiskey sour formula vs the formula adjusted samples.

The absence of cloud in the whiskey sour did not seem to influence the consumer lab panel rating for either the magnitude estimation or the hedonic method of testing. A significant preference for the standard sample was noted by the home panel using magnitude estimation, but not when using the 9-point hedonic scale. Home panel variability was very high ($p < 0.01$) in the hedonic test.

Table 2—Home panel average preference scores of whiskey sour mix variations tested by use of magnitude estimation and the 9-point hedonic test methods, and significance levels of other statistically significant variables in the ANOVA. (n = 50)

Samples[a]	Magnitude Values				Hedonic Values[b]				Statistical significance variables other than mix in ANOVA[c]					
									Magnitude			Hedonic		
	Standard (A) (mean)	Other (O) (mean)	A/O (ratio)	Significance Level	Standard (A) (mean)	Other (O) (mean)	A-O Diff.	Significance Level	P	S:P	MP	P	S:P	MP
AB	1.46	0.97	1.51	*	5.7	5.54	0.2					**		
AC	1.33	0.96	1.39	*	5.6	5.8	−0.2	**			**	**		
AD	1.88	0.77	2.44	*	5.7	4.2	1.5					**		
AE	1.31	0.99	1.32	*	5.6	5.0	0.6	*	*		**	**		
AF	1.10	1.12	0.98		5.2	5.7	−0.5				**	**	**	**
AG	0.96	1.41	0.68	*	5.1	6.4	−1.3	**				**		

[a] A = standard (48° proof) whiskey sour formula; B = no cloud; C = 50% reduction in lemon flavor; D = 50% reduction in sugar; E = 50% reduction in citric acid; F = 25% dilution of standard; G = 35% proof.
[b] 9 = like extremely; 5 = neither like nor dislike; 1 = dislike extremely.
[c] Other ANOVA variables include panelists (P), within panelists (S:P), and mix-panelist interaction (MP).
*$p < 0.05$
**$p < 0.01$

The standard sample (A) was preferred to a significant degree over the lemon reduced sample (C) in one of the consumer lab panel tests and in one of the home panel tests. The standard sample (A) was also preferred to a statistically significant degree over the sugar reduced sample (D), in two of the consumer laboratory panel tests and in both of the home panel tests. The standard sample was preferred to a statistically significant degree over the citric acid reduced sample (E) in one of the consumer laboratory panel tests and both of the home panel tests. The statistically significant preference for the standard sample in the sugar reduced/standard comparisons was particularly significant ($p < 0.01$) showing the importance of an adequate sweetness level in the whiskey sour mix.

Dilution of the standard sample by 25% was included as one of the formula adjustments because preliminary panels had complained of the intensity of the standard mix. In the only test that was statistically significant ($p < 0.05$) the diluted sample was preferred. In all the comparisons but one, the score of the diluted sample was higher. The evaluations by the consumer laboratory panel were carried out neat, no ice was served. In normal consumption such as was practiced in the home panel, serving the standard with ice would produce a natural dilution of the flavor intensity to a more desired level. Seventy-two percent of the home panel reported that they prepared whiskey sours in their home with ice and ordered their whiskey sours served with ice when consuming them outside of their home.

The 35° proof sample was preferred over the 48° proof standard by the home panel using both testing methods, and by the consumer laboratory panel in one of the 9-point hedonic tests. All of the consumer laboratory panel average values were higher for the 35° proof sample, although not to a statistically significant degree. This indicates that the standard could be decreased in alcohol content to achieve increased flavor acceptability.

SUMMARY & CONCLUSIONS

CONSUMER PREFERENCES for different whiskey sour formulations were determined using two different sensory techniques by a consumer laboratory panel and a home panel. Use of the magnitude estimation testing procedure as compared to the 9-point hedonic resulted in more statistically significant differences in preference in both the home panel and the consumer laboratory panel. On this basis magnitude estimation appears to be a more sensitive preference testing instrument.

Many more significant differences in preference were expressed by the home panel that by the consumer lab panel. This was probably due to the fact that the home panel was larger, better motivated, knew more about the scope of the project, and was testing the product under more desirable conditions than was the consumer laboratory panel. The members of the home panel were probably more experienced consumers of alcoholic beverages due to age, background, and alcoholic beverage consumption habits.

The standard whiskey sour formula scored very well in the preference tests in comparison to the formula adjusted samples. In only two of the comparisons were the adjusted samples preferred over the standard, the 25% dilution of the standard and the 35° proof samples.

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Changes in Pectic Substances and Enzymes during Ripening and Storage of "Keitt" Mangos

BONGWOO ROE and JOSEPH H. BRUEMMER

ABSTRACT

The relationship of the distribution of pectic substances to tissue softening was examined in ripening mangos at four stages of ripeness. Water-soluble and alkali-soluble pectin declined and ammonium oxalate soluble pectin increased as the mango lost its firmness and became soft. Polygalacturonase and cellulase activities of cell wall preparations increased markedly during ripening. The decline in alkali-soluble pectin and the increase in polygalacturonase activity correlated well with the loss of firmness. Alkali-soluble pectin declined slowly in ripe mangos stored at 4°C. This decline correlated with loss of firmness of the stored mangos. The cellulase activity of cell wall preparations from ripe mangos increased during 4°C storage and the increase correlated with the decrease in firmness.

INTRODUCTION

THE MANGO (*Mangifera Indica*, L) is second in importance to the banana in production of tropical fruits. Almost 10 million metric tons of mangos are produced annually and most of the fruit is consumed locally as fresh fruit where it is produced (Subramanyam et al., 1975. The fruit is a rich source of vitamins A and C for the less developed countries of India, Pakistan, and the Phillipines where most of the mangos are grown (Subramanyam et al., 1975). The fruit has not gained much popularity in the United States or in the highly developed countries of Europe. Our domestic demand is satisfied by production from about 1500 acres of groves in South Florida and by imports from Mexico and the Caribbean region (McGuire, 1976).

Ripening of the mango fruit is characterized by softening of the flesh. The peak of ripeness is associated with a fairly narrow range of firmness. In peaches (Shewfelt, 1965; Shewfelt et al., 1971), pears (McCready and McComb, 1954) and apples (Bartley, 1976) softening during ripening is accompanied by solubilization of pectin. The enzyme, polygalacturonase has been detected in ripe peaches (Pressey et al., 1971) and pears (McCready and McComb, 1954) but not in unripe fruit. Data are not available on changes in pectic substances in mangos during ripening but softening of mango is assumed to be due to release of pectin depolymerizing enzymes (Azin and Nour, 1978).

Mangos would be a welcome addition to the American diet as a source of vitamins A and C. However, general acceptance of fresh or processed mangos will be slow because the fruit is marketed as firm-unripe, and only consumers experienced with the rich flavor of ripe fruit will buy mangos for home ripening. Marketing firm-ripe fruit requires more knowledge of the ripening process and how to control it. To overcome this information problem, we selected one of the commercial mango varieties and examined the relationship between loss of firmness and changes in cell wall components. We found that ripening brings about dramatic changes in the distribution of pectic substances and showed that changes in ripe fruit are slowed but not suppressed by refrigeration. Our results are presented in this report.

Authors Roe and Bruemmer are with the USDA-SEA Southern Region, U.S. Citrus & Subtropical Products Lab., P.O. Box 1909, Winter Haven, FL 33880.

MATERIALS & METHODS

KEITT MANGOS were obtained from Mitchell Groves, Miami, Florida. Chloramphenicol and cycloheximide were purchased from Sigma Chemical Co., St. Louis, Mo., and other chemicals from Fisher Scientific Company, Fair Lawn, N.J.

Mangos were picked mature green and allowed to ripen at 21°C. Ripeness was based on tissue resistance to measured pressure. On the flat fleshy side of each fruit 6.5 cm^2 of skin were removed, and the Magness-Taylor pressure tester (Magness and Taylor, 1925) equipped with a 0.8 cm diameter plunger was applied to the exposed tissue. Fruit selected for ripening experiments were separated into 4 groups of 5 fruit each based on pressure tester readings: 10 kg, 7-10 kg, 5-7 kg, 3-5 kg, and 1-3 kg. Fruit were selected as ripe for storage experiments when the plunger penetrated the tissue with less than 1.5 kg of pressure on the tester.

Ripening experiment

The 4 groups of 5 mangos each were peeled and quartered. Slices (15 mm x 8 mm^2) were cut from each quarter and assayed for shear resistance. The remaining mango quarters were frozen in liquid N_2. The frozen mangos in each group were separately blended to a fine powder in a high-speed blender. The mango powder was then thawed to form a puree that was used for analyses of alcohol-insoluble solids (AIS), pectic substances and the enzymes cellulase, pectinesterase, and polygalacturonase.

Storage experiment

Nine mangos were selected as ripe. They were peeled to prevent loss of fruit sample from decay mediated through the skin. They were quartered so that each mango would provide samples for all storage periods. They were placed in individual sterile polyethylene bags (3 mil) and stored at 4°C. One-quarter from each of 3 mangos was withdrawn from storage after 0, 2, 4, and 6 wk and evaluated for quality by a panel of tasters. Quarters from the other 6 mangos were withdrawn in the same sequence; slices were taken for assay of shear resistance and the remaining portion of each quarter was separately frozen in liquid N_2 and stored at −70°C. After storage, each frozen quarter section was blended to a fine powder in a high speed blender. The mango powder was thawed to form a puree for analyses of AIS, pectic substances, and the enzymes cellulase, pectinesterase, and polygalacturonase.

Quality evaluation

A panel of 18 untrained tasters, selected from members of the Citrus and Subtropical Products Laboratory, evaluated the flavor and texture of 2-cm^3 portions of mango using a hedonic scale of 1 = like extremely, 9 = dislike extremely. Presentation of samples to the panel followed the recommendation of Larmond (1967).

Shear resistance

Tissue firmness was estimated from shear resistance measurements obtained with an Allo Kramer Press, Model SP-12IMP (Allo Precision Metals Engineering, Inc., Rockville, Md.). Slices of mango tissue (8 mm x 85 mm^2) were cut from each quarter section of test mango and placed in the 65 cm^2 cell box. The plunger stroke was adjusted to a control speed at 3.8 cm/min. Tissue resistance in pounds of force was measured and recorded with an electronic transducer ring with a range of 0-10 lb. All values were expressed as lb per 32 mm thick sample (4 slides).

1—Pectin distribution in ripening mangos[a]

Resistance to shearing force (lb)[b]	AIS (% of dry pulp)	Pectin (% of AIS) Water soluble	Ammonium oxalate soluble	Alkali soluble	Total
33 ± 3	60 ± 2	35.8 ± 0.6a	3.1 ± 0.1a	10.4 ± 0.4a	49.3
22 ± 4	53 ± 1	34.1 ± 0.3b	3.3 ± 0.1b	9.7 ± 0.1b	47.1
14 ± 1	44 ± 2	31.7 ± 0.4c	3.5 ± 0.2c	5.8 ± 0.1c	41.0
4.5 ± 0.2	18 ± 1	29.4 ± 0.2d	4.4 ± 0.1d	4.6 ± 0.1d	38.4

Regression analysis of pectin means vs resistance:

Water-soluble pectin	$r^2 = 0.85$	$y = 29.16 + 0.59x$
Ammonium oxalate-soluble pectin	$r^2 = 0.82$	$y = 4.50 - 0.14x$
Alkali-soluble pectin	$r^2 = 0.91$	$y = 3.35 + 0.64x$

[a] Values are means for 5 fruit ± SD. Means in each column followed by a common letter are not different at the 0.05 level (analysis of variance).
[b] Values are mean resistance forces for 4 slices each from 5 mangos.

Table 2—Enzyme activity in ripening mangos[a]

Resistance to shearing force (lb)[b]	Enzyme activity (units/100g tissue) Polygalacturonase	Pectinesterase	Cellulase
33 ± 3	0.51 ± 0.2a	205 ± 30a	0.7 ± 0.2a
22 ± 4	10.8 ± 0.5b	135 ± 24b	17.9 ± 1.8b
14 ± 1	18.1 ± 1.0c	130 ± 28b	20.3 ± 1.5bc
4.5 ± 0.6	24.3 ± 1.2d	125 ± 22b	24.3 ± 2.3c

Regression analysis of enzyme activity means vs resistance:

Polygalacturonase	$r^2 = 0.99$	$y = 28.6 - 0.83x$
Pectinesterase	$r^2 = 0.76$	$y = 98.3 + 2.73x$
Cellulase	$r^2 = 0.86$	$y = 30.5 - 0.08x$

[a] Values are means for 5 fruit ± SD. Means in each column followed by a common letter are not different at the 0.05 level (analysis of variance).
[b] Values are mean resistance forces for 4 slices each from 5 mangos.

Alcohol-insoluble solids

Alcohol-insoluble solids (AIS) were prepared by the method of Shewfelt (1965). Forty grams of pureed mango were added to 200 ml of boiling ethanol and boiled for 15 min. After it was cooled to room temperature the mixture was filtered through a Buchner funnel. The residue, (i.e., the alcohol-insoluble solids) was washed with ethanol, then with acetone, and dried to constant weight in a vacuum oven at 45°C.

Pectic substances

The AIS preparations were separated into three types of pectic substances by successive extractions with distilled water, 0.75% ammonium oxalate, and 0.05N sodium hydroxide (Rouse and Atkins, 1955). Each type was analyzed by the colorimetric reaction between carbazole and the anhydrogalacturonic acids of the pectin (Dische, 1947) as described by Rouse and Atkins (1955).

Polygalacturonase assay

Mango puree (50g) was homogenized with 50 ml of cold solution of 12% polyethyleneglycol (Carbowax 6000) and 0.2% sodium bisulfite (pH 5) in water for 1 min in a Lourdes homogenizer. The suspension was centrifuged at 7000 x G for 20 min for recovery of the residue. The residue was washed twice by homogenization in 100 ml of cold, distilled water followed by centrifugation. The residue was suspended in 50 ml of 0.5M NaCl. This suspension constitutes the enzyme preparation for assay of polygalacturonase, pectinesterase, and cellulase.

Polygalacturonase activity was measured by a modification of a procedure of Pressey et al. (1971). Two milliliters of the mango residue in 0.5M NaCl, 0.8 ml of 0.2M sodium acetate buffer (pH 4), and 2 ml of 1% sodium polygalacturonate (pH 4) were placed in a 50-ml Erlenmyer flask. An identical sample heated in boiling water for 3 min served as a blank. Chloramphenicol and cycloheximide (100 μg each) were added to both flasks to inhibit growth of microorganisms. The samples were incubated at 30°C in a shaking incubator. After 17 hr the samples were inactivated in boiling water for 3 min, cooled and centrifuged. The supernatants were analyzed for reducing groups by the arsenomolybdate method of Nelson (1944). A unit of polygalacturonase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmole of reducing groups in 17 hr at 30°C. The pH for optimum solubilizing activity of the mango enzyme on protopectin recovered from the alkali-soluble fraction of mango AIS by ethanol precipitation was determined by adjusting substrate and buffer solution to pH 3, 3.5, 4, 4.5, and 6.

Pectinesterase assay

Ten milliliters of the mango enzyme preparation were assayed for pectinesterase by the method of Rouse and Atkins (1955). The unit of activity was defined as the amount of enzyme that hydrolyzes 1 equivalent of ester bonds per minute at 40°C.

Cellulase assay

One milliliter of the mango enzyme preparatin was added to 3 ml of 0.5% sodium carboxymethylcellulose and 1 ml of 0.1M sodium citrate-phosphate buffer (pH 6.0). An identical sample heated in boiling water for 3 min served as a blank. One-tenth milliliter of a solution of chloramphenicol and cycloheximide (1 mg each/ml) was added to each reaction mixture. The samples were incubated at 30°C for 17 hr, then inactivated in a boiling water bath for 3 min. After the samples were cooled and centrifuged, 1 ml of supernatant was withdrawn and analyzed for reducing sugar by the method of Nelson (1944). One unit of cellulase activity was defined as the amount of enzyme that catalyzes the formation of 1 μmole reducing groups in 17 hr at 30°C. The pH for optimum mango cellulase activity was determined by adjusting substrate and buffer solutions to pH 4 through pH 7.

RESULTS & DISCUSSION

SOFTENING of mango flesh as indicated by the decline in resistance to shearing force was accompanied by a rapid decline in the amount of AIS recovered from the fruit (Table 1). From a high value of 60% in mature fruit, AIS decreased to only 18% of the dry pulp in ripe mangos. Most of this decrease can be accounted for by the conversion of AIS starch to soluble sugar. Subramanyam et al. (1972) showed that as mangos ripened the AIS declined rapidly with a concomitant increase in sugar and total soluble solids. However, the decrease in AIS also reflects the conversion of pectic substances to alcohol soluble compounds, because the total pectin content declined from 49.3% to 38.4% of the AIS. The distribution of pectic substances in AIS also changed during ripening. Both the water and alkali-soluble fraction decreased and the ammonium oxalate fraction increased slightly (Table 1). The progressive decline in the alkali-soluble fraction showed a better correlation (0.91) than the other pectin fractions with loss of resistance to shearing force (Table 1). Since the alkali-soluble pectin is structurally bound to the cell wall, these relationships suggest that flesh softening in the ripening process results from disintegration of the cell wall

Table 3–Pectin distribution in ripe mangos stored at 4°C[a]

Storage (wks)	Resistance to shearing force (lb)[b]	AIS (% of dry pulp)	Pectin (% of AIS)			
			Water soluble	Ammonium oxalate soluble	Alkali soluble	Total
0	4.8 ± 1.5	11 ± 1	27.5 ± 2.4a	5.0 ± 1.6a	5.0 ± 0.4a	37.5
2	3.6 ± 0.8	10 ± 1	23.1 ± 4.6b	4.8 ± 0.9a	4.1 ± 0.7b	31.9
4	2.1 ± 0.6	8 ± 1	21.7 ± 3.9b	5.5 ± 1.8a	3.0 ± 0.6c	30.2
6	1.8 ± 0.4	9 ± 1	23.3 ± 4.0b	5.2 ± 1.3a	3.3 ± 0.5c	31.8

Regression analysis of pectin means vs resistance:

Water-soluble pectin	$r^2 = 0.69$	$y = 19.31 + 1.49x$
Ammonium oxalate-soluble pectin	$r^2 = 0.46$	$y = 5.57 - 0.15x$
Alkali-soluble pectin	$r^2 = 0.95$	$y = 1.99 + 0.63x$

[a] Values are means for 6 fruit ± SD. Means in each column followed by a common letter are not different at the 0.05 level (analysis of variance).
[b] Values are mean resistance forces for 4 slices each from 6 mangos.

caused by degradation of the structurally bound pectin.

The distribution of pectic substances in the ripening mango differs from that reported in the peach. The AIS content of ripening peaches did not change but the water-soluble pectin fraction of the AIS increased and the alkali-soluble pectin fraction decreased (Shewfelt, 1965; Pressey et al., 1971). In addition the average molecular weight of the peach water-soluble pectin decreased markedly as the peach ripened and more pectin was solubilized (Shewfelt et al., 1971; Pressey et al., 1971). Thus, conversion of protopectin (alkali-soluble) to water-soluble pectin in the peach is accompanied by polymer degradation but not to the extent observed in the mango. Degradation of mango pectin apparently proceeded to the stage that the product molecules were small enough to be soluble in ethanol and were not precipitated in the AIS.

Enzymes in ripening mangos

Polygalacturonase and cellulase activities increased as the flesh softened in the ripening mango (Table 2). Activities were barely detectable in the firm unripe fruit. The pectinesterase activity declined during the first stage of ripening but then leveled off. The cellulase activity of the mango enzyme preparation was optimum at pH 6.0 similar to the pH (6.5) optimum for peach cellulase enzyme (Hinton and Pressey, 1974). Solubilizing activity of the enzyme preparation using mango protopectin as substrate was optimum at pH 4.0 and therefore the mango enzyme is similar to the endopolygalacturonase isolated from peaches by Pressey and Avants (1973).

Polygalacturonase and cellulase are probably the degrading enzymes responsible for softening of mangos during ripening. In peaches ripening and tissue softening were related to a marked increase in cellulase and polygalacturonase activity (Pressey et al., 1971; Hinton and Pressey, 1974). The decline in pectin fraction of the AIS probably results from polygalacturonase activity for this enzyme hydrolyzes pectin to alcohol-soluble compounds.

The progressive increase in polygalacturonase activity showed a better correlation ($r^2 = 0.99$) than the cellulase activity (0.86) with loss of resistance to shearing force (Table 2). This relationship supports the view that mango softening results from degradation and depolymerization of cell-wall pectin to alcohol-soluble products.

Pectin distribution in ripe mangos stored at 4°C

Six weeks storage of ripe mangos resulted in a progressive loss of firmness as measured by resistance to shearing force (Table 3). The AIS content of the dry pulp was relatively constant, but distribution of pectin in AIS showed that 4°C did not completely inhibit the degradation of pectin initiated by ripening (Table 3). During the initial 2 wk of storage both the water-and alkali-soluble pectin fractions declined without a change in the ammonium oxalate-soluble fraction. As a result the total pectin fraction also declined, continuing the trend toward smaller pectin fractions in AIS. After 2 wk storage the water-soluble and ammonium oxalate-soluble pectin fractions stabilized against further change. After 4 wk storage the alkali-soluble fraction was stabilized at 3% of the AIS. Decrease in alkali-soluble pectin showed a correlation ($r^2 = 0.95$) with the progressive change in resistance of the mango flesh to shearing force. Because the alkali-soluble pectin is identified with the cell wall-bound pectin, the correlation supports the earlier suggestion that flesh softening results from the degradation and depolymerization of cell-wall pectin.

Table 4–Enzyme activity in ripe mangos stored at 4°C[a]

Storage (wks)	Resistance to shearing force (lb)[b]	Enzyme activity (units/100 g tissue)		
		Polygalacturonase	Pectinesterase	Cellulase
0	4.8 ± 1.5	24 ± 1a	124 ± 12a	24 ± 5a
2	3.6 ± 0.8	28 ± 2ab	107 ± 22ab	34 ± 6b
4	2.1 ± 0.6	29 ± 1b	98 ± 18b	46 ± 2c
6	1.8 ± 0.4	32 ± 2c	87 ± 14b	49 ± 3c

Regression analysis of enzyme activity means vs resistance:

Polygalacturonase	$r^2 = .88$	$y = 35.11 - 2.33x$
Pectinesterase	$r^2 = .95$	$y = 70.44 + 10.91x$
Cellulase	$r^2 = 1.0$	$y = 63.62 - 8.25x$

[a] Values are means for 5 fruit ± SD. Means in each column followed by common letter are not different at the 0.05 level (analysis of variance).
[b] Values are mean resistance forces for 4 slices each from 6 mangos.

Table 5–Acceptability of ripe mangos stored at 4°C

Storage (wk)	Resistance to shearing force (lb)[a]	Mean test panel scores[b]	
		Flavor	Texture
0	4.8 ± 1.5	2.8 ± 0.1a	2.2 ± 0.1a
2	3.6 ± 0.8	2.9 ± 0.2a	3.6 ± 0.4bc
4	2.1 ± 0.6	3.1 ± 0.2a	4.2 ± 0.4c
6	1.8 ± 0.4	3.3 ± 0.6a	4.1 ± 1.0c

Regression analysis of panel scores means vs resistance:

Flavor	$r^2 = 0.90$	$y = 3.49 + 0.15x$
Texture	$r^2 = 0.89$	$y = 5.44 + 0.37x$

[a] Values are mean resistance forces for 4 slices each from 6 mangos.
[b] Values are mean scores for 3 mangos, each evaluated by 18 tasters. Means in each column followed by common letter are not different at the 0.05 level (analysis of variance).

The data in Table 3 indicates that between 2-4 wk was required to stabilize the fruit at 4°C against further changes in firmness and pectin distribution. Lowering the temperature to 0°C or near the freezing point for mango tissue might improve storage stability. However, specific control of pectin degradation may be a more promising approach because tissue softening could be controlled during both ripening and storage.

Enzyme activity in ripe mangos stored at 4°C

Enzyme levels showed modest changes in ripe mangos during storage (Table 4). Polygalacturonase and cellulase activities increased and pectinesterase activity decreased over the 6 wk storage period. Cellulase activity increase showed a higher correlation (r^2 = 1.0) than polygalacturonase activity with loss of firmness (resistance to shearing force). The role of cellulase in tissue softening of ripe mangos in storage should be examined by identifying the native substrates and products of the cellulase reactions in the mango. The loss in pectinesterase activity during tissue softening at 4°C is probably not enzyme inactivation. Activity was assayed with cell-wall preparations as enzyme source. Softening of the flesh probably released enough cations to solubilize some of the pectinesterase during preparation of the cell walls. Pectinesterase was shown to be released from cell wall preparations by cations (Jansen et al., 1960).

Acceptability of ripe mangos stored at 4°C

Test panel members did not find the acceptability of mangos (3.3) stored for 6 wk at 4°C much less than that of fresh mangos (2.8). Scores were made with a hedonic scale in which 1 = like extremely and 9 = dislike extremely (Table 5). However, the texture of the mango changed during storage. Mango quarters stored for 2 wk were scored 3.6, and fresh mango 2.2. Storage for 4 and 6 wk resulted in less acceptable scores. The taste panel evaluations confirm the chemical and physical data that mangos lose texture firmness during storage at 4°C. Panel score changes for both flavor and texture correlated with loss of resistance to shearing force.

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DESCRIPTIVE ANAL: MAG EST. VS CATEGORY SCALE . . . From page 181

the values themselves are of little practical comparative value. Because the magnitude estimation scale values have ratio properties, one can put the comparative sweetness on a psychophysical basis. If one wished to place the magnitude values on a category scale, simple procedures are available.

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Papaya Polygalacturonase and its Role in Thermally Injured Ripening Fruit

HARVEY T. CHAN, JR., STEVEN Y.T. TAM, and STANLEY T. SEO

ABSTRACT

The polygalacturonase (E.C. 3.2.1.15) (PGase) activity in papayas increased with fruit ripeness. PGase activity was highest in the placenta with the activity decreasing outwardly from the placenta to the exocarp. Extended hot water treatments (46°C for 65 and 90 min) caused delayed softening of the fruit tissue which was correlated to a decrease in PGase activity. The decrease in PGase activity after the heat treatments was more severe in the riper fruits (quarter and half ripe) than the less ripe fruits (colorbreak stage).

INTRODUCTION

THE SOFTENING of fruits during ripening has been attributed to the action of pectic enzymes on pectic substances in the cell walls. The enzyme polygalacturonase (PGase) (EC. 3.2.1.15) catalyzes the hydrolysis of glycosidic linkages of pectic substances resulting in fruit softening (Dilley, 1970). Fruit quality during the ripening stage of papaya for the fresh and processed markets is frequently determined by the softness of the fruit.

Papayas destined for export are treated with hot (48°C) water for 20 min to control storage decay and fumigated with ethylene dibromide for disinfection (Akamine and Arisumi, 1953). Current research on alternative quarantine treatments include an extended hot water treatment (46°C for 65 min) which disinfects fruit flies but injures the fruit. Prolonged heat treatment causes the fruits to ripen externally with full color development but remain immature internally, retaining a 1- to 1.5-cm-thick area of hard tissue surrounding the seed cavity (Akamine and Seo, 1978).

Of the pectic enzymes, only pectinesterase has been reported in papaya (Chang et al., 1965). Neither the presence of PGase in papayas nor its role in the softening of these fruits has been reported. In this study, we have examined the effect of excessive heat treatments on ripening and PGase activity of papaya fruit. The isolation and characterization of PGase from papaya fruit are to be detailed in another publication (Chan and Tam, 1980).

MATERIALS & METHODS

Preparation of fruit

Papaya (*Carica papaya* L.), Solo variety from a commercial source on the island of Hawaii, were air-shipped on the day of harvest to the USDA-SEA-AR Laboratory in Honolulu. The fruits were sorted according to the amount of yellow coloration on their surface into four stages of ripeness (Akamine and Goo, 1971), colorbreak, quarters, half, and full ripeness.

Extraction and assay of papaya polygalacturonase

Fresh papayas were sliced, their seeds discarded, and the placenta removed and placed into a chilled beaker. The placenta tissue (100g) was blended with 400 ml of cold acetone (−18°C) in a Waring Blendor for 1 min, and the homogenate was filtered *in vacuo* on Schleicher and Schuell 598 filter paper. The residue was extracted twice more by the above procedure and filtered to near dryness on the last extraction. The residue was further dried under nitrogen and stored at −18°C. This yielded 5.3g of acetone powder per 100g of fresh placenta.

PGase activity was measured viscometrically (Arakji, 1968). The reaction mixture consisted of 2.0 ml of enzyme solution in citrate-NaCl buffer (pH 4.6) and 10 ml of 1.2% pectin (Sigma Chemical Co.) in 0.2M citrate buffer (pH 4.6). Citrate-NaCl buffer (0.2M citrate + 1.0M NaCl, pH 4.6) was used throughout the study except where otherwise noted.

The pectin was added to the viscometer (Canon 300) and allowed to equilibrate to the bath temperature (45°C). The enzyme at 45°C was added rapidly to the pectin and mixed by bubbling air through the mixture. Differences in viscosity were determined from measurements of the flow times of the reaction mixture at initial time (t_0) and at 15-min intervals. One unit of PGase activity is defined as 1% loss in viscosity per hour. The results are also expressed as % change in PGase activity to reflect the comparative change in activity from the initial activity.

Effect of maturity on PGase activity at harvest

To measure the effect of maturation on PGase activity, we prepared the acetone powder from the placenta of green, half, and fully ripe papayas. The enzymes were extracted from 5.0g acetone powder by the above procedure with 100 ml of low or high ionic strength buffers. Low ionic strength buffer was made by 1:3 aqueous dilution of 0.2M McIlvaine's buffer (pH 4.6) to 50 mM. High ionic strength buffer was made by the addition of 1.0M NaCl to 0.2M McIlvaine's buffer (pH 4.6). Enzyme activity was assayed as described above.

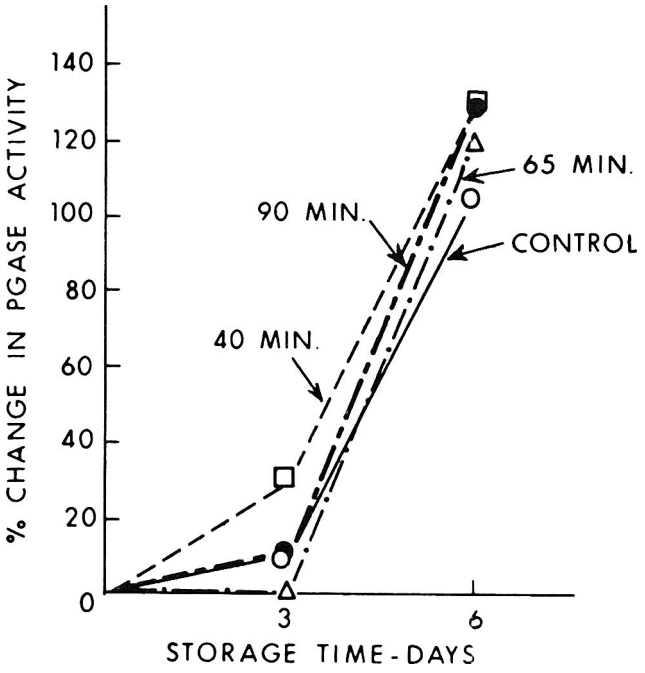


Fig. 1—Effect of length of hot water treatment (46°C) on PGase activity of colorbreak papayas after 3 and 6 days of storage compared to initial activity, 0 days.

Authors Chan, Tam, and Seo are affiliated with the USDA-SEA-AR, P.O. Box 917, Hilo, HI 96720.

Induction of thermal injury

Five hundred forty papayas were segregated into 30 equal lots of increasing ripeness, i.e., colorbreak, quarter, and half ripe. Each lot was heated in water at 46°C for 0 (control), 40, 65, or 90 min and cooled with water to ambient temperature. Polygalacturonase extracts were made of the treated and control papaya placentae, as earlier described, on the day of treatment (day 0) and after 3 and 6 days ripening at 24°C.

RESULTS & DISCUSSION

Effect of maturity, storage, and location on PGase activity

In freshly harvested green, half ripe, and fully ripe fruits, PGase activity increased with ripeness (Table 1). There was about a tenfold difference in PGase activity between green and fully ripe papayas. When PGase was extracted from green, half ripe, and fully ripe fruits with both low and high ionic strength buffers, we found no differences in the relative proportions of activity for the two buffers, even though enzyme activity increased with ripeness. Originally, we thought that this experiment could provide insight into whether the PGase was bound or unbound and also determine if the bound enzymes became solubilized during ripening. However, the relative proportions of PGase activity for low and high ionic strength extraction remained nearly constant even though the total PGase activity increased with ripeness. The differences in PGase activity for the two ionic strengths could be due to cation activation as reported by Pressey and Avants (1971) and Lui and Luh (1978).

Ripening papayas soften gradually beginning with the portion next to the seed cavity. When we measured the PGase activity in the exocarp, measocarp, and placental portions of ripe papayas, we found 27.1, 51.6, and 239.6 units of activity/ml of extract, respectively. The gradation of decreasing PGase activity outward from the placenta to the mesocarp and to the exocarp coincides with the direction of ripening in papayas, that is, the cavity outwards.

Table 1—Effect of fruit ripeness and ionic strength of buffer during extraction on PGase activity

Tissue	PGase[a]	
	Low ionic buffer	High ionic buffer
Green	3	15
0.5 ripe	40	135
Full ripe	80	220

[a]Specific activity units.

Effect of hot water treatment on PGase activity

The effects of hot water treatments on papaya PGase activity are shown in Figures 1, 2, and 3. The effects of duration of hot water treatment, fruit ripeness at treatment, and storage duration after treatment on PGase activity were each independent and significant at the 5% level. In addition, fruit ripeness and storage duration were also

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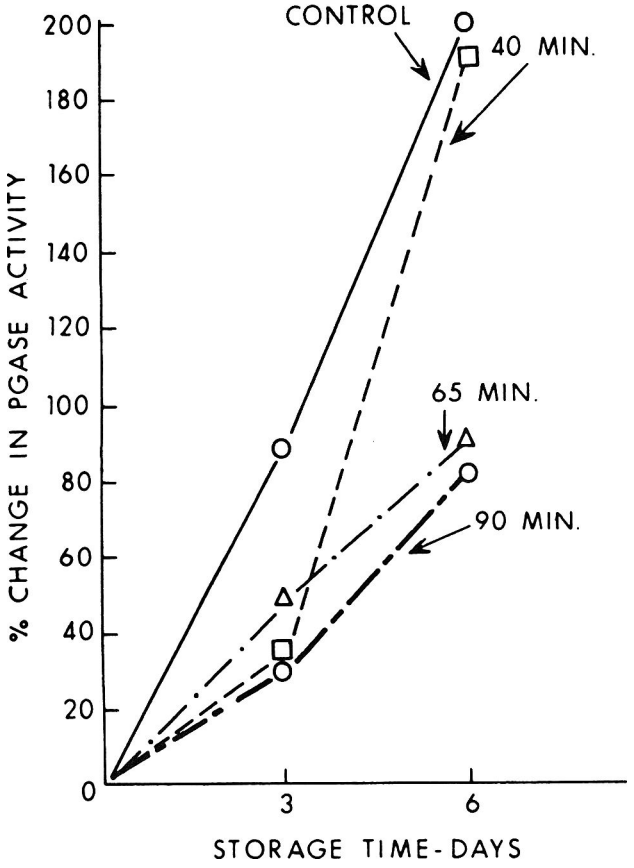


Fig. 2—Effect of length of hot water treatment (46°C) on PGase activity of quarter ripe papaya after 3 and 6 days of storage compared to initial activity, 0 days.

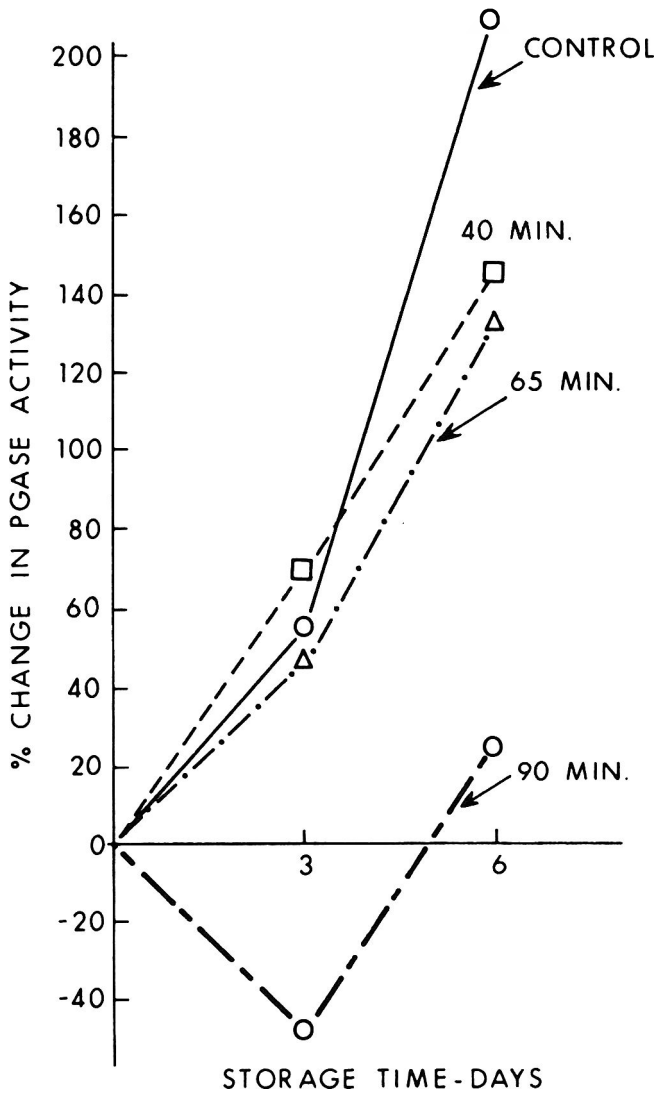


Fig. 3—Effect of length of hot water treatment (46°C) on PGase activity of half ripe papaya after 3 and 6 days of storage compared to initial activity, 0 days.

Development, Evaluation and Industrial Production of a Powdered Soy-Oats Infant Formula Using a Low-Cost Extruder

F. R. DEL VALLE, H. VILLANUEVA, J. REYES-GOVEA, M. ESCOBEDO, H. BOURGES, J. PONCE, and M. J. MUNOZ

ABSTRACT

A low cost powdered infant formula made from soybeans, oats and sucrose, fortified with methionine, vitamins and minerals, has been developed. A dry process for making the formula, which employs a low-cost Brady extruder, has also been developed. The resulting product is of good microbiological quality and disperses readily in water to give a milk-like suspension of satisfactory stability to settling. The essential amino acid pattern of the formula, its PER and NPU values, nitrogen balance data with infants, proximal chemical analysis, calorie distribution and low level of trypsin inhibitor indicate it to be adequate for feeding infants 3 months or older. Clinical trials with infants also showed that the formula was well accepted and tolerated, and produced adequate weight gain. Retail prices of the product, packed in cans and polyethylene bags, are appreciably lower than those of comparable cow's milk and other soya-based infant formulas available in the market. The product is currently being manufactured and sold in several parts of Mexico.

INTRODUCTION

THE USE OF full-fat soy flour in infant feeding has been amply demonstrated. DeMaeyer (1959) used full-fat soy flour in feeding 2- to 24-month old infants in Taiwan, and concluded that full-fat soy products were "practical and satisfactory options to consider with respect to cow's milk-based infant foods." More recently, Gassman et al. (1970) developed a formula consisting of full-fat soy flour supplemented with methionine, vitamins and minerals for milk and lactose intolerant children; test subjects included eight premature infants. The product was found to be well accepted by, and to produce adequate weight gains in all subjects, including those with diarrhea. In Mexico, Suberbie et al. (1972) developed and tested a formula consisting of full-fat soy flour, glucose, fructose and dextrins, fortified with methionine, vitamins and minerals. Again, satisfactory feeding results were obtained with infants of ages 2 months or older, including those with diarrhea. Finally, in Thailand, Tontisirin et al. (1977) tested an infant formula made from full-fat soy flour, rice flour and sucrose, fortified with methionine, vitamins and minerals. Good acceptance of the product was found in all cases, with no signs of intolerance such as diarrhea, nausea or flatulence. The product is currently being produced and sold in Thailand, and its use is expected to extend to other Asian countries including Malaysia, the Philippines and Singapore.

Author Del Valle, formerly with the Chihuahua Institute for Nutritional Research & Development, is now with the School of Chemistry of the Univ. of Chihuahua, P.O. Box 1545, Sucursal C, Chihuahua, Chih., Mexico. Author Villanueva is with the Chihuahua Children's Hospital, Chihuahua, Mexico. Author Reyes-Govea is with Pensiones Civiles del Estado, Chihuahua, Mexico. Author Escobedo is with the School of Medicine of the Univ. of Chihuahua, Chihuahua, Mexico. Author Bourges is with Instituto Nacional de la Nutrición, Mexico City, Mexico. Author Ponce is with Productos Alimenticios Delicias, Delicias, Chihuahua, Mexico. Author Muñoz is a student at the Master of nutrition & Food Science Program at INCAP, Guatemala City, Guatemala.

The State of Chihuahua in Mexico is a producer of substantial amounts of soybeans and oats, whose proteins are of relatively high nutritive value (FAO, 1970). Chihuahua also possesses a large rural and Tarahumara Indian population, who are known to be severely malnourished; the problem is especially acute with Tarahumara Indians, who are facing extinction as a result of diseases caused, directly or indirectly, by malnutrition. As might be expected, those most affected within this ethnic group are infants and children; infant mortality is extremely high, and a recent survey conducted at Chihuahua Children's Hospital revealed that fully 70% of all Tarahumara children admitted were suffering from tuberculosis, caused indirectly by malnutrition (Villanueva, 1979). Due to their own malnutrition, many Tarahumara mothers are incapable of breast-feeding their babies, and infant formulas available in the market are totally outside their economic reach.

In view of the foregoing, the purpose of this work was to develop and test a soy-oats infant formula which would be as inexpensive as possible, and which could be made available to low income groups in Chihuahua, and possibly other parts of Mexico. The work was divided into six parts: (1) Development of the formula; (2) development of an inexpensive process for making the product; (3) nutritional evaluation of the formula; (4) clinical trials; (5) cost evaluation; and (6) data on current use of the product. All are reported in this paper.

EXPERIMENTAL PROCEDURES

Development of the formula

Since this work also involved development of an industrial process, basic ingredients for the formula were whole, raw dehulled soybeans and pearled (dehulled and polished) raw oats which, besides supplying protein also supplied fat and carbohydrate. Vegetable (safflower) oil and sucrose were used as additional sources of fat and carbohydrate, respectively. The proportion of soy and oats was calculated so as to obtain optimum amino acid complementation between proteins. The final formula was calculated, considering the proximal analysis of each raw material, obtained according to AOAC (1965) methods, so as to achieve a calorie distribution between protein, fat and carbohydrate similar to that of commercial cow's milk infant formulas designed for children 3 months or older (see "Results and Discussion").

The amount of DL-methionine required was calculated as that necessary to raise the product's level to that recommended by FAO/WHO (1973) for children.

Vitamins and minerals were also added; the exact amount of each substance required was calculated as the difference between levels recommended for an infant formula as desired (Suberbie et al., 1972; Tontisirin et al., 1977) and those calculated to be contained in the soy-oats formula, from values reported in the literature for each ingredient (Hernández et al., 1977).

Finally, a commercial milk flavor was added in order to make the formula more palatable to infants.

After the formula had been prepared, its proximal analysis was determined according to AOAC (1965) methods, and its calorie distribution calculated from results obtained.

Trypsin inhibitor activity of the final product was also determined according to the method of Rackis (1972).

Development of the process

In existing processes for making powdered soymilk from either

whole soybeans or full-fat soy flour, dry ingredients are usually dispersed in water which, after pasteurization and homogenization, is removed by spray drying (Banzon and Escueta, 1978; Garcés, 1979). Such addition and subsequent removal of water necessarily makes the final product expensive. In this work, a dry process was developed, with a considerably reduced cost. The process, outlined in Fig. 1, was tested at the industrial plant of Productos Alimenticious Delicias, S.A. de C.V. in Delicias, Chihuahua, Mexico, and utilized equipment described by Harper and Del Valle (1979). Additional equipment required to complete the process was purchased by the Chihuahua Institute for Nutritional Research & Development and installed at the Delicias plant. Briefly, the process was as follows. Soybeans were cleaned and dehulled, after which they were mixed in the desired proportion with pearled oats, obtained from a local oats plant. The soy-oats mixture was cooked in a low-cost Brady Model 206 extruder at 160°C for approximately 90 sec. It might be noted that these conditions were chosen because they have been found to be suitable, by previous workers, for cooking mixtures of soybeans and cereals in Brady extruders (Harper, 1977; Tribelhorn et al., 1975, 1976). The product leaving the extruder was cooled and discharged into a 1000-liter horizontal mixer, to which were also added sucrose, vegetable oil and a premix (prepared in a 100-liter rhomboidal mixer) of methionine, vitamins, minerals and flavor, all in the required amounts. Following thorough mixing of the material in the large mixer, it was finely ground in an Alpine Model A-4000W pin mill, after which it was fed to an All-Fill Model B semi-automatic filling machine. This machine was used to dispense 454-g portions into either previously flamed 401 x 411 cans, or into 14 cm x 18 cm polyethylene bags. Before filling, both cans and bags passed through a 2-meter long 15 cm x 30 cm tunnel equipped with ultraviolet lights, designed to reduce surface microbial contamination. The cans were sealed under nitrogen flush using a Poli M-5-V semi-automatic sealing machine, while the polyethylene bags were heat sealed with a Poli M-1 continuous sealer. All filling and sealing operations were conducted inside a "sterile" room which was thoroughly cleaned and disinfected before each use. The room was also provided with ceiling and wall ultraviolet lamps (with guards, so as to avoid direct exposure of personnel), which were lit when the room was in use. All personnel working inside the room wore head caps and masks, and were provided with clean uniforms before each run.

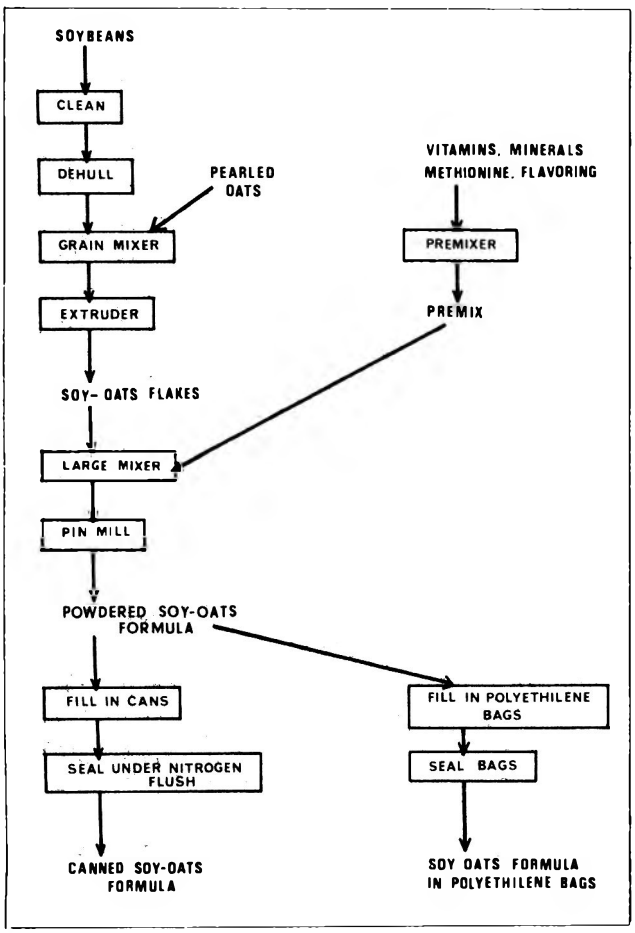


Fig. 1—Outline of dry process developed for making powdered soy-oats infant formula.

In order to ascertain the suitability of the process just described, including asceptic handling conditions from a microbial point of view, a microbiological assay of the product was determined as follows, by the usual methods (Frazier, 1958); total plate count, coliform bacteria, yeasts and molds and salmonellae.

Finally, with the purpose of determining fineness of grinding, and hence stability of aqueous dispersions of the product, the amount of material passing throuhg a U.S. Standard 100-mesh screen was measured.

Nutritional evaluation of the formula

The amino acid analysis of the formula was obtained according to the methods of Spackman et al. (1958) and also of Kohler and Palter (1967). Protein Efficiency Ratio (PER) and Net Protein Utilization (NPU) were determined using the method described by Del Valle and Perez-Villaseñor (1974), using casein as a reference protein. In order to determine the effect of added methionine, PER and NPU values for the formula, with and without methionine, were measured.

A nitrogen balance study was also conducted, comparing the soy oats formula with a commercial cow'w milk formula, utilizing six infants whose ages varied from 3-28 months. Each infant was tested with both the soy-oats and commercial formulas, and in this sense was his own control. Due to budget limitation, the method used involved determination of nitrogen balance at a single equivalent level of nitrogen intake, as described by Hegsted (1974). Data on the commercial cow's milk formula used are reported in the "Results and Discussion" section.

Clinical trials

Clinical trials were conducted with 18 infants from Chihuahua Children's Hospital, and also with 10 infants from a low income section of Chihuahua City. In all cases, weight, number of bowel movements and fecal characteristics were determined and recorded each day. All subjects were also examined daily by an attending pediatrician, in order to detect possible signs of intolerance to the formula. Average length of the test period was 14 days. The formula was fed ad libitum, in substitution of the cow's milk formula normally used.

Results of the trials were evaluated in two ways: growth response and tolerance to the formula. Growth response was measured as percent weight gain (weight gain divided by initial weight x 100) during the test period, while tolerance to the formula was subjectively evaluated by the attending pediatrician, considering daily number of bowel movements, visual fecal characteristics, and presence or absence of other signs of intolerance such as abdominal distension, vomiting and flatulence. Children were classified, both with respect to weight gain and tolerance to the formula, into different groups (i.e. excellent, good, fair, poor; see "Results and Discussion") according to results obtained.

Cost evaluation

The soy-oats formula, whose development is described in this paper, is currently being industrially produced by Productos Alimenticios Delicias, S.A. de C.V. and sold in the State of Chihuahua. A survey was conducted with the purpose of obtaining retail prices of other infant formulas available in the Chihuahua market, and of comparing them with those of the soy-oats formula packed in cans and also in polyethylene bags.

Use of the product

Information on use of the product in Chihuahua was gathered and is reported.

RESULTS & DISCUSSION

Development of the formula

Table 1 reports the ingredient formula developed for the product, including DL-methionine, vitamins, minerals and flavor, while Tables 2 and 3 show proximal analysis and

calorie distribution, respectively, for the product. Table 3, which also lists calorie distirbution for comparable commercial cow's milk formulas available in the Chihuahua market, shows that the soy-oats formula resembled these products quite well. Finally, Table 4 lists calculated amounts of all vitamins and minerals contained in the product.

Concerning trypsin inhibitor activity of the final product, this was found to be 11.3 TIU (Trypsin Inhibitor Units) per milligram, which is extremely low (Rackis, 1979), reflecting efficacy of heat treatment in the extruder and low soy content of the ingredient formula. Such low level of trypsin inhibitor has been reported to be of no significance in soy isolate infant formulas (Liener, 1979).

It might be noted that, while these results look promising, further process and stability studies are contemplated.

Development of the process

The process worked quite well, yielding a light cream-colored powder, 95% of which passed 100-mesh, of agreeable flavor. The powder was found to disperse quite readily in water to yield a milk-like suspension which was fairly stable to settling for approximately 6 hr under refrigeration. It was assumed that this stability was probably due to the protective colloid effect of oat starch, which was pregelatinized when cooked in the Brady extruder. Infants readily accepted this product when presented to them in formula bottles.

Concerning asceptic handling conditions, Table 5 reports results of the microbiological assay of the product. It may be seen that the product was free from salmonellae and coliform bacteria, and that its total plate and mold counts were quite low, within limits prescribed for an infant formula by the Mexican Department of Public Health (1974).

Table 1—Ingredient composition for soy-oats infant formula

Ingredient	Percent
Dehulled soybeans	32.1%
Pearled oats	25.6%
Sucrose	34.1%
Vegetable oil	5.8%
Tricalcium phosphate	1.2%
Sodium chloride	0.5%
DL-Methionine	0.2%
Vitamins and other minerals	0.4%
Flavoring	0.1%

Table 2—Proximal analysis of the soy-oats infant formula

Substance	Percent
Protein	17.5%
Fat	22.2%
Carbohydrate	53.1%
Crude fiber	1.0%
Ash	2.2%
Moisture	4.0%

Table 3—Calorie distribution of soy-oats and some commercial cow's milk infant formulas designed for children 3 months or older

Product	Percent of calories in			Total calories per 100 grams, dry basis
	Protein	Fat	Carbohydrate	
Soy-oats formula	15	35	50	478
Cow's milk formula A	16	37	47	476
Cow's milk formula B	18	36	46	475

Nutritional evaluation

Table 6 reports that amino acid analysis of the product, and compares it with those of mother's and cow's milks and also with the FAO/WHO (1973) children's pattern. With the exception of threonine, the soy-oats formula equalled or exceeded the FAO/WHO pattern; the formula threonine content, on the other hand, was only slightly below that of the FAO/WHO pattern. Generally, the formula tended to resemble the mother's milk pattern, but was inferior to that for cow's milk.

Table 7 contains results of the PER and NPU determinations. Values for the methionine-enriched soy-oats formula closely resembled those for casein, and indeed, comparison of corresponding means by application of t-tests revealed that no significant difference existed between them at the 99% level. However, a significant difference at the same level was found to exist between corresponding values for the methionine enriched and unenriched formulas, reflecting the beneficial effect of methionine fortification.

Concerning the nitrogen balance determination, Table 8 compares the soy-oats and commercial cow's milk formulas utilized in the study, while Table 9 reports results obtained in the study. These tables show that both formulas were quite similar in proximal analysis, calorie distribution and total caloric content per 100 grams, and also in nitrogen retention values obtained, expressed as either mg/kg-day or percent of nitrogen absorbed. Again, comparison of corresponding mean values in Table 9 by application of t-tests revealed that no significant difference existed between them at the 99% level, thus proving equivalency of the animal and vegetable protein formulas.

It might be noted that large standard deviation values, obtained in the case of nitrogen retention expressed as mg/

Table 4—Total vitamin and mineral content per 100 grams of soy-oats formula

Substance	Total content per 100 grams of formula
Vitamin A	1,8000 I.U.
Thiamin	0.45 mg
Riboflavin	0.70 mg
Ascorbic acid	35 mg
Vitamin D	330 mg
Folic acid	25 mcg
Pantothenic acid	2 mg
Pyridoxine	0.30 mg
Niacin	5 mg
Vitamin E	4 I.U.
Vitamin B_{12}	1 mcg
Copper	0.30 mg
Calcium	425 mg
Phosphorus	345 mg
Iron	12.6 mg
Zinc	3 mg
Iodine	53.4 mcg
Potassium	526 mg
Chloride	367 mg
Sodium	115 mg
Magnesium	50 mg

Table 5—Microbiological assay of soy-oats infant formula

Organism	Soy-oats formula	Limits set by Mexican Dept. of Public Health for powdered infant formulas[a]
Total plate count	5,000 per gram	10,000 per gram
Coliforms	Negative	Negative
Yeasts and molds	50 per gram	100 per gram
Salmonellae	Negative	Negative

[a] Mexican Department of Public Health (1974)

kg-day, were due to fairly large variations in response from one subject to another.

Clinical trials

Table 10 summarizes results obtained in the hospital trials, while Table 11 is a similar summary of results obtained in the outpatient trials. In most cases, tolerance to the formula, as well as weight gain, varied from good to excellent. Those cases in which tolerance was fair and weight gain poor were found to correspond to patients with pre-existing pathology, mostly of the gastroenteral infectious type. None of the healthy subjects, however, was found to exhibit, at any time during the trials, signs of intolerance such as diarrhea, abdominal distension, or flatulence.

Figure 2 shows a typical weight and bowel movement chart. The weight may be seen to have increased steadily throughout the length of the study, while the number of bowel movements per day is seen to have remained constant, reflecting good tolerance to the formula.

It might be pointed out that, although hospital trials were conducted with malnourished or sick patients and outpatient trials with essentially healthy children, similar satisfactory results were nevertheless obtained in both cases, thus further corroborating the efficacy of the soy-oats formula in infant feeding.

It was concluded that the formula was generally well accepted and tolerated by most subjects, and that it produced, in most cases, weight gains that varied from good to excellent.

Cost evaluation

Table 12 lists retail prices for the soy-oats formula in cans and also in polyethylene bags, and compares them with those of other infant formulas available in Chihuahua. It is apparent that the soy-oats formula costs from 50-60% in cans, and from 32-40% in polyethylene bags, less than other comprable products. It is felt that, under these circumstances, a positive contribution has been made to combat infant malnutrition in the State of Chihuahua.

Use of the product

The product is already reaching some rural areas, as well as part of the Tarahumara Indian population, since CONASUPO (National Food Distribution Company) has begun to distribute and sell the product in its 176 outlets in Chihuahua, some of which cover remote rural areas and Indian villages. Also, the Chihuahua Government Medical System, which covers all State Government employees as well as state employed teachers, has begun to furnish the formula, free of charge, to children ages 3-12 months, whose families are covered by the System (approximately 50,000 families). This program is closely controlled by pediatricians, who periodically examine children on the formula for signs of intolerance. So far, the product has been very well accepted, and no intolerance has been detected.

The formula is already available in drug stores and other commercial outlets of some cities in Chihuahua, including Chihuahua City, where a number of pediatricians have been using it in their private practice; also, the formula has been tested at, and is currently being used in children's

Table 6—Amino acid analysis of soy-oats infant formula

	Grams amino acid per 100 grams protein			
		FAO/WHO (1973)		
Amino acid	Soy-oats formula	Children's pattern	Mother's milk[a]	Cow's milk[a]
Iosleucine	4.2	4.0	4.1	5.5
Leucine	7.6	7.0	8.6	9.9
lysine	5.6	5.5	6.8	7.2
Methionine	2.5	–	1.6	2.6
Cystein	1.7	–	1.5	0.9
Total sulfur amino acids	4.2	3.5	2.9	3.5
Phenylalanine	4.8	–	3.5	4.8
Tyrosine	3.6	–	3.3	5.0
Total aromatic amino acids	8.4	6.0	6.8	9.8
Threonine	3.5	4.0	4.5	4.2
Tryptophan	1.3	1.0	1.6	1.4
Valine	5.5	5.0	4.5	6.4

[a] Data taken from FAO (1970)

Table 7—Protein efficiency ratio (PER) and net protein utilization (NPU) values for soy-oats formula with and without methionine, and for casein control.

System	Absolute PER[a]	Corrected PER	Absolute NPU[a]
Soy-oats formula without methionine	2.55 ± 0.16	2.0	51.60 ± 6.01
Soy-oats formula with methionine	2.99 ± 0.26	2.4	51.80 ± 4.09
Casein	3.17 ± 0.32	2.5	59.37 ± 9.88

[a] Results are reported as mean ± standard deviation.

Table 8—Comparison of soy-oats and cow's milk formulas used in nitrogen balance study

	Soy-oats formula	Cow's milk formula
Proximal analysis		
Protein	17.5%	20.9%
Fat	22.27%	18.4%
Carbohydrate	53.17%	53.1%
Crude Fiber	1.07%	0.0%
Ash	2.2%	4.6%
Moisture	4.0%	3.0%
Calorie distribution		
Protein	15%	18%
Fat	35%	36%
Carbohydrate	50%	46%
Total calories per 100 grams	478	476

Table 9—Infant feeding study: results of nitrogen balance determinations, comparing soy-oats formula with commercial cow's milk formula[a]

Concept	Soy-oats formula[b]	Commercial cow's milk formula[b]
Nitrogen ingested mg/kg-day	1,046 ± 310	1,137 ± 388
Nitrogen absorbed, mg/kg-day	743 ± 224	826 ± 207
Nitrogen retained, mg/kg-day	232 ± 126	247 ± 110
Nitrogen retained, percent of absorbed	31 ± 8	30 ± 7
Digestibility, percent	71 ± 11	73 ± 13

[a] Infants: Six, ages 3-28 months
[b] Results expressed as mean ± standard deviation.

Table 10—Results of hospital trials with soy-oats infant formula

Distribution by sex		
Males		11
Females		7
Distribution by nutritional status		
Second and third degree malnutrition		13
First degree malnutrition		5
Normal		0
Distribution by hospital admission diagnosis		
Gastroenteritis		13
Bronchopneumonia		4
Meningitis		1
Age range		
3 to 18 months		
Distribution by tolerance to formula (evaluation by pediatrician)		
Excellent		10
Good		4
Fair		4
Distribution by weight gain response		
	Percent weight gain	
Excellent	> 5%	8
Good	2-5%	4
Fair	1-2%	4
Poor	< 1%	2

Table 11—Results of outpatient trials with soy-oats infant formula

Distribution by sex		
Males		4
Females		6
Distribution by nutritional status		
First degree malnutrition		1
Normal		9
Distribution by pathology diagnosis		
All children were considered to be healthy		
Age range		
8 to 24 months		
Distribution by tolerance to formula (evaluation by pediatrician)		
Excellent		8
Good		2
Distribution by weight gain response		
	Percent weight gain	
Excellent	> 5%	4
Good	2-5%	2
Fair	1-2%	4
Poor	< 1%	0

hospitals in Monterray, Tijuana, and Mexico City. In most cases, good results have been obtained, i.e., no signs of flatulence or intolerance, good acceptance, satisfactory weight gain and excellent suitability for milk or lactose intolerant children. In a few cases, the formula has produced striking recoveries of infants who had become extremely ill as a result of milk or lactose intolerance.

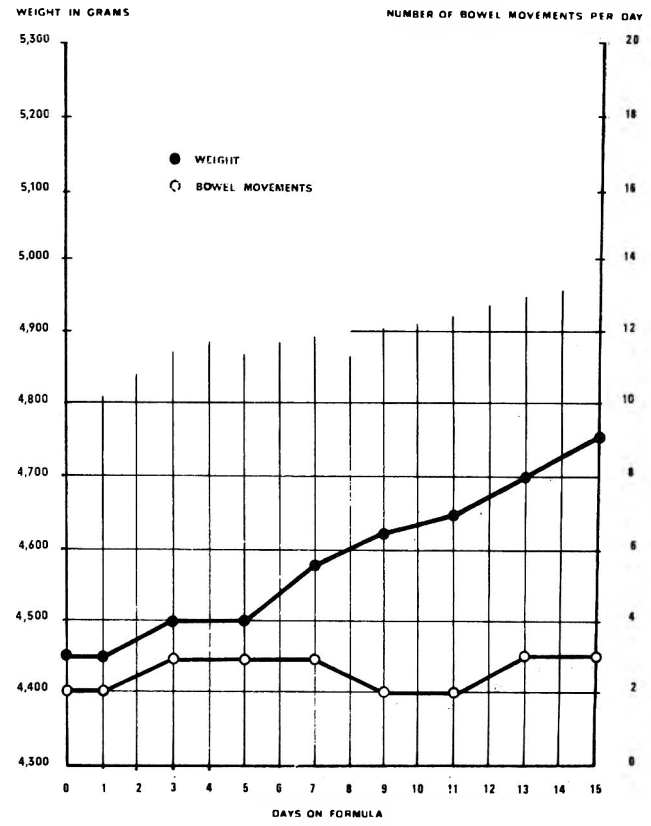


Fig. 2—Typical weight and bowel movement chart for subjects in clinical trials with soy-oats formula.

Table 12—Comparative retail prices of soy-oats and other commercial soy and cow's milk infant formulas in 454-gram portions available in Chihuahua

	Retail price	
Product	Mex. pesos	U.S. dollars
Soy-oats formula in cans	26.50	1.16
Soy-oats formula in polyethylene bags	17.00	0.74
Most expensive cow's milk formula	53.40	2.34
Least expensive cow's milk formula	43.80	1.92
Most expensive soy formula	49.00	2.14
Least expensive soy formula	43.00	1.88

CONCLUSIONS

A LOW-COST soy-oats infant formula has been developed. Nutritional evaluation data, results of clinical trials and current pediatric experience indicate that the formula is probably adequate for feeding infants 3 months or older.

A dry industrial process for making the formula, which utilizes a low-cost Brady extruder, has also been developed and tested. The process appears to be adequate, since it results in a product of satisfactory quality and low cost.

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statistically significant at the 1% level. Multiple regression analysis on the average untransformed data resulted in an $R^2 = 0.800$ and an equation for regression for PGase activity = 9.95 − 0.08 (min heat treatment) + 17.53 (ripeness) + 2.84 (days of storage) that was significant at the 1% level.

Heat treatments caused a departure from the normal increase in PGase activity. Fruits of colorbreak maturity were relatively unaffected by the heat treatments (Fig. 3). However, the quarter and half ripe fruits (Fig. 2 and 3) were less tolerant to heat treatment. The severity of retardation of the progressive increase in PGase activity during storage increased with the duration of heat treatment. The effect of heating on PGase activity was most severe in half ripe papayas heated for 90 min (Fig. 3), which showed a 48% decrease in PGase activity after 3 days of storage. After 6 days the PGase activity recovered to a 25% net gain which was 10% of the activity in the untreated fruits. Our finding that quarter and half ripe were less heat tolerant than fruits at the colorbreak stage agrees with earlier reports that one-third ripe or less ripe papayas had a high degree of tolerance to heat (Akamine and Seo, 1978).

When we examined heat-treated fruits for signs of thermal injury after 3 days of storage, we noted that quarter and half ripe fruits heated for 65 and 95 min were delayed in further ripening and retained a 1- to 1.5-cm-thick area of hard tissue surrounding the seed cavity. Untreated fruits and those that were heated for 40 min ripened normally and had soft tissue surrounding their seed cavities. However, after the sixth day of storage the internal cavity of the fruits heated for 65 and 90 min showed signs of softening. The softening coincided with the partial recovery in PGase activity (Fig. 2 and 3). Thus, the uneven ripening in heat-injured fruits is probably caused by a depression in the PGase activity that delays fruit softening.

It is highly improbable that the heat treatment had a direct deleterious effect on PGase, for the heat treatment of 46°C is nearly identical to the optimum temperature (45°C) of the enzymes. We surmise that the heat treatments depression of the PGase levels is due to an effect on the regulatory mechanism which controls the generation or activation of the PGase enzyme.

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Nutritional and Sensory Properties of Canned and Stored Moin-Moin

AYODEJI O. ADENIJI and NORMAN N. POTTER

ABSTRACT

Canned moin-moin was studied for nutritional and sensory properties after processing and following storage at 22.2°C and 29.4°C for periods up to 6 months. Comparisons were made with freshly prepared, traditional steamed moin-moin. The canned product remained a very good source of thiamin after thermal processing and storage losses. Chemical assays of lysine, availble lysine, methionine, and tryptophan showed no losses of these amino acids from processing or storage, but PER values indicated that thermal processing significantly decreased protein quality of products in rat feeding studies. PER values of canned product then remained stable in storage. After 6 months at both storage temperatures, a Nigerian taste panel ranked overall acceptability of the canned product at 6.5 on a 9-point hedonic scale, compared to 7.5 for the freshly prepared traditional moin-moin.

INTRODUCTION

MOIN-MOIN is a very popular cowpea dish in Nigeria and an important source of plant protein. The traditional recipe and method of preparation, which involves considerable time and labor, has been described by Dolvo et al. (1976). The composition, heat penetration data, and processing of a commercially sterila (F_o = 10.2 min) canned moin-moin which was highly acceptable to a Nigerian taste panel have been reported by Adeniji and Potter (1980).

Cowpea is a good source of thiamin and lysine, adequate in tryptophan, but is deficient in methionine (Block et al., 1956; Bressani et al., 1963; Oyenuga, 1968). The supply and availability of these and other nutrients in a canned moin-moin should not be substantially less than in traditionally steamed moin-moin.

The present study was undertaken to determine the effects of processing and storage on the nutritional and sensory properties of canned moin-moin.

EXPERIMENTAL

Sample preparation and processing

A batch of 200 cans of moin-moin was prepared and processed according to the procedures described by Adeniji and Potter (1980). The cans were heated in the still-retort at 121°C for 57 min and cooled in 48.9°C water for 45 min, giving a sterilization value of F_o = 10.2 min. The processed cans were randomly divided into three groups. One group was used for tests performed immediately after processing and the remaining two groups were separately stored at 22.2°C and 29.4°C for periods up to 6 months.

Chemical analyses

The samples of moin-moin for chemical analyses were sliced into thin pieces, freeze-dried and kept at −40°C until analyzed.

Crude protein (N x 6.25) and thiamin were determined by AOAC methods (1975). Total and available lysine were determined by the method of Finley and Friedman (1973), which involves alkylating the ϵ-amino groups of lysine with methyl acrylate and acid-hydrolyzing alkylated and nonalkylated samples. Lysine contents were then determined on a 119-CL Beckman amino acid analyzer. The difference in lysine content of samples before and after alkylation was the available lysine. Tryptophan was determined by the method of Horn and Jones (1945). Methionine was determined by the method of McCarthy and Paille (1959) as modified by Ascarelli and Gestetner (1962). The amino acid determinations were expressed as grams amino acid residue per 16g nitrogen. All reported analytical values are averages from duplicate samples.

Biological evaluation of protein quality

The protein quality (PER value) of freshly prepared steamed (traditional) moin-moin, and of the canned moin-moin immediately after processing and after 6 months of storage was determined by the method of Campbell and Burroughs (1963) using male Sprague-Dawley weanling rats. Fifty rats, weighing between 50-60g, were randomly assigned to five experimental diets (10% protein level) after feeding them for 3 days on a commercial stock diet. The casein reference diet was prepared with ANRC casein. The moin-moin containing diets were prepared a day before feeding started using newly freeze-dried moin-moin products. Diets were completed with Cerelose-dextrose (CPC International): Jones-Foster salt mix, vitamin fortification mix, Alphacel and corn starch (ICN Pharmaceuticals); and peanut oil (Planters, Standard Brands). All diets were held refrigerated except when used for feeding. The rats were kept in individual wire-bottomed cages and were fed from procelain food cups covered with a wire screen to prevent excessive spillage. Diets and water were provided ad libitum. Individual feed consumption and body weights were recorded at 2- and 5-day intervals, respectively. Experimental feeding lasted 21 days after which PER values were calculated and statistically analyzed by analysis of covariance.

Sensory evaluation

The acceptability of the canned products was assessed by a taste panel of 10 Nigerian students at Cornell University who were familiar with traditional moin-moin. The stored canned moin-moin was directly compared with steamed (traditional) moin-moin prepared fresh for each test. The samples were sliced into equally sized pieces and served as coded randomized duplicates. The comparative preferences were rated on a 9-point hedonic scale (9 = like extremely, 1 = dislike extremely) for appearance, color, flavor, texture, and overall acceptability. The scores were statistically analyzed by a split-plot design and protected Lsd test ($P < 0.01$).

RESULTS & DISCUSSION

THE CRUDE PROTEIN, thiamin and amino acid contents of decorticated cowpea and moin-moin products are presented in Table 1. The data obtained for the decorticated cowpea are comparable to those in the literature. The crude protein content of the moin-moin products ranged from 15.3-17.6%. The lower protein values for the moin-moin products compared to the decorticated cowpea are due to the dilution of cowpea protein by the non-protein ingredients of the moin-moin formula (Adeniji and Potter, 1980). Thiamin retained in the steamed and the canned moin-moin amounted to 91 and 54%, respectively, of the initial thiamin concentration in the unheated moin-moin puree. The small steaming loss is consistent with the findings of Farrer (1955). The substantial thiamin loss due to the heat of retorting also is consistent with losses reported for other foods similarly heated (Feliciotti and Esselen, 1957).

Author Potter is with the Dept. of Food Science, Cornell Univ., Ithaca, NY 14853. Author Adeniji, formerly with Cornell Univ., is now affiliated with the National Cereals Research Institute, Moor Plantation, Ibadan, Nigeria.

Table 1—Crude protein[a], thiamin[b] and amino acid[c] contents of cowpea and moin-moin products

Products	% Crude protein	Thiamin	Total lysine	Available lysine	Methionine	Tryptophan	% Available lysine
Decorticated cowpea	26.6	1.25	6.44	6.31	1.46	1.25	98
Moin-moin puree	17.6	0.85	5.25	4.56	1.50	1.14	87
Steamed moin-moin	16.6	0.77	5.25	4.45	1.48	1.11	85
Canned moin-moin	15.3	0.46	5.51	4.53	1.65	1.08	82

[a] Based on total solids
[b] mg/100g
[c] g/16g N

Since the retort heat treatment employed in this study was conservative (F_0 of 10.2 min), thiamin retention can probably be increased by applying a less severe, yet safe, process. Levels of total and available lysine, methionine, and tryptophan showed no discernible decrease when the moin-moin puree was steamed or canned. The higher level of these amino acids in the decorticated cowpea is due to the dilution effect of the noncowpea solids in the moin-moin products.

Storage stability of thiamin in the canned moin-moin is seen from Table 2. A slight color and textural difference usually observed between the core and the outer ring portions of the sliced canned samples was taken into consideration here and thiamin was determined separately in the two zones to monitor possible differences in thiamin retention during storage. Thiamin in the core declined by approximately 28% within the first 2 months of storage at both temperatures and there was no further appreciable decline up to 6 months at 22.2°C and up to 4 months at 29.4°C. A further decline of approximately 15% of the initial value occurred at 29.4°C between the fourth and sixth month. The thiamin content in the outer ring portion did not change appreciably for the entire storage period at 22.2°C or for the first 4 months at 29.4°C, but there was a 15% decline of thiamin in the ring within the last 2 months at 29.4°C. The thiamin differences in core and ring were not accompanied by an apparent color changes during storage.

The least thiamin retained in the product after the 6-month storage can be assumed to be essentially the average of thiamin in the core and ring of the product stored at 29.4°C. This value was 0.33 mg/100g and it represents a 28% loss during storage. This is within the range observed in baked beans by Cecil and Woodroof (1963). The FAO/WHO recommended daily allowance for thiamin is 0.4 mg/1000 calories. The concentrations of thiamin in the canned moin-moin before and after storage were 0.46 mg/100g and 0.33 mg/100g, respectively. Assuming the 100g of moin-moin supplies approximately 170 calories, canned moin-moin will supply 2.7 mg and 1.9 mg thiamin/1000 calories, respectively, before and after the 29.4°C storage. These projected thiamin supplies are about seven and five times the recommended allowance. The canned moin-moin stored for 6 months at 29.4°C can therefore be regarded as a very good source of thiamin.

Data on the amino acids retained in the stored canned product are presented in Table 3. There were no losses of available lysine, methionine or tryptophan at either temperature over the 6 month period.

The data on average weight gains, food and protein consumed, and protein quality changes determined by the protein efficiency ratio (PER) are presented in Table 4. The raw data on the initial weights and PER values of individual rats were subjected to analysis of covariance which corrected for the differences in initial weights. The results indicate that the PER value of casein was significnatly higher than the PER values of all moin-moin products at $P < 0.05$. The PER value of the steamed moin-moin was significantly higher than those of the canned moin-moin products

Table 2—Thiamin[a] retention in two regions of canned and stored moin-moin

	Months at 22.2°C				Months at 29.4°C		
	0	2	4	6	2	4	6
Core	0.46	0.32	0.33	0.30	0.34	0.35	0.27
Ring	0.46	0.44	0.44	0.44	0.42	0.44	0.39

[a] mg/100g

Table 3—Amino acid[a] contents of canned and stored moin-moin

	Months at 22.2°C				Months at 29.4°C		
	0	2	4	6	2	4	6
Available lysine	4.53	4.67	4.89	4.73	4.96	4.94	4.96
Methionine	1.65	1.73	1.69	1.70	1.76	1.77	1.75
Tryptophan	1.08	1.13	1.10	1.11	1.12	1.13	1.12

[a] g/16g N

Table 4—Growth response of rats fed various moin-moin diets

Experimental diets[a,b]	Wt gain (g)	Food consumed (g)	Protein consumed (g)	PER[c,d]
Casein	84.0	248	24.8	2.50x
Steamed moin-moin	32.0	155	15.5	1.46y
Canned moin-moin (unstored)	15.7	116	11.6	0.95z
Canned moin-moin stored at 22.2°C for 6 months	14.1	126	12.6	0.86z
Canned moin-moin stored at 29.4°C for 6 months	14.5	120	12.0	0.85z

[a] There were ten rats per diet.
[b] Duration of feeding was 21 days.
[c] PER values were adjusted to a casein value of 2.5 and corrected for initial weights of rats.
[d] PER values followed by the same letters are not significantly different at protected Lsd test $P < 0.05$.

at $P < 0.05$, but there were no significant differences between the PER values of the unstored canned moin-moin and those of products stored at 22°C or 29.4°C for 6 months. The values obtained in this study are within the range reported for heat processed cowpeas and similar legume products (Bressani et al., 1963; Elias et al., 1964; Dunlap et al., 1974).

Chemical assay results for the three essential amino acids presented in Table 1 tend to suggest that the thermal process did not have any detrimental effect on protein quality of the canned products. However, the PER test indicates that the thermal process caused a substantial reduction in the protein quality of the canned products. The rats fed the canned moin-moin consumed about 25% less food.

—Continued on next page

Table 5—Treatment preference means for moin-moin products

Treatments	No. of observations	Means[a] Appearance	Color	Flavor	Texture	Overall acceptance
Steam moin-moin[b]	60	6.9	6.9	7.6c	7.1	7.5e
Canned moin-moin stored at 22°C for 6 months	60	6.6	6.8	6.6d	6.7	6.6f
Canned moin-moin stored at 29.4°C for 6 months	60	6.5	6.8	6.6d	6.6	6.5f

[a] Means in the same column followed by the same letter are not significantly different by protected Lsd test $P < 0.01$.
[b] Steamed moin-moin was prepared fresh for each panel test.

The thermal process therefore produced a loss of palatability to the rat and a lowered nutritional quality that was not evident in the chemical analysis. This observed relationship between the chemical values of essential amino acids and the PER values is consistent with results obtained for cooked black bean (Bressani et al., 1963). The similarity of PER values of the unstored and stored canned products indicate that the storage time and temperatures did not have any detrimental effect on the protein quality of the products.

The low PER values of 0.85-0.95 obtained for the canned products and 1.46 for the steamed moin-moin can be substantially improved by supplementing the products with low levels of methionine. Dunlap et al. (1974) was able to increase the PER values of canned pinto beans from 1.0 and 0.95 to 2.0 and 1.8, respectively, with 0.6% methionine supplementation, but undesirable off-flavors were produced in the supplemented products. Bodwell and Womack (1978) have demonstrated a modified PER value of 2.95 for a canned bean and frankfurter product which can supply the critical level of methionine. Hence, the supplementary level of methionine in a canned moin-moin can be achieved by the addition of other protein sources such as meat, poultry or fish to the canning recipe, or when the product is consumed in a meal. The acceptability of canned moin-moin also would be further improved by addition of animal protein sources since they are frequently added in the traditional Nigerian recipe.

Taste panel data are presented in Table 5. The preference means indicate that there were small but significant differences in preferences for flavor and overall acceptance between the steamed (traditional) moin-moin and the 6-month stored canned moin-moin. However, there were no significant differences in preferences for these characteristics between the canned moin-moins stored at the two temperature. Although the steamed moin-moin was preferred over the stored products in favor and overall acceptance, the acceptability of the stored products can be regarded as very favorable since scores for these characteristics in the stored products were always more than half way between 6 (like slightly) and 7 (like moderately) on the 9-point hedonic scale compared to an overall acceptance score for the steamed moin-moin of 7.5. Tasters did not have differential preferences for the appearance, color and texture characteristics between the steamed and the stored canned products. Canned samples at 29.4°C have now been held for 12 months with no visible evidence of spoilage or apparent change in product characteristics.

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Determination of Thermal Process Schedule for Acidified Papaya

NIRANKAR NATH and S. RANGANNA

ABSTRACT

The pH of papaya varies from 4.2-5.65 and is required to be lowered to enable processing at atmospheric pressure. A thermal process has been evolved on the basis of inactivation of pectinesterase (PE), the heat resistant enzyme naturally present. The values for thermal inactivation of PE in pulp were $F_{212}^{26.8}$ = 1.04 and D_{212}^{27} = 0.39 at pH 4.0. In the syrup homogenate of 20% TSS, the values were $F_{207.3}^{27.6}$ = 1.00 and $D_{207.3}^{29}$ = 0.53 at pH 3.8, and $F_{212}^{27.2}$ = 1.23 and $D_{212}^{27.5}$ = 0.8 at pH. 4.0. These F values in syrup homogenate were equivalent to 1.88D at pH 3.8 and 1.53D at pH 4.0. In commercial canning, a 2.5D process at pH 3.8 and 2.0D process at pH 4.0 which are equivalent to the F values of 1.33 and 1.31, respectively, are recomended. Using these F values, process times for different processing conditions were calculated by graphical method and compared with values found by three different procedures of the formula method. The thermal process, thus evolved on the basis of enzyme inactivation, resulted in a safe and acceptable product.

INTRODUCTION

PAPAYA is marketed chiefly as fresh fruit, but the processed products such as canned nectare, pulp, etc. are becoming increasingly more popular.

The pH of ripe papaya ranges between 4.2 and 5.65. Spoilage of such canned products is caused by spore-forming bacteria having high thermal resistance (Hersom and Hulland, 1969). Above pH 4.5, *Clostridium botulinum* can grow and produce toxin. In acid and high-acid foods of pH less than 4.2, pathogens cannot grow and spoilage is caused by nonspore forming bacteria such as lactobacilli, yeasts, and molds (Evans et al., 1949; Murdock et al., 1953; Dastur et al., 1968; Nanjundaswamy et al., 1973). Hence, it is necessary to lower the pH to less than 4.2 for processing at atmospheric pressure.

Papaya contains enzyme systems such as ascorbic acid oxidase, catalase, invertase, papain, peroxidase and pectinesterase (PE) (Garces, 1963; Chan and Kwok, 1975; Chan et al., 1978). Their activity results in the loss of ascorbic acid, inversion of sucrose, development of off-flavor, and gelation of pulp during storage (Garces, 1963; Aung and Ross, 1965; Luh, 1971; Chan et al., 1972; Chan and Kwok, 1975). Activity of these enzymes can be controlled by lowering the pH and/or by heating.

Peralta et al. (1973) found that microflora of underprocessed spoiled cans of acidified papaya (pH 3.6-3.7) to consist of gram-positive nonspore forming bacteria (cocci and rods) and yeasts. The thermal resistance of these organisms was found to be very low as compared to peroxidase or PE (Garces, 1963; Aung and Ross, 1965; Peralta et al., 1973).

Investigations carried out to evolve the minimum safe thermal process for canned acidified papaya, based on enzyme inactivation, are reported in this paper.

Author Ranganna is with the Central Food Technological Research Institute, Mysore, 570013, India. Author Nath, formerly with the Central Food Technological Research Institute, is now affiliated with the Dept. of Food Science & Technology, G.B. Pant University of Agriculture & Technology, Pantnagar, Nainital, India.

EXPERIMENTAL

Studies on thermal inactivation of pectinesterase

Firm ripe fruits weighing 2-4 kg, purchased from the local market, were peeled, sliced, seeds removed, cut into small pieces, and pulped in a Waring Blendor. The resulting pulp was filled into polyethylene bags, sealed, frozen in a plate freezer at −40°F and stored at 0°F.

The activity, thermal inactivation time (TIT) using the viscosity procedure, and the decimal reduction time (D value) of PE were determined in pulp (pH 4.0) and syrup homogenate (Brix 20°; pH 3.8 and 4.0) according to the procedure described earlier (Nath and Ranganna, 1977a).

Heat penetration studies

Prepared fruits were cut into small (3 x 3 cm) or large (5 x 5 cm) pieces. Thickness of the pieces varied between 2.0 and 2.5 cm. Five hundred fifty grams of fruit pieces were filled into each 401 x 411 (A 2½) can and covered with 300g of 40° Brix sucrose syrup. The syrup was filled at room temperature (hereafter referred to as cold-filled cans) or hot, and exhausted (hereafter referred to as hot-filled cans). The canned product was processed in boiling water (207°F, the temperature at which water boils in Mysore situated at an altitude of 750m) or at 212°F in steam. The cans were cooled in water after processing.

The slowest heating point was found to be at about one-tenth of the can height from the bottom, and hence the thermocouples were positioned at this point in the subsequent studies. The thermocouple tips were embedded into the fruit pieces in all the heat penetration studies.

Heat penetration rate into the cans was measured using Ecklund's nonprojecting plug-in needle-type thermocouples and a manually operated Leeds and Northrup potentiometer. The temperature during heating and cooling was recorded every 2 min. Six cans were used for each run and three runs for each processing condition.

Plotting of heat penetration data

To calculate the process time by the graphical method, the temperatures corresponding to various times were plotted on rectangular coordinate graph paper (NCA, 1968).

To calculate by the formula method, heat penetration data were plotted on a semi-log paper (NCA, 1968). The lines through the points were drawn in three different ways: (1) the line of eye-fit; (2) a straight line by joining the initial can temperature and a point within 1° of the final can temperature as suggested by Gillespy (1962); and (3) the line of statistical fit by applying the analysis of least squares as suggested by Hurwicz and Tischer (1956).

There was no come-up time for the water bath at 207°F. It varied from 0.75-1.0 min at 212°F. Hence in drawing the line, no allowance was made for the heating lag and j_h was assumed to be 1.0. The cooling curves were also drawn in the same way.

Calculation of process time

Process time was calculated by the graphical method (Bigelow et al., 1920) and the formula method (Ball, 1923).

The process time by the formula method was calculated using the following three procedures:

Procedure A. In this procedure, the formula $B_B = f_h (\log j_I - \log g)$ of Ball (1923) was used. The slope of the heating curve (f_h) was obtained from the heating curve drawn by "eye-fit" and was assumed to be equal to the slope of the cooling curve (f_c). The value of g corresponding to the actual log of the cooling curve (j_c) was found from f_h/U:g tables of Purohit and Stumbo (1972).

Procedure B. Same as 'A' except that the f_h value found by plotting the heat penetration curve according to the procedure suggested

by Gillespy (1962) was used in the calculation.

Procedure C. Same as 'A' except that f_h found from the heat penetration curve drawn by applying the analysis of least squares was used in the calculation. There was no come-up time when the cans were processed in the boiling water. When the processing was done in steam in a retort, an allowance of 42% of the come-up time was given to calculate the pseudo-initial temperature (Ball and Olson, 1957).

Microbiological examination of processed cans

The hot-filled cans of acidified papaya (pH 4.0) were processed for 6, 8, 10, and 12 min (calculated process time being 10.3 min) at 207°F. The processed cans were incubated at 86, 98.6, and 122°F for 4 wk. In another study, fully ripe papaya (pH 5.4) was processed for 30 min at 207°F. The cans were incubated similarly. The microbiological examination of the canned products was carried out according to the procedure of NCA (1968).

The cans of ripe papaya processed for 30 min bulged on incubation. To test the ability of bacteria isolated from the bulged cans to survive below pH 4.6, they were sub-cultured into sterile tubes of papaya broth adjusted to pH 3.8, 4.2 and 4.6 prior to autoclaving. The inoculated tubes were incubated and examined.

NCA (1968) has recommended the use of *B. coagulans* and *C. pastuerianum* as the test organisms in evolving the thermal process for canned acid foods of pH below 4.5. The lyophilized cultures of these bacteria were purchased from the American Type Culture Collection.

To determine whether or not papaya supported the growth of these two bacteria, pH of pulp and syrup homogenate was adjusted to 4.0, 4.5, 5.0, and 5.5, and sterilized in screw-capped tubes for 20 min at 240°F. Three tubes of each were inoculated with the spores of *B. coagulans* or *C. pasteurianum*, incubated at the optimum temperature for 15 days and examined periodically for any sign of growth.

Hot-filled cans of acidified papaya (pH 4.0) were inoculated with the spores of *C. pasteurianum* or *B. coagulans* at the rate of 5 million spores per can. The cans were processed for 8, 10, and 12 min at 207°F. They were incubated at room temperature and at the optimum temperature for the growth of inoculated bacteria for 7 and 30 days, and examined at intervals for external signs of spoilage.

Examination of canned product during storage

The hot-filled cans of large pieces of papaya (pH 3.8) were processed for 17 and 30 min (commercial practice) at 207°F, and stored at room temperature for an extended period and at 93.2-98.6°F and 122-131°F for 30 days. Cut out examination of the canned product was carried out according to the procedure described by Ranganna (1977). The canned papaya were analyzed for total carotenoids and β-carotene according to the procedure of the Association of Vitamin Chemists (1966).

Texture of the fresh and processed fruits was determined using a recording shear apparatus developed by Voisey and Hansen (1967). The texture is expressed as maximum force (grams) required to shear a piece that fits into the triangular slot of one test plate. An average of 10 readings was taken as the texture of the fruit.

Sensory evaluation of the canned products was carried out by a panel of 10 members The panel members rated the products on a hedonic scale. Data were analyzed by t-test (Larmond, 1977).

RESULTS & DISCUSSION

PEROXIDASE and PE are the two heat-resistant enzymes reported in papaya (Garces, 1963). Earlier workers (Seagrave-Smith and Sherman, 1954, Garces, 1963; Aung and Ross, 1965; Stafford et al., 1966; Chan et al., 1972; Brekke et al., 1973) developed a thermal process for papaya puree based on the inactivation of PE. In the material used in the present study, peroxidase was not found and hence PE was used as the test enzyme.

TSS, acidity, pH, and the activity of PE in the edible portion of the fresh fruit varied considerably (Table 1). Analytical data of the pulp and the syrup homogenate used in the TIT studies are given in Table 1.

Table 1—Total soluble solids, acidity, pH and pectinesterase activity in papaya and its preparation used in heat inactivation studies

Particulars	Edible portion[a]	Pulp[b]	Preparation for TIT studies: Syrup homogenate[c]	
TSS (%)	10.5-12.0	10.5-12.0	20.0	20.0
Acidity as anhydrous citric acid (%)	0.80-0.24	0.42-.46	0.38-0.43	0.32-0.34
pH	4.20-5.65	4.0	3.8	4.0
Pectinesterase activity ($PE.ux10^4$/ml)	2.67-15.00	7.0-15.0		1.36-2.45

Prepared by (a) hand peeling and blending the edible portion; (b) adjusting the pH of the pulp prepared in (a) with citric acid; and (c) blending the pulp with sucrose syrup in the ratio of 11:6 so as to get a final Brix of 20°.

F, D, and z values of pectinesterase

The processing schedule required for papaya at the natural pH (4.2-5.65) has a deteriorative effect on the texture and flavor. The pH is required to be lowered to enable processing at atmospheric pressure. TIT studies were, therefore, carried out after adjusting the pH of the pulp to 4.0, and of syrup homogenate to 3.8 and 4.0.

Mean value of the come-up time in the determination of the TIT of PE in pulp is 4.72 min of which 52.27% had lethal effect. The come-up time in the syrup homogenate was 5.25 min of which 49.67 and 48.96% had a lethal effect at pH 3.8 and 4.0, respectively. The lethal effect increased slightly with the lowering of the pH.

The TIT curves of PE in pulp and syrup homogenate are shown in Figure 1. The F and z values are given in Table 2. The TIT of PE in the syrup homogenate were higher than the values in the pulp. According to the unpublished data of Garces (1963), the time required for heat inactivation of PE was 7.5 min at 194°F. Whether this time is the actual or the uncorrected F value, or the time required under pasteurization conditions, is not clear. Brekke et al. (1973) have reported the pasteurization requirement as 2 min at 205°F between pH 3.3 and 3.5. The TIT value of $F_{212}^{26.7} = 1.04$ for PE in papaya pulp at pH 4.0 found in the present study (Table 2) was higher than the value of $F_{200}^{11} = 1.0$ at pH 4.2 found by Aung and Ross (1965).

The F values of PE in the syrup homogenate (Table 2) were made use of in evolving the thermal process of canned papaya in syrup.

The thermal resistance curves are shown in Figure 1, and the D and Z values in Table 2. The z values found from the thermal resistance curves were higher by 0.29-1.36°F than the values found from TIT curves. The difference is attributed to the different procedures used in the determination of F and D values.

Process calculation

The heat transfer into canned papaya in syrup was by convection (Fig 3) (see Table 4 for f_h values). The TIT values of PE, $F_{207.3}^{27.6} = 1.0$ at pH 3.8 and $F_{214.4}^{27.2} = 1.0$ at pH 4.0 in syrup homogenate, were made use of for calculation of the process times by the graphical and the formula methods.

Process calculation by graphical method. The heat penetration and the corresponding inactivation rate curves for small pieces of papaya filled cold or hot and processed at 207°F are shown in Figure 2. The process time equivalent to the desired F value was found from graphical interpolation curves of F value versus process time (Fig. 3). The process time by the graphical method was calculated making use of two procedures: (i) the curves parallel to the cooling portion of the heat penetration curves were first

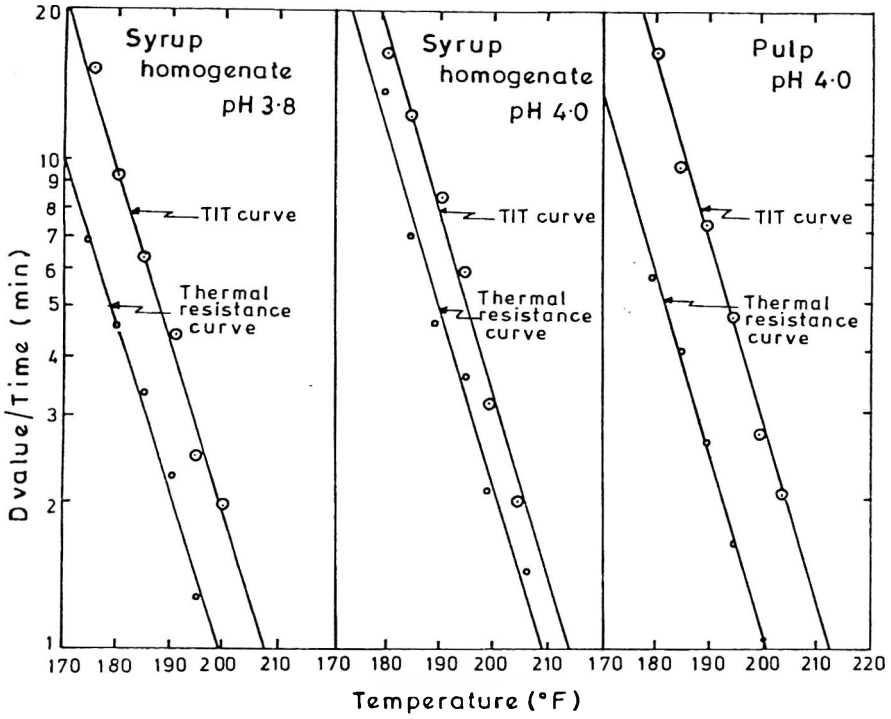


Fig. 1—TIT and thermal resistance curves of PE in papaya preparations. Y axis represents time in min for the TIT curve and D value for the thermal resistance curve.

drawn and then the corresponding inactivation rate curves were drawn, and (ii) curves parallel to the cooling part of the inactivation rate curve were drawn. The difference in the processing time between these two procedures was negligible. Therefore, only the second procedure was used in the calculation of the process time. Time required to achieve the desired F value in hot-filled cans was considerably less (7.6 min when the pH was 3.8) as compared to cold-filled cans (16.6 min) (Table 3).

Process time equal to F value may prove inadequate when the enzyme concentration in the material used for canning is higher than that used for TIT studies as discussed earlier (Nath and Ranganna, 1977a, b, c). To account for such variations, it is advisable to use a process with decimal reductions slightly higher than that equivalent to the F value of the enzyme. F values at pH 3.8 and 4.0 in syrup homogenate are equal to 1.88D and 1.53D, respectively (Table 2). Therefore, in commercial processing, use of a process equivalent to 2.5D at pH 3.8, and 2.0D at pH 4.0 is suggested. The recommended increase in the decimal reductions would ensure complete inactivation of PE in the canned product and would take into consideration variations in fill-in weight, initial temperature, etc. The corresponding increase in the process time is very small (1.0 and 1.3 min for hot-filled cans) (Table 3).

Heat penetration, inactivation rate, and graphical interpolation curves were drawn similarly for cans processed under different initial and processing temperatures. The process times calculated for a 2.5D process at pH 3.8 and 2.0D process at pH 4.0 are given in Table 5.

Process calculation by formula method. The graphical method is an accurate method of process calculation as no assumption about the nature of heating and cooling curves is made. The only possible source of error is in the manual integration of lethality. However, this method can be used only when the processing conditions such as can size, and initial and processing temperatures are identical.. The formula method overcomes this disadvantage.

Lenz and Lund (1977) have compared newer methods of process calculations with the well established graphical and formula methods. But the comparative data of the process times evolved by the graphical and formula meth-

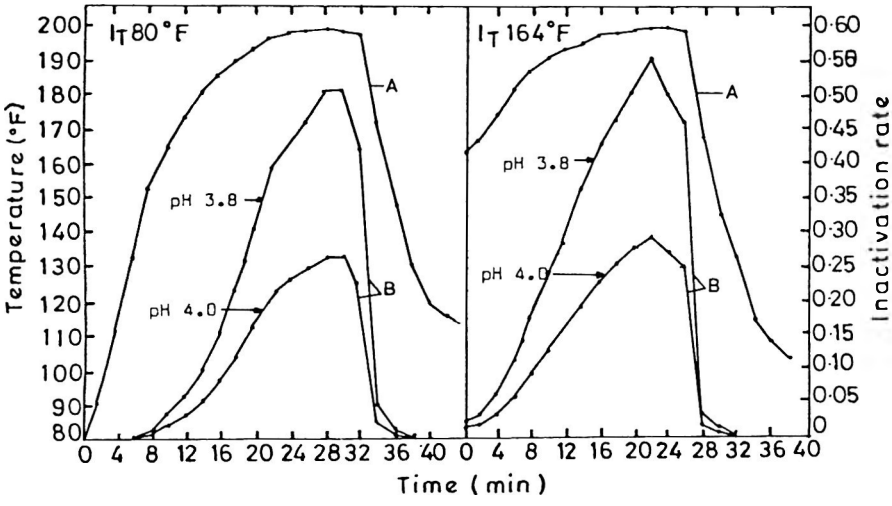


Fig. 2—Heat penetration curves (A) for small pieces of papaya canned in 401 x 411 (A 2½) cans processed at 207°F, and (B) corresponding inactivation rate curves.

Table 2—F, D and z values of pectinesterase

Preparation	pH	F value from TIT curve	D value from thermal resistance curve	Decimal reductions (F/D)
Pulp	4.0	$F_{212}^{26.7} = 1.04$ (r=−0.9972)	$D_{212}^{27.1} = 0.39$ (r=−0.9997)	2.67
Syrup homogenate	3.8	$F_{207.3}^{27.6} = 1.00$ (r=−0.9961)	$D_{207.3}^{29} = 0.53$ (r=−0.9952)	1.88
	4.0	$F_{212}^{27.20} = 1.23$ (r=−0.9884)	$D_{212}^{27.49} = 0.80$ (r=−0.9924)	1.53

Table 3—Process time calculated by graphical method for different F values.

pH	F value for process calculations	Process equivalent	Process time[a] in min for initial temperature of 80°F	164°F
3.8	$F_{207.3}^{27.6} = 1.0$	1.88 D	16.6	7.6
	= 1.07	2.00 D	16.9	7.9
	= 1.33	2.50 D	17.9	8.6
	= 1.60	3.00 D	18.6	9.6
4.0	$F_{214.4}^{27.2} = 1.00$	1.53 D	19.5	10.3
	= 1.31	2.00 D	20.8	11.6
	= 1.63	2.50 D	22.1	13.0

[a] For small pieces (3 x 3 cm) of papaya covered with 40° Brix syrup and processed at 207°F.

ods, particularly for convection-heating acid and high acid foods, is scarce.

Plotting of heat penetration data. The semilog plots of heating and cooling curves for small pieces of papaya filled cold and processed at 212°F are shown in Figure 4. Similar curves were drawn for cans processed under other processing conditions. The f_h and f_c values found by visual-fit as compared to the values found by statistical-fit did not vary much but the f_h values found by Gillespy's procedure were generally higher (Table 4).

Comparison of process time calculated by graphical and formula methods. For the purpose of comparison, when the process time calculated by the formula method was within 1.0 min of the value found by the graphical method, the value was considered satisfactory. The limits chosen are arbitrary. A higher variation was considered inadvisable as the process time required for the canned product is short. Process times calculated using f_h from Gillespy's plot (Procedure B) were beyond the limits of acceptability (Table 4). The values found using proce-

Table 4—Process time calculated by the formula method by different procedures and graphical method to achieve $F_{207.3}^{27.6} = 1.33$ (2.5 D process) at pH 3.8 for papaya canned in 401 x 411 (A 2½) cans

		Formula method					Graphical method
		Process parameters[b]				Process time (min)	Process time min
I_T °F	Procedure[a]	f_h	j_h	f_c	j_c		
		Small pieces processed at 207° F					
80	A	19.6	1.21	16.8	1.30	18.08	17.9
	B	23.2	1.00	18.8	1.30	18.63	
	C	18.6	1.16	17.3	1.26	17.65	
164	A	22.0	1.16	15.6	1.72	7.63	8.6
	B	27.6	1.00	15.6	1.72	6.55	
	C	23.3	1.13	15.5	1.77	7.32	
		Large pieces processed at 207° F					
82	A	22.4	1.44	19.6	1.67	20.34	21.1
	B	26.8	1.00	19.6	1.67	19.24	
	C	22.9	1.40	18.3	1.65	20.44	
162	A	34.4	1.22	33.2	1.14	13.41	13.4
	B	44.4	1.00	33.2	1.14	11.43	
	C	33.0	1.25	33.3	1.15	13.39	
		Small pieces processed at 212° F					
80	A	22.0	1.43	25.6	1.10	19.98	20.6
	B	28.0	1.00	25.6	1.10	20.11	
	C	22.4	1.39	25.0	1.12	20.02	
159	A	36.8	1.26	25.2	1.09	14.55	16.2
	B	48.4	1.00	25.2	1.09	12.45	
	C	36.4	1.27	26.4	1.07	14.74	
		Large pieces processed at 212° F					
80	A	28.8	1.26	24.4	1.00	24.36	25.2
	B	34.0	1.00	24.4	1.00	24.41	
	C	28.9	1.26	30.0	1.00	24.48	
168	A	48.4	1.09	39.2	1.00	10.77	13.3
	B	55.2	1.00	39.2	1.00	8.46	
	C	49.1	1.10	38.8	1.04	10.66	

[a] See text for details.
[b] f_c values have not been used in the calculations. Correlation coefficient (r) for heating (f_h) and cooling (f_c) curves varied from 0.9918-0.9993.

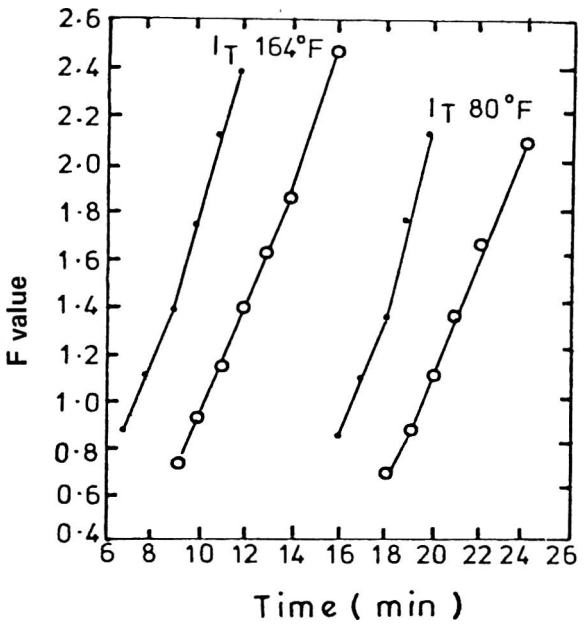


Fig. 3–F value vs process time plot corresponding to Fig. 2: • — •, pH 3.8; ○ — ○, pH 4.0

dures A and C were generally closer to the values found by the graphical method. In a few other fruits, procedure C yielded values very near to those found by the graphical method (Nath, 1978). Hence process calculations were made using this procedure. The results are given in Table 5.

Tests for microbiological safety of process

The bulged cans of overripe papaya (pH 5.4) processed at 207° for 30 min contained gram-positive spore and gas-forming mesophilic rods. On subculturing into sterile papaya broth adjusted to different pH levels, the growth was positive at pH 4.6 and 5.0 but not at pH 3.8 and 4.2 indicating that mere lowering of the pH prevented the growth of the spoilage organisms. Further confirmation was made by inoculating into hot-filled 401 x 411 cans and processing for 10 min at 207°F. The canned products having pH 4.9 bulged but not those having pH 4.0. Subculturing studies of the latter revealed no viable microorganisms.

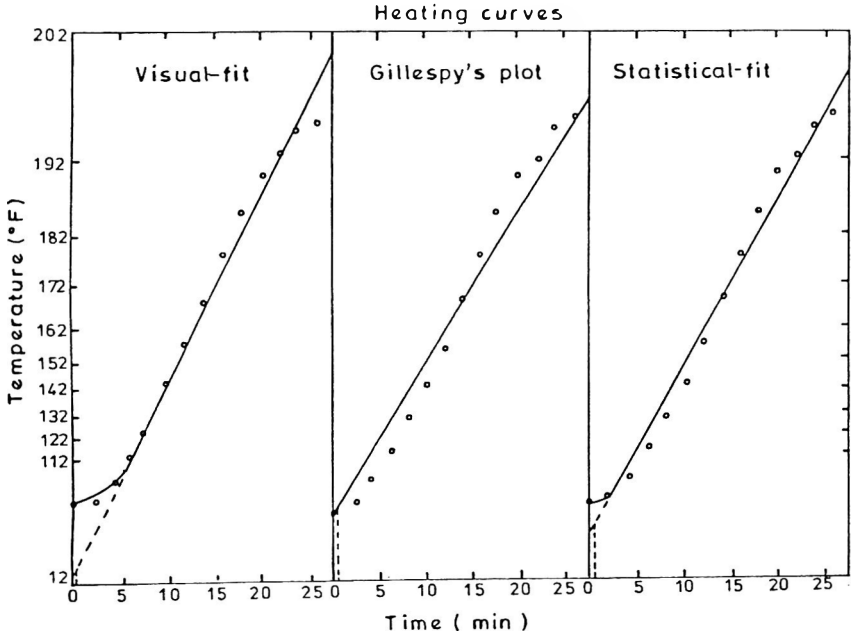
The calculated process time at 207°F for hot-filled cans containing small pieces of papaya and pH adjusted to 4.0 is 11.6 min. The cans bulged when processed for only 4 min but not when processed for 6 min. The microflora in the bulged cans consisted of gram-negative cocci and gram-negative as well as gram-positive nonspore forming rods. The mixed culture inoculated into the fresh hot-filled cans did not survive the process time of 6.0 min at 207°F.

Since no heat-resistant spore-forming bacteria could be isolated from underprocessed cans of acidified papaya, the possibility of spoilage by *B. coagulans* and *C. pasteurianum* was investigated. The spores of these bacteria were inoculated into the tubes of sterile papaya pulp and syrup homogenate adjusted to pH 4.0, 4.5, 5.0, and 5.5, and incubated at their optimum temperature (122-131°F for *B. coagulans* and 82.4-86.0°F for *C. pasteurianum*) for growth. The organisms did not grow indicating that papaya does not support their growth. The above results were further confirmed by the inoculated pack studies. Hot-filled cans (pH 4.0) were inoculated with the spores (5×10^6) of *B. coagulans* or *C. pasteurianum*, sealed and processed for 8, 10, and 12 min at 207°F. Uninoculated cans were processed along side as control. Sub-culturing studies revealed no viable organism in the inoculated packs. These studies confirm that the process evolved on the basis of PE inactivation renders the canned product microbiologically safe.

Quality of the canned product

Changes in the canned product during storage are given in Table 6. No residual PE activity was found in the canned product which had received a process equivalent to 2.5D at pH 3.8 either immediately after processing or after storage for 6 months at room temperature. The vacuum decreased slightly during storage while TSS, acidity and pH remained constant. The drained weight varied from 59.41-63.19% and the cut-out Brix of the drained syrup was 19.5°. Texture of the fresh fruit was 399.3g and reduced to 298.9 and 186.4g on processing for 17 and 30 min at 207°F, respectively. During storage there was further reduction in the texture values.

The absorption spectra of the carotenoid pigments in fresh and processed papaya either immediately or after storage for 6 months at room temperature were superimposable indicating that the processing had not brought about trans-



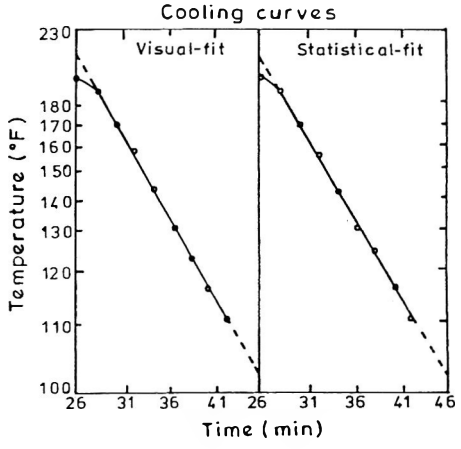


Fig. 4–Semilog plot of heating and cooling curves for small pieces of papaya with initial temperature of 80°F, processed at 212°F, and cooled in water at 80°F.

Table 5—Process time for papaya canned in syrup in 401 x 411 (A 2½) cans

Piece size	Temp[a] P_T °F	Temp[a] I_T °F	f_h (min)	j_h	j_c	Process time pH 3.8 $F_{207.3}^{27.6} = 1.33$ Formula method (min)	Process time pH 3.8 $F_{207.3}^{27.6} = 1.33$ Graphical method (min)	Process time pH 4.0 $F_{214.4}^{27.2} = 1.31$ Formula method (min)	Process time pH 4.0 $F_{214.4}^{27.2} = 1.31$ Graphical method (min)
Small	207	80	18.6	1.16	1.26	17.65	17.9	20.71	20.8
		164	23.0	1.13	1.77	7.32	8.6	10.72	11.6
Large	207	80	22.9	1.40	1.65	20.44	21.1	23.80	23.8
		162	33.0	1.25	1.15	13.39	13.4	17.65	17.7
Small	212	80	22.4	1.39	1.12	20.05	20.6	22.90	23.6
		159	36.4	1.27	1.07	14.74	16.2	18.64	20.1
Large	212	80	28.9	1.26	1.00	24.48	25.2	27.83	28.6
		168	49.1	1.10	1.04	10.66	13.3	15.44	17.4

[a] P_T = Processing temperature; I_T = Initial temperature.

formations in the carotenoid pigments. The papaya pieces had retained natural color and shape. The syrup was clear and the flavor was full. Analyses of the sensory evaluation data showed that the canned papaya processed for 17 min were significantly superior to those processed for 30 min at 2.5% level.

Recommendation for commercial processing

The values for thermal inactivation of PE in syrup homogenate were $F_{207.3}^{27.6} = 1.00$ and $F_{212}^{27.2} = 1.23$ at pH 3.8 and 4.0, respectively, which were equivalent to 1.88D and 1.53D, respectively. From the point of view of microbial spoilage, the lower the pH the safer is the product. In commercial practice, a 2.5D process at pH 3.8 and 2.0D process at pH 4.0 would ensure a microbiologically safe and organoleptically acceptable product. The time required to achieve the same for small and large pieces at different initial and processing temperatures are given.

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Table 6—Results of the cut-out examination of papaya canned in syrup in 401 x 411 (A 2½) cans and processed at 207°F (initial can temperature 160°F)

Storage period (month)	Process time (min)	Vacuum (in. Hg)	Drained (%)	TSS (%)	Acidity (%)	pH	Texture (g)
0	17	18	62.1	19.5	0.43	3.74	298.9
	30	16	63.2	19.5	0.43	3.74	184.4
3	17	14	59.4	19.5	0.43	3.74	251.1
	30	16	61.8	19.5	0.43	3.74	176.1
6	17	14	63.1	19.5	0.43	3.74	170.9
	30	11	61.8	19.5	0.43	3.74	140.9

—Continued on page 211

Some Rheological Characteistics of Soy Extrudates in Tension

J. W. FINKOWSKI and M. PELEG

ABSTRACT

Experimental soy flour extrudates, prepared under different processing conditions were tested in tension by an Instron Universal Testing Machine. All the extrudates exhibited a nonlinear stress-strain relationship that is indicative of progressive development of structural failure. Complementary relaxation curves at three prefailure deformation levels were linearized and used for calculation of asymptotic relaxed moduli (also called equilibrium moduli). The relationship between the moduli and the strain or the initial stress indicated that both the break elongation and the ultimate strength could independently be estimated from the relaxation data. A mathematical and computer procedure for such predictions is described and its applicability demonstrated with experimental results.

INTRODUCTION

RHEOLOGICAL CHARACTERIZATION of solid polymers and many engineering materials is mainly done through tensile tests. The advantage of tension over compression is that inherent experimental artifacts, most notably friction along the supporting surfaces, do not have a decisive or significant effect on the results (Marin, 1962; Dieter, 1976). In food research, application of tensile methods for textural or theological evaluations is fairly rare. This is mainly due to the obvious difficulties in obtaining suitable specimens for tension and the problems that are associated with proper grasping of the specimen in the testing machine.

In a few cases, such as in extrudates, these technical obstacles do not really exist and tensile analysis can be performed by the same standard procedures that are applicable to polymers or metals. Most extruders, with the appropriate die can produce specimens that have perfect dimensions and shape for tensile testing. This of course is only true in a certain range of operational conditions, especially with respect to the extrudate moisture and the extrusion temperature profile. Within this range (i.e. where the extrudated specimens have sufficient strength and continuous structure) tensile parameters can be a useful tool in quantifying the textural properties of extrudates with distinctly different microstructures (Cumming et al., 1972).

This work has been focussed on the capabilities of tensile analysis to characterize the mechanical behavior of soy extrudates without entering the structural and chemical mechanisms that are responsible for it. These aspects have been extensively studied elsewhere and are widely reported and discussed in the food literature (e.g. Harper, 1979; Stanley and deMan, 1978; Kinsella, 1978; Hermansson, 1978).

Since the emphasis was on the tensile test methodology no effort has been made to evaluate or optimize the extruder performance nor the product's quality. In fact, after the range of conditions for texturization had been found, the extruder was only used as a means of providing extrudates with different textural properties and no further textural modification (such as humidification, for example) has been attempted.

Authors Finkowski and Peleg are with the Dept. of Food Engineering, Univ. of Massachusetts, Amherst, MA 01003.

EXPERIMENTAL

DEFATTED SOY FLOUR (Cargill Inc., Minneapolis) of 200 mesh was admixed with distilled water at levels that produced a final moisture content of 22-29% (wet basis). The mixture was left to equilibrate at 3-8°C for 24 hr prior to the extrusion run. The wet flour was then extruded through a Rheodrive extruder/mixer Model 3000 (Haake, Inc., Saddlebrook, N.J.) with the following characteristics:

Barrel diameter – 3/4 in.
Length to diameter ratio – 25:1
Compression ratio – 2
Die shape – round
Diameter – 1/8 in.

The extruder has four, independently controlled heating/cooling zones (including the die zone) and a gear for variable screw speed in the range of up to 150 rpm.

The extrusion conditions were set as follows:

Feed zone temp – 50°C
Die temp – 100-140°C
Screw speed – 50 and 100 rpm

The extrudate was carefully collected, let to cool overnight in sealed plastic bags, and then tested in tension.

Tensile tests

The extrudates (Fig. 1) were tested in an Instron Universal Testing machine Model TM. The specimens were gripped by the standard Instron fiber jaws (type 2A) as shown in Figure 2. The specimens, 4 cm in length, were stretched at a deformation rate of 1 cm min^{-1} until failure or to three levels of a preset deformation for recording the force relaxation curves.

Ultimate strength (failure) results are based on 10-15 successful replicates. (The very few samples in which failure occurred at or in the vicinity of the gripping jaws were discarded). The relaxation data are based on at least three curves at each deformation level. Since the overall strain levels were small and cross-sectional area changes insignificant (see Fig. 1a), stressed (σ) and strains (ϵ) are reported in apparent (or engineering) terms, i.e.:

$$\sigma = \frac{F}{A_o} \tag{1}$$

where F is the force and A_o is the original cross-sectional area and:

$$\epsilon = \frac{\Delta L}{L_o} \tag{2}$$

where ΔL is the elongation and L_o the original length.

RESULTS & DISCUSSION

Tensile stress-strain relationship

Typical stress-strain curves of soy extrudates produced under different processing conditions are shown in Figure 3. All the curves, despite the small strain levels, had a clear concave downward shape with considerable curvature. As can be seen in Figure 3a, the curvature could not be attributed to necking (a common source of curvature in apparent stress-strain relationships of engineering materials) nor to the nonlinearity of the true strain. its proper inter-

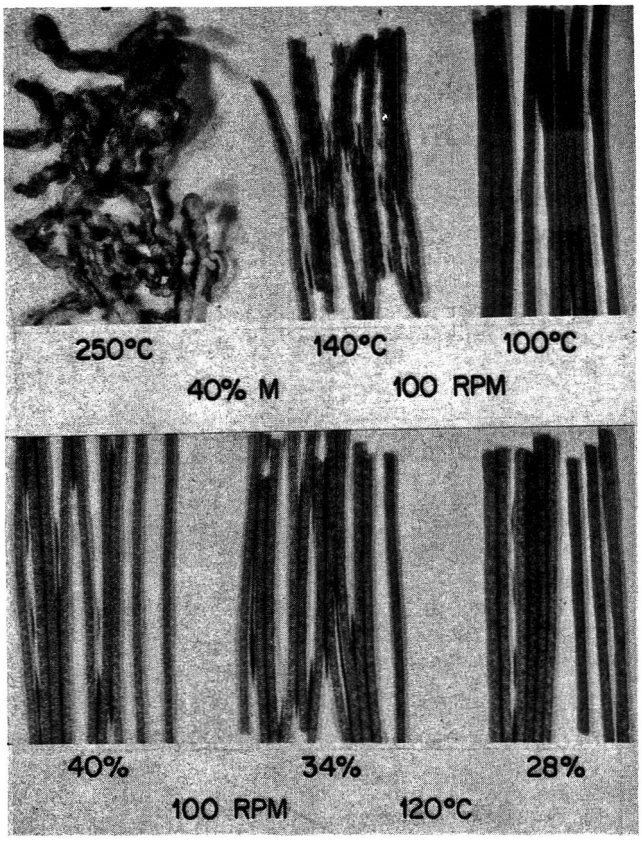


Fig. 1—Samples of soy extrudates extruded under various conditions.

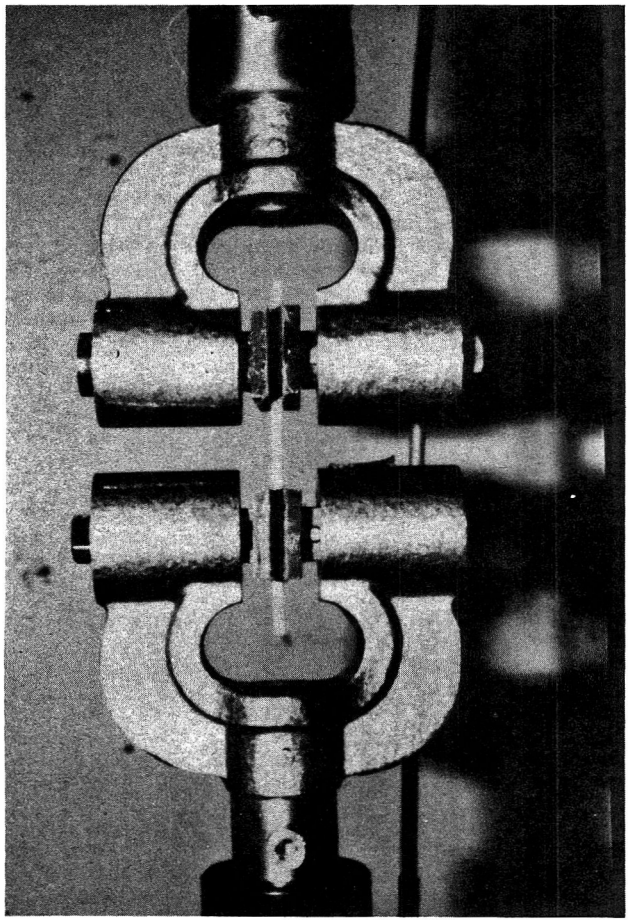


Fig. 2—A specimen in tension test.

pretation is continuous and progressively increasing disintegration of the internal physical structure that results in a continuous loss of mechanical strength.

As could be expected, extrudates with different shear and temperature histories also differed in the magnitude of their ultimate strength (Cumming et al., 1972). Under the experimental conditions of this work, moisture was the most influential factor outweighing the mutually dependent effects of the temperature history and the screw speed. Generally, increasing the flour moisture resulted in increased extrudate plasticity which was reflected in lower ultimate strength and larger prefailure elongation. It should be noted that moisture does not necessarily produce this same effect at any extrusion conditions. At higher temperatures, for example, puffy or porous structures may be the result (Fig 1) but these were not tested in the course of this work.

Experimental scatter

The coefficient of variance in ultimate strength of extrudate produced at die temperatures of 100 and 120°C was between 4.0-12.5% irrespectively of the moisture content or the screw speed. At die temperature of 140°C the scatter in terms of the coefficient of variance increased to 12.5-28.7%. In testing spaghetti of two commercial brands by the same procedure the coefficient of variance was only 1.3-1.4%. This, as well as the fact that in more than 90% of the other runs the scatter was less than 10% suggests that the variability in this case is a textural characteristic and not a measure of the test reproducibility. Apparently, discontinuities in the matrix or other structural defects within the extrudate were responsible for this experimental scatter and this again is supported by the overall decrease of both the ultimate strength and the break elongation.

Since the scatter in tensile properties of extrudates seems to carry information that is relevant to the structure uniformity and strength, it ought to be treated as a quality factor in itself. It, therefore, may not be advisable to disregard the scatter after averaging the results, especially if its magnitude largely exceeds the experimental error. (It would also be worth investigating how the textural nonuniformity is affected by different methods of product rehydration.)

Stress relaxation pattern

Deformed specimens were let to relax and their force-relaxation curves were recorded for 5-8 min. For each type of extrudate the test was replicated and repeated at three different levels of prefailure deformation. In order to avoid premature failure these levels were selected and set as not to exceed about 60% of the break elongation. Schematic view of the curves is shown in Figure 4.

The recorded curves were normalized and linearized in the form of (Peleg, 1979a; 1980):

$$\frac{F_o \cdot t}{F_o - F(t)} = k_1 + k_2 t \qquad (3)$$

where F_o is the initial force, F(t) the force after time t and k_1 and k_2 constants.

If equation 3 is applicable (i.e. the data when plotted are indeed on a straight line as demonstrated in Fig. 5) then the reciprocal of k_1 depicts the initial relaxation rate and that of k_2, the asymptotic or equilibrium value of the parameter $[F_o - F(t)]/F_o$. The latter represents the portion of the stress that will be relaxed after a very long time. The theoretical aspects and physical significance of this parameter when applied to biological materials (which are biochemically active or may dehydrate during the test) are discussed elsewhere (Peleg, 1980). In any case, for char-

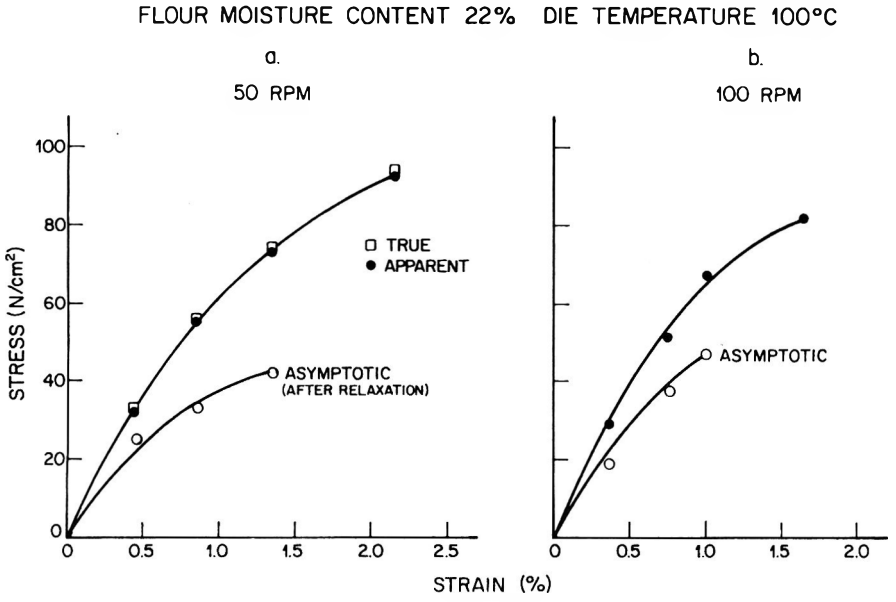


Fig. 3—Typical stress-strain and relaxed stress-strain curves of soy extrudates.

acterizating short-time rheological properties, the most useful parameter is the asymptotic *modulus* (E_A) which is calculated from:

$$E_A = \frac{F_o}{A\epsilon}\left[1 - \frac{1}{k_2}\right] \qquad (4)$$

In linear viscoelastic materials E_A ought to be independent of the strain and the strain history. In materials which are nonlinear it can provide indications as to whether the deformation involves internal fracture, compressibility or strain hardening and can be employed to predict failure (Peleg, 1978; 1979b; 1980). If during deformation, internal fracture or disintegration is dominant, and the shape of the stress-strain curves clearly indicates that this is the case with soy extrudates, then plotting E_A vs both the strain (ϵ) or the initial stress (σ) must result in a smoothly descending curve with progressively decreasing slope. A schematic view of these curves is given in Figure 4c. Additional property of these relationships between E_A and ϵ or σ is that the failure strain ϵ_f (break elongation) or the failure stress σ_f (ultimate strength) ought to be on a smooth continuation of the curve as shown in the figure. (In this case, and unlike the case of compression (Pollak and Peleg, 1980), E_A at failure must have zero value).

Typical experimental curves of E_A vs ϵ or σ of various soy extrudates are shown in Figures 6 and 7. These demonstrate that the tensile failure of the extrudates was indeed a continuous process that involves progressive rather than instantaneous structural breakdown.

This kind of behavior is similar to that of certain solid polymers in tension. For the latter, the failure conditions could be predicted by employing the following simple empirical equations (Peleg, 1978; 1978b):

$$E_A = B_1 - B_2\,\epsilon^{n_1} \qquad (5)$$

or

$$E_A = B_3 - B_4\,\sigma^{n_2} \qquad (6)$$

where the coefficients B_1, B_2, B_3, B_4 and the powers n_1 and n_2 are empirical constants derived from curve fitting

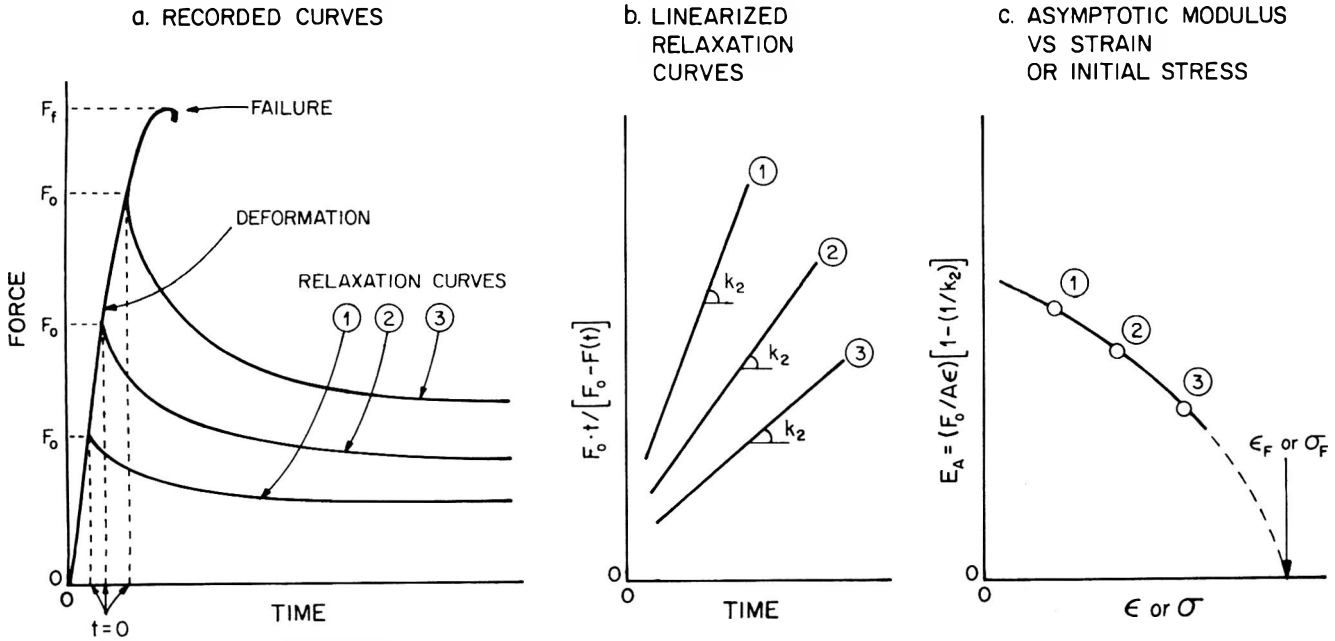


Fig. 4—Schematic presentation of the relaxation tests and the interpretation of their data.

of the E_A vs ϵ and E_A vs ϵ relationships, respectively. (Accelerated loss of strength and consequently loss of residual modulus is expressed by $n > 1$.) Since the tensile failure the residual relaxed modulus is zero, the failure conditions can be calculated by extrapolating the curves to zero modulus or algebraically from the expressions:

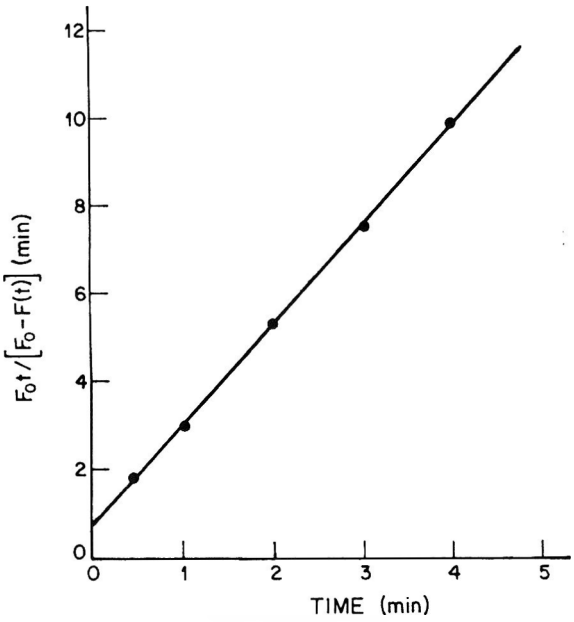


Fig. 5—Demonstration of the applicability of Eq. 3 to normalization and linearization of relaxation curve to soy extrudates.

$$\epsilon_F = \left(\frac{B_1}{B_2}\right)^{\frac{1}{n_1}} \quad (7)$$

$$\sigma_F = \left(\frac{B_3}{B_4}\right)^{\frac{1}{n_2}} \quad (8)$$

where ϵ_F and σ_F are the break elongation and the failure stress (ultimate strength) respectively.

Testing the applicability of those equations for the soy extrudates was done as follows. The experimental data points (see Fig. 4, 6 and 7) were fitted to Equations 5 and 6 by the SPSS nonlinear regression program. The program was run with several preset values for the power constants n_1 and n_2 so that the corresponding coefficients (B_1 - B_4) and subsequently ϵ_F and σ_F could be calculated for each. (This procedure was adapted in light of the limited number of data points that resulted from the technical difficulty in spacing more than three strain levels within the small range of absolute deformation.) It can be shown that due to the mathematical nature of Equations 5 and 6, changing the power n has only very minor effect on the statistical fit. It does have, however, a significant effect on the extrapolated values of the intercept, i.e. the calculated values of ϵ_F and σ_F (Peleg, 1979b).

The applicability of the equations is demonstrated in Tables 1 and 2. It shows that the break elongation could be predicted with reasonable accuracy at powers n_1 of about 1-2. Independently, the ultimate strength could also be satisfactorily predicted by varying the power n_2 in the neighborhood of 3-5.

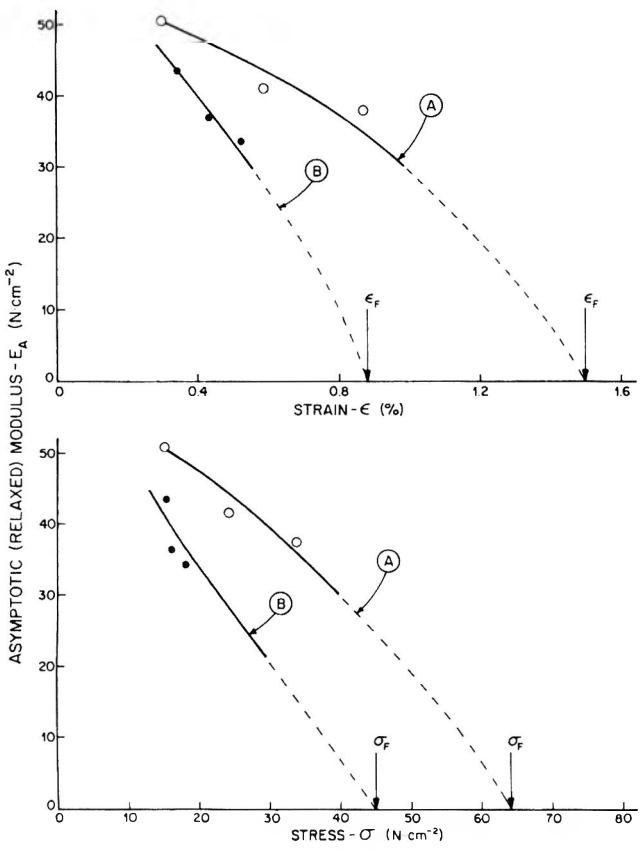


Fig. 6—Experimental plots of E_A vs ϵ and E_A vs σ of two soy extrudates (marked A and B) with relatively small break elongation. (Note: that the failure conditions (ϵ_F and σ_F) are on a smooth continuation of the curves).

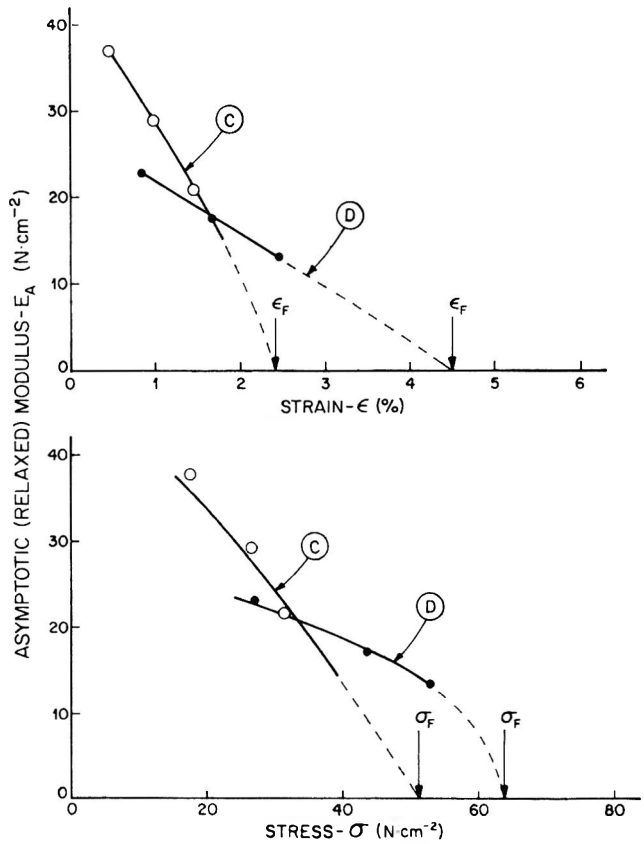


Fig. 7—Experimental plots of E_A vs ϵ and E_A vs σ of two soy extrudates (marked C and D) with relatively large break elongation. (Note that the failure conditions (ϵ_F and σ_F) are on a smooth continuation of the curves).

Table 1—Demonstration of the applicability of Equation 5 to estimation of the break elongation (ϵ_F) of soy extrudates from relaxation data[a]

Extrudate No.[b]	Calculated ϵ_F (%) n=1	n=1.5	n=2	Measured ϵ_F (%)
1	4.7	4.0	3.7	4.2
2	4.5	3.8	3.4	3.7
3	2.3	2.1	2.0	2.4
4	2.7	2.3	2.1	2.4
5	2.4	2.1	2.0	2.2
6	2.6	2.0	1.7	1.5
7	1.5	1.3	1.2	1.2
8	1.7	1.3	1.1	0.9
9	7.3	5.7	5.1	5.1
10	4.4	3.9	3.7	4.0

[a] Underlined are the figures that are closest to the measured values.
[b] Extruded under different processing conditions as described in the text.

Table 2—Demonstration of the applicability of Equation 6 to estimation of the ultimate strength (σ_F) of soy extrudates from relaxation data[a]

Extrudate No.[b]	Calculated σ_F (N.cm^{-2}) n=2	n=3	n=4	n=5	Measured σ_F (N.cm^{-2})
1	75.5	69.2	65.7	63.6	63.3
2	77.7	71.3	66.2	63.8	61.5
3	50.1	46.9	45.1	44.0	47.1
4	61.3	56.0	53.2	51.5	52.3
5	100.7	92.7	88.6	85.8	92.2
6	126.5	106.3	97.0	91.5	91.9
7	81.3	73.1	68.9	66.4	63.6
8	71.0	59.2	53.5	50.2	44.9
9	47.4	41.3	38.2	36.4	34.8
10	34.5	31.8	30.5	29.7	30.0

[a] Underlined are the figures that are closest to the measured values.
[b] Extruded under different processing conditions as described in the text and are the same as in Table 1.

Obviously, these powers as well as the other equation constants largely reflect the textural differences among the various extrudates. The fact, however, that the same type of equation was found predictive despite the noticeable differences in strength and break elongation indicates that the suggested theory regarding the existence of early indications of failure is not only internally consistent but also experimentally confirmed.

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Effect of Tumbling and Tumbling Temperature on Total Aerobic Plate Counts (Incubated at 25°C) and Quality of Boneless, Cured Hams

CURTIS LYNN KNIPE, R. F. PLIMPTON and H. W. OCKERMAN

ABSTRACT

The effects of intermittent tumbling (up to 18 hr) and processing temperature (25°C or 3°C) on internal temperature and exudate total aerobic plate counts incubated at 25°C (ATPC) in boneless, cured hams were determined. The design used 80 hams in five replicates involving four treatment groups (tumbled at 25°C, tumbled at 0°C, control held at 25°C and control held at 0°C). The significant ($P<0.01$) tumbling effect on the rise in internal ham temperature occurred after 3 hr, but only accounted for a difference of 1.26°C-1.32°C, independent of processing temperature. While tumbling significantly improved the cooked yield by 3.45%, processing at 25°C rather than at 0°C resulted in a significant ($P<0.05$) yield reduction of 2.15% after 9 hr of processing. The exudate ATPC was significantly ($P<0.01$) reduced after 18 hr of tumbling.

INTRODUCTION

TUMBLING and massaging are recent innovations in meat curing now used to improve yield and quality of boneless, cured pork (Maas, 1963; Rust and Olsen, 1973; Krause et al., 1978b; Cassidy et al., 1978).

Krause et al. (1978b) reported that tumbling increased tenderness, cohesiveness, and product yield. According to Cassidy et al. (1978) and Theno et al. (1976) tumbling caused a significant increase in muscle sarcolemma disruption. Krause et al. (1978a) showed that disrupting the muscle sarcolemma promoted the migration of curing ingredients between and into muscle fibers, which resulted in a quicker and more uniform distribution of the curing ingredients.

Tumbling and massaging involve the imparting of mechanical energy to the meat. Maas (1963) reported that the impact energy of tumbling leads to the transfer of kinetic energy to the muscle resulting in a rise in temperature and that is the basis for producing sufficient protein exudate on the surface of the meat pieces to produce a cohesive, formed product (Siegel et al., 1978a; Cassidy et al., 1978).

Tumbling may involve continuous activity for short periods (1-3 hr) or may involve an intermittent cycle for quite long periods; i.e., 10-15 min of every hour for 18-24 hr (Ockerman et al., 1978). In an attempt to cure meat quickly, a temperature above refrigerated temperatures would be expected to increase the rate of cure migration, but perhaps, also decrease the shelf life (Moulton and Lewis, 1940). This may be of concern since hams used in tumbling and massaging are usually boneless and thus have been subjected to significant contamination during processing.

The addition of alkaline phosphates to curing solutions, which is important to increased yields and improved cohesiveness of tumbled meats (Siegel et al., 1978a; Krause et al., 1978, b; Ockerman et al., 1978) complicates matters by increasing the pH and therefore slowing down the reduction of nitrite to nitrous acid, which in turn, should inhibit bacterial growth (Lawrie, 1974). Krause et al. (1978a) reported that tumbling increased the rate and extent of cure migration and simultaneously reported a significant increase in nitrite residues. These alkaline phosphates also increase the water-holding capacity (Wierbicki et al., 1976). Since microbial growth increases with both increased pH and a higher water-activity ratio (a_w) (Lechowich, 1971), the addition of phosphates could very likely result in an increase in microbial number.

The objectives of this research were to determine the effect of tumbling time and tumbling temperature on internal temperature changes during processing, on product quality and yield and on aerobic total plate counts (25°C incubation).

EXPERIMENTAL

IN TOTAL, 80 fresh hams weighing 6.4-7.2 kg were boned, closely trimmed of external fat and visible connective tissue, and assigned to one of four treatment groups within each of five replicates. Each ham was multiple stitch pumped to 120% of its green weight with a curing solution that included 14.30% salt, 2.75% sugar, 0.29% sodium erythorbate, 0.11% sodium nitrite, 2.75% sodium tripolyphosphate, and 79.80% water. Within each replicate, 4 hams were tumbled at each of two temperatures (25°C and 3°C). These temperatures were chosen to represent room temperature and cooler temperature, respectively. The hams were tumbled for 10 min of every hour for 18 hr. Following the 18 hr processing period, all hams were placed in fibrous casing and cooked in a smokehouse to an internal temperature of 68.3°C. In addition, 4 control hams were stored without tumbling at each temperature (room temperature, 25°C and cooler temperature, 3°C).

The tumblers were buit at The Ohio State University Meat Laboratory and consisted of stainless steel drums with three baffles positioned around the inside of the drums. The baffles carry the hams up to a position where the hams fall to the bottom of the drum, giving the tumbling effect. The drums were turned with a 3/4 horsepower, 3 phase, 220 volt, 1725 rpm motor. The actual tumbler speed was 11 rpm. The drum was 81 cm long and had a diameter of 58 cm. Baffles had a depth of 7.6 cm.

Two of the four hams in each of the four treatment groups were selected at random to be used throughout the processing for all microbial sampling. Samples were taken at nine stages: prior to brine injection and at processing time 0, 3, 6, 9, 12, 15, and 18 hr and after cooking to an internal temperature of 68°C. At these times, all hams were also weighed and internal temperatures recorded.

At each sampling time, swab samples of a 3.3 cm^2 area from both the external surface of the ham and the internal surface (exposed during boning) of the ham were obtained and then combined. Swabs pre-moistened with the dilutant (double distilled water) were used. These swab samples were diluted and subsequently plated with a tryptone glucose extract agar. These plates were then incubated for four days at 25°C (Ockerman, 1978). Total aerobic microbial plate counts at this incubation temperature were then determined.

A trained, six person sensory panel evaluated each ham for tenderness, color, and cohesiveness, using 1/8" cold slices of the finished product. Hams were rated on a Hedonic scale from 1-10 (Color score, 1 = unacceptable to 10 = acceptable; Tenderness score, 1 = extremely tough to 10 = extremely tender; and Cohesiveness score, 1 = not cohesive to 10 = very cohesive).

Analysis of results was accomplished using the Least Squares Maximum Likelihood General Purpose Program (Harvey, 1968).

Authors Plimpton and Ockerman are with the Ohio State Univ., Columbus, OH 43210 and the Ohio Agricultural Research & Development Center, Wooster, OH 44691. Author Knipe, formerly with Ohio State Univ., is now located at Iowa State Univ., Ames, Iowa.

RESULTS

Product quality

There was no significant interaction between temperature and tumbling. As expected, tumbling with temperature effects statistically absorbed did significantly ($P<0.01$) improve internal color and slice cohesiveness (Table 1). These results agree with the previous reports of Vartorella (1975), Krause et al. (1978b) and Siegel et al. (1978b). Tenderness was found to be improved by tumbling, but only at the 8% probability level. Vartorella (1975) did not find a highly significant improvement in tenderness of canned, cured hams due to tumbling. However, he found tumbling to significantly improve tenderness when evaluated by th Warner-Bratzler Shear method.

Temperature did significantly affect both internal color and tenderness. Processing at room temperature (25°C) significantly ($P<0.01$) improved the internal color of the final cooked product (Table 2). This agreed with Moulton and Lewis (1940) who reported that higher than normal temperatures accelerate the curing rate and color development.

Processing at room temperature also resulted in a significant increase ($P<0.01$) in tenderness compared with processing at a lower temperature (3°C). This increase in tenderness could be the result of improved cure migration and subsequently an increase in the salt's effectiveness in increasing the water-holding capacity.

The processing temperature did not have a significant effect on cohesiveness. Comparing the data in Tables 1 and 2 would indicate that the mechanical action of tumbling and not the temperature increase caused the improved cohesiveness.

Internal temperature

The relationship between ham internal temperature and sampling time was significant ($P<0.01$) and quadratic (Fig. 1). However, there was a significant ($P<0.01$) interaction between the treatment temperature and sampling time effect on ham internal temperature ($P<0.01$).

As illustrated in Figure 1, processing (tumbling or storage) hams at room temperature (25°C) resulted in a curvilinear increase in internal temperature for both tumbled and nontumbled (stored) boneless, cured hams while processing at a refrigerated temperature of 3°C resulted in a curvilinear decrease in internal temperature. The greatest rate of temperature change occurred within the first 3 hr and subsequently, temperature leveled off after 9 hr.

There was no significant interaction between tumbling and treatment temperature (Fig. 1). With treatment temperature effects statistically absorbed, the analysis of variance showed that tumbling significantly increased the ham internal temperatures overall and at 3, 6, 9, 12, 15, and 18 (Table 3). For the hams processed at 25°C the maximum tumbling effect of 1.32°C was achieved after 3 hr and this temperature difference between tumbled and stored hams gradually was reduced to about 0.63°C after 18 hr. For hams processed at 3°C, the maximum difference of 1.26°C was not achieved until after 18 hr (Fig. 1).

These data confirm that the effects of tumbling or massaging on color, exudate release, cohesiveness and subsequent product slicability are the result of the physical breaking up of normal cellular structure and subsequent ease of movement of salt and nitrite as reported by Cassidy et al. (1978), Theno et al. (1976), and Krause et al. (1978a) and not the result of the subsequent small rise in muscle temperature.

Yield

The weight of the ham was recorded at each sampling time and again after cooking and chilling. In Figure 2, this weight was expressed as a percent of the green, uncured weight and is referred to as yield. For this yield, there was no significant interaction between processing temperature and tumbling treatment. The relationship between sampling time and yield (Fig. 2) was significantly ($P<0.01$) quadratic as would be expected.

Final cooked, chilled yield was significantly affected by tumbling when the effects of processing temperature were

Table 1—Least-squares means ($\bar{X}$) and standard errors (SE) showing the effect of tumbling on sensory evaluation of cured hams[a]

	Tumbled[b]	Nontumbled	
	$\bar{X}$	$\bar{X}$	S.E.
Internal Color[c]	6.26**	5.30	0.18
Tenderness[d]	7.20	6.88	0.13
Cohesiveness[e]	6.71	5.92	0.15

[a] Processing temperature effects absorbed. Number of hams = 40 per treatment.
[b] Tumbled for 10 min out of every hour for 18 hr.
[c] Color score: 1 (unacceptable) to 10 (acceptable)
[d] Tenderness score: 1 (extremely tough) to 10 (extremely tender)
[e] Cohesiveness score: 1 (not cohesive) to 10 (very cohesive)
**Significant ($P<0.01$)

Table 2—Least-squares means ($\bar{X}$) and standard errors (SE) showing the effect of processing temperature on sensory evaluation of cured hams[a]

	Processing temperature		
	25°C	3°C	
	$\bar{X}$	$\bar{X}$	S.E.
Color[b]	6.14**	5.42	0.18
Tenderness[c]	7.34**	6.75	0.13
Cohesiveness[d]	6.20	6.43	0.15

[a] Tumbling effects absorbed. Number of hams = 40 per treatment.
[b] Color score: 1 (unacceptable) to 10 (acceptable)
[c] Tenderness score: 1 (extremely tough) to 10 (extremely tender)
[d] Cohesiveness score: 1 (not cohesive) to 10 (very cohesive)
**Significant ($P<0.01$)

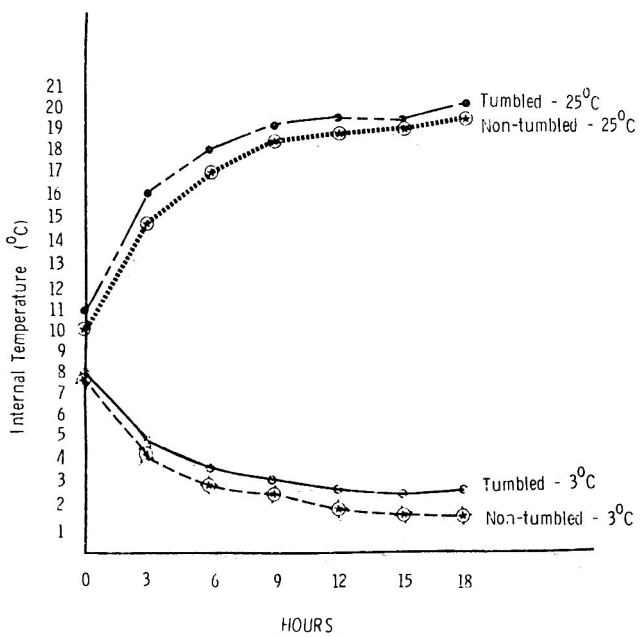


Fig. 1—Effect of tumbling and treatment temperature of tumbling and processing on internal temperatures of boneless, cured hams.

statistically absorbed (Table 4). Tumbling improved the yield of boneless, cured, cooked hams by 3.45%. These results agreed with data reported by Krause et al. (1978b) and Cassidy et al. (1978).

After the first 3 hr of tumbling these hams were 2.15% heavier than the nontumbled product. This advantage was gradually lost, such that after 15 and 18 hr of tumbling the different in yield was not significant. This is easily understood since no cover pickle was added to the tumbler and evaporative weight loss occurred in these hams. This much evaporative loss would not be expected in industry because of the practice of adding pickle to the tumbler and adequately sealing it.

However after cooking to an internal temperature of 68°C the ability of the tumbling process to assist the muscle in holding onto water again materialized. This in part may be due to the more uniform distribution of cure (including phosphate) and to the exudate of myofibrillar protein sealing in moisture as it coagulates on the surface (Rust and Olsen, 1973).

Processing temperature did significantly affect final, cooked yield independent of tumbling (Table 5). Processing at 25°C resulted in a significant ($P<0.05$) reduction in yield of 2.15%. The effects of processing at 25°C became significant ($P<0.05$) at the 9-hr treatment time.

Table 6 presents the effect of tumbling over time on the aerobic total plate counts (ATPC) (incubation of 25°C) when the effects of processing temperature was statistically absorbed. The aerobic total plate counts of the exudate on the surface of the hams were significantly decreased ($P<0.01$) after the completed 18 hr of tumbling. There was no significant interaction between tumbling and processing temperature.

The trend in ATPC (25°C incubation) from 3-9 hr of processing showed a consistent but nonsignificant reduction in ATPC for the tumbled hams. After 9 hr there was a significant ($P<0.05$) decrease in ATPC due to tumbling. The difference in ATPC between tumbled and nontumbled hams at the 12 hr sampling time, approached significance ($P<0.05$). After 12 hr all counts increased, indicating the end of the lag phase and the beginning of the log phase in the growth pattern. After 15 hr, the exudate ATPC was still increasing for the nontumbled hams, but the ATPC for the tumbled seemed to have leveled off. This resulted in a significant ($P<0.01$) difference in exudate ATPC between tumbled and nontumbled ATPC at both the 15 hr and the 18 hr sampling times.

Physical detachment of bacteria from the hams during tumbling would probably not be expected to decrease growth in the exudate as long as the bacteria remained in contact with nutrients. The salt content in this protein exudate, resulting from tumbling, could be high enough, however, to inhibit growth of bacteria detached from the ham surface during tumbling.

Tumbling, combined with the resultant disruption of the muscle sarcolemma could, however, force microorganisms into the internal regions of the hams, thus increasing the subsurface bacterial numbers while apparently reducing the surface exudate numbers. Unfortunately, the veracity of this hypothesis was not determined in this experiment.

As shown in Table 7, with tumbling effects statistically absorbed, processing temperature had no significant influence on ATPC (25°C incubation) in the surface exudate until the 15 hr time period. Again there appeared to be a 9 hr lag phase as evidenced by the declining ATPC during this period. At the 12 hr time, the ATPC at both temperatures increased by about 0.25% of a log unit, but there was no significant processing temperature effect at that time.

After 15 hr of processing, ATPC (25°C incubation) of the surface exudate from the refrigerated hams (3°C) leveled off, but it increased in the hams processed at room temperature (25°C), resulting in a significant difference ($P<0.05$) between the two temperature treatments at that time.

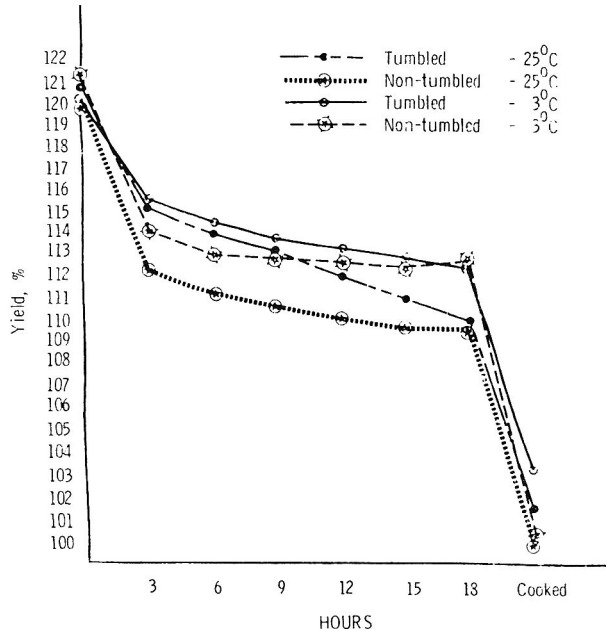


Fig. 2—Effect of tumbling and processing temperature on yields of boneless, cured hams over time.

Table 3—Least-squares means ($\bar{X}$) and standard errors (SE) showing the effect of tumbling on internal temperature[a] of boneless, cured hams[b]

	Tumbled[c] $\bar{X}$	Nontumbled $\bar{X}$	S.E.
0 hr	9.48	9.04	0.53
3 hr	10.78**	9.74	0.38
6 hr	11.19**	10.21	0.23
9 hr	11.51**	10.77	0.22
12 hr	11.48**	10.66	0.22
15 hr	11.36**	10.70	0.28
18 hr	11.82**	10.83	0.29

[a] Temperatures in °C
[b] Processing temperature effects absorbed. Number of hams = 40 per treatment.
[c] Tumbled 10 min of every hour for 18 hr.
**Significant ($P<0.01$)

Table 4—Least-squares mean ($\bar{X}$) and standard errors (SE) showing the effect of tumbling on yield[a] over time[b]

	Tumbled[c] $\bar{X}$	Nontumbled $\bar{X}$	S.E.
Yield, % – pumped	120.42	120.74	0.25
Yield, % – 3 hr	115.52**	113.37	0.41
Yield, % – 6 hr	114.38**	112.30	0.44
Yield, % – 9 hr	113.52*	111.89	0.45
Yield, % – 12 hr	112.98*	111.54	0.47
Yield, % – 15 hr	112.21	111.11	0.47
Yield, % – 18 hr	111.67	111.29	0.48
Yield, % – cooked	102.36**	98.91	0.62

[a] Yield is the weight at particular sampling time divided by green (initial) weight. Number of hams = 40 per treatment.
[b] Processing temperature effects absorbed
[c] Tumbled 10 min out of every hour for 18 hr
**Significant ($P<0.01$)
*Significant ($P<0.05$)

Table 5—Least-squares mean (X) and standard errors (SE) showing the effect of processing temperatures[a] on yields of boneless, cured hams over time

	Processing temperature		
	25°C $\bar{X}$	3°C $\bar{X}$	S.E.
Yield, % — pumped	120.52	120.64	0.25
Yield, % — 3 hr	113.93	114.95	0.41
Yield, % — 6 hr	112.77	113.91	0.44
Yield, % — 9 hr	111.97*	113.44	0.45
Yield, % — 12 hr	111.41*	113.11	0.47
Yield, % — 15 hr	110.61**	112.71	0.47
Yield, % — 18 hr	110.30**	112.66	0.49
Yield, % — cooked	99.56*	101.71	0.62

[a] Tumbling effects absorbed. Number of hams = 40 per treatment.
**Significant (P<0.01)
*Significant (P<0.05)

Table 6—Least-squares mean ($\bar{X}$) and standard errors (SE) showing the effects of tumbling on total aerobic plate counts[a] (incubated at 25°) from boneless, cured hams[b]

	Tumbled[c] $\bar{X}$	Nontumbled $\bar{X}$	S.E.
Number of Hams	20	20	
Initial[d]	4.10	3.94	0.14
0 hr	4.32	3.97	0.15
3 hr	3.79	4.02	0.12
6 hr	3.72	3.88	0.88
9 hr	3.64*	3.90	0.09
12 hr	3.90	4.09	0.09
15 hr	3.90**	4.30	0.09
18 hr	3.81**	4.26	0.11

[a] Total aerobic plate counts converted to log numbers per cm^2
[b] Processing temperature effects absorbed
[c] Tumbled 10 min out of every hour for 18 hr
[d] Sampled after skinning and boning but prior to curing
[e] Sampled after curing but prior to beginning of treatment time
**Significant (P< 0.01)
*Significant (P<0.05)

Table 7—Least-squares mean (X) and standard errors (SE) showing the effect of processing temperature on the total aerobic plate counts[a] (incubated at 25°C) on boneless, cured hams[b]

	Processing temperature		
	25°C $\bar{X}$	3°C $\bar{X}$	S.E.
Number of Hams	20	20	
Initial[c]	3.96	4.08	0.14
0 hr[d]	4.18	4.11	0.15
3 hr	3.93	3.88	0.12
6 hr	3.78	3.83	0.08
9 hr	3.74	3.79	0.09
12 hr	4.01	3.97	0.09
15 hr	4.23*	3.97	0.09
18 hr	4.22*	3.85	0.11

[a] Total plate counts converted to log numbers per cm^2
[b] Tumbling effects absorbed
[c] Sampled after boning but prior to curing
[d] Sampled ater curing but prior to beginning of treatment time
*Significant (P<0.05)

In the final 3 hr, the ATPC of the hams at room temperature stabilized while the ATPC of the refrigerated hams showed a slight decline as would be expected, thus a significant difference (P<0.05) resulted due to temperature treatments.

Since the tumbling effect on internal temperature only resulted in a 1.26°C difference, potential microbial problems hypothesized for tumbled hams prior to cooking would not materialize.

The exudate ATPC (incubation 25°C) differences due to either tumbling or processing temperature were small and would have little practical significance unless the hams were held for a time prior to entrance into the smokehouse. However, these results might raise questions both relative to the microbial situation within the hams and to the significance of pathogens if present. As would be expected, cooking to an internal temperature of 68.5°C resulted in a mesophilic ATPC of log 0.06 → log 0.11.

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Textural Characteristics of Beef: Effects of the Heating System

PAMELA L. BRADY and MARJORIE P. PENFIELD

ABSTRACT

Textural characteristics of beef semitendinosus roasts heated in 93 and 149°C ovens to 60 and 70°C were compared with those of small samples of semitendinosus heated as cylindrical cores in glass tubes in a water bath. The small samples were heated to the same endpoint temperatures as the roasts were at rates comparable to heating at the two oven temperatures. Cooking losses varied with heating method and in some cases with endpoint temperature and rate of heating. Heating method did not affect the textural parameters of penetration hardness, cohesiveness, and chewiness and shear cohesiveness and firmness. Hydroxyproline solubilization was greater in water bath samples than in intact samples heated at the slower rate. The extent of heat-related changes and their effects on final meat quality partially is determined by the heating system.

INTRODUCTION

MANY STUDIES on the effects of heating meat have involved the use of a technique in which small samples of meat are heated in test tubes or plastic bags in a water bath. A small sample technique was used by Machlik and Draudt (1963) who noted that previous workers (Marshall et al., 1960) had reported temperature variations of as much as 33°C within a 4.5-kg top round roast during the first few hours of heating. Machlik and Draudt (1963) concluded that small samples, in which the heat transfer is rapid, should be used in studies to obtain definitive information on heat effects. They used a technique in which cylindrical cores of meat were heated in test tubes in a water bath for varying lengths of time at temperatures ranging from 59-90°C. Temperatures at which various alterations in the proteins of meat occur were defined in that study.

Other groups have attempted to simulate oven roasting of meat by heating small samples in water baths that were periodically adjusted to produce the desired heating rate. Laakkonen et al. (1970) simulated conditions at the center of a steamship round of beef heated in a 121°C oven by heating slices of meat sealed in plastic bags and submerged in a water bath. Oven roasting of semitendinosus roasts at 163°C was simulated in the water bath heating of semitendinosus and biceps femoris strips (Paul et al., 1973). In several studies (Penfield and Meyer, 1975; Penfield et al., 1976; Hearne et al., 1978a, b), cylindrical samples of semitendinosus were heated in test tubes in a water bath that was controlled to simulate oven roasting of top round roasts at 93 and 149°C. Heat-related changes in meat quality have been described and explained on the basis of data derived from these studies.

Berry (1975) cited a need to determine whether conclusions based on small sample studies can be extrapolated to roasting and other cooking methods, which are employed by consumers. The present investigation was designed to compare textural characteristics and related chemical properties of beef semitendinosus roasts heated in an oven with those of small samples of semitendinosus heated in test tubes in a water bath. Roasts and samples heated at two rates and to two endpoint temperatures were compared.

Author Penfield is with the Dept. of Food Science, Nutrition & Food Systems Administration, Agricultural Experiment Station and College of Home Economics, Univ. of Tennessee, Knoxville, TN 37916. Author Brady, formerly with the Univ. of Tennessee, is presently affiliated with the Dept. of Food, Nutrition & Institution Administration, Univ. of Maryland, College Park, Md.

EXPERIMENTAL

TWELVE paired USDA Choice grade rounds were obtained from carcasses weighing 400-500 lb. Semitendinosus muscles were excised from the rounds. Muscle pH was determined (Rogers et al., 1967) and values were found to range from 5.50-5.82 with a mean of 5.69. Muscles were wrapped in freezer paper, blast frozen at −30°C, and then stored in a freezer at −15°C for periods of no longer than 6 wk.

Sample preparation and heating

Three pairs of muscles were assigned randomly to each of the two heating rates. One muscle from each pair was assigned to an endpoint temperature of 60°C and the other to a 70°C endpoint temperature. Frozen muscles were divided in half across the muscle fibers. One-half was designated for heating as an intact roast in an oven, and the other was designated for heating as small samples in a water bath system.

Each intact roast was thawed for 48-72 hr at 4°C prior to testing. Epimysial connective tissue and adhering fat were removed. A copper-constantan thermocouple was placed in the geometric center of the roast and the roast was placed in an unheated electric oven. The oven control was adjusted to achieve the designated temperature. Roasting temperatures of 93 and 149°C were used and will be referred to as slow and fast heating, respectively. When the desired endpoint temperature was reached, the roast was removed from the oven and allowed to cool to 26°C at room temperature (23°C) for 1-2 hr. Following overnight refrigerator storage (4°C), roasts were cut across the fibers into 5.7-cm segments. Cylindrical cores, 2.5 cm in diameter, were removed for analysis. Cores were taken parallel to the muscle fibers.

Frozen, raw muscle halves designated for core heating were sawed with a handsaw into 5.7-cm long sections across the muscle fibers. The meat was allowed to partially thaw to facilitate removal of cores 2.5 cm in diameter. Muscle fibers were parallel to the length of each cylindrical core. Each core was placed in a preweighed 50-ml glass centrifuge tube and the tubes were placed in a shaker water bath containing cold water (2-5°C). The water bath temperature control was adjusted every 8 min so that the heating curve of the cores matched the heating curve of the intact roast from the same muscle. When a copper-constantan thermocouple placed in the center of one core measured the desired endpoint temperature, the tubes were removed from the water bath and the cores were allowed to cool to 26°C in the tubes at room temperature (23°C) for 1-2 hr. Cooled cores were wrapped in foil and refrigerated (4°C) overnight prior to further analysis.

Cooking losses

Appropriate weights were determined before and after heating for use in calculations of losses.

Expressible moisture index

Samples (300 mg) of ground heated meat were placed on sheets of Whatman No. 1 filter paper. Papers with samples were placed between acrylic sheets and subjected to pressures of 2272 kg over a 5-min period with a Harco Hydraulic Press (FSNFSA, 1976). The Expressible Moisture Index (EMI) was defined as the ratio of the meat area to the juice area.

Evaluation of tenderness

Following overnight refrigeration storage, the heated cores were allowed to warm to 23°C. Tenderness components of texture, measured as penetration and shear parameters, were evaluated with an Instron Universal Testing Machine (Model 1130). For measurement of penetrability characteristics, a flat plunger (0.63 cm in diameter) was driven vertically 80% of the way through a core. The samples were presented so that the fibers were perpendicular to the direction of the plunger penetration (Bouton et al., 1971). The plunger was driven into the meat twice at each of two locations on each of three cores per heating treatment. A 50-kg load was used with a crosshead speed of 50 mm/min and chart speed of 100 mm/min. The work force penetration curve for each of the two cycles was recorded.

Penetrability parameters were determined as outlined by Bouton et al. (1971). "Hardness," or the force in kilograms required to achieve the first penetration, was measured as the peak height of the curve. "Cohesiveness" was defined as the ratio of the work done during the second penetration to that performed during the first. Work was determined by using a compensating polar planimeter to trace the area bounded by the baseline, the upward sweep of the curve, and the line drawn perpendicular to the baseline through the maximum point on the curve. "Chewiness" was defined as the product of "hardness" and "cohesiveness" and expressed in kilograms of force.

Shearability parameters were measured with a Warner Bratzler shear attachment to the Instron. Load cell, crosshead speed, and chart speed were as described for penetration testing. The work force curve for each of six shear operations per treatment was recorded. Evaluations of the curves produced the textural parameters of "firmness" and "shear cohesiveness" as defined by Larmond and Petrasovits (1972). "Firmness" was calculated as the slope of a line drawn from the origin of the curve to its peak and was reported in kilograms per minute. "Shear cohesiveness," expressed in kilograms, was measured as the peak force recorded on the shear-deformation curve.

Hydroxyproline solubilization

Samples for determination of hydroxyproline solubilized during heating were prepared according to the method of Paul et al. (1973) as modified by Penfield and Meyer (1975). The term hydroxyproline solubilized rather than collagen solubilized is used since hydroxyproline values were not corrected for elastin content. Hydroxyproline analysis was performed according to Method II described by Woessner (1961).

Statistical analysis

A 2 x 2 factorial experimental design with three replications was used for studying each of two heating rates. Analysis of variance was performed to study the effects of the independent variables, heating system and endpoint temperature, and their interactions. When preliminary analysis showed no significant differences due to interactions of main effects, the interactions were pooled into the error term for final analysis. Mean separation was accomplished by the Student-Newman-Keuls Test (Sokal and Rohlf, 1969). Because of differences inherent in mean values from different animals, samples heated at the two rates were compared with a t-test assuming unequal variances. Correlation coefficients were used to determine relationships between penetration and shear parameters.

RESULTS & DISCUSSION

Cooking losses

With the fast heating rate, drip and evaporative losses were affected by heating system (Table 1). Oven roasting resulted in lower ($P<0.001$) drip losses and greater ($P<0.001$) evaporative losses than water bath heating. Oven roasting results in exposure of the meat and the cooking pan to the hot air of the oven allowing losses to be dissipated through evaporation. In water bath heating the samples were enclosed to a greater extent. Therefore, most loss remained in the tube and was measured as drip. Endpoint temperature affected drip loss but did not affect evaporative and total losses of samples heated at the fast rate (Table 1).

In slowly heated meat the effect of heating system on drip and evaporative losses varied with endpoint temperature. Drip losses in samples heated in the oven to 60 and 70°C did not differ significantly, whereas drip losses in water bath heated samples increased ($P<0.01$) with endpoint temperature. The reverse was seen with evaporative losses. As endpoint temperature increased, the evaporative loss from oven-heated samples increased ($P<0.05$), but samples heated in the water bath to differing endpoint temperatures were similar with respect to evaporative losses. The patterns of greater drip and less evaporation with the water bath system and greater evaporation and less drip with oven roasting observed in the samples heated at the fast rate were observed also with the slow heating rate. Total cooking losses of slowly heated samples varied with heating method ($P<0.001$) and endpoint temperature ($P<0.01$)

Mean total cooking losses of slowly heated samples were greater ($P<0.05$) than those for the samples heated at the fast rate (Table 1). Mean drip and evaporative losses were similar for the two heating rates.

Expressible moisture index

The EMI's of samples heated at both the fast and slow rates were not significantly affected by heating system or endpoint temperature. Although not significant, there was a trend for the EMI's of oven-roasted samples to be lower than those of samples heated in the water bath (Table 1).

–Continued on next page

Table 1–Cooking losses and expressible moisture indices of beef semitendinosus muscle heated as intact roasts in an oven and as cores in a water bath to two endpoint temperatures at two rates[a,b]

Source of variation	Cooking losses: Drip %	Evaporative %	Total %	EMI[c]
Fast heating rate				
Heating system				
Oven	1.97x	18.08x	20.05	0.236
Water bath	23.76y	0.26y	24.02	0.271
Endpoint temp (°C)				
60	10.35a	9.15	19.49	0.247
70	15.39b	9.19	24.58	0.259
System X Endpoint (°C)				
Oven-60	1.45	18.09	19.54	0.242
Oven-70	2.49	18.07	20.56	0.229
Water bath-60	19.24	0.20	19.44	0.252
Water bath-70	28.28	0.31	28.59	0.289
Slow heating rate				
Heating system				
Oven	1.32x	21.55p	22.87x	0.276
Water bath	32.47y	0.66q	33.14y	0.284
Endpoint temp (°C)				
60	14.10p	8.00a	22.10x	0.267
70	19.69q	14.22b	33.91y	0.293
System X endpoint				
Oven-60	1.01p	15.72a	16.73	0.261
Oven-70	1.63p	27.38b	29.01	0.290
Water bath-60	27.18q	0.27c	27.47	0.273
Water bath-70	37.75r	1.05c	38.80	0.295
Heating rate				
Fast	9.17	12.87	22.03a	0.253
Slow	11.11	16.89	28.00b	0.280

[a] Means of three replications.
[b] Means in the same column within a variation grouping followed by different letters differ at $P<0.05$ (a,b,c); $P<0.01$ (p,q,r); or $P<0.001$ (x,y).
[c] Expressible Moisture Index.

Table 2—Penetration and shear parameters and hydroxyproline solubilization in semitendinosus muscles heated as intact roasts in an oven and as cores in a water bath to two endpoint temperatures at two rates[a,b]

Source of variation	Penetration			Shear		Hydroxyproline solubilized
	Hardness kg	Cohesiveness	Chewiness kg	Cohesiveness kg	Firmness kg/min	%
Fast heating rate						
Heating system						
Oven	3.271	0.538	1.698	7.42	75.81	5.65
Water bath	2.960	0.528	1.395	7.16	73.16	5.09
Endpoint temp (°C)						
60	2.937	0.518	1.467p	7.14	70.11	5.80
70	3.294	0.549	1.626q	7.44	78.86	4.94
System X Endpoint						
Oven-60	3.208	0.499	1.537	7.19	71.18	5.72
Oven-70	3.333	0.577	1.859	7.64	80.44	5.58
Water bath-60	2.665	0.536	1.396	7.09	69.04	5.88
Water bath-70	3.255	0.520	1.393	7.23	77.27	4.29
Slow heating rate						
Heating system						
Oven	2.647	0.540	1.276	7.35	71.80	6.71a
Water bath	2.709	0.476	1.329	6.57	62.71	9.16b
Endpoint temp (°C)						
60	2.260a	0.581a	1.276	6.79	60.74a	7.19
70	3.097b	0.435b	1.329	7.13	73.76b	8.69
System X endpoint						
Oven-60	1.889a	0.700a	1.253	6.84	61.34	6.01
Oven-70	3.405b	0.379b	1.229	7.86	82.25	7.41
Water bath-60	2.630c	0.462b	1.299	6.74	60.14	8.36
Water bath-70	2.788c	0.490b	1.358	6.40	65.27	9.96
Heating rate						
Fast	3.115	0.533	1.621x	7.30	75.33p	5.37p
Slow	2.678	0.508	1.276y	6.86	65.61q	7.94q

[a] Means of three replications.
[b] Means in the same column within a variation grouping followed by different letters differ at $P<0.05$ (a,b,c); $P<0.01$ (p,q); or $P<0.001$ (x,y).

Tenderness

Penetrability values are presented in Table 2. In meat heated at the fast rate, neither heating system nor endpoint temperature had a significant effect on hardness and cohesiveness. In meat heated at the slow rate, however, hardness and cohesiveness were affected ($P<0.05$) by endpoint temperature in oven-roasted samples. Meat heated in an oven to 70°C had higher hardness values and thus offered more resistance to penetration than samples heated to 60°C in an oven. In meat heated at the slow rate, cohesiveness (penetration) of oven-roasted samples decreased with increased endpoint temperature. Cohesiveness and hardness were unaffected by endpoint temperature in meat heated in a water bath. Heating rate did not significantly affect hardness or cohesiveness.

Chewiness was altered ($P<0.01$) by endpoint temperature but was not affected by heating system when samples were heated at the fast rate. In meat heated at the slow rate, neither heating system nor endpoint temperature had an effect on chewiness. Chewiness was lower ($P<0.001$) in samples heated at the slow rate than in samples heated at the fast rate.

Bouton et al. (1975) observed that penetration measurements of the type reported in the present study correlated better with connective tissue properties than with muscle fiber properties. However, the data reported here may be indicative of the generally accepted changes that occur in meat during heating. An increase in hardness during heating from 60 to 70°C may reflect coagulation or hardening of muscle fibers. Although the change at the fast rate was not significant there was a trend toward increased hardness. The prolonged heating time of the slow method may have contributed to increased hardening of the muscle fibers.

The decrease in cohesiveness or reduction in internal bonding strength that occured as samples were heated from 60 to 70°C at the slow rate may have resulted from increased granulation or solubilization of collagen (Penfield and Meyer, 1975). The length of the heating period may not have been long enough to permit significant collagen solubilization or granulation at the fast rate.

Regardless of heating rate, neither heating system nor endpoint temperature had an effect on the shear parameter, cohesiveness. Values presented in Table 2 show that with both heating rates there was a slight but nonsignificant increase in cohesiveness with increasing endpoint temperature. Since shear cohesiveness was defined as the maximum height in kilograms of the shear force-deformation curves (Larmond and Petrasovits, 1972), these findings are consistent with Warner Bratzler shear values reported by Laakkonen et al. (1970), Penfield and Meyer (1975), and Hearne et al. (1978a).

Firmness was not affected by heating system or endpoint temperature when meat was heated at the fast rate. Treatment means however, revealed a trend toward increased firmness with increasing endpoint temperature. With slow heating, a sample's firmness or resistance to deformation increased ($P<0.05$) with increased endpoint temperature.

Mean shear values for the heating rates showed that, while the mean for cohesiveness was slightly greater for the fast heating rate than for the slow one, this difference was

not statistically significant. Firmness values, however, were higher ($P<0.01$) with fast heating than with slow.

Correlation coefficients between penetration and shear values (Table 3) indicated that in meat heated in a water bath all parameters were related except shear cohesiveness and penetration hardness. With oven roasting, shear cohesiveness was related only to the penetration parameter, chewiness.

Hydroxyproline solubilization

In meat heated at the fast rate, hydroxyproline solubilization was not affected by heating system (Table 2). In slowly heated samples, hydroxyproline solubilization was greater ($P<0.05$) in water bath samples than in oven-roasted ones. Since hydroxyproline solubilization has been shown to increase with increased heat application (Paul et al., 1973; Penfield and Meyer, 1975), this increased solubilization suggests that in slowly heated samples, water bath heating resulted in a more severe heat treatment than oven roasting.

Hydroxyproline solubilization was not affected by endpoint temperature at either heating rate (Table 2). This is in agreement with the work of Williams and Harrison (1978) but is in contrast to the findings of Paul et al. (1973) and Penfield and Meyer (1975) who reported increased hydroxyproline solubilization with increased endpoint temperature.

Hydroxyproline solubilization was greater ($P<0.01$) in samples heated at the slow rate than in samples heated at the fast rate (Table 2). These findings are in agreement with those of Penfield and Meyer (1975).

In conclusion, textural characteristics and related chemical changes of meat heated as cores in a water bath may be affected by endpoint temperature. This factor also may affect the tenderness of meat heated as intact roasts in an oven. The extent of the heat-related changes and their effect on final meat quality is, at least in part, determined by heating system. In the present study, mechanical parameters of texture were not influenced by heating system even though cores from meat heated as intact roasts inherently represent more tissue than is represented by cores heated as small samples. However, cooking losses and hydroxyproline solubilization were affected. In general, for each parameter studied, variation among data within a heating system was similar for both oven and water bath heating. If conclusions from research studies using water bath heating are to be extrapolated to meat heated as intact roasts in an oven, it will be necessary to further define the similarities and differences of samples heated in the two systems.

Table 3–Correlation between shear parameters and penetration parameters of beef semitendinosus muscles heated as intact roasts in an oven and as cores in a water bath

	Shear cohesiveness		Shear firmness	
	Intact n=12	Core n=10	Intact n=12	Core n=9
Penetration				
Hardness	0.542	0.198	0.828***	0.639*
Cohesiveness	−0.414	0.654*	0.665*	−0.674
Chewiness	0.595*	0.578*	0.739*	0.873***

* $P<0.05$
** $P<0.01$
*** $P<0.001$

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Effect of Bone Source and Storage on the Role of Mechanically Deboned Pork in Rancidity Development in a Cooked and Smoked Sausage

H. W. OCKERMAN, J. H. HOUBEN, B. KROL, R. F. PLIMPTON JR. and M. SCHAD

ABSTRACT

The chemical and physical differences found in this study between mechanically deboned pork and hand deboned pork agree with the data reported in the literature except for fatty acid analyses which suggest little difference between these two types of tissue. The type of bone (ham versus vertebrae) had no significant effect on color, flavor, odor or tenderness of the sausage product, but mechanically deboned tissue from vertebrae did not influence color, but did increase rancidity. However, the evaluation panel did not object to this flavor in this type of sausage product. Mechanically deboning tissue significantly darkened the color of the raw material and the finished product. It also decreased the tenderness desirability of the sausage. Type of antioxidant significantly improved external color, but rancid taste was not significantly affected. Color became darker, tenderness desirability decreased, and flavor and odor deteriorated with product storage.

INTRODUCTION

AS THE WORLD searches for increased protein for human consumption, one protein source that is often overlooked is that of mechanically deboned meat. The term mechanically deboned meat (MDM) is intended to include meat tissue from which the bone portion has been removed by mechanical, as opposed to manual, techniques (Ackroyd, 1976). With mechanical deboning it would be possible to recover 2,090,757 metric tons of mechanically deboned tissue per year (Field, 1976a). Pork carcasses would each contribute from 3-4 pounds per carcass (Goldstrand, 1975; Fried 1976) if all bones were processed. This would amount to 374,000,000 pounds of mechanically deboned pork for human consumption (Pietraszek, 1975). Under present industry practices (Goldstrand, 1975) picnic, ham and blade bones are the bones available in large volume for deboning. These bones represent 60-65% of the bones of a pork carcass and will produce 2.0-2.8 pounds of deboned meat. The weight of protein recovered is 0.22-0.30 pounds per carcass. This tremendous quantity of currently under utilized protein is not without its problems, since mechanically deboned meat is usually a very fine minced product, making it highly susceptible to bacterial growth. The temperature encountered during mechanically deboning (which can be somewhat controlled by control of equipment) also encourages bacterial growth and oxidation of the tissue.

At the present time, meat bones are treated as by-product with little value. These bones comprise some 16-20% of the carcass (Katz and Ackroyd, 1976), and high labor costs make it economically unfeasible to further manually separate lean tissue from bones. Certain bones designated for by-products can contain in excess of 50% lean meat (Ackroyd, 1976). Field (1976) reported that bones from the vertebral column, ribs and sternum were most suitable for mechanically deboning because they usually have more lean attached and thus will yield a greater percentage of mechanically separated tissue. Round bones were least suitable for mechanical deboning since they have very little lean attached and the marrow is primarily fat. The composition of mechanically deboned meat has been reported (Field, 1974) as dependent upon the material entering the boning machine and the setting at which the machine was operating. Field et al. (1976) also reported that the percent protein in mechanically deboned meat was lower and the fat content was higher from similar hand deboned meat. This difference in composition was due to bone marrow which is extracted as deboned meat and to connective tissue which is removed from the meat during mechanically deboning (Field et al., 1976). According to Gillett et al. (1976) mechanically desinewing removed approximately half of the connective tissue and reduced the tendency for formation of gelatin pockets in cooked product. Field (1976b) stated that the calcium content in mechanically deboned meat was always higher than that for hand deboned meat because some fine bone particles go through the holes in the deboner and Chant et al. (1977) found the ascorbic acid content of mechanically deboned meat to be 1.37 μg/100g of tissue. This tissue is darker in color than whole muscle (Meiburg et al., 1976; Field, 1986b) and Goldstrand (1975) reported that the color level of mechanically deboned pork and beef was 25-35% higher than that of pork and beef trimmings of similar protein and fat level. The color of mechanically deboned meat was a bright red when fresh and properly chilled, but a dull brownish red after pigment oxidation had occurred (Field, 1976b). Marshall et al. (1977) found that consumer panelists generally preferred, or did not dislike, the flavor, juiciness, and texture of frankfurters containing 10% mechanically deboned pork when compared to control frankfurters. However, Field (1976a) reported that bologna with high percentages of mechanically deboned meat had a spicier flavor than product made with hand deboned meat. In other work, a triangle test indicated no significant flavor difference ($P>0.05$) existed in bologna containing 10% machine deboned versus 10% hand deboned meat (Field and Riley, 1972). When using mechanically deboned beef in a cooked salami product, flavor profile and panel results indicated higher aroma and flavor intensities and more rancid flavor with increased levels of mechanically deboned meat and with increased lengths of storage (Joseph et al., 1977, 1978). Field et al. (1974a) and Anderson and Gillett (1974) found that there was no significant difference in emulsifying capacity or emulsifying stability between hand deboned meat and mechanically deboned meat from the whole carcass. In cooked meat items, heme is the predominant catalyst, but significant lipid oxidation can occur by nonheme iron-type catalyst even in the presence of heme (Kwoh, 1971). Mechanically deboning increases the hemoglobin and nonheme iron content, but has little influence on the amount of myoglobin in deboned fish flesh. Myoglobin has a greater catalytic effect than hemoglobin on the oxidation of oleic acid

Authors Ockerman, Plimpton, and Shad are affiliated with the Dept. of Animal Science, The Ohio State Univ., Columbus, OH 43210, and The Ohio Agricultural Research & Development Center, Wooster, OH 44691. Author Houben is with The State University, Faculty of Veterinary Medicine, Utrecht, The Netherlands. Author Krol is with The Central Institute for Nutrition and Food Research TNO, Zeist, The Netherlands.

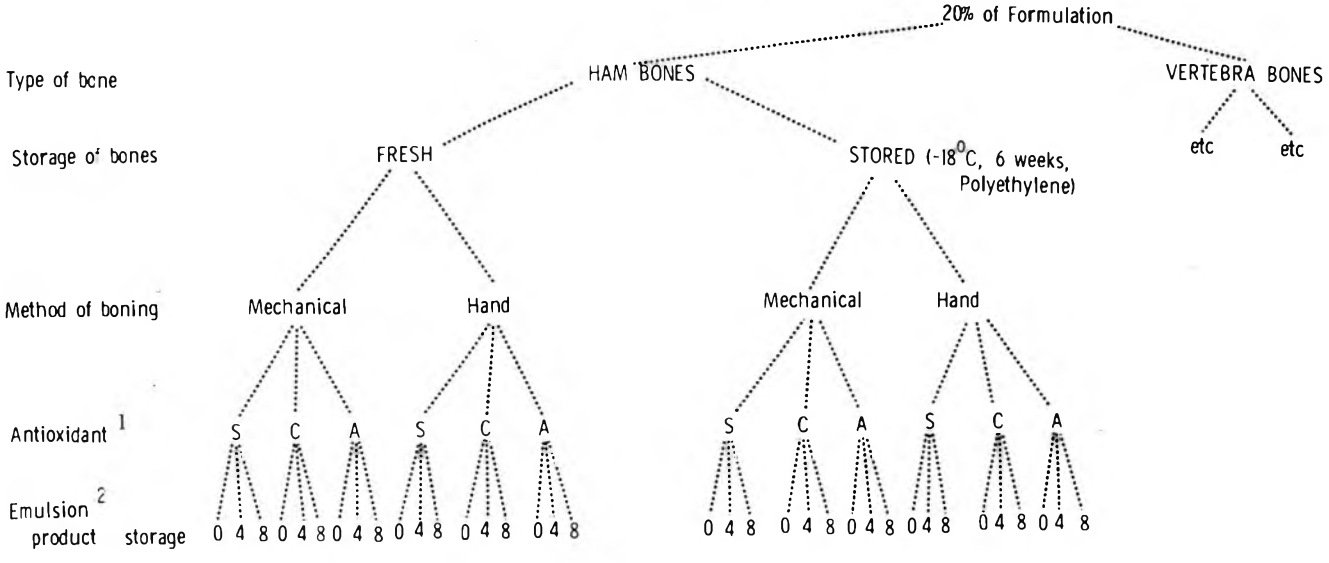
(Silberstein and Lillard, 1978). Lipid oxidation can be inhibited by using anti-oxidants and chelating agents, especially polyphosphates. In raw meat, propyl gallate and BHA protect meat pigments and inhibit lipid oxidation. Nitrite can also be an effective antioxidant. Carpenter (1976) reported that the limitation on keeping time on mechanically deboned poultry meat in frozen storage is established by the rate of rancidity. Because of this, the present recommendation for length of storage time of frozen stock is not more than 6 months at −23.3°C from the date of manufacture. Froning (1976) stated that a slight decline in the quality of summer sausage made from 50% mechanically deboned poultry meat occurs when the product is stored at 25°C. This was evidenced by corresponding TBA values and sensory panel scores. Maxon and Marion (1970) found that changes in lipid in mechanically deboned turkey meat occurred during storage and TBA demonstrated a linear increase with storage time. Uebersax et al (1978) found TBA numbers increased with increased amounts of mechanically deboned turkey meat. Goldstrand (1975) reported that although mechanically deboned red meat was less stable than hand deboned meat, the large decrease in flavor during storage, as noted by Dimick et al. (1972), for mechanically deboned poultry, was not present in mechanically deboned red meat. Meiburg et al. (1976) found that TBA values remained low and acceptable for frankfurters made from cow and mutton mechanically deboned meat after 18 wk of frozen storage and Kunsman et al. (1978) and Cross et. al. (1978) found mechanically deboned meat from beef bones (aged or fresh) oxidized at about the same rate as lipids in ground beef. Mechanically deboned red meat had a much higher level of hemo-proteins (hemoglobin and myoglobin) and a lower polyunsaturated fatty acid content than does mechanically deboned chicken meat. Therefore, the relative concentration ratio of polyunsaturated fatty acids to hemo-proteins was much different in red meat than it was in mechanically deboned chicken meat. Changes in the ratio of polyunsaturated fatty acids to hemo-protein were undoubtedly responsible for the difference in storage characteristics between mechanically deboned poultry and mechanically deboned red meat (Field, 1976b). Meiburg et al. (1976) found that mechanically deboned pork was between mechanically deboned beef and mechanically deboned poultry as far as stability was concerned and recommended that mechanically deboned pork should be stored only for a short period of time in the frozen state before TBA values become too high and taste panelists object to the flavor of this product.

The purpose of this experiment was to study any oxidation problems encountered when using mechanically deboned pork as one of the raw materials in making a cooked and smoked sausage product. Other variables in the experiment were types of bone (ham bones in contrast to vertebra), frozen storage (−18°C) of bones prior to deboning and type of anti-oxidant. The level of mechanically deboned pork used was 20%. Samples were stored after manufacturing for 0, 4 and 8 wk at 12°C.

EXPERIMENTAL

THIS RESEARCH was conducted on a product called Boterhamworst which, in many respects, is similar to bologna. It is a cooked and smoked emulsion-type sausage that has a fine texture, except for rather large fat particles (pork backfat) that are evenly distributed throughout the product. The sausage, which contains both beef (1 part) and pork (3 parts), salt, phosphate, spices, nitrite and 4% wheat starch, was formulated to process 30% fat and a 4:1 moisture to protein ration in the finished product. Twenty percent (calculated on total weight) of the pork portion of this formulation was distributed as shown in Figure 1.

All bones were from Dutch breeds of hogs of approximately 6 months of age that were slaughtered in the same plant and the bones were obtained at 2-3 days postmortem (stored at 4°C). The vertebra bones were obtained as a by-product of a pork loin boning operation and the ham bone consisted of the femur, patella, and tibia. One-half of the bones used in this experiment were stored at −18°C for 6 wk in polyethylene bags. The other half were used immediately. The bones were subjected to two types of boning, either mechanical or hand deboning. The mechanical deboning was done with a Protecon MRS-40 machine which consisted of a filter system with orifices of 0.8 mm in size. The deboning was



[1] S = No antioxidant, C = sodium ascorbate, A = α-tocopherol and ascorbyl palmitate

[2] 0 = Zero weeks, 4 = four weeks, 8 = eight weeks at 12°C

Fig. 1—Experimental design (replicated 3 times) for the manufacture and storage of boterhamworst.

performed at a pressure of 280 kgf/cm^2. A rise in temperature of mechanically deboned product ranged from 6-15°C for fresh product and from 0-4°C for frozen product. Hand deboning consisted of removal of all visible skeletal muscle tissue and soft connective tissue from the bones.

Sausage processing

During sausage processing, beef, the deboned pork (either hand or mechanical) and ice was added first and chopped in a silent cutter for approximately 30 sec. Next salt, nitrite, phosphate, spices (and sodium ascorbate, if used) were added. Then a blend of α-tocopherol and ascorbyl palmitate, if used, were added. Next, the starch was added to the chopper. Pork backfat was then added and chopping was completed at a slow speed for a total of 5 min. The final emulsion temperature was approximately 9°C. The product was then stuffed into a fibrous cellulose casing and was smoked and cooked to an internal temperature of 70°C (see Table 1).

Analysis of raw material

Analysis of meat from the bones was characterized by determining the moisture (drying to constant weight at 103°C) and protein (modified Kjeldahl) of the tissue by the methods according to the Dutch Food law. Fat content was determined by the butyrometric fat (acid digestion) determination technique. The pH was determined by using a pH meter on a slurry of the tissue. The percentage of bone was determined by heating the tissue in an alcoholic KOH solution for 1.5 hr and then filtering through previously dried filter paper. Hydroxyproline (ISO/DIS 3496/2, 1976) and gas chromatography (IUPAC II.D.19 preparation of fatty acid methyl esters; IUPAC II.D.25 gas chromatography of the mixture) analysis of the methyl esters of fatty acids from these tissues was determined at the Central Institute for Nutrition & Food Research TNO in Zeist, The Netherlands. The copper, calcium, and iron determination was accomplished by means of atomic absorption (Perkin-Elmer Corporation).

Analysis of manufactured product

After the sausage product was manufactured, the zero storage time product, the product that has been stored for 4 wk and products that had been stored for 8 wk was sensory evaluated (Table 2) and pH determined. This evaluation was carried out by a trained panel consisting of seven judges (facilities were designed for meat product evaluation and facilities and panel members are the same as those used at TNO to evaluate meat products for export). Panel members were all experienced and trained Dutch male food researchers and evaluated the sausage for external and internal color; odor-flavor, and tenderness. The scores were based on a scale of 1-10. In addition, panel members were verbally instructed to make a notation if they noted any rancid taste or odor (see step 3, Table 2).

Sausage product data were analyzed using least square analysis of variance procedure for unequal subclass numbers as described by Harvey (1968).

Table 1—Smoking and cooking schedule

Cycle	Time (min)	Dry bulb	Wet bulb	Temp (°C)
Drying	40	40°	37°	–
Smoking	60	40°	40°	–
Cooking	40	70°	60°	–
Cooking[a]	90	–	–	75°

[a]This cycle was done in hot water.

Table 2—Emulsion product sensory panel sheet

Product Date Judge's Name

Step 1 Rank the samples in order from excellent to poor for judgment of external and internal color.
Step 2 Use a scale of 1 (poor – dark) to 10 (excellent – bright) to describe the properties in Step 1.
Step 3 If you detect anything unusual, write it down.

	External color	Internal color
Number		
Score		
Remarks		

Product Date Judge's Name

Step 1 Rank the samples in order from excellent to poor for judgment of flavor-odor and tenderness
Step 2 Use a scale of 1 (poor – little flavor-odor, lacking desirable tenderness, tough or mushy) to 10 (excellent – characteristic boterhamworst flavor-odor, characteristic tenderness) to describe the properties in Step 1.
Step 3 If you detect anything unusual, write it down.

	Odor & flavor (rancidity)	Tenderness
Number		
Score		
Remarks		

RESULTS & DISCUSSION

Raw material

Preliminary information on chemical composition, color, and fatty acid analysis of hand and mechanically deboned meat from ham and vertebra bones can be found in Tables 3 and 4. Percent moisture was higher, percent protein was lower, percent fat was very similar, and percent ash was higher in mechanically deboned meat than in hand deboned meat. The vertebra bones yielded a higher protein value than the ham bones in both the hand and mechanically deboned meat categories. While the ham bones yielded a higher percent fat than the vertebra bones, little difference in chemical composition could be seen between the meat obtained from fresh bones and those stored for 6 wk at −18°C.

The percent hydroxyproline and the collagen/protein ratio were higher in hand deboned than in mechanically deboned meat and the hydroxyproline content and collagen/protein ratio was higher in tissue from ham than in vertebra bones. This agrees with the work reported by Field et al. (1976). The difference has been attributed to the fact that mechanically deboning separates bone and collage from the muscle (Satterlee, 1975; Field, 1976a; Field and Riley, 1972, Field et al., 1974b). In general some connective tissue is removed by mechanically deboning and some bone particles and bone marrow are added to the deboned meat (Field, 1976a). The amount of collagen in mechanically deboned meat is related, not only to the bone content of mechanically deboned meat, but also to the amount of lean on the bone prior to deboning (Chant et al., 1976). Little difference was noted in hydroxyproline and collagen/protein ratio between meat obtained from fresh bones and meat obtained from bones stored for 6 wk at −18°C.

The percent of calcium and percent of iron were higher, while the percent of copper was approximately equal in mechanically and hand deboned product. As would be expected, the bone content is higher in mechanically deboned meat. Field (1976b) stated that the calcium content of mechanically deboned meat was always higher than that of hand deboned meat. The increase in iron content in mechanically deboned meat can be explained by the contribution of heme pigments from the bone marrow incorporated into meat during this process (Froning and Johnson, 1973).

A higher pH was found in mechanically deboned meat, which agrees with the report of Chant et al. (1977). The pH readings were higher in mechanically deboned tissue from ham than from mechanically deboned tissue from vertebra

Table 3—Chemical composition and color characteristics of meat from bones

	Fresh bones				Stored 6 wk at −18°C			
	Hand boning		Mechanical boning		Hand boning		Mechanical boning	
Chemical composition	Ham bones	Vertebrae	Ham bones	Vertebrae	Ham bones	Vertebrae	Ham bones	Vertebrae
Moisture, %	47.8	59.4	52.2	60.6	48.1	58.7	52.7	60.0
Protein, %	14.3	17.2	9.6	14.4	14.7	17.9	11.0	15.5
Fat, %	38.5	22.5	36.5	23.3	38.4	23.2	33.8	23.0
Ash, %	0.51	1.21	1.25	1.44	0.58	1.44	2.74	1.76
Hydroxyproline, %	1.03	0.32	0.17	0.15	0.88	0.20	0.26	0.14
C/P ratio[a]	0.58	0.15	0.14	0.08	0.48	0.09	0.19	0.07
Calcium, %	90 ppm	0.20	0.25	0.27	0.04	0.19	0.80	0.38
Copper, ppm	1.1	0.9	1.0	1.0	–	–	–	–
Iron, ppm	10.0	10.0	41.0	39.0	–	–	–	–
Bone, %	0.10	1.41	1.57	2.00	0.08	0.47	2.07	0.93
pH	6.79	6.06	7.07	6.56	7.17	6.51	7.23	7.07
Color[b]	5.0	3.7	3.0	3.0	–	–	–	–

[a]C/P ratio = collagen/protein ratio = 8 x % hydroxyproline/% protein
[b]Sensory panel evaluation—Hedonic scale: 1 = dark, 10 = light.

Table 4—Fatty acid analysis[a] of meat from fresh (not stored prior to processing) bones

	Hand boning		Mechanical boning	
	Ham bones	Vertebrae	Ham bones	Vertebrae
10:1	0.1	–	–	–
12:0	0.1	0.1	–	–
14:0	1.4	1.4	1.2	1.3
16:0	23.0	24.6	24.5	25.0
16:1	2.8	1.9	1.7	1.9
17:0	0.3	0.4	0.4	0.4
17:1	0.4	0.3	0.3	0.4
18:0	10.1	14.7	15.8	14.2
18:1	48.2	44.2	44.6	47.0
18:2	10.0	9.4	8.6	7.9
18:3	0.7	0.5	0.5	0.2
20:0	–	–	0.1	–
20:1	0.8	1.2	1.2	1.3
20:2/20:3	–	–	0.2	0.4
20:3	0.4	0.5	0.1	–
22:0	0.8	–	0.1	–
22:1	–	–	–	–
24:0	–	–	–	–
24:1	0.7	0.5	0.5	–

[a]Weight percent of methyl esters of fatty acids

bones. These same researchers claim that bone marrow undoubtedly made the major contribution to the higher pH readings. Ham bones contain more marrow than vertebra bones (Field, 1976a). Anderson and Gillett (1974) observed that pH of marrow extracts was more than a full pH unit higher than that of muscle extracts from the same animal and they also found that the addition of marrow to muscle increased the pH of the muscle. The pH also seemed to increase during storage when the fresh product was compared with the product derived from bones stored for 6 wk at −18°C.

The mechanically deboned meat was much darker in color than the corresponding hand deboned meat from the same type of bone. This agrees with the reports of other researchers (Goldstrand, 1975; Meiburg et al., 1976; Field, 1976b). The difference in color has been attributed to the contribution of heme pigments from the bone marrow incorporated into the meat (Froning and Johnson, 1973) and to the elimination of connective tissue (which is devoid of pigment) during mechanically deboning (Field, 1976b).

The results of the fatty acid analysis in this project (Table 4) did not agree with the literature. It was expected that there would be more unsaturated fatty acids in the mechanically deboned meat than in hand deboned meat. Some authors have stated that bone marrow lipids contain more unsaturated fatty acids than subcutaneous or intermuscular fat (Moerck and Ball, 1973; Mello et al., 1976). These data would suggest little difference in fatty acids between hand and mechanically deboned meat and also little difference between ham and vertebra deboned meat composition in this respect.

Manufactured product

Cooked and smoked sausage sensory and chemical characteristics were studied. These included external and internal (outside and inside) color, flavor and odor desirability, rancid taste, tenderness, and pH. The least square means and standard errors are shown in Table 5 and the least square means and standard errors for two-way interactions that were significant are shown in Table 6.

Type of Bone

Type of bone (ham versus vertebra) did not have a significant effect on either inside or outside color, flavor, and odor desirability or tenderness in the sausage product (Table 5).

The type of bone, however, did have a significant influence on the pH of the sausage product with sausage made from ham bones yielding a higher pH than sausage made from vertebra bones. This same difference was also noted in the deboned tissue prior to making the sausage product (Table 3). Differences in pH due to different pork muscles within the animal are not uncommon (Price and Schweigert, 1971) however, probably of greatest importance is the effect of marrow on pH. Even though these differences in pH in sausage products were significant ($P<0.01$) it is doubtful that this significance is of biological importance.

Type of bones did not have a significant influence on rancid taste (Table 5), but there was a significant interaction between type of bone and method of deboning (Table 6), with mechanically deboned vertebra tissue yield a larger percentage of taste panel members indicating a rancid taste than with hand deboned vertebra tissue. It is possible that red bone marrow present in the vertebra had a higher concentration of heme than the marrow in ham bones which is not considered to be red bone marrow (Field, 1976a) thus, the higher concentration of heme may have had a catalytic effect on oxidation.

Storage of bones prior to processing

Frozen storage (−18°C) for 6 wk prior to deboning did not have a significant influence on inside or outside color

Table 5—Least square means[j] and standard errors (S.E.) showing effect of type of bone, storage (−18°C) of bones prior to processing, method of boning, type of antioxidant and storage (12°C) after processing on the sensory and pH characteristics of cooked and smoked sausage

	Color[a]		Flavor and odor desirability[a]	Rancid taste[b]	Tenderness	pH
	Inside	Outside				
Type of bone[c]						
Ham	5.43NS	5.27NS	5.74NS	11.09NS	5.33NS	6.13**
Vertebrae	5.52	5.36	5.79	11.97	5.31	6.09
Storage of bones prior to processing[d]						
0 week	5.47NS	5.24NS	5.57**	6.05**	5.19*	6.08**
6 weeks	5.48	5.39	5.97	17.31	5.44	6.15
Method of boning[e]						
Hand[f]	5.84**	5.68**	6.07**	9.72NS	5.44*	6.09**
Mechanical[g]	5.11	4.94	5.47	13.64	5.20	6.14
Type of antioxidant[h]						
Control	5.36a	5.10a	5.86a	10.45a	5.43a	6.13a
Sodium ascorbate	5.51a	5.40ab	5.67a	11.04a	5.35a	6.12a
α-tocopherol and ascorbyl palmitate	5.56a	5.44b	5.77a	13.54a	5.17a	6.10a
Storage following processing[i]						
0 week	6.98A	6.88A	6.37A	7.00A	6.10A	6.12ab
4 weeks	5.21B	4.86B	5.77B	26.75B	5.31B	6.13a
8 weeks	4.24C	4.20C	5.16C	1.25A	4.54C	6.10b
S.E. for type of bone, storage of bone prior to processing and method of boning	0.09	0.09	0.07	1.85	0.08	0.01
S.E. for type of antioxidant and storage time following processing	0.11	0.11	0.09	2.21	0.10	0.01

[a] Sensory panel evaluation—hedonic scale: 10 = excellent; 1 = poor.
[b] Based on percent of sensory panel members indicating rancid taste.
[c] Effect of storage prior to processing, method of boning, type of antioxidant, and storage following processing are absorbed.
[d] Effect of type of bone, method of boning, type of antioxidant, and storage following processing are absorbed.
[e] Effect of type of bone, storage prior to processing, type of antioxidant, and storage following processing are absorbed.
[f] Manual removal of all soft tissue from bone.
[g] Mechanical deboning with Protecon MRS-40.
[h] Effects of type of bone, storage prior to processing, method of boning, and storage following processing are absorbed.
[i] Effects of type of bone, storage prior to processing, method of boning, and type of antioxidant are absorbed.
[j] Means with the same letter within a vertical group do not differ significantly: lower case (P<0.05). upper case (P<0.01).
NS = Not significant
*Significant (P<0.05)
**Significant (P<0.01)

of this sausage product (Table 5).

Denotation of rancid taste by the sensory panel occurred significantly (P<0.01) more frequently in cooked and smoked sausage which was made from bones that had been frozen 6 wk at −18°C than in sausage products manufactured from deboned meat from fresh bones. This would be expected due to oxidative processes producing rancid flavor during frozen storage. This rancid flavor can be carried over into the final sausage product. This is especially true since some products of oxidation (hydroperoxides) are accelerators of the oxidation cycle (Ockerman, 1975). Incorporation of rancid meat containing a relatively high concentration of hydroperoxides into a cooked and smoked sausage, therefore, would act as a prooxidant in this product.

Tenderness scores were also significantly (P<0.05) increased by 6 wk frozen storage of the bones prior to deboning. The pH also increased during frozen storage and it is postulated that the increase of pH could have increased the water-holding capacity due to the higher pH values, and consequently, improved the tenderness scores.

Flavor and odor desirability, and pH both received a significant interaction score (Table 6) between storage prior to processing and antioxidants used in the manufacture of these products. The antioxidant combination of α-tocopherol plus ascorbyl palmitate significantly reduced the flavor and odor desirability score when compared to the control tissue for products made from bones that were boned fresh (0 wk). For product made with deboned meat from bones that had been frozen 6 wk, neither antioxidant had a significant influence when compared with the control for flavor and odor desirability scores. This would suggest, along with the rancidity scores, that the panel could detect a rancidity developing from product made from bones that had been frozen 6 wk, but they did not object to this flavor.

In pH values, the α-tocopherol plus ascorbyl palmitate also significantly reduced the pH values of product made from freshly boned tissue; however, for tissue obtained from bones that had been stored 6 wk, neither antioxidant had a significant influence on the pH value of the product.

Method of boning

Method of boning had a significant influence (P<0.01) on inside and outside color (Table 5). This agrees with data on deboned meat prior to manufacturing and shows that mechanically deboned meat is darker in color than hand deboned meat. These same results were reported by Goldstrand (1975) who found that mechanically deboned pork and beef was 25-30% higher in color values than beef and pork trimmings at the same protein and fat levels. It also agrees with Meiburg et al. (1976) who found that excessively dark frankfurter color was caused by using high levels of cow meat which had been mechanically deboned. Field (1976b) also observed that bologna made from mechanically deboned meat was darker in color than hand deboned product and he emphasized that this was due to the contribution of heme in the bone marrow (Froning and Johnson, 1973) and the elimination of connective tissue by mechanically deboning (Field, 1976b).

Tenderness ratings were significantly (P<0.05) more desirable for cooked and smoked sausage product made with hand deboned meat than for sausage product manu-

factured from 20% level of mechanically deboned meat. These findings are in agreement with most of the reported literature. Field et al. (1974a) indicated that panels prefer bologna made from hand deboned meat in the area of tenderness. Joseph et al. (1977) found consumers prefer the tenderness and texture of hand deboned meat used to make cooked salami. Marshall et al. (1977) also found that frankfurters containing 20-40% mechanically deboned pork were very mushy. This less desirable tenderness score may be due to the extremely fine grinding to which the mechanically deboned meat is subjected and also from the elimination of connective tissue in this product, creating a very tender or mushy type of product.

Mechanically deboned meat was found to produce a sausage product with a higher pH than hand deboned meat ($P<0.01$). These same differences were found in deboned meat of the raw material used in making this sausage product (Table 3).

Method of boning interacted significantly with storage after processing when flavor and odor desirability scores were compared (Table 6). Flavor and odor desirability decreased in both cases for hand and mechanically deboned meat as storage time progressed through 0, 4 and 8 wk following manufacturing. However, the mechanically deboned meat started at a much lower level than the hand deboned meat and showed a nonsignificant lowering between 0 and 4 wk, whereas in the hand deboned product each storage period (0, 4, and 8 wk following processing) decreased significantly.

Rancid taste also showed a significant interaction between type of bone and method of boning and this has already been discussed.

Table 6—Least square means[a] and standard errors (S.E.) for two-way interactions that were significant after processing on the sensory and pH characteristics of cooked and smoked sausage

	Rancid taste[b]	Flavor and odor desirability[c]	pH
Type of bone X Method of boning[d]			
Ham			
Hand[e]	12.94NS	–	–
Mechanical[f]	9.83	–	–
Vertebrae			
Hand[e]	6.50**	–	–
Mechanical[f]	17.44	–	–
S.E.	2.59	–	–
Storage prior to processing X Antioxidant[g]			
0 week			
Control	–	5.77a	6.13A
Sodium ascorbate	–	5.58ab	6.07AB
α-tocopherol + ascorbyl palmitate	–	5.36b	6.05B
6 wk			
Control	–	5.95ab	6.13A
Sodium ascorbate	–	5.76a	6.17A
α-tocopherol + ascorbyl palmitate	–	6.18b	6.14A
S.E.	–	0.12	0.02
Storage prior to processing X Storage following processing[h]			
0 wk prior			
0 wk following	0.14a	–	–
4 wk following	1.42b	–	–
8 wk following	0.25a	–	–
6 weeks prior			
0 wk following	1.26A	–	–
4 wk following	3.93B	–	–
8 wk following	0.00C	–	–
S.E.	0.32	–	–
Method of boning X Storage following processing[i]			
Hand[e]			
0 wk following	–	6.88A	–
4 wk following	–	5.96B	–
8 wk following	–	5.34C	–
Mechanical[f]			
0 wk following	–	5.86A	–
4 wk following	–	5.59A	–
8 wk following	–	4.96B	–
S.E.	–	0.12	–

[a] Means with the same letter within a vertical subgroup do not differ significantly: lower case ($P<0.05$); upper case ($P<0.01$)
[b] Percent of sensory anel members indicating rancid taste
[c] Sensory panel evaluation—Hedonic scale: 10 = excellent; 1 = poor.
[d] Effect of storage prior to processing, type of antioxidant and storage following processing are absorbed.
[e] Manual removal of all soft tissue from bones
[f] Mechanical deboning with Protecon MRS-40
[g] Effect of type of bone, method of boning and storage following processing are absorbed.
[h] Effect of type of bone, method of boning and type of antioxidant are absorbed.
[i] Effect of storage prior to processing, type of bone, and type of antioxidant are absorbed.
NS = Not significant ($P>0.05$)
** Significant ($P<0.01$)

Type of antioxidant

The type of antioxidant did not have a significant influence on inside color (Table 5). A significant influence was noted, however, on outside color of product made with α-tocopherol and ascorbyl palmitate being significantly improved in color when compared with the control. Values for inside color were also in this same direction, but not large enough to be significant.

The values obtained for rancid taste and tenderness, showed a nonsignificant influence of type of antioxidant.

Flavor and odor desirability and pH both had an antioxidant X storage prior to processing interaction (Table 6) and both of these scores have been previously discussed.

Storage time following processing

Effect of storage time following processing, at 12°C and a relative humidity of 65%, was evaluated after 0, 4, and 8 wk. The inside and outside color changed significantly ($P<0.01$) at each storage period with the color becoming darker as storage time progressed (Table 5). This is likely due to dehydration of external surface and/or oxidation of the meat pigments.

Tenderness also decreased significantly as storage time progressed and at each stage tenderness was significantly reduced when compared to the preceding stage. The pH also decreased significantly from 4 to 8 wk of storage.

There was a significant interaction (Table 6) for flavor and odor desirability due to method of boning and storage time following processing and this has already been discussed.

Rancid taste also received a significant interaction value due to storage prior to processing and storage following

Table 7—Simple correlation between acceptability characteristics and pH of a cooked and smoked sausage

	Inside color[a]	Outside color[a]	Flavor & odor[a]	Rancid taste[b]	Tenderness[a]	pH
Inside color[a]	1.00	–	–	–	–	–
Outside color[a]	0.94**	1.00	–	–	–	–
Flavor & odor[a]	0.35**	0.64**	1.00	–	–	–
Rancid taste[b]	0.01	−0.02	0.11	1.00	–	–
Tenderness[a]	0.71**	0.66**	0.76**	0.18	1.00	–
pH	−0.07	−0.09	0.13	0.05	0.19	1.00

[a] Sensory panel evaluation — Hedonic Scale: 10 = excellent; 1 = poor.
[b] Based on percent of sensory panel members indicating rancid taste.
**Highly significant ($P<0.01$).

processing. It would appear that few taste panel members gave rancid taste notation on 8 wk of storage. This could have been a communication problem since suggestions for notations were given verbally or other off-flavors may have masked an eventual "rancid" taste since this certainly is not in the direction that would be expected.

Table 7 gives simple correlation values between evaluations of smoked sausage products. Inside and outside color are significantly, positively correlated as would be expected. Flavor and odor are also significantly correlated with both inside and outside color, suggesting that both changed in the same direction during storage. Tenderness is correlated with inside and outside color and flavor and odor, again suggesting changes in the same direction for these parameters.

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Effect of Heat Processing in Cans and Retort Pouches on Sensory Properties of Fowl Meat

B. G. LYON and A. A. KLOSE

ABSTRACT

Fowl meat processed in cans and flexible retortable pouches was evaluated for heat effects on sensory properties. A trained panel evaluated texture and off-flavor of canned boned chicken, uncooked fowl meat retorted in flexible pouches, cooked meat retorted in flexible pouches, and simmered fowl meat (control). Results indicated that the retort pouch process may offer a method for improving the texture of processed fowl meat from spent hens by adequately cooking to tenderize the meat but not overcooking it to the extent that meat chunks are reduced to fibrous, shredded, or stringy components. Off-flavor development was related to precooking before retorting.

INTRODUCTION

SENSORY PROPERTIES of canned boned chicken products prepared from fowl meat (spent hens) and produced for commercial retail market, school lunch, welfare, and military programs were evaluated by Lyon (1980) and Lyon and Klose (1980), and differences in sensory properties were found to be related primarily to texture and off-flavors. Considerable variability was found in textural properties of canned boned chicken produced under USDA school lunch specifications by different processors, even though all products evaluated met the objective requirements for fat, moisture, protein, and drained weight stated in the specifications. Commercial products in 5-oz cans were rated better than either school lunch or military products in 29-oz cans. Small cans require shorter sterilization periods than large cans and thus are subjected to less destructive heating.

Spent hens are generally precooked for tenderizing and more efficient deboning before canning. MacDougal and Gibbons (1947) investigated open- and pressure-cooking methods for effects on moisture content and found that more moisture was lost by pressure-cooking. However, moisture loss due to canning was greater for open-cooking than for pressure-cooking.

An alternative to the can is the retort pouch, a flexible laminated package that withstands thermal processing and combines many of the advantages of conventional metal cans, glass jars, and frozen-food packages. The pouch has been used commercially in Europe and Japan since the early 1970's but was not approved for use in the United States until 1978. The benefits of the pouch were reviewed by Mermelstein (1978) and Hopkins (1977). Less time is needed for products to reach sterilization temperatures; thus less energy is required for product processing and product quality (i.e., color, texture, flavor) is maintained. The pouch, like the can, provides a product that needs no refrigeration or freezing. Because pouches weigh less than cans, jars, or trays, storage and transportation space and distribution costs can be reduced.

In this study, we compared the sensory properties of cooked fowl meat sterilized in cans with those sterilized in retortable pouches.

Authors Lyon and Klose are with the USDA-SEA Animal Products Composition and Utilization Research Unit, Richard B. Russell Research Center, P.O. Box 5677, Athens, GA 30604.

MATERIALS & METHODS

FOUR PRODUCTS were used: canned boned chicken (29-oz cans), obtained from a processor of canned boned chicken for USDA school lunch programs; uncooked fowl meat retorted in flexible pouches; cooked fowl meat retorted in flexible pouches; and fowl meat simmered in water to serve as a control.

Preparation of cooked fowl meat for retorting

Raw meat (light and dark) and skin from spent fowl were obtained from a poultry processor in northern Georgia. Light and dark meat and unground skin were weighed and cooked separately in large vessels for 2 hr on institutional ranges with water levels that just covered the meat or skin. After cooking and cooling, meat and skin were drained and weighed. Meat was diced manually into 1- to 1.5-in. pieces, and skin was ground. The pouches were filled with 156g light meat (34%), 150g dark meat (33%), 45g skin (10%), 20g fat collected from the cooled broth of the cooked skin (4.4%), 61 ml broth from light and dark cooked meat (13.4%), and 22 ml 20% sodium chloride solution (4.8%).

Preparation of uncooked fowl for retorting

For the meat that was uncooked before retorting, raw meat was manually diced into 1- to 1.5-in. pieces. Uncooked skin was first diced and then ground. Each pouch was filled with 198g light meat, 198g dark meat, 45g ground skin, and 22 ml 20% sodium chloride solution. These ingredient amounts were calculated on the basis of a yield, after cooking, of 78% for light meat, 76% for dark meat, and 99% for skin. Based on these yields, the composition of the uncooked product after retorting was expected to be the same as the precooked retorted sample: 68% meat, 10% skin, 1% salt, and 21% fat and liquid.

Retorting

Rectangular retort pouches measuring 6½ in. x 8½ in. (16.5 cm x 21.5 cm) made at the Reynolds Metals Company Laboratory, Richmond, Va., were used. Pouches were made from a laminate of an outer layer of polyester for strength, a middle layer of 0.00035-in. thick aluminum foil, and an inner layer of polypropylene for heat-sealing properties. The uncooked and cooked products were retorted in separate batches. For four of the pouches in each batch, thermocouples of copper constantan wires were placed in the center of a diced piece of chicken located in the center of the pouch. Pouches were evacuated to 70 cm (76 cm full vacuum) and heat sealed in a 3-step process.

Retorting was conducted in a modified vertical Dixie Canner retort with compartmentalized trays that provided an individual compartment for each pouch. The 4 thermocoupled pouches were placed on top racks and thermocouples were connected to lead-in thermocouples attached to a recorder. The retort was an air-water type equipped with automatic controls and a temperature gauge, pressure gauge, and sight glass. The retort was operated at 15 lb (gauge) of steam, 121.1°C (250°F) steam-water temperature with an override air pressure of 10 lb (gauge) to give a total pressure of 25 lb, which was maintained during the cooling period by automatically increasing air pressure to prevent pouch damage. Warm-up was started with cold water and steam. Total accumulated lethality was followed by adding each lethality increment, from a table based on a lethality of 0.1 at 232°F (111.1°C) and a z of 18°F (10°C), for the average temperature of the slowest heating thermocouple over each successive minute, beginning at a temperature of 232°F (111.1°C). Heating curves are shown in Figure 1. Cooked samples

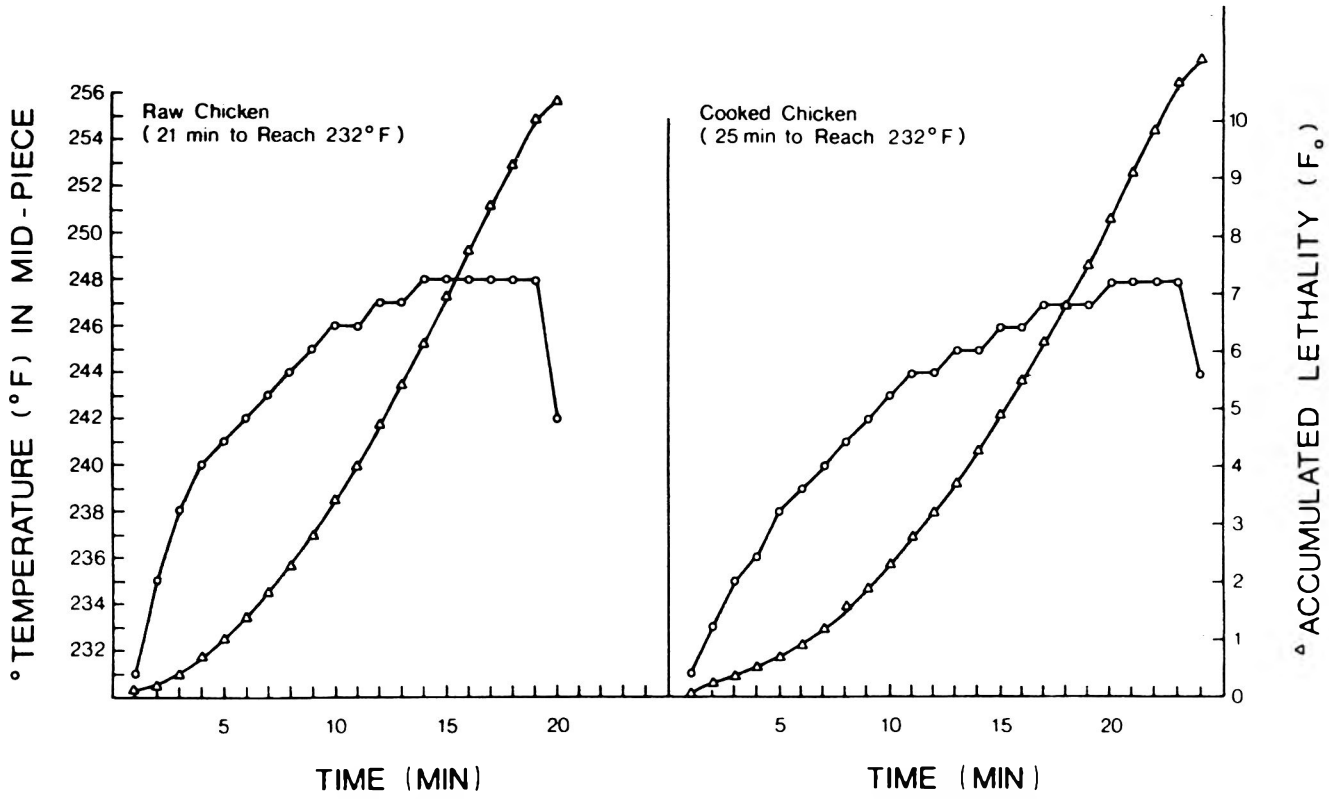


Fig. 1—Temperature (○ ——— ○) and lethality (Δ ——— Δ) curves of retort pouch products processed in 6½" x 8½" pouches, at 250°F (121°C), 15 psig steam, 10 psig air.

took about 6 min longer than uncooked samples to reach the maximum temperature (248°F, 120°C). Steam was turned off when an accumulated F_0 of 9 was reached. Accumulated lethality reached 10.4 for uncooked chicken and 11.1 for cooked chicken during the cooldown phase. A single retort trial for each treatment was conducted. Pouches were held at 28-30°F (-1 to -2) before evaluation. For panel evaluations, pouches were heated to boiling water for 15 min, opened, and drained.

Preparation of control

Light and dark meat and ground skin from spent fowl were simmered for 2 hr in separate vessels with water level to cover. Broth was drained, meat diced, and ingredients weighed. Approximately 156g light meat, 150g dark meat, 15 ml 30% sodium chloride solution, and 45g skin blended with 100 ml broth from cooked skin were combined and simmered on low heat for approximately 20 min before being drained and served to the panel.

Preparation of canned boned chicken

Canned boned chicken was heated for 20 min in a double boiler and drained.

Sensory evaluation

Definitions of terms and techniques for sensory evaluation are explained in detail in two companion papers (Lyon, 1980; Lyon and Klose, 1980). A six-member panel trained in texture profiling and Quantitative Descriptive Analysis (QDA) (Stone et al., 1974) participated. At each of the 6 sessions, panelists evaluated the 4 products. The order of tasting the 4 products was balanced for panelists and sessions.

Random samples of the warm, drained meat were placed in coded containers and served warm to panelists in individual booths equipped with incandescent lighting. Room temperature water and unsalted crackers were provided to the panelists for use before and after each sample. For each sample, trained panelists evaluated 11 sensory attributes of texture and taste. These attributes were visual moisture, initial moisture, proportion of light and dark meat, rate of breakdown, particle size/shape, tenderness, persistence of moisture release, cohesiveness, seasoning, off-flavor, and predominant mass (ranging from ground to chunky). A line scale for each attribute, anchored at the ends and center, was used. Each panelist marked his/her response with a vertical line across the horizontal line scale. Responses were converted to numerical scores from 1-60 by use of a transparent overlay.

Proximate composition

Moisture, fat and protein contents were determined on all samples according to AOAC methods (1965), Contents of containers were ground 3 times and aliquots were taken for analyses.

Statistical analyses

ANOVA procedures of the SAS computer programs were employed to determine individual and panel group performance and product differences for each attribute. Where applicable, means were tested by Duncan's multiple range test (Steel and Torrie, 1960). Sensory data were also factor analyzed.

RESULTS & DISCUSSION

Proximate composition

Percents of moisture, fat, and protein are given in Table

Table 1—Percentage moisture, fat, and protein of fowl meat[a]

	Moisture	Fat	Protein
Pouch-retorted			
Uncooked	72.9	4.4	20.9
Cooked	70.8	5.6	21.4
Canned	65.3	10.7	20.8

[a] Values are means of 4 observations.

Table 2—Percent responses for predominant mass

	Control	Pouch-retorted		Canned
		Uncooked	Cooked	
Ground	0	0	0	0
Shredded	0	0	2.8	13.9
Stringy	2.8	0	2.8	30.6
Fibrous	5.6	11.1	27.8	47.2
Chunky	91.6	88.9	66.6	8.3

Table 3—F-values from analysis of variance of sensory data[a]

Attributes	I. F-values vs error			II. F-values vs interaction	
	Treatment	Panel	Treatment x Panel	Treatment	Panel
Visual moisture	11.5	19.8	1.8	6.5	11.1
Initial moisture	13.0	20.7	2.7	4.9	7.8
Proportion	0.4NS	3.9	1.9	0.2NS	1.9NS
Rate of breakdown	13.1	1.5NS	1.4NS	9.6	1.1
Particle size/shape	3.2	10.2	1.8	1.7NS	5.5
Tenderness	16.1	4.6	1.7NS	9.4	2.7NS
Persistence of moisture release	3.3	12.5	1.5NS	2.2NS	8.3
Cohesiveness	1.8NS	2.3NS	1.9	0.9NS	1.2NS
Seasoning	18.7	35.3	3.4	5.5	10.4
Off-flavor	15.4	7.8	2.9	5.4	2.8NS

[a] NS denotes not significant at the 5% level. All other values are significantly different ($P \leq 0.05$).

Table 4—Mean sensory scores[a,b] *for fowl products*

Sensory attributes	Control	Pouch-retorted		Canned
		Uncooked	Cooked	
Visual moisture	34.1b	33.4b	31.2b	41.2a
Initial moisture	26.8b	28.2b	30.1b	39.0a
Proportion	25.9a	25.6a	27.1a	27.4a
Rate of breakdown	32.8a	20.4b	22.1b	22.0b
Particle size/shape	29.3a	25.3a	24.2a	23.2a
Tenderness	33.3a	23.4b	21.5b	19.1b
Persistence/moisture release	24.2a	27.5b	23.4a	30.5a
Cohesiveness	27.0a	21.3a	23.8a	23.9a
Seasoning	11.8b	14.0b	15.0b	20.5a
Off-Flavor	10.1c	11.3bc	18.7ab	22.6a

[a] Each value is the mean of 36 observations. Values in rows followed by different letters are significantly different ($P \leq 0.05$).

[b] Numerical range of scores is 1-60: Visual moisture = dry to moist; initial moisture = dry to moist; proportion = light to dark; rate of breakdown = rapid to slow; particle size/shape = small to long; tenderness = tender to tough; persistence = low to high; cohesiveness = none to much; seasoning = slight to much; off-flavor = slight to much.

1. The pouch product that was uncooked before retorting had the highest moisture content, followed by the cooked pouch and canned chicken. Both pouch products had significantly higher moisture contents than the canned product. The canned product had the highest fat content (10.7%) but was within the stated limits in the specifications. The pouch products contained significantly less fat. Protein content was similar for all products.

Sensory evaluation

Responses for predominant mass (Table 2) were tabulated and expressed as the percent responses for five descriptive terms (chunky, fibrous, stringy, shredded, and ground) and were scored 1 = ground to 5 = chunky for other analyses. Control and raw retorted samples were rated as predominantly chunky; the cooked retorted sample was rated chunky (67%) and fibrous (28%). The canned product was rated as predominantly stringy (31%) and fibrous (47%). If these products were ranked according to extent of heat treatment involved in their preparation, these data suggest that the more intense the heat treatment, including precooking and retorting, the more the cohesive meat chunks broke down into fibrous, stringy, shredded pieces. The retort pouch process offers an advantage over canning in that the product is subjected to high heat for a shorter period of time and meat chunks stay more intact.

Analysis of variance showing treatment, panel, and treatment x panel effects for each sensory attribute is given in Table 3. Treatment effects were significant for all sensory attributes except proportion and cohesiveness. When treatment effects were tested by treatment x panel interaction, there were nonsignificant treatment differences for persistence of moisture release and particle size/shape, in addition to proportion and cohesiveness.

Of the 11 scales used by the trained panel to evaluate sensory properties, only 6 showed significant differences among the samples. When panel performance was analyzed by one-way analysis of variance, only the proportion and cohesiveness scales indicated poor discriminating ability by several panelists. The other scales showing nonsignificant treatment differences (persistence of moisture release and particle size/shape) had good panel agreement, and panelists showed good discriminating ability on these scales. Therefore all scales except proportion and cohesiveness were appropriate scales for evaluating the products, and the products were differentiated by the panel on the basis of 6 sensory attributes, namely visual and initial mois-

Table 5—Correlation coefficents of sensory attributes[a]

	Visual moisture	Initial moisture	Proportion	Rate of breakdown	Particle size/shape	Tenderness	Persistence of moisture release	Cohesiveness	Seasoning	Off-flavor
Initial moisture	0.47									
Proportion	0.25	0.39								
Rate of breakdown	0.04	−0.02	0.02							
Particle size/shape	0.21	0.22	0.20	0.54						
Tenderness	−0.08	−0.14	0.01	0.75	0.42					
Persistence/moisture release	0.36	0.67	0.29	0.07	0.29	−0.12				
Cohesiveness	0.14	0.28	0.12	0.52	0.51	0.47	0.18			
Seasoning	0.21	0.38	0.18	−0.08	−0.11	−0.05	0.18	0.13		
Off-flavor	−0.07	0.01	0.01	−0.18	−0.33	−0.08	−0.24	−0.15	0.12	
Predominant mass	−0.17	−0.24	−0.12	0.18	0.14	0.26	−0.15	0.04	−0.31	−0.29

[a] Correlation had to exceed r = 0.20 to be significant at the 1% level.

ture, rate of breakdown, tenderness, seasoning, and off-flavor.

Mean sensory scores are given in Table 4. Duncan's multiple range test was applied to treatment means, using either the error term or treatment x panel interaction, as appropriate. Of the sensory attributes relating to texture, only rate of breakdown and tenderness showed significant treatment differences. The control was significantly less tender and slower to breakdown. The pouch product that was uncooked before retorting was less tender, but not significantly so, than the pouch product cooked before retorting and the canned product. Thus, precooking fowl meat before pouch retorting may not be necessary for tenderizing effects.

The canned product was rated more moist on appearance and on initial chew. Analytical moisture contents, however, were the reverse. For sensory evaluations, visual moisture was defined as degree of superficial luster and initial moisture was defined as fluids released on the first 3-5 chews (Lyon, 1980). These definitions would not necessarily restrict sensory moisture evaluations to water content. Sensory scores may well have been influenced by fat content as well as the state of water, i.e. bound or free. (Swift et al., 1954; Ritchey and Hostetler, 1964). Analyzed fat content of the canned product was higher (11%) than fat content of the pouch products (<6%).

The canned product was scored highest in seasoning intensity and off-flavor. The control and the uncooked pouch were the least off-flavored. Precooking of fowl meat before retorting in cans or pouches may enhance off-flavor development because of the prolonged cooking in open vats and exposure to oxidation. The retorting process is a second heating of the product and may further enhance off-flavor development.

Correlations and factor analysis

Sensory data were correlated and factor analyzed to examine the interrelationships of the attribute scales. Associations of the sensory attributes to each other are given in Table 5. All correlations between terms relating to texture were significant. The highest correlation coefficient was for rate of breakdown with tenderness (r = +0.75). High correlations indicate either redundancy or relative trends of individual components of a basic underlying attribute. Significant correlations for other texture attributes ranged from r = +0.42 to +0.54. These values are relatively low for conventional reporting, probably due to panel disagreement and nonsignificant treatment differences noted in Table 3 for some texture attributes.

The best correlation between attributes relating to the moisture in the products was between initial moisture and persistence of moisture release (r = +0.67). The degree of moisture released during chewing was related to the moisture perceived during the initial 3-5 chews. Apparently the moisture perceived visually was not an indication of juiciness of the products perceived on chewing.

Factor analysis was applied to the sensory data. Using all sensory attributes in the factor analysis, three factors were extracted that accounted for 61.8% of variance in the data (Table 6). The variation explained in the data was improved to 76.8% by eliminating proportion, particle size/shape, persistence of moisture release, cohesiveness, and seasoning (Table 7). In the analysis of variance, all of these attributes, except seasoning, had shown no significant differences among treatments.

In factor analysis, the first factor extracted represents the best single linear composition explaining the most variance in the data. The second factor, independent of the first, represents the next best single summary of the data. Subsequent factors are extracted until all the variance in the data is accounted for.

In the analysis explaining 61.8% variance in the data, rate of breakdown, particle size and shape, tenderness and cohesiveness loaded high in factor I. All these terms relate to texture. In factor II, terms loading high included visual moisture, initial moisture, and persistence of moisture release. Factor III included seasoning, off-flavor and predominant mass. In the analysis explaining 76.8% variance in the data that eliminated nondiscriminating attribute scales, factor I included rate of breakdown and tenderness; factor II included visual and initial moisture; and factor III included off-flavor and predominant mass.

Terms that load high in a factor have some common relationship; either they are redundant and measuring the same thing or they are individual components of a common characteristic. By examining both factor patterns (61.8% and 76.8%), 3 primary categories of sensory attributes emerge as important discriminators of differences among the 4 products, namely texture, moisture (juiciness), and off-flavor. When nondiscriminating attribute scales were eliminated in the 76.8% analysis, factor I (texture) accounted for the greatest portion of the variance explained in the data (33.5%), an increase of 6.6 percentage points from the initial factoring including all sensory attributes. Factor II, which included moisture terms, increased by 1.8 percentage points from the 61.8% analysis in the proportion of variance explained in the 76.8% analysis, and factor III (off-flavor) increased by 6.6.

The results of this study show that both the conventional canning procedures and the retorting procedures of the flexible food packages adequately tenderize meat from spent hens. The retort pouches offer an improvement in the texture of processed spent fowl by adequately cooking to tenderize the meat but not overcooking to the extent that meat chunks are broken up and reduced to ground,

—Continued on page 233

Table 6—Rotated factor pattern using all sensory attributes and explaining 61.8% of variance in the data

	FACTOR		
	I	II	III
Visual moisture	0.04	0.66	0.07
Initial moisture	0.04	0.85	0.21
Rate of breakdown	0.88	–0.02	–0.13
Particle size/shape	0.65	0.37	–0.34
Tenderness	0.88	–0.22	–0.04
Persistence of moisture release	0.01	0.82	–0.13
Cohesiveness	0.75	0.26	0.04
Seasoning	0.04	0.35	0.61
Off-flavor	–0.10	–0.27	0.75
Predominant Mass	0.16	–0.25	–0.66
Proportion	0.10	0.53	0.15
Cumulative % variance explained	26.9	50.0	61.8

Table 7—Rotated factor pattern using only discriminating sensory attribute scales

	FACTORS		
	I	II	III
Visual moisture	0.02	0.85	–0.04
Initial moisture	–0.06	0.83	0.11
Rate of breakdown	0.92	0.07	–0.13
Tenderness	0.93	–0.12	–0.07
Off-flavor	–0.03	–0.17	0.87
Predominant mass	0.18	–0.33	–0.70
Cumulative% variance explained	33.5	58.5	76.8

Sensory Scores and Shear Force Values for Beef Roasts Cooked Either Before or After Chilling

B. W. BERRY, E. E. RAY and D. M. STIFFLER

ABSTRACT

Sensory and physical characteristics were determined on beef semimembranosus and semitendinosus muscles removed and cooked either 1.5 hr post-exsanguination or after 7 days of chilling by either steam, hot water vat or convectional electric cookery. The effects of cookery method on sensory and physical characteristics were not statistically significant ($P>0.01$). Hot-boned roasts from both muscles served as cubes had higher shear force values, higher amounts of connective tissue (sensory panel), and lower tenderness and higher juiciness scores than cold-boned cooked roasts. However, no differences in sensory characteristics were found between hot- and cold-boned cooked semitendinosus roats evaluated in a thinly sliced form. These results suggest that acceptable tenderness in beef roasts cooked hot-boned may be achieved when the product is used in a thinly-sliced or similar form.

INTRODUCTION

ENERGY CONSERVATION is of vital importance to the meat industry which is one of the major consumers of energy among food industries. For meat products that require only minimal or no reheating, prerigor cooking of hot-boned muscle would appear to be a feasible method to lower unit energy cost. Marsh (1977) cited improved tenderness and reduced cooking losses as advantages to prerigor cooking with alterations in shape during cooking as a possible disadvantage. Ramsbottom and Strandine (1949), Paul et. al. (1952), Weidemann et al. (1967) and Cia and Marsh (1976) have found prerigor cooked muscle to be more tender than postrigor cooked muscle. However, Ray et al. (1980) and Montgomery et al. (1977) reported decreased tenderness in prerigor cooked muscle.

Sensory and physical characteristics of hot-boned, cooked beef muscle (especially thin-sliced products) have not been extensivley documented. Thus, in this study we evaluated those characteristics on hot- and cold-boned semimembranosus and semitendinosus muscles cooked by three methods.

EXPERIMENTAL

Muscle boning and cooking procedures

Specific procedures detailing the muscle boning and cooking phases of this study have been previously reported (Ray et al., 1980). In summary, those procedures consisted of removing the semimembranosus and semitendinosus muscles from one side of 30 short-scrotum bull carcasses within 1.5 hr post-exsanguination, while the same muscles were removed from the opposite sides after 7 days of cooler storage (2°C). Ten carcasses were randomly assigned to each of the three cooking systems. A commercial roast beef seasoning (B. Heller Co.) was applied to the muscles. The ingredients in the spice mixture in descending order by amount were: salt, monosodium glutamate, dextrose, sugar, hydrolyzed vegetable protein, caramel powder, pepper and other spices including onion, and garlic powder. Next, the roasts were placed in PVDC (polyvinylidene chloride) bags, vacuumized and cooked by either steam, hot water vat or convectional electric oven to an internal temperature of 68°C. The packaging material had an oxygen transmission rate of 30 cc/m^2/24 hr. The packages were evacuated by use of a chamber-type vacuum packaging machine which was set to achieve a vacuum of 29.5 of Hg. The cooking schedule for all cooking systems was: 1st hr at 68°C; 2nd hr at 74°C; and the remaining time at 80°C. Subsequently, when the roasts had cooked at 25°C, 475-525g sections were removed from the interior of each muscle, vacuum packaged (same O_2 transmission rate and vacuum as described above) and frozen at −28°C. Sections were held at −28°C for 45-60 days and then evaluated for sensory and physical characteristics. This range in storage time was necessary for the sensory panel to evaluate all samples.

Author Berry is with the Meat Science Research Laboratory, SEA-AR, USDA, Beltsville, MD 20705. Author Ray is with the Dept. of Animal & Range Sciences, New Mexico State Univ., Las Cruces, NM 88003. Author Stiffler, formerly with New Mexico State Univ., is with the Meats and Muscle Biology Section, Dept. of Animal Sciences, Texas A & M Univ., College Station, TX 77843.

Sensory panel and physical evaluations

Roast sections were thawed at 3°C for 36 hr before they were served to the panelists. Thaw loss percentages were determined from pre- and post-thawing weights. Degree of doneness was scored by two evaluators using an 8-point photographic scale (8 = very rare, 1 = very well done). Samples were approximately 7°C at the time of serving. Meat cubes (1.2 cm) were served in paper cups to panelists stationed in individual booths. Six samples were evaluated at each session: paired hot- and cold-boned samples from each of the three cooking methods. The order of the samples within a session was completely at random. Panelists received samples from the various locations within a roast at random. A 10-member descriptive attribute panel trained according to procedures outlined by AMSA (1978) was used to evaluate samples. Panelists assigned scores according to the following attributes and scales: tenderness, 8 = extremely tender, 1 = extremely tough; juiciness, 8 = extremely juicy, 1 = extremely dry; connective tissue amount, 8 = none, 1 = abundant, and roast beef and seasoning intensity, 8 = extremely intense, 1 = extremely bland.

Since differences in tenderness were detected between hot- and cold-boned roasts served as cubes, the sensory panel also evaluated thinly sliced (2 mm) semitendinosus muscle. The strips of sliced muscle were rolled to a dimension of 1.2 x 1.2 x 3.8 cm and held together with toothpicks. The samples were served au jus; just before serving, they were dipped for 3 sec in a hot (77°C) solution prepared from a dry mix (The R. T. French Co.). The ingredients in the au just dry mix in descending order by amount were: modified cornstarch, salt, hydrolyzed vegetable protein, dextrose, beef fat and artificial flavor, guar gum, caramel color, silicon dioxide, onion, monosodium glutamate, lactic acid, beet powder and spices. The dry ingredients were mixed with water (on a 1g to 30 ml ratio) that was heated to boiling.

Samples were sheared with the Instron and Warner-Bratzler shear machines according to procedures outlined in the AMSA (1978) guidelines. All cores were 1.27 cm in diameter. Each core was sheared twice with eight cores per roast subjected to Instron maximum single blade shear force and two cores used for Warner-Bratzler shear force.

Statistical analyses

Data were analyzed according to analysis of variance procedures (Steel and Torrie, 1960). The effects of hot- vs cold-boning and method of cookery were evaluated for each muscle and type of sample.

RESULTS & DISCUSSION

SENSORY AND PHYSICAL characteristics for hot- and cold-boned semimembranosus and semitendinosus roasts are listed in Table 1. Comparisons of means in Table 1 are

Table 1—Sensory and physical characteristics of beef roasts as influenced by muscle, sample form at evaluation and postmortem boning temperature

Characteristic	Cube				Thin Slice	
	Semimembranosus		Semitendinosus		Semitendinosus	
	Cold boned	Hot boned	Cold boned	Hot boned	Cold boned	Hot boned
Tenderness[a]	6.4[b]	5.3[c]	6.6[c]	5.2[c]	7.7	7.7
Connective tissue amount[a]	7.1[b]	6.2[c]	7.4[b]	6.5[c]	7.9	7.8
Juiciness[a]	5.2[b]	5.7[c]	4.8[b]	5.4[c]	6.5	6.6
Roast beef flavor intensity[a]	4.7	4.6	4.1	4.0	5.3	5.3
Seasoning flavor intensity[a]	3.1	3.4	2.6	2.7	4.4	4.6
Thaw loss, %	8.3	7.2	9.6	9.6	12.4[b]	10.5[c]
Degree of doneness score[a]	3.0	2.9	2.7	2.7	2.7	2.9
Instron maximum single blade shear force (kg)	6.5[b]	9.8[c]	6.1[b]	9.2[c]	–	–
Warner-Bratzler shear force (kg)	4.3[b]	7.3[c]	3.9[b]	6.5[c]	–	–

[a] Scoring systems based on 8 = extremely tender, juicy and intense in roast beef and seasoning intensity; none in connective tissue amount and very rare in degree of doneness. 1 = extremely tough, dry and bland in roast beef and seasoning intensity, abundant in connective tissue amount and well done in degree of doneness.
[bc] Means on the same line under a muscle and sample configuration category possessing different superscripts are significantly different ($P < 0.01$). Where no superscripts exist, differences between means were nonsignificant ($P > 0.01$).

only between hot- and cold-boned roasts within a muscle or sample form (cubes and thin slices). The interaction effect between boning temperature and cooking system was nonsignificant ($P > 0.01$) for all characteristics. For both muscles served as cubes, cold-boned cooked roasts were rated as being more tender, lower in the amount of connective tissue and less juicy than hot boned cooked roasts. Shear force values obtained from both the Universal Instron and Warner-Bratzler shear machines support the tenderness differences found by the sensory panel between hot- and cold-boned roasts. Shear force values obtained after cooking and before freezing on adjacent muscle samples to those used in this study also reflected greater tenderness for cold-boned than for hot-boned roasts (Ray et al., 1980). Also, decreased tenderness of prerigor cooked pork loin chops and roasts has been reported by Montgomery et al., 1977). However, several studies have shown that prerigor cooked muscle is more tender than muscle cooked after the completion of rigor (Ramsbottom and Strandine, 1949; Paul et al., 1952; Weidemann et al., 1967). Cia and Marsh (1976) found that beef sternomandibularis muscle was quite tender when cooked prerigor by either water or microwave techniques. They suggested that the improved tenderness of prerigor cooked muscle was the result of supercontraction which produced fiber shattering; whereas when increased toughness has been found in prerigor cooked muscle, (Montgomery et al., 1977), results have been attributed to heat-stimulated contraction of heat rigor without fiber shattering. It is possible that in our study, the slower cooking methods employed produced extensive heat rigor rather than supercontraction. Panelists commented that many of the particles of completely masticated hot-boned cooked product were "hard" and "gritty" in texture. Panelists probably associated this "grittiness" with more connective tissue in hot-boned samples, when in fact it was probably extensive areas of supercontraction nodes produced under heat rigor. The shear force values for hot- and cold-boned roasts in our study are very similar to those found on adjacent roasts after cooking and before freezing (Ray et al., 1980). Thus, the freezing and thawing of these roasts exerted no effect on shear force values.

Although, sensory panelists detected reduced tenderness

Table 2—Sensory and physical characteristics of beef roasts as influenced by method of cooking[a]

Characteristic	Cube						Thin Slice		
	Semimembranosus			Semitendinosus			Semitendinosus		
	Steam oven	Hot water vat	Conventional electric	Steam oven	Hot water vat	Conventional electric	Steam oven	Hot water vat	Conventional electric
Tenderness[b]	5.7	5.7	6.1	5.8	5.9	6.1	7.7	7.7	7.7
Connective tissue amount[b]	6.5	6.6	6.9	6.9	6.9	7.1	7.9	7.8	7.8
Juiciness[b]	5.5	5.4	5.4	5.2	5.1	5.0	6.6	6.5	6.6
Roast beef flavor intensity[b]	4.5	4.7	4.7	4.0	4.0	4.1	5.2	5.5	5.3
Seasoning flavor intensity[b]	3.3	3.2	3.3	2.9	2.4	2.6	4.5	4.4	4.6
Thaw loss, %	8.5	6.6	8.1	10.4	9.0	9.4	12.0	11.2	11.1
Degree of doneness score[a]	3.0	2.8	3.0	2.8	2.8	2.5	2.9	3.0	2.6
Instron maximum single blade shear force (kg)	8.3	8.4	7.9	7.7	7.7	7.4	–	–	–
Warner-Bratzler shear force (kg)	5.9	5.8	5.7	5.3	5.3	5.1	–	–	–

[a] Method of cookery exerted nonsignificant ($P > 0.01$) effects on all sensory and physical characteristics.
[b] Scoring systems based on 8 = extremely tender, juicy and intense in roast beef and seasoning intensity; none in connective tissue amount and very rare in degree of doneness. 1 = extremely tough, dry and bland in roast beef and seasoning intensity, abundant in connective tissue amount and well done in degree of doneness.

in hot-boned prerigor cooked semitendinosus roasts served as cubes compared to cold-boned semitendinosus samples, no palatability differences were found between cold- and hot-boned roasts when samples were served as slices. Thus, it would appear that, regardless of the cooking method, the palatability of prerigor cooked beef probably is quite acceptable when it is served thinly sliced. Because we were unable to obtain consistant and uniform samples for shearing, no Instron or Warner-Bratzler shear force values are given for sliced product. Sensory scores for cubes and thin slices were not compared statistically due to (1) obvious differences in product form and (2) the two product forms were evaluated in different sensory panel sessions.

The cooking methods used on the roasts exerted no influence ($P>0.01$) on any of the sensory and physical measurements (Table 2). Likewise, no interaction effects were detected between boning temperature and method of cocking. Even though the hot water vat cooking system required less time per unit weight to reach an internal roast temperature of 68°C than the other systems, this method did not affect palatability. Cia and Marsh (1976) found that microwave cooking improved the tenderness of prerigor muscle compared to boiling, whereas Montgomery et al. (1977) reported that conventional roasting produced greater tenderness than microwave cooking. Buck et al. (1979) found that roasts cooked in a water bath postrigor were more tender than paired roasts cooked by oven roasting.

The cooking of hot-boned semitendinosus and semimembranosus muscles by cooking methods similar to those of our study may result in tenderness reductions. However, when this nonreheated product is used in a thinly sliced or similar form, any tenderness problems should be eliminated. Cooking of hot-boned muscle can result in significant energy savings, expecially if the product does not require reheating. Recent studies (Anon., 1977; Willardson, 1978) with beef would seem to indicate that with the internal temperature of 68°C used in our study, no problems should exist with *Salmonella* and *C. perfringens* especially if proper food handling procedures are followed.

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FOWL MEAT IN CANS AND RETORT POUCHES . . . From page 230

shredded, or stringy components. Differences in moisture (juiciness) evident in the samples may be more related to fat content than to processing. In this study the two products precooked before retorting (i.e. cooked pouch and canned products) were significantly more off-flavored than the two products that were not precooked. It is likely that the second heating of the product during retorting enhances the development of off-flavors.

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Fish Protein Concentrate (FPC) from Bombay Duck Isolated by Radiation-Heat Combination Procedure: Functional and Nutritional Properties

S. B. WARRIER and V. NINJOOR

ABSTRACT

A process involving gamma-irradiation (200 Krad) and heat treatment (60°C, 10 min), for the preparation of fish protein concentrate (FPC) from a tropical fish Bombay duck (*Harpodon nehereus*) is described. The procedure enabled the precipitation of 75% of proteins from Bombay duck muscle which accounted for 80% of myofibrillar proteins. The solubility of FPC was 3% in water and 15% in 5% NaCl. However, the preparation could be solubilized by treatments with alkali (0.2N NaOH) or proteolytic enzymes. Among the enzymes tested for FPC solubilization, pronase was found to be the most effective both with respect to the extent and the rate of hydrolysis. The FPC displayed good functional properties in terms of emulsifying capacity and wettability. The PER was better than that obtained for casein when fed to rats at 11% protein level.

INTRODUCTION

IN THE THICKLY POPULATED developing countries the protein requirements could be successfully met with high quality proteins isolated from inexpensive varieties of fish. In India, among other fish varieties Bombay duck (*Harpodon nehereus*), a tropical fish, merits consideration for the preparation of fish protein concentrate (FPC) since it is available in plenty throughout the year (Marine Fisheries Information Services, 1979) and conventional conservation methods other than sun-drying are not suitable for its preservation (Kumta and Sreenivasan, 1970). Further, it offers another advantage for making FPC in that its fat content is low (Sawant et al., 1967). Sen et al. (1966) were the first to describe a method for the preparation of FPC from Bombay duck based on solvent extraction process. Moorjani and Lahiry (1970) while summarizing the efforts made in India on the preparation and nutritional evaluation of FPC prepared from a variety of fish, including Bombay duck demonstrated that production of FPC is both feasible and economical. However, there is no report on the functional properties of FPC prepared from Bombay duck.

During our earlier studies, it was observed that while exposure to gamma-radiation (250 Krad) precipitated 30% of the proteins from Bombay duck muscle homogenates, a combination of radiation (250 Krad) and heat treatment (60°C for 10 min) resulted in the precipitation of 75% of the total proteins (Warrier et al., 1973). This suggests that a radiation-heat combination procedure can be used in the isolation of fish proteins obviating solvent extraction.

Although many processes for making FPC have been advocated, production of FPC for supplementing the existing staple foods and food products has not fulfilled its promise due to several limitations including loss in functional properties (Holden, 1971; Warrier et al., 1975). For the successful application of FPC in fortifying foods it is desirable that the protein should be inexpensive and possess good functional and nutritional properties. The present paper defines the process parameters for the preparation of Bombay duck FPC and describes some of its functional and nutritional properties.

Authors Warrier and Ninjoor are with the Biochemistry & Food Technology Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085, India.

MATERIALS & METHODS

Fish

Freshly caught Bombay duck (*Harpodon nehereus*) were brought to laboratory in iced condition. They were cleaned, eviscerated, filleted and stored in crushed ice until used. The skeletal muscle free from skin and bone was finely minced with scissors and set aside for homogenization.

Preparation of muscle homogenate

Skeletal muscle homogenate (10%) was prepared in 0.01M phosphate buffer (pH 7.5) containing 5% sodium chloride (Kumta and Gore, 1970). The homogenization was carried out at 0-4°C in a commercial Waring Blendor having a stainless steel jar of 5 liter capacity with an intermittent speed of high and low for 3 min.

Isolation of fish protein concentrate

Irradiation. The muscle homogenate packed in polyethylene bags were exposed to a gamma-radiation dose of 200 Krad at a dose rate of 2.2 Krad/min in a Package irradiator (Atomic Energy of Canada Limited) having a ^{60}Co source of 40,000 Ci strength. The irradiation was carried out in ice temperature.

Heat treatment. The irradiated fish homogenate was subjected to heat treatment in a stainless steel vessel maintained at 60°C for 10 min. After the thermal treatment the homogenate was cooled and the precipitate formed was filtered through three layers of muslin cloth. The precipitate was dried for 8 hr in a vacuum dryer maintained at a constant temperature of 70°C. The dried FPC was powdered mechanically and packed in polyethylene bags and stored at room temperature.

Proximate composition of FPC

Nitrogen was determined by Kjeldahl digestion of FPC followed by nesslerization (AOAC, 1965) and the protein calculated (N x 6.25). Protein measurement was also made from suitable aliquots of FPC dissolved in 0.2N NaOH by the procedure of Lowry et al. (1951). Total lipids were determined following extraction of FPC in chloroform for a refluxing period of 16 hr in a soxhlet apparatus (AOAC, 1965). The chloroform extract of FPC was used for the determination of phospholipid (Frampton et al., 1948), cholesterol (Mann, 1961) and total glyceride (Van Handel, 1961). Moisture and ash contents were measured according to AOAC method (1965).

Amino acid composition

Acid hydrolysis of FPC was carried out by autoclaving a known amount of FPC in 6.0N HCl under vacuum at 110°C for 24 hr. After hydrolysis, the acid was evaporated and the residue dissolved in citrate buffer (pH 2.2). One milliliter of the sample containing 1 mg protein was used for amino acid analysis which was carried out in a Beckman/Unichrom Amino Acid Analyser Model 4231. Tryptophan was determined by a colorimetric method (Spies and Chambers, 1949).

Solubilization of FPC

This was ascertained in different solvents and under varied pH conditions. Influence of various hydrolytic enzymes in solubilizing FPC was also studied.

Solubility of FPC in different solvents. Protein concentrate (1g)

was added to 50 ml of distilled water in a 250 ml beaker and agitated continuously for 60 min, at room temperature using a magnetic stirrer. Following this the suspension was centrifuged at 750 x G for 15 min. The protein content in the supernatant as obtained after filtration was determined (Lowry et al., 1951). This procedure was adopted for testing the solubility of FPC in different solvents. The protein content (N x 6.25) of original FPC was estimated by Kjeldahl procedure (AOAC, 1965).

pH induced solubilization of FPC

The protein was tested for solubility over a pH range 3.0-9.0. The protein, 500 mg, was dispersed in 25 ml of 0.2N NaOH and agitated for 30 min at room temperature using a magnetic stirrer. This treatment dissolved the protein completely. The pH of the resulting solution was 12.8 which was altered to various values by the addition of 1.0N HCl with continuous stirring. The solutions were then centrifuged at 12,000 x G for 10 min. The extent of solubility was determined by measuring the protein content in the supernatant (Lowry et al., 1951; AOAC, 1965).

Solubilization by proteolytic enzymes

Procedures described by Hale (1969) and Cheftel et al. (1971) were employed with minor modifications to examine the solubilization of FPC by enzymes such as papain (BDH, England), pronase (BDH), pepsin (E. Merck, Germany) and bromelain (Sigma, USA). The enzymes were dissolved in distilled water, held at room temperature for 10 min and centrifuged at 12,000 x G for 10 min in cold. The supernatants stored in ice were used as enzyme sources. The standard conditions for the solubilization study were as follows: Temperature 37°C, pH 8.0, pronase; temperature 37°C, pH 8.0, cysteine (0.005M) and EDTA (0.002M), papain; temperature 35°C, pH 7.5, cysteine (0.15M), bromelain; and temperature 37°C, pH 2.0, pepsin. The ratio between the substrate and the enzyme was maintained at 60:1.

FPC (600 mg) was suspended in 20 ml distilled water and heated in a boiling water bath for 15 min with intermittent agitation. The contents were cooled and the buffers of desired pH were added (20 ml, 0.1M). Before treating with the enzyme the pH was adjusted to the optimum level by the addition of 0.05N HCl or NaOH. The overall procedure of enzyme hydrolysis consisted of treating FPC with the enzyme in a stoppered Erlenmeyer flask which was incubated in shaking water bath maintained at appropriate temperatures. Where the reaction was allowed to proceed for 24 hr, a bacteriostatic reagent was included in the assay mixture. On the completion of proteolysis the contents were centrifuged at 12,000 x G for 10 min at 0-4°C, the pellets washed and recentrifuged. The clear supernatants were combined and the solubilized protein content was estimated (Lowry et al., 1951; AOAC, 1965). For the determination of rate of proteolysis, FPC enzyme samples in duplicate and a blank consisting only FPC were incubated as above. A known amount of aliquot was removed at different intervals of time and the reaction terminated by the addition of appropriate stopping reagents. After centrifugation, α-amino acids from the aliquots were determined (Rosen, 1957).

Since the preliminary experiments indicated that maximum solubilization of FPC was obtained by treatment with pronase, proteolysis at different pH levels and the influence of enzyme concentration on the digestibility were studied. The pH range studied included 7.5-8.5 and the ratio of enzyme to substrate varied from 1:300 to 1:60.

Determination of emulsifying capacity

The method described by Dubrow et al. (1973) was used with the following modifications. FPC varying from 10-500 mg were separately homogenized in a Sorvall Omnimixer with 20 ml of 5% NaCl containing 0.02M $NaHCO_3$ for 3 min at low speed. Til oil (20 ml) was then slowly added to the blender and the mixture homogenized further for 2 min at low speed. The entire mixture was poured into 3 test tubes in 10 ml portions and the tubes were placed in a boiling water bath for exactly 30 min. After cooling in an ice bath, volume of both aqueous and oil layers were measured. Emulsion capacity was calculated as ml of oil emulsified per mg of protein.

Wettability

This evaluation was subjective and based on the extent of wetting when particles were applied to the surface of a water medium. The sample that was wet within 2 min or less was considered as excellent; between 2-5 min good; between 5-15 min fair, and any sample that took more than 15 min to become wet was rated poor (Rasekh and Metz, 1973).

Water absorption

The method used for this determination was essentially that of Smith and Circle (1972). In 20 ml distilled water, 1g of FPC was added gradually to avoid formation of clumps. The tubes were centrifuged at 750 x G for 10 min. The supernatant was poured through a funnel containing a small amount of glass wool into a calibrated tube. The volume of the supernatant was substrated from the original 20 ml. The results are reported in terms of ml of water absorbed by 1g of protein.

Determination of protein efficiency ratio

Animals. Weanling male albino rats of Wistar strain weighing 50-55g were used in feeding studies. They were housed individually in wire bottomed aluminium cages in a room maintained at 25°C. The animals had free access to water and food throughout the day.

The rats were divided into 2 groups, each consisting of 6 animals. The first group (control) was given a basal diet (Ninjoor and Kumta, 1974) containing 11% casein and 0.1% methionine. The second group was reared on a diet containing an amount of FPC that provided 11% protein. The composition of basal diet, salt mixture, and vitamin mixture was as described by Padwal-Desai et al. (1969). Measurement of body weight and dietary intake was made daily for a period of 28 days.

RESULTS & DUSCUSSION

BOMBAY DUCK MUSCLE contains 85-90% moisture, 8-10% protein and 0.1-0.3% fat. The proportion of sarcoplasmic and fibrillar proteins in the muscle is 20:80 (Kumta and Gore, 1970). The method shown in Figure 1 accomplishes the selective extraction of myofibrillar proteins by a combination treatment of gamma-radiation and heat (Warrier et al., 1973). The procedure results in the precipi-

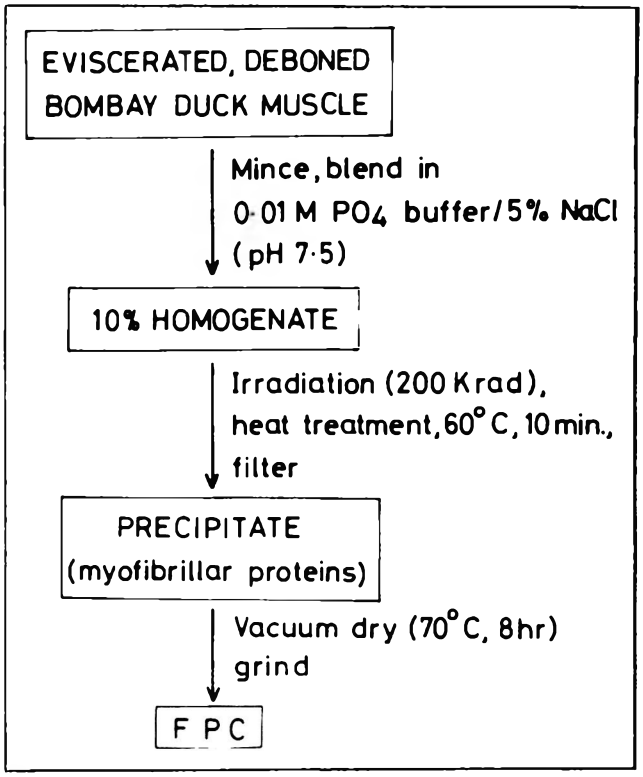


Fig. 1—Preparation of Bombay duck FPC employing radiation-heat combination treatment.

tation of 75% of the total proteins of Bombay duck muscle which accounts for 80% of the structural proteins. In recent years the importance of utilizing structural proteins of fish muscle in preparing functional isolates and derivatives has been emphasized by various workers (Spinelli et al., 1972a; Cobb and Hyder, 1972; Groninger, 1973; Meinke and Mattil, 1973; Spinelli et al., 1975; Groninger and Miller, 1979). Since sugars and majority of odor forming compounds associated with the sarcoplasmic protein fraction (Spinelli et al., 1975) are removed, browning and reversion of acute fishy flavor do not arise in the present preparation even during storage. The FPC thus possesses a light color and mild fishy flavor. No attempt has been made in the present investigation to eliminate the odor since FPC samples exhaustively extracted with petroleum ether or hexane were found to retain fishy smell (Sen et al., 1966). Although ethanol at boiling temperature appears to remove the odor associated with FPC (Sen, 1966) such treatment seemed to drastically reduce the functional properties (Dubrow et al., 1973).

A typical preparation of FPC has a composition of 76% protein, 12% ash, 6.5% moisture, and 4% lipids (Table 1). The bulk of the lipids appear to be comprised of phospholipids. Although Bombay duck has been categorized as a lean fish (Sawant et al., 1967) the higher lipid content in FPC apparently results because of nonutilization of solvents for extracting proteins. Radiation heat combination treatment procedure for obtaining FPC provides a preparation devoid of viable organisms. Duplicate batches of FPC examined gave similar results.

Table 1—Composition of Bombay duck fish protein concentrate[a]

Constituents	%
Protein (N x 6.25)	75.90 ± 1.52
Ash	12.40 ± 0.50
Moisture	6.66 ± 0.11
Total lipids	4.33 ± 0.19
Phospholipids (mmoles/100g FPC)	4.66 ± 0.18
Cholesterol (mmoles/100g FPC)	0.94 ± 0.03
Total glycerides (mmoles/100g FPC)	0.10 ± 0.004

[a] Values represent the average ± SEM of 5 batches of FPC preparations. The value for total glycerides was calculated employing coconut oil as standard.

Table 2—Composition of essential amino acids in Bombay duck FPC[a]

Amino acid	g/16g of N
Lysine	10.52 ± 0.03
Histidine	1.98 ± 0.05
Arginine	5.98 ± 0.06
Threonine	4.33 ± 0.07
Valine	4.70 ± 0.02
Methionine	2.14 ± 0.01
Isoleucine	4.52 ± 0.04
Leucine	7.10 ± 0.07
Phenylalanine	3.53 ± 0.03
Tryptophan	0.81 ± 0.05

[a] The values represent the average ± SD of three independent estimations. Tryptophan was determined by Spies and Chamber (1949) method.

Table 3—Solubility of FPC in different solvents[a]

Solvent	% Extractable protein
Distilled water	3
Hot distilled water (60°C)	10
NaCl, 5%	15
Acetate buffer (0.10M), pH 3.8	Nil
NaOH, 0.2N	100
NaOH, 1.0N	gel formation

[a] All solubility experiments excepting that in hot distilled water were carried out at 25°C as stated in the text.

The profile of essential amino acids from FPC is included in Table 2. The amino acid composition compared favorably with those reported for solvent extracted FPC and chemically modified fish proteins (Groninger and Miller, 1979).

Although the utility of FPC as an excellent source of protein has been recognized (Stillings, 1974), its use as a food ingredient is limited because of poor functional properties including lack of solubility (Spinelli et al., 1972a; Cheftel et al., 1971). Several approaches have been made in solubilizing FPC by treatments including acid, alkaline and enzyme hydrolysis (Cobb and Hyder, 1972; Meinke and Mattil, 1973; Tannenbaum et al., 1970a, b; Hale, 1969; Cheftel et al., 1971; Hevia et al., 1976). Table 3 provides data on solubility of FPC in different solvents. While the product was soluble to the extent of only 10% in distilled water at 60°C, it was completely soluble in 0.2N NaOH. However, when the concentration of NaOH was increased to 1.0N, gel formation was observed. Sodium chloride did not appreciably alter protein solubilization. An alkaline process described earlier (Tannenbaum et al., 1970a) for solubilization of solvent extracted FPC utilized a condition of high pH (12.5) and temperature (95°C). In contrast, the present study shows that Bombay duck FPC prepared by a combination of radiation and heat could be solubilized to the extent of 80% in alkaline pH (8.0-9.0) without heat treatment. When the pH was lowered, the extent of solubilization decreased leading to isoelectric precipitation in the region of pH 4.0 (Fig. 2) indicating the retention of functionality of the protein (Tannenbaum et al., 1970b). Similar findings have been reported both for fish (Spinelli et al., 1972b; Rasekh and Metz, 1973) and vegetable (Sekul et al., 1978; Lawhon et al., 1972) proteins.

Direct use of commercially available proteolytic enzymes in solubilizing FPC has been investigated by Hale (1969), Cheftel et al. (1971) and more recently by Hevia et al. (1976). Table 4 includes data on the capacity of different enzymes to solubilize FPC. Pronase and pepsin hydrolysed FPC to the extent of 96 and 85%, respectively, during a 24 hr period. Among the various proteolytic enzymes tested pronase appeared to be more effective both in terms of extent (Table 4) and rate (Fig. 3) of solubilization of FPC which corroborate earlier findings (Hale, 1969; Cheftel et al., 1971; Hevia et al., 1976). In agreement with the report of Hevia et al. (1976) the data on the peptide bond splitting as measured by the ninhydrin method apparently correlated well with solubility (Fig. 3).

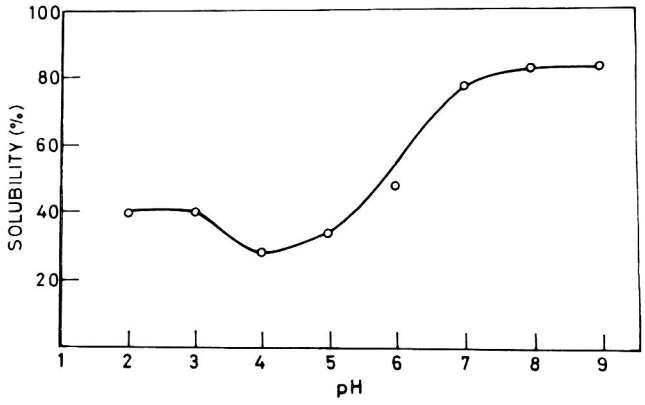


Fig. 2—Influence of pH on the solubilization of FPC.

A detailed assessment was carried out on the influence of pronase on FPC solubilization at different pH levels (Fig. 4) and at different concentrations (Table 5). The results revealed that in the pH range tested (7.5-8.5) there was little difference in the degree of hydrolysis. Among the six enzyme to substrate ratios tested, 1:300 resulted in 75% solubilization. When the enzyme to substrate ratio was increased fivefold, there was only a further increase of 21% in the solubilization, indicating that this procedure is feasible even at low concentration of the enzyme.

Unlike solvent extracted FPC (Dubrow et al., 1973), the present protein concentrate was wettable. When compared with defatted casein, the wettability could be judged to be excellent. The water absorption ability (ml water per g protein) was found to be 7.0 which is considerably less than those reported for modified fish proteins (Miller and Groninger 1976).

Figure 5 depicts the data on the emulsifying capacity of FPC. The ability of the protein to emulsify oil per unit of protein decreased as the concentration of protein in solution increased. Similar results for the oil emulsifying efficiency were reported for different muscle proteins including those of fish (Acton and Saffle, 1972; Cobb and Hyder, 1972; Tsai et al., 1972). However, the data on emulsifying capacity of model system can not be correlated with the true functionality of a protein (Tsai et al., 1972). Nevertheless, it may be indicated that the emulsifying capacity of Bombay duck FPC is comparable with that of soy protein isolate and other modified proteins (Groninger, 1973).

—Continued on next page

Table 4—Solubilization of FPC proteins by proteolytic enzymes[a]

Enzyme	% Protein solubilized
Bromelain	45
Papain	75
Pepsin	85
Pronase	96

[a] The extent of solubilization was determined as described in the text during 24 hr incubation. The values are averages of two experiments.

Table 5—Influence of pronase concentration on the solubility of FPC proteins[a]

Enzyme: FPC (W/W)	% Protein solubilized
1 : 300	75
1 : 200	75
1 : 150	82
1 : 100	89
1 : 75	92
1 : 60	96

[a] Solubilization studies in duplicate were carried out as described in the text for 24 hr.

Table 6—Nutritive quality of Bombay duck FPC[a]

	Total wt gain (g)	Total food intake (g)	Total protein intake (g)	Protein efficiency ratio (PER)
FPC	182.33 ± 3.10	485.42 ± 2.63	53.40 ± 0.29	3.41 ± 0.05
Casein	174.00 ± 3.37	492.92 ± 2.96	54.22 ± 0.32	3.21 ± 0.04

[a] Values are the average ± SEM of six animals. In control group rats were given diet containing 11% casein and in FPC group casein was replaced by 11% FPC protein. Each group consisted of 6 male rats. The feeding period was 28 days.

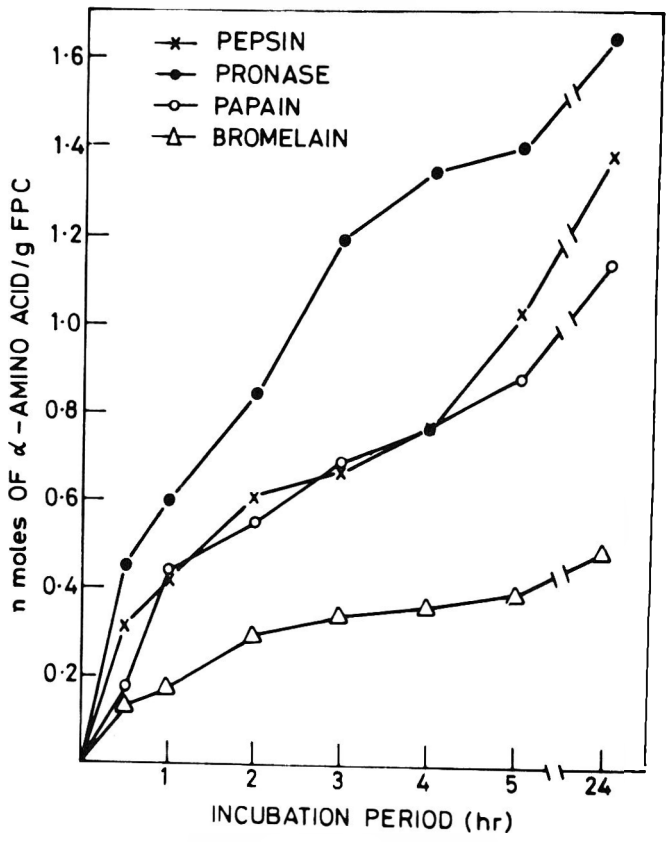


Fig. 3—Rate of solubilization of FPC by various proteolytic enzymes.

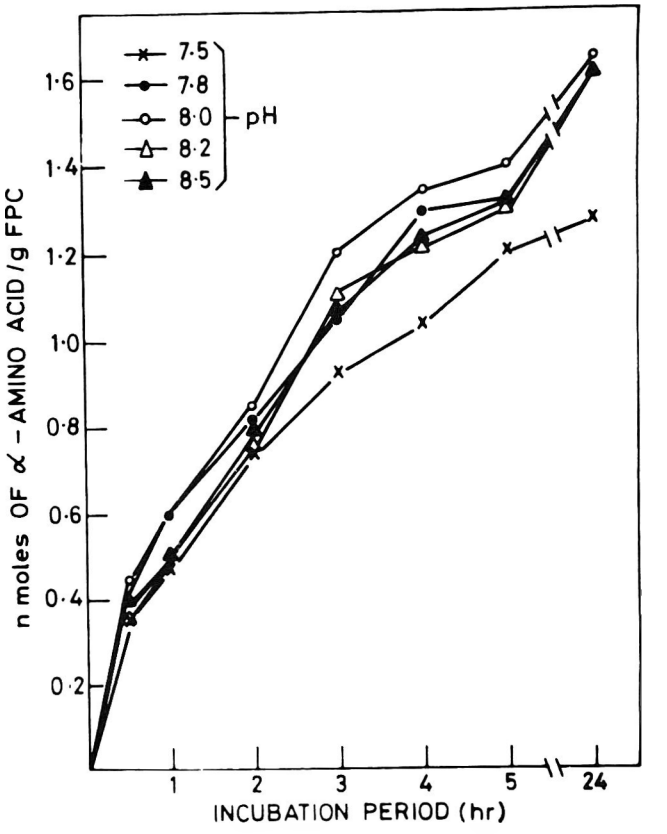


Fig. 4—Rate of solubilization of FPC by pronase at different pH.

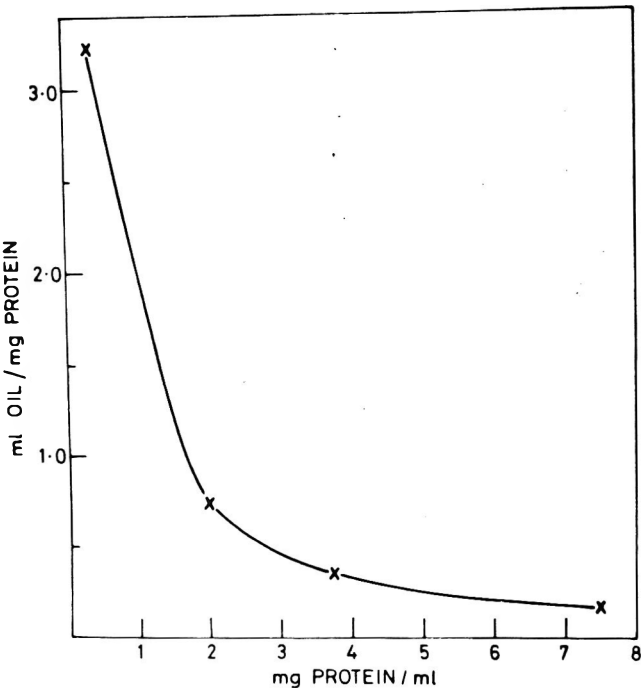


Fig. 5—Emulsifying capacity (ml oil emulsified/mg protein) of different concentrations of Bombay duck FPC.

The data on the growth pattern of rats fed Bombay duck FPC (Table 6) revealed that the product supports the normal growth of animals. When fed at 11% protein level, a PER of 3.41 was obtained as compared to 3.21 for casein. Also, the PER data are comparable with those reported for FPC in the literature (Groninger, 1973; Moorjani and Lahiry, 1970; Stillings, 1974). Thus the functional and nutritional properties of Bombay duck FPC point to its potential use as a suitable protein supplement in food systems.

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Yield and Quality of Soymilk Processed by Steam-Infusion Cooking

L. A. JOHNSON, C. W. DEYOE and W. J. HOOVER

ABSTRACT

Traditional soymilk is boiled for approximately 60 min at 99°C, pH 6.7, which reduces trypsin inhibitor (TI) activity by more than 90% to improve nutritional value. We evaluated continuous, direct steam-infusion cooking that facilitated higher temperatures (99-154°C) than that traditionally used for cooking soymilk. At temperatures above 120°C in steam-infusion cooking we observed consistent temperature-dependent patterns in yields of soymilk, solids, and protein, characterized by an initial decrease, followed by a rise to maximum recovery, then by a final decrease. At 154°C, pH 6.7, maximum recovery occurred at the same point as did adequate inactivation of TI. As much as 90% of the slurry, 86% of the soybean solids, and 90% of the protein were recovered as soymilk after centrifuging at 1050 x G for 5 min. Under optimum conditions for steam-infusion cooking the soymilk also retained less than 8% residual TI activity, and less chemical browning. Using traditional cooking methods, about 72% of the slurry, 61% of soybean solids, and 73% of the protein are recovered.

INTRODUCTION

THERE IS considerable interest in soy-based milk analogs as substitutes for bovine or human milk in areas where bovine milk is expensive or unavailable and to feed to infants who are allergic to animal milk. Lack of commercial acceptance of soymilk prepared from whole soybeans, however, continues as a result of undesirable, lipoxygenase-catalyzed flavors; and failure to achieve a high yield of a stable dispersion while inactivating trypsin inhibitors. Soymilk has been prepared for hundreds of years in the Orient by soaking the beans in water for several hours, draining, grinding with fresh water, filtering to remove the insoluble residue, and boiling for 30 min (Smith and Circle, 1972). The traditional method produces soymilk with a green, beany flavor caused by lipoxygenase activity during wet grinding. Toasting raw intact seeds at 200°C for 30 min will inactivate lipoxygenase (Wilkens et al., 1967); doing that, however, will greatly reduce protein extraction and yield of soymilk and will cause nutty flavor, chalky texture, and darkened color. Grinding soybeans in hot water (> 80°C) greatly improves the flavor of soymilk. Lipoxygenase is inactivated within 10 min when the seed is wet ground at 80-100°C (Wilkens et al., 1967); therefore, activity and reaction time are significantly reduced. Nelson et al. (1975, 1976) patented a process, now known as the Illinois Process in which soybeans are blanched before being ground. After soaking 12 hr in a solution of $NaHCO_3$, the beans are drained, boiled in fresh 0.5% $NaHCO_3$ for 30 min, drained again and ground with sufficient water to make 12% bean solids. The slurry is then homogenized in two stages. Water is added and the slurry neutralized and heated to 82°C and rehomogenized. Nelson et al. (1976) reported good stability of a dispersion of dehulled soybeans during storage; but no filtration or centrifugation was conducted. Johnson and Snyder (1978) showed that blanching intact soybeans fixed protein bodies and resulted in high losses in yields of soymilk and protein when the bean slurry was centrifuged at 642 x G. Although the Illinois Process produces large amounts of waste water, requires sophisticated equipment, and is relatively energy intensive, it produces the most acceptable soymilk that can be produced with current technology. In this paper we report a new approach to producing a remarkably stable soy beverage with improved yields, and less chemical browning while achieving greater than 90% inactivation of trypsin inhibitors.

Author Deyoe is affiliated with the Dept. of Grain Science and Industry, Kansas State Univ., Manhattan, KS 66506. Author Johnson, formerly with Kansas State Univ., is now with the Food Protein R&D Center, Texas A&M Univ., College Station, TX 77843. Author Hoover, formerly with Kansas State Univ., is now with the American Institute of Baking, Manhattan, KS 66502.

MATERIALS & METHODS

Preparing soy flour slurries

Whole seed-grade Columbus soybeans were ground in a laboratory hammermill through a 0.125-mm opening screen. One-part soy flour was hand mixed with four parts water until homogenous, requiring about 10 min. The slurry was cooked within 15 min after mixing. Sodium hydroxide was used to adjust selected slurries to pH 9.5 prior to cooking.

Soymilk processing

A control sample was prepared by cooking the soy flour slurry in the traditional manner at 99°C. Approximately 30 sec were required to reestablish the cooking temperature after adding the soy flour. The slurry was agitated continuously during cooking, after which the slurry was rapidly cooled by using an ice-bath heat exchanger.

A Penick and Ford laboratory jet cooker was used to heat soy flour slurries to 99°C. Samples were discharged into preheated 2,000 ml round-bottom flasks which were immediately connected to a condenser and heating mantle. Cooked samples were cooled by using the ice-bath heat exchanger.

For experimental batches, the laboratory jet cooker was modified (Fig. 1) to process soy flour slurries for variable times at temperatures of 121, 132, 143, and 154°C. Continuous cooking for 0-34 sec was achieved by increasing the length of the holding tube. For process times greater than 34 sec, the system was operated in a semi-batchwise fashion because it was not practical in our pilot plant to extend the holding tube beyond 37 ft. In semi-batchwise operation, the slurry was sealed in the holding tube for the desired period beyond 34 sec. Approximately 1,000 ml of cooked slurry was collected in a stainless-steel container in an ice bath. Temperature did not vary by more than ±1.7°C from the desired temperature with respect to time and position within the holding tube. Instantaneous heating to desired cooking temperatures and cooling to 99°C were achieved. Cooling from 99°C to 30°C required about 120 sec. Samples cooked at pH 9.5 were neutralized immediately after cooling. All samples were adjusted to 10% solids with distilled water after slurry solids had been determined by AOAC (1975) procedure 16.032. All samples were stored at 5°C until analyzed.

Analyses

About 1,500g of cooked slurry (5°C) was centrifuged at 1,050 x G for 5 min. Four samples, 250 ml each, were used to quantify soymilk fraction yield, solids yield, and protein yield. Solids were determined by AOAC (1975) procedure 16.032 to determine solids balance. Additional samples of residue and supernatant were lyo-

philized and analyzed for nitrogen and moisture by using AACC (1969) methods 46-11 and 44-15, respectively, to determine a protein balance. Protein was estimated by using N x 6.25.

Color was measured by a Gardiner Color Difference Meter. The Rd values were converted to Hunter L values by standard formulas.

Trypsin inhibitor (TI) activity was assayed by the procedure of Swartz et al. (1977) as detailed in Johnson et al. (1980a). Percentage residual TI activity in processed soymilk was calculated as the number of TIU/g soymilk divided by the number of TIU/g in raw soymilk times 100%.

RESULTS

Product yields

The terms % soymilk fraction yield, % solids yield, and % protein yield were used to denote amounts of sediment-free milk recovered and proportions of the solids and protein that were present in the milk fraction. The following formulas for calculation were used:

$$\%\ \text{soymilk fraction yield} = \frac{\text{wt of supernatant}}{\text{wt of cooked slurry}} \times 100\% \quad (1)$$

$$\%\ \text{solids yield} = \frac{\%\ \text{soymilk fraction yield}}{} \cdot \frac{\%\ \text{solids in supernatant}}{\%\ \text{solids in soy slurry}} \quad (2)$$

$$\%\ \text{protein yield} = \frac{\%\ \text{soymilk fraction yield}}{} \cdot \frac{\%\ \text{protein in supernatant}}{\%\ \text{protein in soy slurry}} \quad (3)$$

Traditional processes for preparing soymilk involve cooking wet-ground soybeans for 60 min at 99°C to stabilize microbiological degradation and improve nutritional quality and flavor. In the absence of wet-grinding equipment, we simulated traditional processing by grinding whole soybeans to <0.125 mm, slurrying the flour in boiling water and cooking at 99°C. The yield of soymilk fraction decreased, during 5-60 min cooking, from 72.4-62.4%. The yield of soybean solids in the soymilk fraction decreased from 46.1 to 37.2% after 5 and 60 min of cooking, respectively (Fig. 2). This decrease in yield during cooking was presumably due to protein denaturation and decreased emulsification.

Greater yields of dispersed solids were accomplished by passing the soy flour slurry through the hydro-heating valve and then cooking at 99°C (Fig. 2). Yields of soymilk fraction, solids, and protein decreased less substantially during cooking than in the initial experiment. During the 60 min of cooking, soymilk fraction yield decreased from 75.3 to 72.1%; solids yield, from 63.8 to 61.2%; and protein yield, from 76.9 to 73.2% (Table 1). Adjusting the pH of the slurry to 9.5 prior to cooking and back to 7.0 after cooking increased yields of soymilk fraction, protein, and solids by 2-3% over the entire 60 min cooking period. Because the reaction rate of TI inactivation is greatly increased by cooking at pH 9.5 versus 6.7 (Johnson et al., 1980a), cooking time for adequate TI inactivation would be much less than 60 min.

In that the kinetics of TI inactivation may differ from the kinetics of yield reduction, the effects of steam-infusion cooking were studied in an attempt to maximize yields while providing adequate inactivation of TI. Steam-infusion cooking is the continuous direct injection of steam into the product to provide instantaneous heat transfer and severe agitation. Flashing the product through pressure release provides instantaneous cooling to 99°C. Therefore, in a pressurized system, high-temperature (121-154°C), short-time (0-4 min) processing is possible. Hackler et al. (1965) reported that 90% of the original TI activity was inactivated within 4 min at 121°C by using conventional heat exchangers. Therefore, we examined the yields of soymilk fraction, protein, and solids over 4 min of steam-infusion cooking at temperatures ranging from 121-154°C.

The yield of soybean solids as soymilk when cooked at 121°C decreased to a minimum (63.4%) after 150 sec and then increased to the initial level of 66% after 265 sec of cooking (Fig. 3). The same initial decrease in solids recovery was also observed at 132, 143, and 154°C. At 132°C the yield of solids decreased for the first 90 sec and then increased linearly to 83.3% after 270 sec. At 143°C solids recovery decreased for the first 30 sec and then rapidly increased to maximum recovery after 150 sec of cooking.

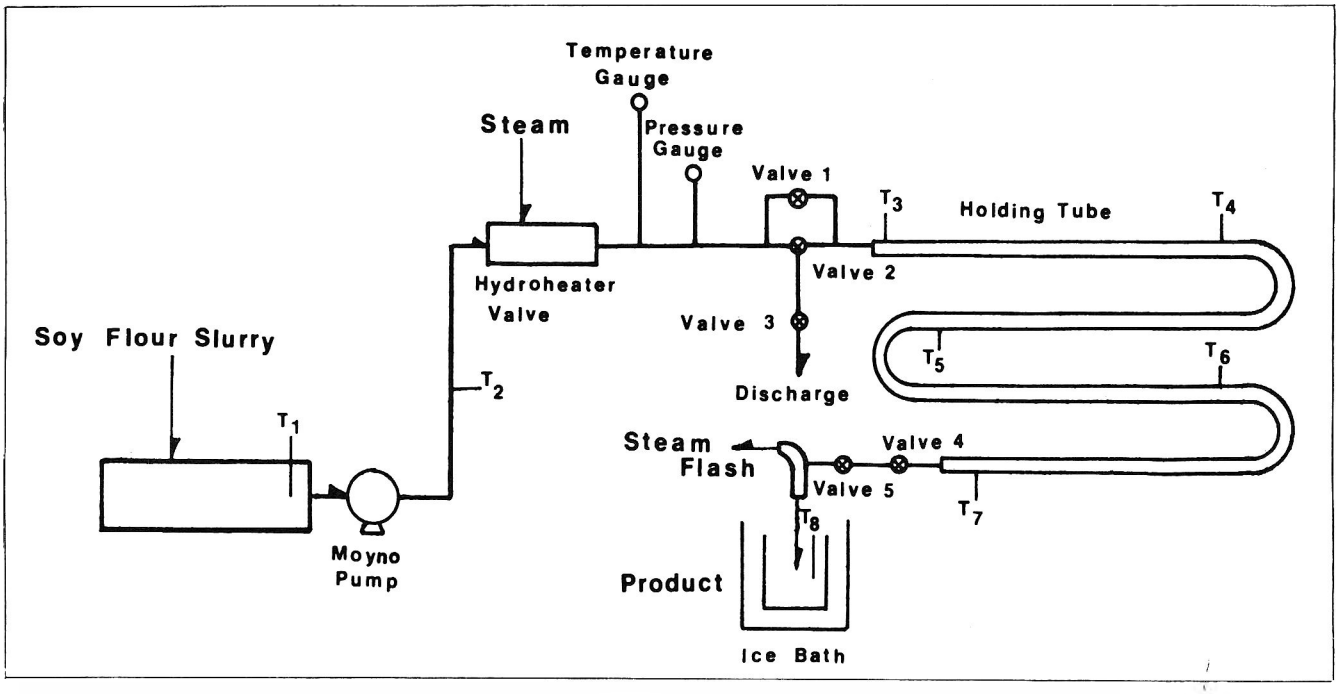


Fig. 1—Flow scheme for processing soymilk by steam-infusion cooking: T_1-T_8 Thermocouples; Value 1 by-pass throttle (needle valve); Valve 2 flow control (3-way ball valve); Valve 3 discharge pressure control (gate valve); Valve 4 flow control (2-way ball valve); Valve 5 back pressure control (gate valve).

At 154°C solids recovery decreased for 11 sec and then increased to a maximum of 86.0% after 34 sec. After 34 sec solids yield decreased rapidly. Yields of milk fraction and protein followed similar trends as solids yields (Table 1). Fukushima (1959) has also reported similar patterns in protein solubility when heating defatted soy flour at 110-120°C with equal quantities of water.

Similar patterns in solids yield recovery were observed at pH 9.5 as at pH 6.7 except that the curves for pH 9.5 were shifted to the left as if cooked at 11°C higher than actual conditions used. Process times to minimum solids recovery were halved by using the pH 9.5 versus 6.6 (Fig. 4). At 121°C maximum solids recovery were also more rapidly approached. At pH 9.5 initial solids recovery were 3-5% higher than at pH 6.7. Yields of milk fraction and protein also followed the same trends as solids yield (Table 1).

–Continued on next page

Table 1–Yield and quality of soymilk processed by steam-infusion cooking at pH 6.7 and 9.5

Cooking conditions		Soymilk[a]					
pH	Temp	Time (sec)	Fraction yield (%)	Solids yield (%)	Protein yield (%)	Residual TI (%)	Color (Hunter L)
6.7	99°C	5	75.3	63.8	76.9	70.0	77.1
		300	74.7	63.3	75.6	23.2	76.9
		900	73.9	62.7	75.1	11.5	76.2
		1800	73.7	62.9	75.0	9.6	75.2
		2700	72.9	61.8	74.2	8.0	73.6
		3600	72.1	61.2	73.2	7.6	73.0
6.7	121°C	5	76.4	65.9	78.1	73.3	78.3
		19	ND	ND	ND	57.0	ND
		55	ND	ND	ND	21.3	ND
		85	74.9	65.6	77.0	13.2	79.0
		145	71.0	63.4	74.4	11.6	78.5
		205	72.9	64.6	74.9	8.9	78.2
		265	73.8	66.0	76.2	8.0	77.9
6.7	132°C	5	75.3	64.8	75.8	72.0	78.3
		58	72.6	64.1	75.0	13.0	80.0
		88	69.9	63.4	72.0	9.4	79.4
		148	74.9	69.9	76.2	7.0	78.0
		208	81.7	76.7	82.4	6.5	77.0
		268	87.2	83.3	87.2	6.0	76.0
6.7	143°C	5	74.0	63.6	75.5	71.8	78.2
		9	ND	ND	ND	53.0	79.3
		30	69.4	61.2	69.0	14.8	79.1
		60	84.3	79.8	85.1	8.6	78.1
		90	89.0	84.7	89.3	7.7	76.7
		150	86.1	85.2	82.7	6.5	74.2
		210	85.3	78.7	82.0	5.6	73.0
		270	84.2	76.8	80.0	4.4	71.5
6.7	154°C	5	76.0	65.8	77.7	71.1	79.0
		11	72.9	64.6	76.4	32.5	79.8
		25	88.2	84.8	85.8	10.9	79.0
		34	90.2	86.0	89.4	8.5	77.4
		64	89.1	83.4	86.3	6.1	74.4
		94	87.0	79.1	80.5	5.1	72.9
		154	84.7	73.3	78.1	4.7	70.5
		214	81.5	71.9	73.2	2.9	67.2
9.5	99°C	5	78.5	66.5	79.1	45.6	76.2
		300	74.6	65.9	75.3	5.5	73.5
		900	72.9	64.8	77.2	3.0	70.8
		1800	74.2	67.6	73.9	1.8	67.1
		2700	74.4	66.6	73.9	0.9	65.8
		3600	73.7	63.7	73.9	Trace	63.6
9.5	121°C	5	79.7	69.0	76.7	47.5	77.2
		25	74.3	64.8	74.9	12.8	77.2
		40	74.8	65.8	75.7	9.0	77.2
		85	72.0	63.0	71.9	7.2	76.1
		145	73.2	65.4	73.0	5.9	74.9
		205	76.7	70.1	76.7	4.1	73.6
		265	81.3	76.2	82.0	3.8	71.1
9.5	143°C	5	78.9	68.7	79.8	27.2	77.3
		9	73.3	63.5	74.2	15.1	74.5
		22	74.9	67.8	76.6	11.9	75.9
		30	80.0	74.6	80.8	8.3	74.9
		60	81.6	76.7	80.1	6.0	72.6
		90	86.1	82.1	84.5	3.0	69.4
		150	89.4	86.0	87.5	Trace	66.6
		210	90.5	87.0	87.9	Trace	64.3

[a] ND denotes not determined.

Table 2–Properties of soymilk processed by steam-infusion cooking temperatures to inactivate 92-93% original TI activity

Cooking conditions			Soymilk			
pH	Temp	Time (sec)	Fraction yield (%)	Solids yield (%)	Protein yield (%)	Color (Hunter L Value)
6.7	99°C	3600	72.1	61.2	73.2	73.2
6.7	121°C	280	74.1	66.4	76.6	77.6
6.7	132°C	165	77.1	71.6	77.9	78.8
6.7	143°C	100	88.0	84.0	88.0	76.6
6.7	154°C	40	90.2	86.0	89.4	76.8
9.5	99°C	150	76.6	66.2	77.2	74.9
9.5	121°C	80	71.6	63.2	72.3	76.7
9.5	143°C	40	80.5	75.3	80.6	74.0

Chemical browning

Effects of heat treatment on chemical browning were also studied. At 99°C the Hunter L value decreased from 77.1 to 73.0 at pH 6.7 over 60 min of cooking (Table 1). Chemical browning was much more rapid at pH 9.5, decreasing from an initial value of 76.2 to 63.6 over 60 min of cooking. The Hunter L value after 6 min of cooking at pH 9.5 was equivalent to 60 min of cooking at pH 6.7.

Color of soymilk was also noted when cooked at hypobaric temperatures and pH 6.7 (Table 1). Initially the Hunter L value increased at all temperatures. Increased whiteness corresponded to the initial decrease in solids yield and therefore could have been the result of decreased dispersed solids. As process temperature increased, the rate of chemical browning increased. Soymilk could be processed for about 600 sec at 121°C, 400 sec at 132°C, 210 sec at 143°C, or 90 sec at 154°C before equivalent chemical browning to 60 min at 99°C would occur.

DISCUSSION

IN GENERAL, soymilk has been separated from insoluble residue by plate-and-frame filters, laboratory cheese-cloth filters, and various centrifugation techniques. Variations in filter pressure and centrifugal force are common. Although no standard separation technique has been established, one wants to remove only that portion that will not remain dispersed over a reasonable storage period and that would contribute to a throaty, raspy texture. It is desirable to remove at least the hull material, which accounts for 8% of the solids and 2% of the protein in whole soybeans. In this work all samples were centrifuged at 1050 x G for 5 min. This procedure was more stringent than that used in other investigations, such as those by Johnson and Snyder (1978) who compared yields of soymilk prepared by various process techniques by centrifuging samples at 642 x G.

Using finely ground soybeans in lieu of wet grinding resulted in yields of soymilk and solids lower than those traditionally obtained. Traditional soymilk processes yield about 62% of the soybean solids in the milk fraction (Lo et al., 1968). We obtained greater yields of dispersed protein and solids by passing the soy flour slurry through the hydroheating valve and then cooking at 99°C. Apparently the shear from steam infusion produced a more stable dispersion/emulsion.

Hand et al. (1967) reported maximum possible yields to be 65% of the soybean solids and 83% of the protein. Wilkens and Hackler (1969) found that 62% of the solids and 76% of the protein of whole soybeans could be recovered

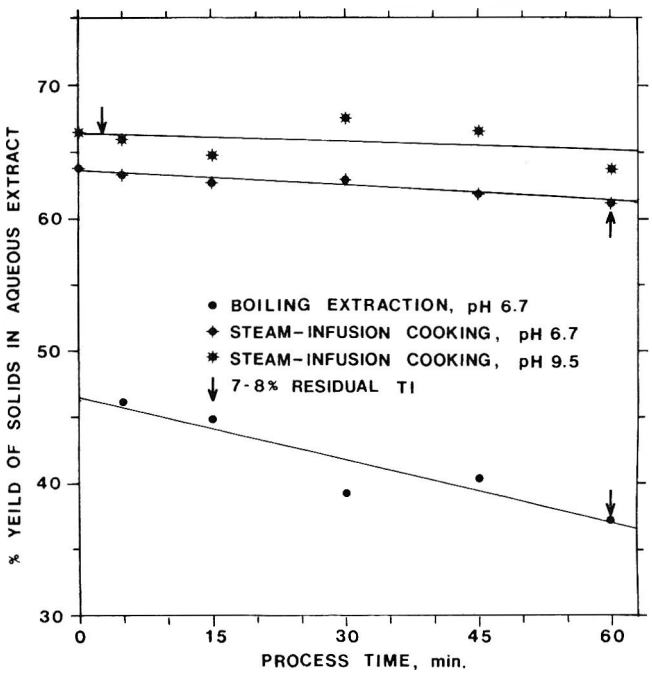


Fig. 2–Effect of cooking method, process time, and pH on yields of solids in milk fractions cooked at 90°C.

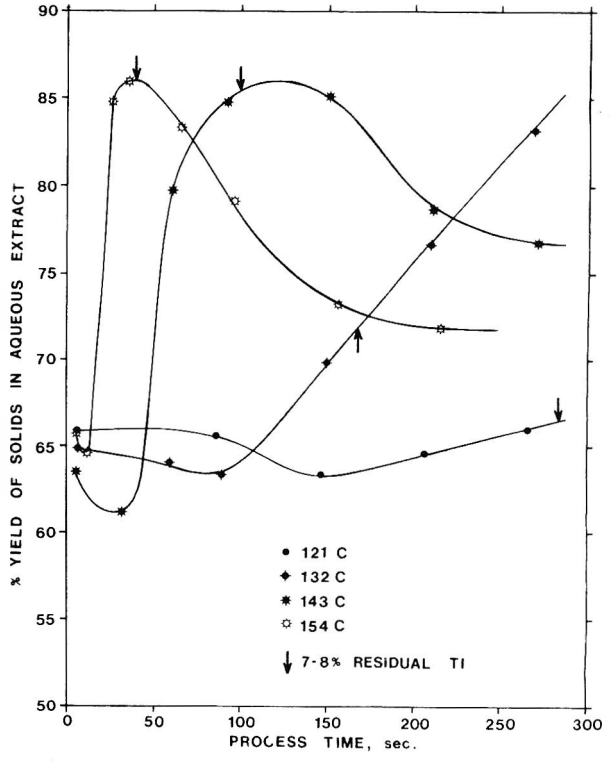


Fig. 3–Yield of solids in milk fraction processed by steam-infusion cooking at pH 6.7.

in nonheat-treated aqueous extracts of soybeans. Johnson and Snyder (1978) reported that the maximum yield of soymilk fraction was 68.7%, solids yield was 62%, and protein yield was 75% by extracting soybeans by wet grinding, homogenizing at 8,000 psi, 75°C, and centrifuging at 642 x G. Yields from steam-infusion processing of soymilk at 99°C are similar to the former observations.

At temperatures 121-154°C, all samples exhibited similar but unique patterns in recovery of soybean solids in the milk fraction: an initial decrease followed by a rapid increase to a maximum yield and then a final decrease. These patterns were consistent; only the length of the trend was temperature-dependent. Maximum solids recovery as soymilk was 86% of the total soybean solids. The maximum yield of the soymilk fraction was 90% and the maximum recovery of protein, 89.5%. No sediment was observed in the soymilk at 5°C for 14 days. In that 8% of the whole soybean is seed coat, that would not be expected to be recovered in the soymilk upon centrifugation, these yield values are quite high despite the high centrifugal force used for separation. Purportedly only by the Illinois Process can greater yields be recovered (89% of solids and 95% of protein). However, in the Illinois Process, there is no filtering or centrifuging of the cooked slurry to reduce yields. When Johnson and Snyder (1978) subjected soymilk prepared by the Illinois Process to centrifugation at 642 x G, they recovered only 49% of the solids in the supernatant fraction.

High yields associated with steam-infusion processing are difficult to explain. Presumably several chemical and physical phenomena are involved that have different reaction kinetics. A certain amount of heat is required to dissociate protein bodies. Heat insolublization of soy protein is not always predictable because partial denaturation (dissassociation of subunits) may increase solubility and emulsification; however, extensive denaturation causes insolublization. Greater yields of stable emulsion are probably the result of optimum heat treatment and extreme shear encountered in steam infusion and flashing.

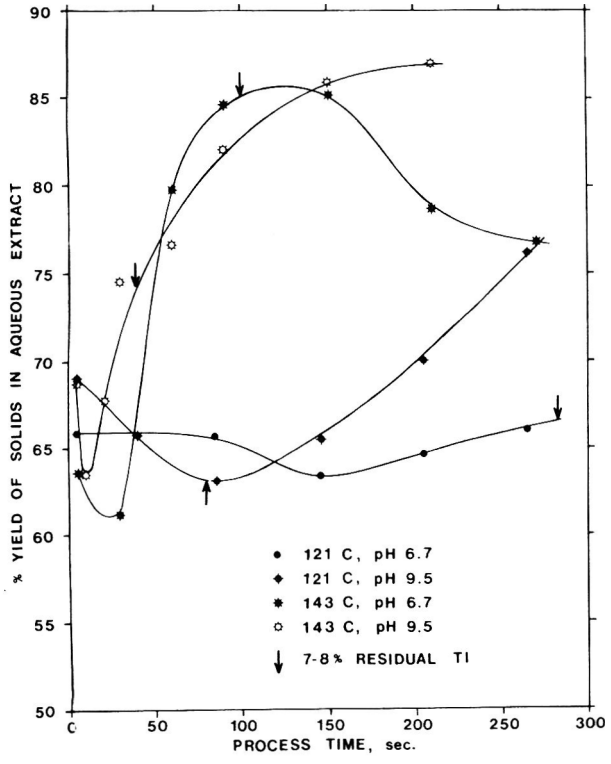


Fig. 4—Solids yield in milk fraction processed at pH 6.7 and 9.5.

While the effect of heat on yields of dispersible solids and protein is interesting, it would be of even more practical value to identify those conditions that achieve maximum solids and protein recovery and also provide for adequate inactivation of TI and maximum nutritional value. All soy products require heat treatment to have high nutritional value. Heat inactivates TI and increases susceptibility of protein to enzyme attack. Hackler et al. (1965), Hackler and Stillings (1967), and Van Buren et al. (1964) have shown that maximum nutritional value of soymilk is attained by heating soymilk for 60-120 min at 93°C and 4-5 min at 121°C. Maximum protein efficiency ratio (PER) is generally observed when more than 90% of native TI activity is inactivated. Whereas a previous report describes kinetics of TI inactivation in steam-infusion cooking (Johnson et al., 1980b), we have indicated (on appropriate figures) points at which 92-93% of original TI activity was inactivated. We discovered that as temperature increases process times at which maximum yields occurred approach the point where adequate TI inactivation also occured. At 154°C these points occurred after the same cooking time (Table 2).

Hackler et al. (1963) reported the PERs of dehulled soybeans, water-insoluble residue, and traditional soymilk as 2.51, 2.71, and 2.11, respectively. Therefore, any increase in soymilk of normally insoluble residue should enhance PER. Van Buren et al. (1964) showed that the extent of chemical browning was a good indicator of heat damage. Optimally, steam-infusion processed soymilk had much less chemical browning than did traditional soymilk. Hackler et al. (1965) have also shown slightly better PER of soymilk optimally processed at 121°C than soymilk optimally processed at 99°C.

CONCLUSIONS

TRADITIONALLY soymilk is heat-treated for approximately 60 min at pH 6.6, 99°C to reduce TI activity by about 92% to improve nutritional value. We evaluated a continuous direct steam-infusion process facilitating aseptic processing with shorter heat exposure times and elevated temperatures. At temperatures above 121°C in steam-infusion cooking, consistent temperature-dependent patterns were observed in yields of soymilk, solids, and protein. An initial decrease was followed by a rise to maximum recovery, then by a final decrease. At 154°C maximum recovery occurred at the same point as did adequate inactivation of TI. We recovered as much as 90% of the slurry, 86% of the solids, and 90% of the protein as soymilk with improved color and low TI activity after processing at 154°C for 40 sec at pH 6.7. Using traditional cooking at 99°C for 60 min to produce equivalent TI inactivation, the soymilk fraction accounted for 72% of the slurry, 61% of the solids and 73% of the protein from whole soybeans.

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Relative Importance of Perceptual Factors to Consumer Acceptance: Linear vs Quadratic Analysis

HOWARD R. MOSKOWITZ

ABSTRACT

This paper introduces a new approach to evaluating the relative importance of sensory characteristics to acceptance, using quadratic relations. The analysis illustrates that the relative importance of any specific characteristic cannot be defined as a single number, but depends upon the level of that characteristic as perceived by the consumer, and upon the other sensory factors of the product.

INTRODUCTION

THIS PAPER PRESENTS an approach to assessing the relative importance of sensory characteristics. Traditionally, researchers have used linear-based statistics (such as correlation and linear regression), to relate liking to different sensory characteristics of foods. However, common-sense and every day observation reveals that liking does not relate in a simple linear fashion to sensory intensity, per se. Rather, as sensory intensity increases, liking increases. However, at an intermediate level (called here the "bliss point") acceptability maximizes. Any further increase in sensory intensity will produce no additional increment in liking. Rather, it may well produce a decrement in liking.

The psychophysics of this relation between liking and physical ingredient level have been well documented in the literature [see Moskowitz (1977) for a full review of the literature on taste and smell vs liking and ingredient level]. Although some researchers continue to investigate individual differences in panelist acceptance of foods vs ingredient levels and find no such intermediate optima for some panelists (e.g., Pangborn, 1970), nonetheless, behaviorally, there probably is an intermediate optimal level on every continum for every individual. The *location* of that optimum may differ by panelists.

Given this parabolic relation between ingredients and liking, there is probably a similar relation between sensory attribute levels and liking. To date, most researchers seem to avoid exploring this relationship, and prefer to use linear-based methods for their analysis of how attributes impact on liking. The Appendix lists representative studies published in the two years 1978-1980 in the *Journal of Food Science* alone, most of which use linear correlations of liking vs sensory intensity ratings or physical measures.

This paper illustrates an alternative approach to relative importance, analyzing the data by mean of curvilinear function. It will use the study by Ennis et al. (1979) as the data base. The study by Ennis et al. is particularly valuable because it was done on a large scale panel of consumers, in a joint industrial R&D setting, with a large number of product variations. Since the authors reported the data, it is a straightforward matter to use these data for the reanalysis reported here. The reader should be aware, however, that the comments and approaches pertaining to that study just as well apply to the literally dozens of similar papers which have used the linear analysis.

Author Moskowitz is affiliated with Developmetrics, Inc., Division of Weston Group, Inc., 60 Wilton Road, Westport, CT 06880 and Taste and Smell Consulting Group, Inc., 320 Davis St., Evanston, IL.

ANOTHER APPROACH TO IMPORTANCE: THEORY

Nature of the acceptance function

Let us consider more closely the issue of sensory correlates with liking. In traditional studies researchers have assessed the relative importance of sensory factors to acceptance by correlating the liking ratings, separately with the separate sensory ratings, to generate a simple correlation matrix. Table 1 presents this simple correlation matrix for the beverage data. It shows that the perception of sweetness correlates 0.73 with liking, the perception of sourness correlates 0.69, and the perception of acceptable appearance correlates 0.50 with liking.

These simple correlations, however, mislead. Let us consider what the correlation actually implies. When the statistician correlates two variables he or she assumes that the two variables are linearly related. On the basis of that linearity assumption, the statistician calculates the Pearson correlation coefficient to assess the degree of relatedness. In actual evaluation of products, where the attributes are sensory characteristics (such as sweetness or sourness) we have no reason to conclude that either sensory characteristic is linearly related to, or even monotonically related to acceptability. A simple analogy will illustrate this point. An individual who likes sugar in his or her coffee will downrate coffee with very little or no sugar. As one adds sugar, the coffee increases in acceptability, until at some point the sugar is 'just right' and the coffee becomes optimally acceptable to that particular individual. Further increases in sugar level only diminish acceptability, with the too-sweet coffee often rejected on the basis of its overly sweet taste. Similarly with sourness and sweetness in orange drinks, or in any other product with such *nonevaluative* sensory characteristics. As the orange beverage (of a single type) becomes increasingly sweet, panelists will find the beverage increasingly acceptable until the beverage reaches the optimum sweetness. Any sweetness higher than that optimum or "bliss point" will diminish acceptability, down from the optimum to a lower level. Hence, the Pearson correlation coefficient relating liking to sweetness will vary. On the ascending portion of the curve, increasing sweetness will show a positive correlation with liking. On the decending portion of the curve, increasing sweetness will show a negative correlation with liking. If, by chance, the researcher has included points around the optimal level, equally sampling the higher and lower values around the optimum, the correlation will dramatically decrease, perhaps close to 0. (See Fig. 1).

In light of this re-statement of correlates of attributes with liking, the food scientist and sensory researcher require an alternative approach to assessing which particular sensory characteristics are most important to consumers. One can do what Ennis et al. did—fractionate the sample of products into relatively homogeneous subgroups of stimuli which lie on one side of the acceptance curve (in terms of sensory attributes), and proceed to apply linear regression and correlation to the ratings, for each subsample at each point of the curve. That approach will generate nongeneral correlates for each "cluster" of products, in terms of how

specific characteristics correlate with overall liking. The shape of the curve, and the location of the clusters on the curve (which relates sensory characteristics and liking), will determine the magnitude of the correlation.

Quadratic acceptance function

One can, however, consider the problem from another point of view. Let us assume that general nonlinear curve exists, relating acceptability to liking. The curve must *allow*, but not force, a peak point in the middle range of the ingredient variable. Owing to the dispersion of the products on that surface, and the fact that the independent predictors are themselves sensory ratings vs. liking, we expect a fairly good, but probably far from perfect fit of that surface to the empirical data. Both the independent and the dependent variables are subject to substantial experimental error, since each is obtained from panel data.

We can describe such a surface in many ways. Let us, however, express it by means of the general second order, or quadratic equation, which comprises linear, square and cross product terms:

$$\begin{aligned}\text{Liking} = {} & k_1(\text{Sweetness}) + k_2(\text{Sweetness})^2 + k_3(\text{Sourness}) \\ & + k_4(\text{Sourness})^2 + k_5(\text{Appearance}) + k_6(\text{Appearance})^2 \\ & + k_7(\text{Sweetness})(\text{Sourness}) + k_8(\text{Sweetness}) \\ & \quad (\text{Appearance}) \\ & + k_9(\text{Sourness})(\text{Appearance}) + k_{10}\end{aligned}$$

We could have chosen more complicated functions, as did Ennis et al. including logarithmic equations, reciprocals, ratios, etc. The second order quadratic equation is the simplest equation. Furthermore, the quadratic form is most tractable for other uses, such as optimization. The quadratic equation takes into account linear and nonlinear relation of attributes vs liking, as well as taking into account simple pairwise interactions of attributes as determinants of liking.

Generally, the quadratic equation will describe the data reasonably well and parsimoniously. One can determine how well this liking equation fits the Ennis data, as shown in Table 2. As a first approximation the equation fits fairly well, with a multiple correlation of 0.93 (with all terms = 86% of the variability accounted for). Using only sourness and sweetness yields a multiple R = 0.87 (76% of the variability accounted for). In light of the statistical significance of these values, we can accept the equations as *reasonable* first approximation of the results. *Other equations might fit better, but would be less parsimonious.* Table 2 compares three specific equations for the orange beverage data, showing how the multiple correlation varies, with increasing number of terms.

- Simple linear equation (no square, nor interaction terms)
- Complete quadratic equation, including terms for appearance
- Complete quadratic equation, without appearance terms

RELATIVE IMPORTANCE AS THE DERIVATIVE OF THE LIKING EQUATION: CALCULATIVE RELATIVE IMPORTANCE OF ATTRIBUTES BY USING SLOPES

ONE CAN CALCULATE the relative importance of attributes by using a linear equation to indicate how rapidly overall liking changes with unit changes in the independent variables. For the orange beverage data, the linear equation has three coefficients (one each for sweetness, sourness and appearance, equalling 0.68, 0.16, and −0.47, respectively). The rank order of the absolute values of the slopes indicate the relative importance of the characteristics (assuming that the units of measurement of the three independent variables are all equal, which they are, since the same scale was used for each). One could also use the simple correlation coefficient between liking and sensory characteristics. This would rank order the attributes as: (1) sweetness (r = 0.73), (2) sourness (r = −.69), and (3) appearance (r = 0.50).

On the other hand, as we saw, the linear equations do not adequately represent how liking varies with sweetness, sourness, and appearance. Rather, liking increases, perhaps peaks at a middle level, and then diminishes with further increases in the sensory characteristics. Table 2 shows that by describing liking as a quadratic equation and optimizing that equation there are intermediate levels in attributes which generate optimal liking. Therefore, the linear equation does not apply, and the slopes or coefficients are not euqal all along the continuum.

An approach using partial derivatives of the liking equation

The partial derivative of an equation indicates the momentary rate of change of the function with respect to that particular variable (all other variables held constant). For linear equations, the partial derivative of the equation equals the slope of the equation. Thus, for liking as linearly related to sweetness by the equation:

Table 1—Intercorrelation matrix of attributes

	Liking	Sweetness	Sourness	Appearance
Liking	1.00			
Sweetness	0.73	1.00		
Sourness	−0.69	−0.95	1.00	
Liking of Appearance	0.50	0.85	−0.75	1.00

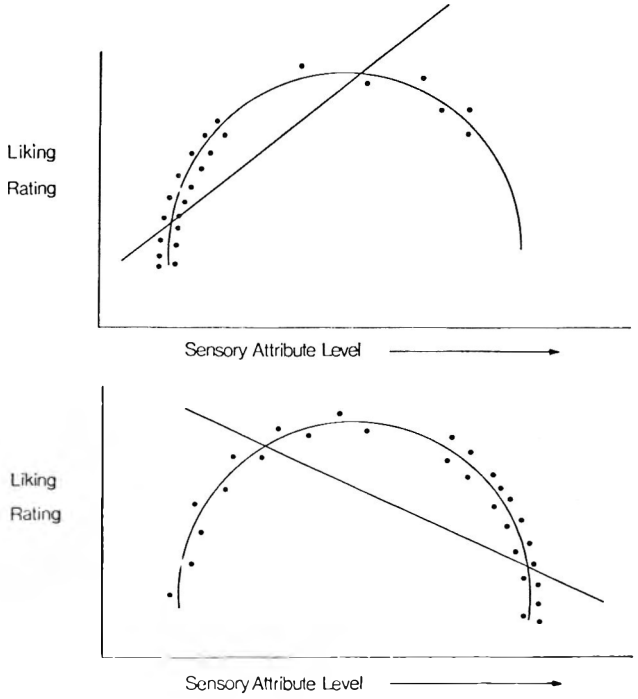


Fig. 1—Shows schematically how a parabolic or inverted U shaped curve for liking vs sensory level can generate two different appearing equations, depending upon the location of the points on that curve. The equation can be expressed by the equation: Acceptability = 0.50 + 2.25(S) − 0.15(S)² (S = Sensory Attribute Level). A line through the data has a correlation of −0.25. A line through the lower values of S has a positive correlation of 0.84. A line through the upper values of S has a negative correlation of −0.87.

Table 2—Comparison of linear and nonlinear equations relating liking of orange beverages to sensory attributes[a]

Liking =	Linear	Quadratic (with appearance)	Quadratic (without appearance)
k_o	3.85	1.54	−17.18
$+k_1$(Sweetness)	0.68 (0.73)	−3.21 (0.73)	6.03 (0.73)
$+k_2$(Sweetness)^2	–	−0.87 (0.67)	−0.29 (−0.67)
$+k_3$(Sourness)	0.16 (−0.69)	−0.75 (−0.69)	8.97 (−0.69)
$+k_4$(Sourness)^2	–	−0.55 (−0.69)	−0.70 (−0.74)
$+k_5$(Appearance L/D)	−0.47 (0.50)	4.47 (0.50)	–
$+k_6$(Appearance L/D)^2	–	−2.16 (0.50)	–
$+k_7$(Sweet)(Sour)	–	−1.08 (0.34)	−0.73 (0.34)
$+k_8$(Sweetness)(Appearance L/D)	–	2.79 (0.67)	–
$+k_9$(Sour)(Appearance L/D)	–	1.77 (−0.64)	–
Multiple R	0.77	0.93	0.87
Optimal Level			
Sweetness	Cannot determine	1.50 (lowest point)	6.44*
Sourness	Cannot determine	3.20*	3.02*
Appearance (Liking)	Cannot determine	3.32*	–
Highest Liking Realized By Optimum		5.37	5.98

[a] Numbers in parentheses = Partial correlations
*True Intermeidate Optimum

$$\text{Liking} = k_0 + k_1 \text{ (Sweetness)}$$

the partial derivative of liking with respect to sweetness equals k_1, the slope. (In fact, the partial derivative is identical to the derivative of the equation for a univariate function.) *The partial derivative indicates the expected rate of change in liking at that point, for a unit change in sensory level (or ingredient level).* In turn, if a function has a large slope, then unit changes in the independent variable generate larger changes in liking than would occur if the function has a small slope. We can assume, therefore, that the important attributes are the ones with higher slopes-viz., those attributes whose changes entail larger changes in liking.

For simple, linear functions comprising one or more independent variables, the partial derivative of the equation equals the component slopes. In the orange beverage data, where one has different sensory attributes as predictors, the general form becomes:

$$\text{Liking} = k_0 + k_1 \text{ (Atrribute 1, e.g., sweetness)} + k_2 \text{ (Attribute 2, e.g., tartness), etc.}$$

The partial derivatives, in fact, equal the coefficients. The coefficients thus provide the properties of the relative importance of the characteristic assuming that each sensory attribute is measured with the same scale. (For instance when the magnitudes of the sensory attributes are not commensurate—viz., where the experimenter uses an entirely different scale to rate sweetness vs rating sourness, the sensory analyst should equate the scales by standardization, or other equivalent procedures.)

Liking is not simply a linear function of sensory characteristics. As psychophysicists have shown over the past fifty years (Moskowitz, 1977; Beebe-Center, 1932), overall liking first increases with sensory intensity of a single attribute, peaks at an intermediate bliss point, and then with further increases in that sensory attribute diminishes, first towards neutrality and then into the negative zone of disliking.

The quadratic equation possesses partial derivatives which do not remain constant. Rather, a partial derivative of the simple quadratic equation varies as a function of predictor level. Specifically, if one expresses overall liking/disliking as a quadratic equation of two variables (A, B),

$$\text{Liking} = k_0 + k_1 A + k_2 A^2 + k_3 B + k_4 B^2 + k_5 AB$$

then the partial derivative with respect to A ($\partial/\partial A$) is written as:

$$k_1 + 2k_2 A + k_5 B$$

Note that the partial derivative, or the measure of importance of the attribute:

- Varies as a function of the level of A, and thus does not remain constant.
- Takes into account the existence and levels of other sensory variables, such as B, because variables interact with each other.

Following this logic, one can calculate the relative importance of the different sensory characteristics, by calculating the absolute size of their partial derivatives. The slope of the partial derivative varies according to the sensory level. The slope changes signs, depending upon whether at that point increments in the sensory values show increasing liking or decreasing liking.

Table 3 shows the partial derivatives using only sweet and sour as the predictors, as well as some estimated relative importance values at different points on the curve. The points are 2, 4 and 6 on the sensory scale. (We choose not to use the hedonic attribute of liking of appearance, because sweetness and sourness are sensory attributes, whereas liking is a hedonic attribute.)

DISCUSSION

THIS DEMONSTRATION of nonlinear equations of sensory characteristics vs liking, and the use of the partial derivatives as mesures of relative importance, brings up the issue of the validity of other, linear-based approaches, as well as the ability of panelists to directly estimate relative importance in sensory evaluation terms.

Linear-based approaches

If, in fact, sensory ratings of liking (or purchase interest) conform to an inverted U shaped function for any specific attribute, or to an inverted bowl-like surface for combinations of two attributes, then this calls into question analyses which assume the relative importance of characteristics to be linearly related to overall acceptability. When

Table 3–Partial derivatives of liking vs sweet and sour

$$\frac{\partial (\text{Liking})}{\partial (\text{Sweet})} = 6.03 - 0.58\ (\text{Sweet}) - 0.73\ (\text{Sour})$$

$$\frac{\partial (\text{Liking})}{\partial (\text{Sour})} = 8.97 - 0.73\ (\text{Sweet}) - 1.40\ (\text{Sour})$$

	ESTIMATED RELATIVE IMPORTANCE VALUE								
	Sensory level of the attributes								
Sweet =	2	3	3	4	4	4	6	6	6
Sour =	2	4	6	2	4	6	2	4	6
	Relative Importance of the attributes (viz Partial derivatives)								
Sweet	3.41	1.95	0.49	2.25	0.79	−0.67	1.09	−.37	−1.83
Sour	4.71	1.91	−0.89	3.25	0.45	−2.35	1.79	−1.01	−3.81

the researcher correlates attributes either singly or by means of multiple linear regression, to overall acceptability, the researcher has implicitly assumed that the relative importance remains constant throughout the entire range. Furthermore, those characteristics which show optimal or bliss points at unreachably high levels (e.g., naturalness; for beverage) will most highly correlate with liking, and be thus deemed most important. In fact, these highly correlated attributes may be really inactionable characteristics, and may be restatements of liking (Moskowitz, et al., 1980). In contrast, sweetness, tartness, and other sensory, and nonevaluative characteristics which show lower simple correlations singly or in concert with liking, may really be the key, actionable characteristics. They score lower on the correlation analysis and in terms of multiple linear regression because they show intermediate peaks or bliss point optima. Such inverted U shaped curves for level of sensory characteristics vs overall acceptability will not fare well if the ingoing hypothesis is that acceptability and sensory characteristics are linearly related.

If the foregoing hypothesis relating the actual nature of the liking function is shown to be valid, then this hypothesis calls into question the analysis of relative importance vs sensory intensity. It may well turn out that relative importance as a fixed value must be abandoned. Consumers may not assign specific weights to different sensory inputs as determinants of quality. The researcher assumes that they do, searches for predictors, and forces a linear equation onto an intrinsically quadratic (or otherwise nonlinear) system.

Direct estimates of importance by panelists

How should one interpret direct estimates of importance by panelists. Had the panelists in the orange beverage study directly estimated the relative importance of the characteristics, would they have agreed with estimated importance derived from correlations? Paradoxically, the ability of panelists to rate relative importance using a single number contradicts the hypothesis that relative importance continuously changes as a function of the level of the sensory characteristic, and as a function of the distance of the sensory characteristic from the bliss point level. If panelists do, however, estimate the relative importance of characteristics to overall acceptability, and if they can generate a data base of sensory attributes vs acceptance, then one ought to be able to estimate the sensory profile of characteristics which correspond to the relative importance ratings. To the knowledge of this author, that has not yet appeared in the published literature, but it provides an intriguing possibility. In theory, at the bliss point the relative importances of all attributes should be equal, and if the bliss point truly represents an intermediate optimum, where the curve reaches its highest point. If so, then non-equal relative importance values suggest that the panelist is basing his or her judgment upon a linear equation, or upon another sensory profile, not necessarily the most acceptable one. If so, then what is that sensory profile towards which the panelist targets?

CONCLUSIONS

THIS PAPER has presented a different analysis of relative importance data, taking as a point of departure previously published data by Ennis et al. (1979). It suggests that behaviorally, one can construct a nonlinear (quadratic) function relating sensory characteristics and overall liking.

Furthermore, with this quadratic function, relative importance is defined as the momentary rate of change of overall liking with respect to each separate sensory characteristic. In terms of the differential calculus, this becomes the partial derivative of the liking function at the specific sensory profile level.

APPENDIX

List of studies using linear as well as nonlinear (quadratic) models attributes or acceptance and physical measures

Independent variable	Statistics	Source
Flavor, juiciness, adhesion, tenderness, cook yield vs palability of steakettes	Correlation	Ford et al. (1978)
Cooking time, shear, flavor, juiciness, tenderness vs palatibility of meat	Correlation	Moody et al. (1978)
Size, shape, color intensity, firmness, heat, flavor vs acceptability of Jalapeno pepper cultivars	Correlation	Weisenfelder et al. (1978)
Mouthfeel, gumminess vs preference for ice cream	Regression and Correlation	Pearson & Ennis (1979)
Shape, external color, crispiness, placenta size vs	Regression equation Correlation	Ennis & O'Sullivan (1979)

Appendix continued

Independent variable	Statistics	Source
overall quality of cucumbers		
Freezer burn flavor vs acceptability	Correlation	Golkamp et al. (1979)
Wine flavor attributes vs quality	Correlation, Factor analysis	Kwan & Kowalski (1980)
Flavor vs storage time (canned foods)	Quadratic Regression and partil derivative analysis to determine rate of change flavor with time	Hayakawa et al. (1978)
GLC components vs flavor acceptance (desirability) of tomatoes	Regression	Watada & Aulenbach (1979)
Ingredients vs acceptance (sausage from mullet)	Quadratic regression (Response Surface)	Daley et al. (1978)
Kinematic viscosity vs quality of okra	Correlation	Uzo & Oziako (1980)
Objective measures of sardine quality vs attribute acceptance	Correlation	Slabyj & True (1978)
Objective measures of green beans vs acceptability and other attributes	Correlation	Godwin et al. (1978)
Storage time vs resulting physical changes and acceptability of pork patties	Correlation Linear regression	Campbell and Mondogo (1978)
Concord grape pigment (objective measures) vs color acceptability	Correlation	Clydesdale et al. (1978)

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Mushroom Response to Postharvest Hyperbaric Storage

HENRY A. ROBITAILLE and ARTHUR F. BADENHOP

ABSTRACT

Vessels suitable for storing mushrooms [*Agaricus bisporus* (Lange) Singe] at absolute pressures to 35 atm were constructed from 43 liter (1A) gas cylinders and plumbed for conducting static or continuous-flow experiments. Neither pressurization nor gradual depressurization over 6 hr injured mushrooms. Pressure did not affect respiration, but significantly reduced moisture loss during storage. Mushrooms depleted O_2 to very low levels and showed high tolerance to CO_2. Pressure was used to safely increase CO partial pressure (p) 35-fold. At 0.035p, CO reduced mushroom browning but not respiration. A completely autonomous storage system with CO_2 removal and automatic O_2 replenishment was developed.

INTRODUCTION

MARINE DEPTHS constitute the only natural habitat where plants are exposed to pressures significantly greater than one atmosphere. Reduced light availability limits these pressures to several atmospheres (atm) for photoautotrophic plants. The few studies of hyperbaric effects on plants have been reviewed by Vidaver (1972). Even fewer studies on hyperbaric storage of detached plant tissues and organs are available. Barker (1960) decreased the CO_2 output of potatoes and apple by increasing the absolute pressure to 4 atm. He attributed this result to mechanical tissue distortion. Stoll (1975), after observing that CO_2 scrubbers in apple controlled environment storage rooms operated 20% less during periods of high barometric pressures, designed experiments to maintain apples at: (a) constant hypobaric pressure at the low end of the normal fluctuation range (−30 mm H_2O); (b) constant hyperbaric pressure at the high end of the normal fluctuation range (+30 mm H_2O); and (c) normal fluctuating barometric pressures. Although fruit quality was slightly improved under hypobaric conditions, constant hyperbaric conditions resulted in the best quality, highest taste panel scores, least scalding and yellowing, and significantly lower storage ethylene levels (although storage ethylene levels were slightly higher than under hypobaric conditions). These significant hyperbaric effects were ascribed to the maintenance of a constant lower level of O_2 inside the fruit.

Direct pressure effects on metabolism and growth of microorganisms have been reported only under very high pressure conditions. For example, pressures above 240 atm substantially extended the refrigerated storage life of highly perishable biological material in a nonfrozen state by inhibiting microbial growth probably by slowing a critical enzyme reaction (Charm et al., 1976). A pressure of 272 atm at 296°K reduced the reaction rate of peroxidase as much as did a temperature drop of 7.2°C. In another study (Schmid et al., 1975), yeast glyceraldehyde-3-phosphate dehydrogenase was reversibly deactivated, but only at 500 atm pressure. Rabbit muscle lactic dehydrogenase was unaffected at that pressure. Total deactivation of both enzymes occurred only at 2000 atm.

There is considerable interest in the use of CO in storage to reduce both decay incidence and severity (Kader et al., 1978), and tissue respiration (Kader et al., 1973). Because the activity of a gas is determined by its partial pressure (p) and not by its concentration, increasing pressure in storage would allow effective use of toxic gas at safer concentrations than would be otherwise possible.

Objectives of this study were to determine pressure effects on moisture loss, respiration rate, and keeping quality of mushrooms, and to demonstrate the use of pressure to boost safe levels of CO to effective partial pressures.

EXPERIMENTAL

STANDARD 1A (43 liter) gas cylinders were cut off 30 cm from the valve end, and the cut ends were flanged and machined to accommodate a butyl rubber O-ring (Fig. 1A). When the two flanges were bolted tightly together, a gas tight seal was effected at absolute pressures to 35 atm. One chamber was further modified by cutting off the cylinder 6 cm from the valve end to allow addition of a polycarbonate (Lexan) window so storage trials could be monitored visually (Fig. 1B). The 7.5 cm diam x 2.5 cm thick window was sealed with an O-ring, and fastened with a hold-down ring and bolts. Leaks were detected by hydrostatic testing at 70 atm. Four storage cylinders were connected through a valved manifold, to allow for independent or multiple pressurization (Fig. 1C). Cylinders were equipped to hold one large sample up to 40 liters, or two small samples (replications) up to 1/2 liter each (Fig. 2A). In the latter case, widemouth canning jars equipped with independent outlets were pressurized together with the void cylinder volume. Inlet lines extended to the bottoms of the jars, while the entrances to the outlet lines were among the mushrooms in the center of the jars. Outlet lines were connected to Nupro fine-metering valves for flow rate adjustment accurate to 1 ml/min. These valves were connected in line to the sampling loop of a Carle Instruments 8700-804B gas chromatograph for measurement of O_2, CO_2, and CO. Flow rates were measured with a bubble tube and stop watch at the sampling loop exit. In static experiments it was necessary to bleed the entire outlet line before each reading. To minimize gas volume loss, internal (pressurized) and external outlet lines were small diam; 0.159 cm (1/16 in.) i.d. tygon, and 0.0584 cm (0.023 in.) i.d. stainless steel tubing, respectively. Total outlet line volume was 3.24 ml at 1 atm.

Oxygen and N_2 were mixed in an empty 1A cylinder to give the desired pO_2 at storage pressure. A supply tank pressure more than twice the final desired storage pressure was required to provide sufficient gas to flush and then pressurize the storage chamber. Oxygen was added using a mercury manometer (± 0.5 mm), and the final pressure was attained with nitrogen. When CO was included, the supply cylinder was evacuated and the calculated amount of CO was drawn into the tank. Remaining vacuum was released in a N_2 environment before O_2 and N_2 were added as described above. Carbon monoxide was generated by dehydrating formic acid with sulfuric acid, and collecting the gas by water displacement in an inverted graduated cylinder. Supply mixtures were checked by gas chromatography and allowed to equilibrate to the experimental temperature before use. All exhaust gas in CO experiments was passed through a cuprous chloride-hydrochloric acid mixture.

Turnover in continuous-flow experiments was at the rate of 10 liters kg^{-1} of mushrooms atm^{-1} of pressure hr^{-1}. Both high pressure and control gas mixtures were adjusted to give equivalent pO_2 during storage. To reduce N_2 waste, static experiments were

Author Robitaille is with the Dept. of Horticulture, Purdue Univ., West Lafayette, IN 47907. Author Badenhop, formerly with Purdue Univ., is now with the Dept. of Food Science & Technology, Oregon State Univ., Corvallis, OR 97331.

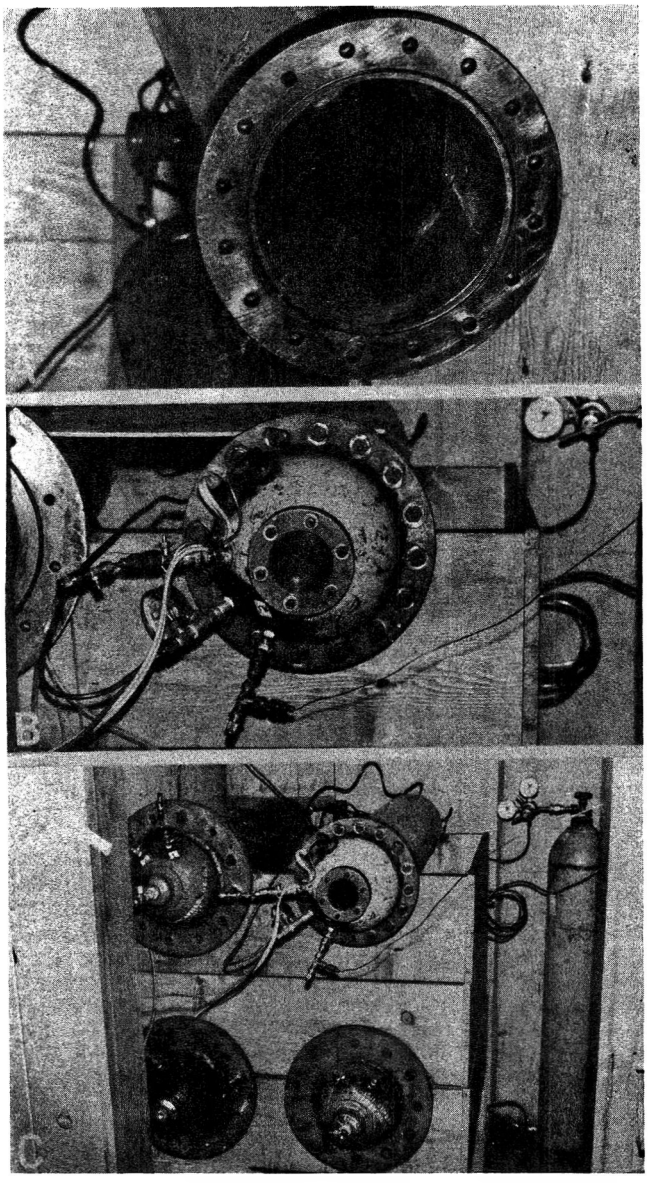


Fig. 1—High pressure storage vessels constructed from empty 1A gas cylinders: (A) Flange welded to the cylinder showing the butyl rubber O-ring for gas-tight sealing. An identical flange (minus the O-ring) was welded to the cap; (B) Cap bolted to the cylinder, showing the polycarbonate (Lexan) window and gas-flow connections including the fine metering valves; (C) Four cylinders arranged for simultaneous or independent pressurization.

used to measure rates of O_2 utilization so that O_2 alone could be supplied. Nitrogen use was further reduced by filling the void volume of the storage chamber with water-filled steel cans. Partial pressures were calculated as follows: p = μliters liter^{-1} x 10^{-6} x atm.

Later experiments were conducted using an O_2 analyzer (Series 326B O_2 Analyzer and Micro-fuel Cell Class C-3, Teledyne Analytical Instruments), which automatically maintained pre-set pO_2 by controlling a solenoid valve in line to an O_2 cylinder (Fig. 2B). No gas mixing was necessary. Pressurization was done with N_2; the analyzer then supplied O_2 to the selected partial pressure. Because the O_2 sensor deteriorated rapidly in the pressure of high CO_2, and because it was desirable to mix rapidly the incoming O_2, a fan was installed inside the cylinder to maintain a circulation pattern through the mushrooms and over a series of shelves moistened with 10% (w/v) KOH. This effectively removed all detectable CO_2 from the system.

Pressurization rate was not critical for mushrooms. Rapid pressurization, however, did crack tomatoes, a fruit in which gas exchange is restricted to the scar. Depressurization was much more critical, especially after long storage periods when tissues were saturated with gas. Six hour linear depressurization through a pressure regulator, was adequate for mushrooms.

Mushrooms were ideal for these experiments because of their porosity, small size, ready availability from commercial outlets at any time, high respiration rate, high water loss rate and absence of response to, or production of, ethylene. Experiments were conducted in insulated rooms with precise temperature control. To speed results, experiments were conducted at 20°C unless stated otherwise. Gas pressures, measured with mechanical guages, were accurate to ± 5%. Storage pressures were limited to 35 atm absolute.

RESULTS

Continuous flow experiments

When comparing mushrooms stored for 4 days at equivalent temperature, pO_2, and flow rate, those at 35 atm evolved CO_2 at the same rate but lost significantly less total weight than did those at 1 atm (Table 1, Fig. 3). To examine the interaction of temperature with pressure on CO_2 release, mushrooms were stored at 20°C for 1 day and then lowered to 5°C for the remainder of the experiment. Again, there was no pressure effect on CO_2 evolution, but after 13 days (12 at 5°C) pressurized mushrooms again had lost significantly less moisture (Table 2).

Static experiments

Rate curves for O_2 depletion and CO_2 release by mushrooms at 20°C and 35 atm showed respiration to be linear to 0.005 pO_2 (Fig. 4). It was previously noted that reduced O_2 levels had little or no value in modified storage atmospheres for mushrooms (Morris and Kader, 1977). Decarboxylation continued at the same rate after O_2 depletion as when mushrooms were stored in a N_2 atmosphere. In agreement with results of continuous flow experiments, analysis of variance indicated no difference between slopes of O_2 depletion curves at 20°C, 0.20 pO_2, and either 35 (slope = 0.91) or 17.5 (slope = 0.78) atm pressure. The CO_2 curve in Figure 4 did not start at zero due to respiration occurring during pressurization. When pressurization to 35 atm was rapid (5 min) with continuous gas flow to time zero, CO_2 accumulation did start precisely at zero. The lack of any lag in CO_2 accumulation suggested that there was no detectable dissolving of CO_2 within the tissue during pressurization. Rapid pressurization caused no visible tissue injury or change in respiration rate.

Mushroom respiration was not affected by 0.035 pCO (Fig. 5). However, when used at the same partial pressure in longer term, continuous flow experiments, CO did result in observable reductions in browning.

O_2 replenishment experiments

The rate of O_2 depletion by mushrooms at 20°C, the slope of the regression line in Figure 5, was used to calculate the correct flow rate to maintain 0.05 pO_2 in storage from a supply tank of 10% O_2 in N_2. Oxygen was maintained close to 0.05p for 120 hr, until pCO reached 1.60 (Fig. 6). At that point aerobic respiration apparently ceased. The decrease in CO_2 and increase in O_2 levels after 160 hr were due to the continuing influx of O_2-rich supply gas. Control mushrooms in this experiment were held as purchased in pressed paper containers sealed with Saran Wrap adjacent to the pressure storage cylinders. At 210 hr, pO_2 and pCO_2 within the control packs were approximately 0.0004 and 0.08, respectively. At this time outlet and inlet valves to the storage cylinder were closed and both pressurized and control mushrooms left an additional 183 hr. At 393 hr total fresh weight loss was 9 and 44% for pressurized and control mushrooms, respectively. Adult and larval flys were numerous in control packs, but were inhibited by pressure or by the high pO_2 and pCO_2 in the pressure cylinders. Larval flies were visible on pressure-

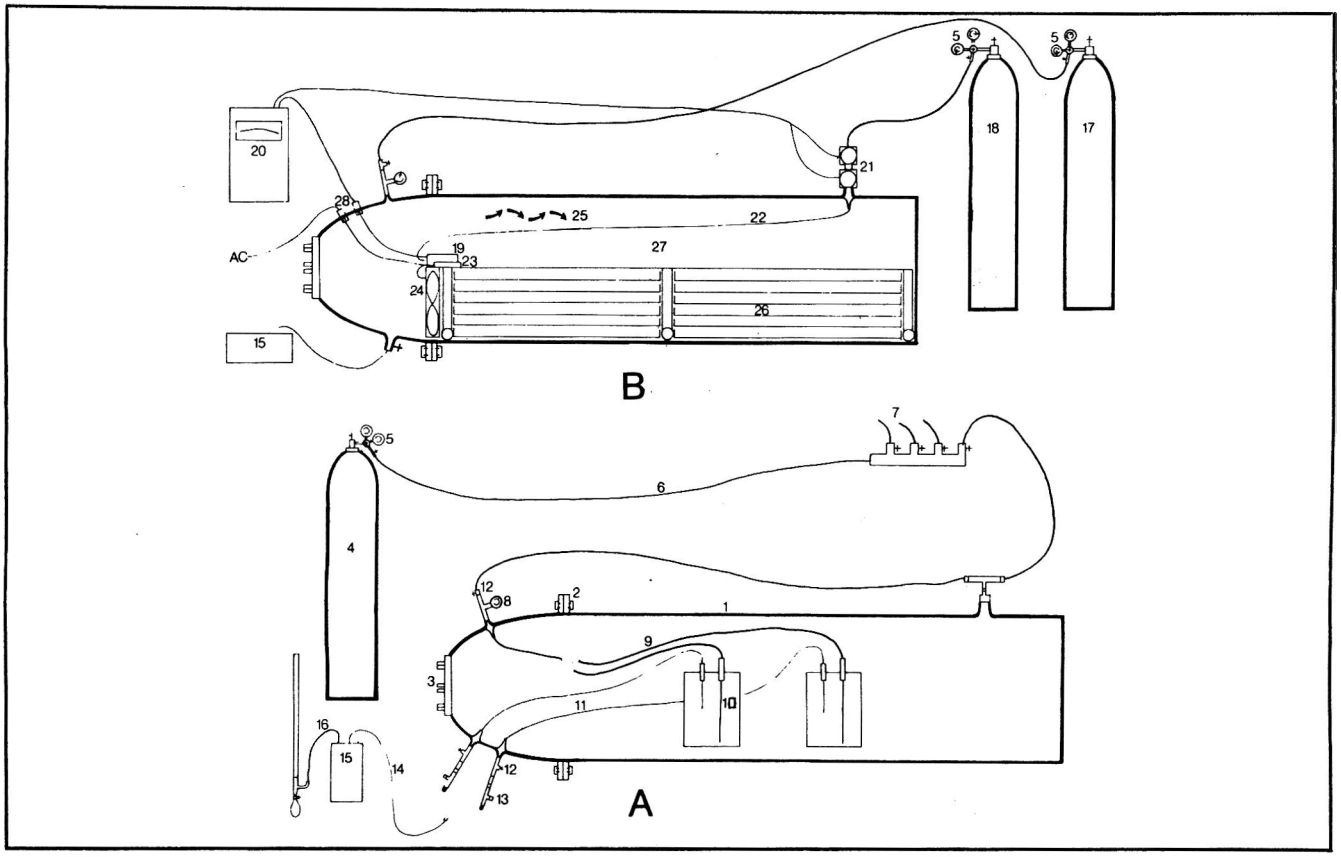


Fig. 2—Diagram of the high pressure laboratory model (A) as used for continuous flow and static experiments, and (B) as modified for O_2 replenishment only. Components include: (1) standard 1A gas cylinders, cut off near the top to make high pressure storage vessels; (2) bolted flanges with O-ring seal between them; (3) Lexan window; (4) gas supply: N_2 pre-mixed with the correct amount of O_2 to give the desired pO_2 at final storage pressure; (5) high pressure regulators (Matheson model 3); (6) external gas inlet lines (0.635 cm o.d. Cu tubing); (7) manifold to allow for independent or simultaneous pressurization of the 4 storage vessels; (8) gauge for vessel internal pressure reading; (9) internal gas inlet lines (0.635 cm o.d. tygon tubing); (10) wide-mouth canning jars (0.5 liter) for storing mushrooms; (11) internal gas outlet lines (0.159 cm i.d. tygon tubing); (12) needle valves for shut-off, and for gross flow rate adjustment; (13) Nupro fine metering valves for accurate flow adjustments at storage gas exit ports; (14) gas chromatograph (GC) sample loop inlet, 0.0584 cm i.d. stainless steel tubing with Swagelock connection for attachment to either Nupro valve; (15) GC with sampling loop (Carle model 8700, 804-B) and recorder; (16) GC sample loop exit connected to a bubble tube for monitoring gas flow rates; (17) N_2; (18) O_2; (19)micro-fuel cell (O_2 sensor) class C-1; (20) Series 326 B Oxygen Analyzer, both from Teledyne Analytical Instruments; (21) high pressure solenoid valves (second valve for back-up only); (22) internal O_2 inlet line, 0.0584 cm i.d. stainless steel tubing, terminating in front of the O_2 sensor; (23) A.C. receptacle. (24) circulation fan; (25) direction of atmospheric flow; (26) CO_2 scrubber (stainless steel shelves lined with absorbant paper saturated with 10% (w/v) KOH); (27) mushroom storage compartment (approx. 20 liter capacity); and (28) bulkhead fittings, one for 120v A.C., and another for the O_2 sensor connection. Internal wires unplug for vessel cap removal.

stored mushrooms 1 wk following depressurization.

Further experiments were conducted with the automatic O_2 analyzer and CO_2 scrubbing system at 20° and 0.21 pO_2. Controls were held in open, shallow, enamel pans adjacent to the pressure cylinders. The percent of initial water and dry weight lost after 168 hr of storage is shown in Table 3. Open storage resulted in rapid, almost complete dehydration, with little respiration possible and, therefore, little dry weight loss. Mushrooms stored in cylinders at 1 and 35 atm lost equal amounts of dry weight, confirming earlier results indicating no pressure effects on respiration. Again, pressure significantly reduced moisture loss.

DISCUSSION

AN ABSOLUTE PRESSURE of 35 atm had no effect on respiration as measured by CO_2 evolution or O_2 uptake. This was expected since effects on metabolism have occurred only at pressures approximately one order of magnitude higher (Charm et al., 1976; Schmid et al., 1975). However, experiments did indicate a significant effect of 34 atm on moisture loss. This effect occurs for the same reason that low pressure enhances diffusivity out of tissues. For a detailed treatment of the latter phenomenon see Burg (1975). Deterioration in storage, especially of porous tissues like mushrooms, leafy vegetables, or cut flowers, results from both respiratory and water losses. Temperature and atmosphere control can significantly reduce the former but not the latter. Low pressure improves the storage quality of some crops by reducing pO_2 and enhancing dif-

Table 1—Percent total weight loss of mushrooms, and pCO_2 in the effluent gas, after 4 days storage at 20° C and 1 and 35 atm[a]

Absolute pressure (atm)	pCO_2 (atm)	Weight loss (%)
1	0.019	8.75 ± 0.25[b]
35	0.020	3.75 ± 0.25

[a] A mixture of N_2 plus the calculated % of O_2 to give the desired pO_2 at final storage pressure was used for initial flush, pressurization, and continuous flow at a rate of 10 liters kg^{-1} hr^{-1} atm^{-1}. Pressurization was linear over 2.5 hr.
[b] Standard errors of the mean

fusive removal of tissue ethylene (Burg, 1975), but high cost, the need for humidification, and accelerated losses of volatile flavor components are disadvantages. Under high pressure, atmosphere and temperature can be controlled and moisture loss reduced with the product surface remaining dry. This might be advantageous for disease control.

Reduction of mushroom browning by CO was previously noted by Besser and Kramer (1972). With other crops, pCO in the range 0.01-0.20 reduced decay incidence and severity (Woodruff, 1977; Kader et al., 1977; 1978), reduced respiration rates (Kader et al., 1973), and, in a mixture with a lowered pO_2 and p ethylene, inhibited enzymes and microorganisms in a wide range of foods during packing operations (Dunkley, 1948) Mammalian CO toxicity is concentration dependent, 0.1 and 1% saturating 60 and 90% respectively of the hemoglobin in 1-4 hr depending on rates of respiration and circulation (Sayers and Davenport, 1930). Although mushroom respiration rates were unaffected by 0.035 pCO, we did demonstrate the feasibility of using pressure to boost the pCO of our mixture from 0.001 to 0.035 within the safe confines of the storage cylinder. Upon depressurization, pCO returns to 0.001. Still higher partial pressures of CO or any other biologically active gas could be easily and safely achieved by further increasing total pressure. Of course the absence of dissolved gas or possible reaction products following depressurization would have to be demonstrated.

No production of or response to ethylene was noted. With some crops, however, tissue ethylene accumulation is expected to be a problem since diffusivity is inversely related to absolute pressure (Burg, 1975). Another difficulty with hyperbaric systems is to pressurize and, especially, to depressurize without tissue injury. In tomatoes, a fruit where gas exchange occurs primarily at the scar, cuticular cracking with subsequent fungus growth occurred even when pressurization to 35 atm was accomplished linearly over a 4-hr period. Decompression injury was not a problem with mushrooms, if decompression was linear (through a pressure regulator) over a 6-hr period. However, decompression tables may have to be developed for each individual product.

The major obstacles to pressurized systems are structural. At 35 atm there is a force of 1,603 kg on the 7.5 cm diameter polycarbonate window on the front of the storage cylinder. Although there may be applications for small, pressurized, storage vessels especially for high cash commodities, the most promising application for pressurized storage appears to be underwater. Mitsuda et al. (1972) stored rice, wheat, and soybeans at a depth of 30m

Table 2–Percent moisture loss of mushrooms, and pCO_2 in the effluent gas of a continuous-flow experiment[a]

Temp (°C)	Absolute pressure (atm)	pCO_2 (atm)	Moisture loss (%)
20	1	0.0174[b]a	–
	35	0.0173 a	–
5	1	0.0034 b	5.74 a
	35	0.0039 b	3.54 b

[a] Twenty and 5°C data collection, and the temperature change, were made at 26, 126 and 32 hr, respectively, and moisture loss was determined after terminating the experiment at 13 days. Zero time occurred when experimental pressures were attained, after a 2.5 hr linear pressurization. A mixture of N_2 plus the correct % of O_2 to give a final pO_2 of 0.20 at final storage pressure, was used for initial flush, pressurization, and continuous flow at a rate of 10 liters kg^{-1} hr^{-1} atm^{-1}.
[b] Mean separation by Duncan's multiple range test, 5% level

Table 3–Percent of the initial water and dry wt lost in open or closed (high pressure) storage after 168 hr at 20°C and 0.21 pO_2[a]

Storage	Total pressure (atm)	% of initial wt lost in storage H_2O	Dry wt
Open	1	99.0	1.0
Experimental vessel	1	61.5	35.6
Open	1	98.5	0
Experimental vessel	35	49.2	35.0

[a] Closed storage was in sealed cylinders at 1 or 35 atm (pressurized with dry N_2), with a fan for circulating the internal atmosphere over KOH for CO_2 removal. O_2 was added automatically as needed to maintain 0.21 pO_2. Open storage mushrooms were atop the cylinder one layer deep in shallow enamel pans.

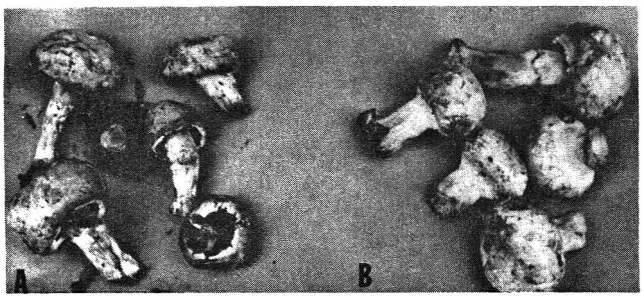


Fig. 3–Mushrooms following 4 days of storage at 20°C and either 1 atm (A) or 35 atm (B). Flow rates at both pressures were 10 liters kg^{-1} hr^{-1} atm^{-1}. Pressurization and depressurization were linear over 2.5 and 6 hr, respectively.

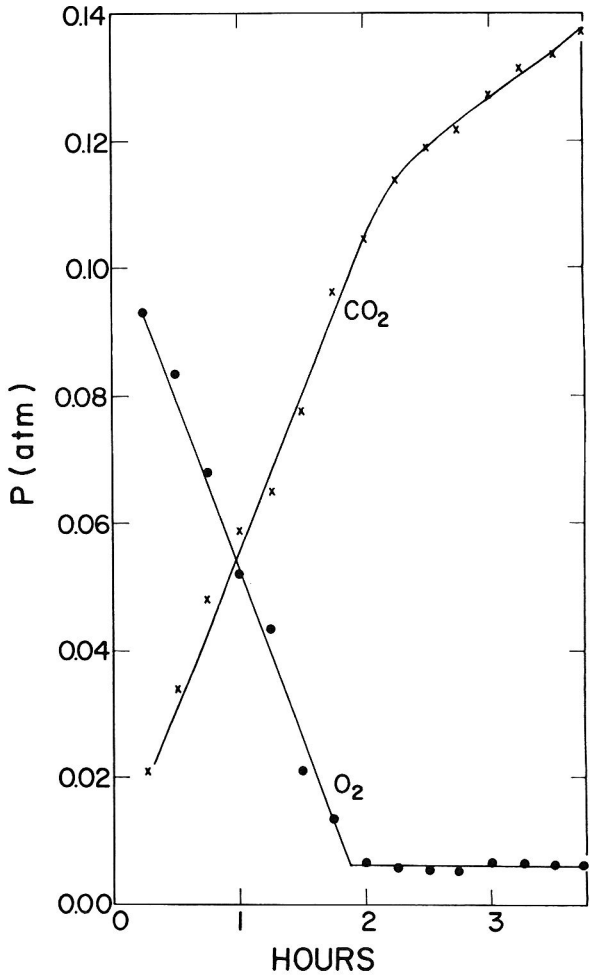


Fig. 4–Rate curves for O_2 uptake and CO_2 release by mushrooms at 20°C. Curves unaffected by pressures to 35 atm.

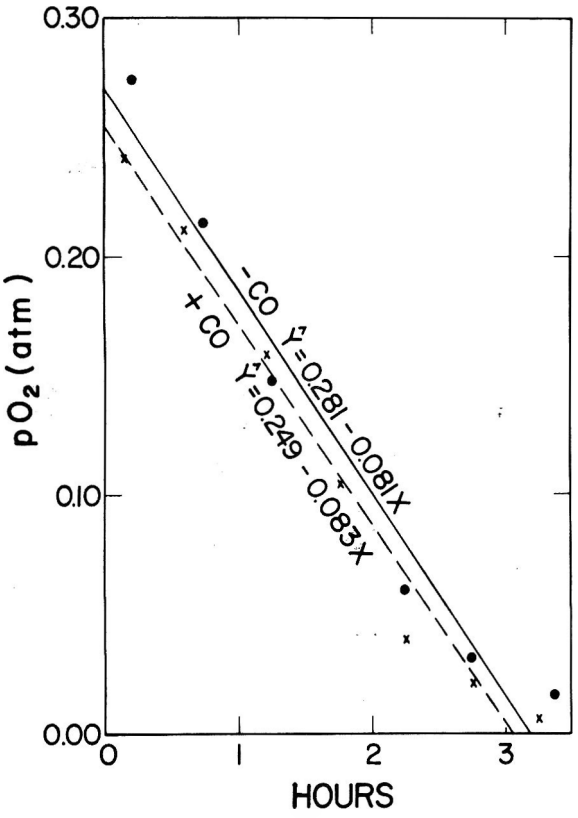


Fig. 5—Linear regression curves for mushroom respiration at 20°C in the presence and absence of 0.035 pCO.

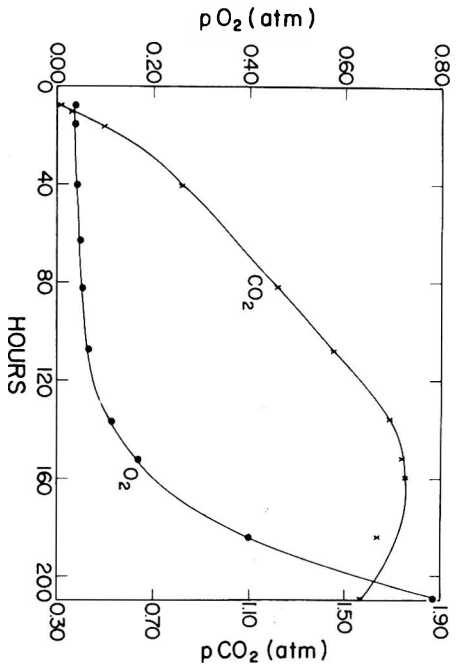


Fig. 6—Mushroom respiratory response to increasing CO_2 levels at 20°C. Oxygen maintained at approximately 0.05 pO_2 for 100 hr by precise flow rate adjustment.

in a fresh water lake for over 1 yr in specially designed laminated plastic containers. Changes in seed moisture, fatty acids, vitamin B_{12}, and reducing sugars were less, and germinative capacity, palatability and cooking quality were better for rice stored underwater than for rice stored in the conventional manner under atmospheric conditions. Benefits resulted from the constancy of ideal temperature and humidity conditions. At 305m, the deepest point in the Great Lakes, the temperature is a constant 1°C with a pressure of 35 atm. This temperature is close to ideal for many products currently stored in energy-intensive refrigerated buildings. The packaging technology for underwater storage is available (Mitsuda et al., 1972), and this study demonstrates the practicality of automatic atmosphere control. The economics of transport and submersion of pressurized storage containers have not been considered.

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Rheology of Enzyme Modified Cheese

Y. C. JAO, A. H. CHEN, R. V. CHAUDHARI, and W. E. GOLDSTEIN

ABSTRACT

The rheological properties of commercial enzyme modified cheese products (Dariteen®, Miles Laboratories, Inc.) are investigated with a micro-rheolog type of viscometer with cone-and-plate geometry. These products exhibit thixotropic shear-thinning behavior, where viscosity changes with the duration and the rate of the applied shear. A viscosity model invoking gradually increasing and decreasing shear rate is investigated. The model involves expression of viscosity as a function of shear rate in the range 1.92-384 sec^{-1} for the case of increasing shear rate and 384-1.92 sec^{-1} for the case of decreasing shear rate. In these investigations, temperature and moisture content range from 24-72°C and 42.3-59.3%, respectively. Hysteresis curves representative of the phenomena are also illustrated.

INTRODUCTION

IN THE PAST 10 YEARS, world cheese production has more than doubled (Morris, 1979). Addition of cheese flavor to various food products is commonly accepted. As a consequence, the cheese industry faces an increasing demand for cheese with high flavor intensity from the prepared food industries. Many prepared food products require sources of cheese such as Cheddar, Swiss, Blue, Romano and others which impart typical flavor characteristics. Research work directed toward accelerating cheese curing was reported by Kristoffersen et al. (1967) who developed a process to obtain a cheese slurry type of product with typical Cheddar, Brick, or Romano flavor from fresh curd in 4 or 5 days. Recently, Miles Laboratories, Inc. has developed technology to produce natural cheese flavors at an accelerated rate with considerable success. A new generation of products designated as enzyme modified cheese is being marketed in the U.S.A. as flavor ingredients under the Dariteen® trademark. These products contain a concentrated source of the major notes of various aged cheese. The principal steps involved in enzyme modified cheese (EMC) manufacturing are mixing, fermentation, and pasteurization. The structure and properties of EMC are complicated due to the co-existence of water, fat, protein and other components in the system, potentially affecting the physical and chemical properties. Therefore, process and quality control of the product becomes vital to maintain desirable structure, texture, and rheological properties. For example, coalescence of the dispersed components may result in a poor emulsion and create undesirable phase separation if product material is not properly handled during manufacturing and delivery.

To develop a manufacturing process and to select equipment for a complicated food system as EMC, study of the rheological properties of the product is necessary.

Authors Jao and Goldstein are affiliated with the Department of Chemical Engineering Research and Pilot Services, Biotechnology Group, Miles Laboratories, Inc., P.O. Box 932, Elkhart, IN 46515. Author Chen is with Anderson Clayton Foods, P.O. Box 226165, Dallas TX. 75266. Author Chaudhari is with Milupa Corp., 48 Church St., Canajoharie, NY 13317.

MATERIALS & METHODS

Enzyme modified cheese

The enzyme modified cheese products are commercially available as Dariteen NCF (Natural Cheese Flavor) ingredients from Miles Laboratories, Inc. The typical compositions of Dariteen NCF 131, and 238 which is shown in parenthesis, are: moisture content, 60% (44%); butter fat, 22% (26%); protein, 12.5% (16%); and total free fatty acid, 1.35% (3.9%). Samples used in this rheological study are Dariteen NCF 131 with moisture content of 59.3%, Dariteen NCF 238 with moisture content of 42.3%, and their one to one mixture with moisture content of 50.8%.

Instrumentation

A Micro-Rheolog viscometer with cone-and-plate geometry from the Brookfield Engineering Laboratories Inc. was employed in the study. This unit is equipped with a jacketed sample cup connected to a Colora Ultra Thermostat (Model N) for temperature control. The cone spindle can be driven at eight different speeds by a synchronous motor through a calibrated spring. The deflection of this spring caused by viscous drag opposing the rotation of the cone is a measure of torque. Operating air pressure of the viscometer is 20 psi. A back-sensing fluidic device converts the spring torque to air pressure as a linear output signal. The signal is then fed into a pneumatic panel-mounted recorder from Foxboro (Model 5310 F-E) for a continuous viscosity monitoring. The recorder is calibrated by using zero and span adjustments to set the pen to 0% at 1/3 psi and to 100% at 2-1/3 psi, respectively.

The principle of the cone-and-plate viscometer and its applications have been described in detail by Wells et al. (1961) and Rand et al. (1964) more than 15 years ago. In this study, cones of 1.565° divergence angle with diameters of 24 and 48 mm were used. Viscosity data (Table 1) at various shear rates was obtained using standard fluids from Brookfield Engineering Laboratories, Inc. The quantity of sample required for each measurement is 2.5 ml, sufficient to fill the space between the cone and plate and also to cover the outer edge of the cone to about 1 mm. To obtain the viscosity in centipoise at a particular cone speed, the chart reading is multiplied by the full scale viscosity at the speed (shown in Table 1) and then divided by 100. Prior to the viscosity measurement, a viscosity standard is used to fine tune the proper position of the micrometer adjusting ring against the index mark so that the percent reading on the recording chart is accurate.

Experiment procedure

Refrigerated enzyme modified cheese (EMC) samples are prewarmed in a thermobath prior to the measurement. Water at a predetermined temperature is circulated from the Colora Ultra Thermostat to the jacket of the sample cup to maintain the cone-and-plate system at the desired temperature. A 2.5-ml sample is allowed to reside in the cup for 3 min to insure temperature equilibration. For rheological modeling studies, the measurement begins at a low shear rate and proceeds gradually to higher shear rates. Viscosity values are calculated from the reading within 2 min. Usually a plateau can be reached within this time. Increase in either the operating temperature or sample moisture will shorten the time for obtaining the steady state value. The measurement is conducted continuously from lowest speed (0.5 rpm) to highest speed (100 rpm) and then back to the lowest speed using the increments noted in Table 1.

RESULTS & DISCUSSION

A PRELIMINARY EXAMINATION of the viscosity of enzyme modified cheese (EMC) was initiated with testing of dariteen NCF 238 with a 24 mm diameter cone spindle

at 10 rpm and 24°C. A 12,000 cps standard fluid was first used to adjust the clearance between cone and plate so that 12.6% was indicated on the chart as shown in the plateau part of the curve in Case A of Figure 1. The EMC viscosity measurement was initiated after this calibration had been completed. The viscosity was determined to be time dependent (thixotropic) as exhibited by Case A. A peak at 37,200 cp appears first followed by a secondary peak at 31,476 cp and then a subsequent decrease at a rate of 222 cp per second for the first minute. Three samples showed exactly the same profile, in contrast to the smooth plateau observed with a standard Newtonian fluid. The profile shown in Case B (Fig. 1) was obtained at 50 rpm (48 mm cone) at 50°C with a duplicate measurement. The viscosity again showed time dependent behavior with the peak at 1137 cp followed by a rate of reduction in viscosity of 6.6 cp per second in the first minute. A secondary peak is not apparent in Case B in comparison with Case A. In his study of mechanical treatment on the hardness of margarine and butter. DeMan (1969) also detected a similar type of curve for the decrease of the hardness of margarine during agitation with a mixer equipped with an electronic recorder. In this EMC study, it is likely that both fat and casein govern the shear sensitive behavior observed.

Vasic and Deman (1965) studied the melting behavior of milkfat and indicated that solid fat content is 100% close to −40°C while at 24°C, only 15% remains. The fat content woult be further reduced with temperature elevation. It is believed that butterfat appears mostly in liquid form at 50°C and provides lubrication for other components in the system. Therefore, a lower viscosity of the system at 50°C when compared with that at 24°C is likely due to the existence of higher amount of liquid fat.

Further investigation was conducted to examine the relationship between shear-thinning phenomenon and the applied shear rate. Samples were subjected to increasing shear rates incrementally from 1.92 sec^{-1} (0.5 rpm) to 384 sec^{-1} (100 rpm) and then using the same increments reduced to 1.92 sec^{-1} (0.5 rpm). The up-and-down cycling process continued until the viscosity remained at same value at the same shear rate (Fig. 2). The viscosity curves derived from Figure 2 are plotted in Figure 3.

Comparison of data at 10 rpm against that shown in Case A (Fig. 1) reveals the viscosity at the peak is 14,307 cp as opposed to 37,200 cp and the viscosity reduction rate is 111 cp per second in comparison with 222 cp per second. Nevertheless, a secondary peak was not apparent in comparison to Case A (Fig. 1). The gradual increase in applied shear rate to the EMC system results in both a lower viscosity value and a lower viscosity reduction rate when compared with the same shear rate applied instantaneously to the system; this result suggesting the thixotrophic nature of the EMC system. In some cases, viscosity values were calculated from the average in two minutes after the shear rate was applied.

According to data depicted in Figure 3, enzyme modified cheese showed thixotropic shear-thinning properties with a viscosity of 1.25 × 10^5 cp at a shear rate of 1.92 sec^{-1}, reduced to 2 × 10^3 cp when the shear rate was increased to 384 sec^{-1}. Reduction of the shear rate back to 1.92 sec^{-1} resulted in a viscosity of 5 × 10^4 cp which is 40% of the original value. The shear rate was then increased again to 384 sec^{-1}, a viscosity of 1845 cp resulted and then the shear rate reduced again down to 1.92 sec^{-1} with a viscosity of 8500 cp which is only 6.8% of the original viscosity. The up-and-down process for the particular test concluded when the third "up" (shear rate increasing)

Table 1—Full scale viscosity of 1.565° cone with 24 and 48 mm diameter

Speed (rpm)	Shear rate (sec^{-1})	24 mm Viscosity (cp)	48 mm Viscosity (cp)
100	384.00	10,252	763
50	192.00	19,672	1,422
20	76.80	47,525	3,487
10	38.40	95,383	6,780
5	19.20	187,438	13,326
2.5	9.60	367,346	26,682
1.0	3.84	874,074	65,332
0.5	1.92	1,668,958	128,188

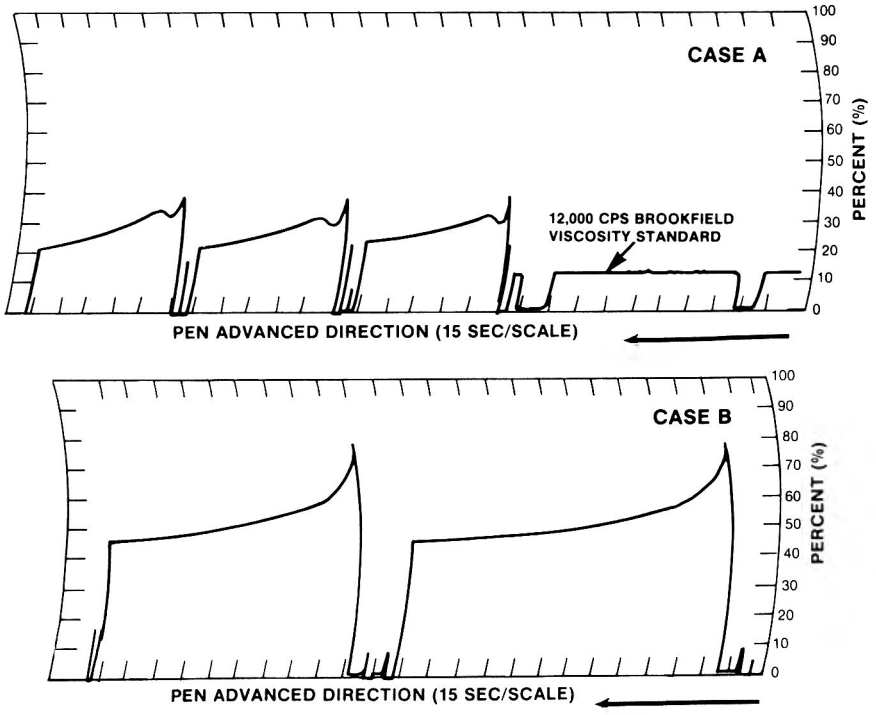


Fig. 1—Time dependent flow characteristic of enzyme modified cheese: (A) 42.3% moisture, 24°C, 10 rpm, 24 mm; (B) 42.3% moisture, 50°C, 50 rpm, 48 min.

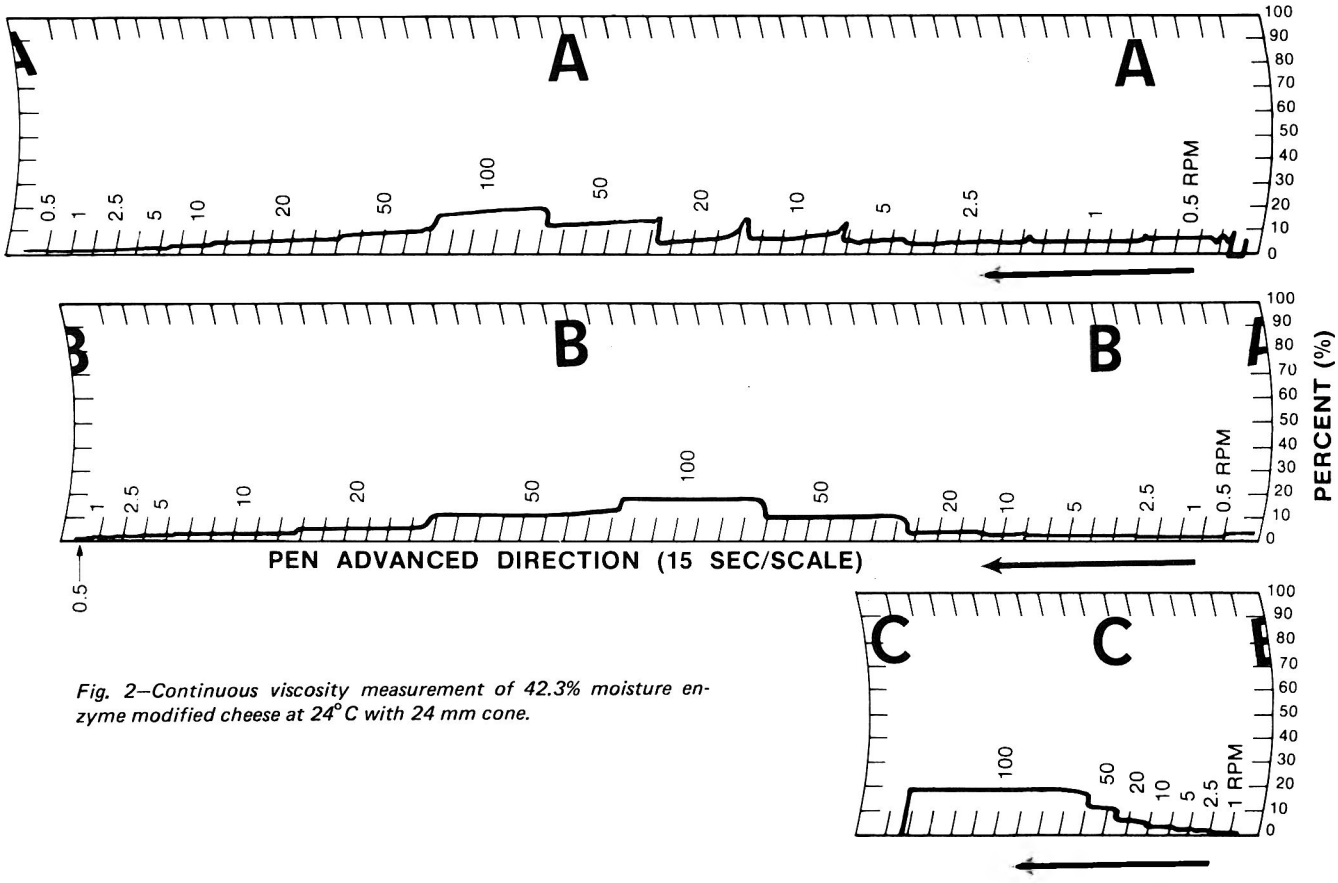


Fig. 2—Continuous viscosity measurement of 42.3% moisture enzyme modified cheese at 24°C with 24 mm cone.

curve coincided with the second "down (shear rate decreasing) curve.

Knoop and Samhammer (1961) presented the concept of the monoclinic structure of butterfat crystals with the triglycerides arranged in a lattice. It was reported (Van den Temple, 1961) that weak London-van der Waal's forces and another stronger force exist as attraction forces to hold fat crystals together. Those crystals interact with liquid butterfat and water to produce a liquid crystalline phase which forms a submicroscopic gel-like network structure. Water and casein particles are also dispersed in the gel-like network in EMC system. A micro-structure examination of the EMC samples at 5°C reveals that broken and deformed fat globules, protein and some small air pockets are dispersed in a continuous phase, this continuous phase consists of free fat in both liquid and crystalline forms with water

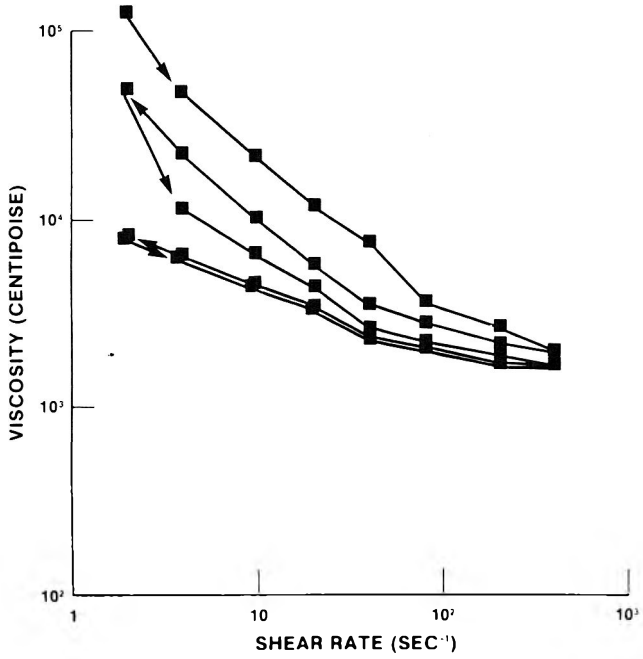


Fig. 3—Hysteresis effect of Miles' Dariteen NCF 238 with 42.3% moisture at 24°C.

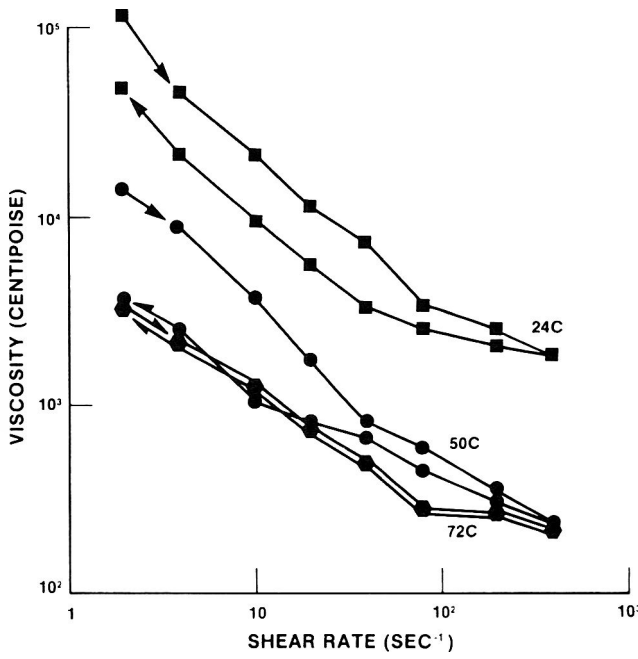


Fig. 4—Viscosity curves of Miles' Dariteen NCF 238 with 42.3% moisture at various temperatures.

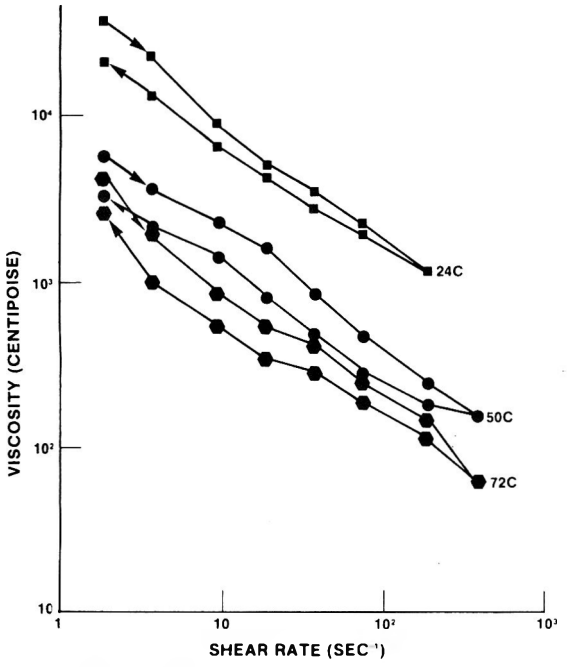


Fig. 5—Viscosity curves of one to one mixture of Miles' Dariteen NCF 238 and 131 with 50.8% moisture at various temperatures.

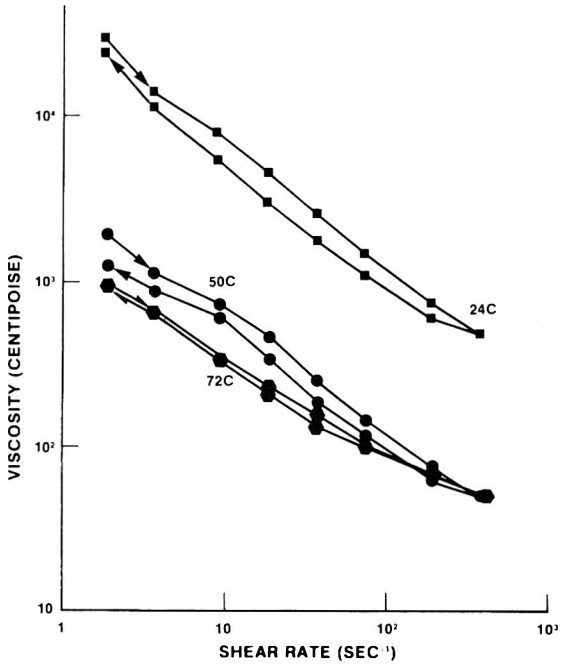


Fig. 6—Viscosity curves of Miles' Dariteen NCF 131 with 59.3% moisture at various temperatures.

interspersed in the continuous phase. It is believed that attraction forces also occur between casein particles and fat crystals. When shear is applied to the system, it tends to break up those aggregates of fat and casein. Destruction of aggregates and flocs of food materials under shear is quite common (Rha, 1975). It is highly possible that the EMC system exhibits a gel-like structure with thixotropic shear-thinning properties at 24°C prior to shear application. However, after the application of various shear rates for a certain period of time, the system may convert to a sol-like structure retaining only shear-thinning properties. The reversible phenomena of gel-sol-gel transformation produced by shear and by rest respectively are quite well known in pharmaceutics (Schott, 1975; Sheath, 1976).

The rheological properties of EMC were further examined at 24, 50, and 72°C with application of increasing and decreasing shear rates. Viscosity curves developed from this test are shown in Figures 4, 5, and 6, with moisture contents of 42.3, 50,8, and 59.3%, respectively. All samples at 24 and 50°C behaved as thixotropic shear-thinning material with the profile of typical hysteresis. Samples with 42.3 and 59.3% moisture at 72°C only showed a shear-thinning property, possibly due to lack of crystalline fat and due to the lubrication effect of liquid fat on the system. It should be noted that samples of 50.8% at 72°C exhibited thixotropy. This behavior is unusual when compared with the other two samples tested at the same condition. This sample was prepared by mixing equal amounts of 42.3 and 59.3% moisture samples with mechanical agitation. It is highly possible that the applied shear during the mixing further emulsified and dispersed components of the sytem resulting in another type of structure that tended to sustain thixotropy even at 72°C.

Study of curves shown in Figure 4, 5, and 6 suggests that the viscosity of EMC is inversely related to the moisture content, to the temperature and to the applied shear rate. Similar findings for soy dough rheology in extrusion have been reported also by Jao et al. (1978).

A semi-empirical model (Harper et al., 1971; Chen et al., 1979) was used to describe the viscosity of the enzyme modified cheese system expressing as a function of shear rate, temperature and moisture. The model is presented in

Table 2—Coefficients of viscosity (Centipoise) Model[a] as a function of shear rate $\dot{\gamma}$ (sec^{-1}), temperature T (°C)[b] and moisture H (%)[c]

μ	A_0	A_1	A_2	A_3	A_4
Shear rate from 1.92-384 sec^{-1}	10.9390	56,331	−0.7129	94.6174	−0.0762
Shear rate from 384-1.92 sec^{-1}	9.8216	18,427	−0.6071	88.5375	−0.0637

[a] Form 1: $\mu = A_1 \dot{\gamma}^{A_2} e^{A_3/T} e^{A_4 H}$; Form 2: $\ln\mu = A_0 + A_2 \ln\dot{\gamma} + A_3/T + A_4 H$
[b] Temperature range 24-72°C
[c] Moisture range 42.3-59.3%

Table 3—Coefficients of viscosity (Centipoise) Model[a] as a function of shear rate $\dot{\gamma}$ (sec^{-1}), temperature T(°K)[b] and moisture H (%)[c]

μ	A_0	A_1	A_2	A_3	A_4
Shear rate from 1.92-384 sec^{-1}	−4.6502	0.0096	−0.7150	5752.33	−0.0762
Shear rate from 384-1.92 sec^{-1}	−4.6531	0.0095	−0.6093	5346.63	−0.0637

[a] Form 1: $\mu = A_1 \dot{\gamma}^{A_2} e^{A_3/T} e^{A_4 H}$; Form 2: $\ln\mu = A_0 + A_2 \ln\dot{\gamma} + A_3/T + A_4 H$
[b] Temperature range 297-345°K
[c] Moisture range 42.3-59.3%

Table 4—Analysis of variance for viscosity model with shear rate from 1.92-384 (sec^{-1}) and temperature expressed in °C

Source	Degree of freedom	Sum of squares	Mean squares	F
Regression	3	223.50	74.50	1079.7
Error	73	5.03	0.069	
Total	76	228.53		

1079.7 = F – Ratio, A 100% Value
0.9889 = Multiple Correlation Coefficient

Table 5—Analysis of variance for viscosity model with shear rate from 384-1.92 (sec^{-1}) and temperature expressed in °C

Source	Degree of freedom	Sum of squares	Mean squares	F
Regression	3	175.59	58.530	498.8
Error	73	8.56	0.11734	
Total	76	184.15		

498.8 = F-Ratio, A 100% Value
0.9765 = Multiple Correlation Coefficient

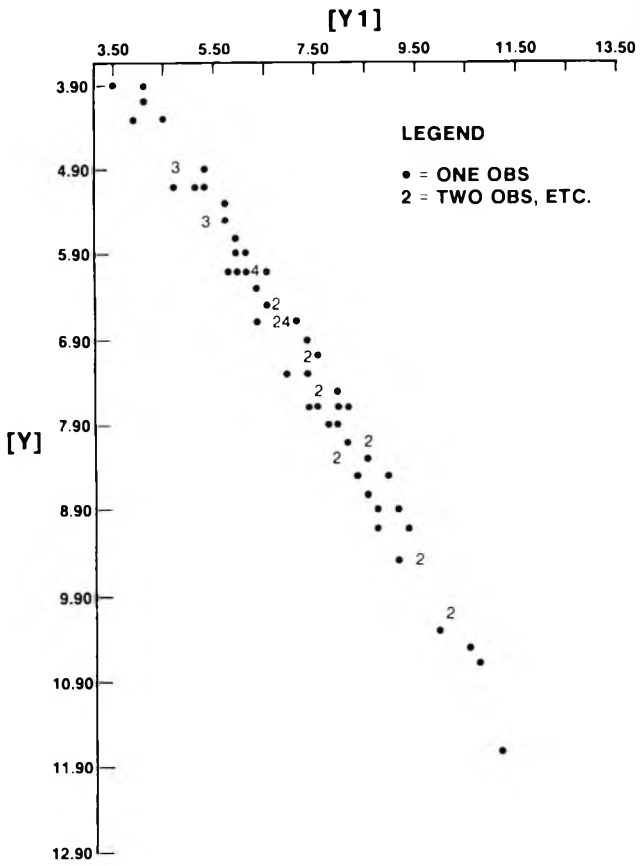


Fig. 7—Plot of computed viscosity from model vs observed (OBS) viscosity ($\dot{\gamma}$: sec^{-1}, T: °C, H: %). [Y1] = ln μ_{comp} = 10.9390 - 0.7129 1 n$\dot{\gamma}$ + 94.6174/T - 0.0762H. [Y] = ln μ_{obs}.

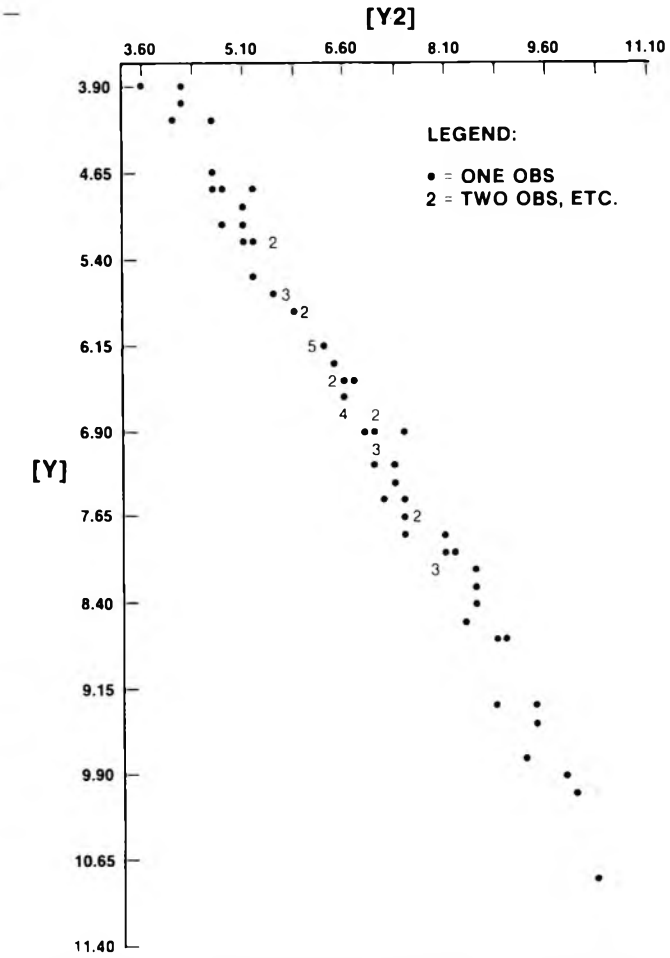


Fig. 8—Plot of computed viscosity from model vs observed (OBS) viscosity ($\dot{\gamma}$: sec^{-1}, T: °C, H: %). [Y2] = ln μ_{comp} = 9.8216 - 0.6071 ln$\dot{\gamma}$ + 88.5375/T - 0.0637H. [Y] = ln μ_{obs}.

the following equation with A_1, A_2, A_3, and A_4 as coefficients.

$$\mu = A_1 \dot{\gamma}^{A_2} e^{A_3/T} e^{A_4 H}$$

This model combines power law, logarithmic mixing rule and Eyring kinetic theory. Due to the thixotropic nature of EMC, two equations derived from the model, were developed to separately describe the viscosity with shear rate gradually increased from 1.92 to 384 sec^{-1} and the viscosity with shear rate decreased from 384 to 1.92 sec^{-1}. Table 2 lists coefficients of these equations with temperature expressed in °C, while Table 3 lists those with temperature expressed in °K. The analysis of variance for the viscosity model with increasing shear rate is shown in Table 4, noting a multiple correlation coefficient (R value) of 0.99, and F ratio of 1079 (at a 100% significant level). When temperature is expressed in °K, the R value becomes 0.98 and F ratio, 565 (at 100% significant level). Table 5 lists the analysis of variance for the viscosity model of decreasing shear rate with R value of 0.98, and F ratio of 499 (at a 100% significance level). The R value is 0.96 and F ratio is 309 (also at 100% level) when temperature is expressed in °K. It is evident that within the narrow range of the temperature involved in this study, the data fit the model better when temperature is expressed in °C rather than in °K. Figure 7 and 8 reveal the relationship of computed viscosity and the measured viscosity. These comparisons suggest that the model properly represents the data.

Examination of the viscosity model reveals that increase in the shear rate, temperature, and moisture will cause reduction in viscosity of the enzyme modified cheese system. The exponent of the shear rate of the increasing shear rate model is −0.72 and that for the decreasing shear rate model is −0.60. Corresponding flow indices for these cases are 0.28 and 0.39, respectively, suggesting the shear-thinning

—Continued on page 262

A Study of Solvent Extraction of Lipids from Whole Peanut Kernels Using All-Purpose Automatic Solvent Extractor

V. M. BHUCHAR, A. K. AGRAWAL, and S. K. SHARMA

ABSTRACT

Extraction of lipids from whole peanut kernels can be accomplished much faster in an automatic solvent extractor with a lower solvent: seed ratio than by immersion or in a Soxhlet extractor. A possible explanation of the function of moisture in the extraction of lipids from the whole peanut kernels has been put forward. This mode of extraction is expected to produce economically fat-lean kernels, while oil is obtained as a co-product.

INTRODUCTION

A SINGLE PEANUT kernel consists of cells (0.07 mm diam) containing the oil surrounded by fairly strong cell walls (Stein et al., 1976; Fan et al., 1948) which are impervious to oil. The oil is expressed mechanically by breaking the cell wall; heat aids in the expression of oil by reducing its viscosity; heat and humidity also assist in this through coagulation of the proteins.

In solvent extraction, the concentration gradient between the oil solution in hexane in the cell and the oil solution surrounding it, controls the dissolution rate and therefore the mass transfer; humidity inhibits this process, and coagulation could retard the intimate mix-up of the solvent and the solute and transfer of the oil through the coagulated mass. However, at a comparatively lower temperature the coagulation may not be significant.

To reach the oil faster in an extraction process the seed is comminuted into thin flakes about 0.32 mm thickness (Fan et al., 1948). For fast transfer of oil solution, however, the higher temperature and constant maintenance of high concentration gradient does help. Except for the diminutive size one could obtain these parameters in the all purpose automatic solvent extractor (AASE) developed by us (Bhuchar et al., 1979). The apparatus is an allied development to the multipurpose solvent extractor (MSE) described earlier by Bhuchar and Agrawal (1975a). Varied extractions, viz solid-liquid, and liquid-liquid (heavier/lighter), extractions at any specified temperature can be performed automatically in this extractor (Fig. 1). The automation has been achieved by introducing an angular stopcock on the exit end of the extractor. The continuous flow of the extract can be thereby regulated. Easiest of the extractions, viz solid-liquid extraction, could have been carried out without further development. However, when liquid-liquid extractions are to be performed in AASE, an additional duck-settling bulb (Fig 1C) is introduced at the end of the long exit-tube. The length of this tube provides additional liquid head in opposition to the back pressure of the vapors that are produced in the distillation flask. The additional liquid head helps to maintain the continuous flow of the extract. The arrangement of other parts in case of liquid –heavier liquid is shown in Fig. 1A and in case of liquid-lighter liquid in Fig. 1B. The exit end of the extract in the settling bulb is placed near its bottom. The settling bulb retains any heavier liquid drops that may accompany the lighter extract.

Authors Bhuchar, Agrawal, and Sharma are with the National Physical Laboratory, Hillside Road, New Delhi–10012, India.

For smaller capacity, an AASE of cylindrical type (Fig. 1) is suitable. For larger capacity, the spherical AASE (Fig. 2) is more suitable. The fabrication and repair of the spherical AASE in this shape is easier and the cost of the apparatus is less.

This AASE apparatus has the merits of conserving solvent and possibly energy. For ensuring intimate contact of the solute with the solvent, it is not necessary to incorporate a mechanical or electrical stirrer because the vapors of the solvent perform this function. There is continuous outflow of the solution from the extractor, and a high concentration gradient is maintained throughout the extraction.

In view of these advantages of the Automatic Solvent Extractor developed by Bhuchar and Agrawal, a study on the extraction of whole peanut kernels appeared interesting. In this study, the efficiency of the automatic solvent extractor was compared with that of the Soxhlet extractor (Soxhlet and Szombathy, 1879) and also with that of a Multipurpose Solvent Extractor (MSE) (Bhuchar and Agrawal, 1975a) which has the advantage of extraction at higher temperature. In the Soxhlet extraction as well as in MSE the concentration gradient decreases gradually in a single extraction. In an All-purpose Automatic Solvent Extractor (AASE), one obtains a uniformly high concentration gradient at a higher temperature.

EXPERIMENTAL

Materials, apparatus, and process

Peanuts were obtained from the market and are trivially known

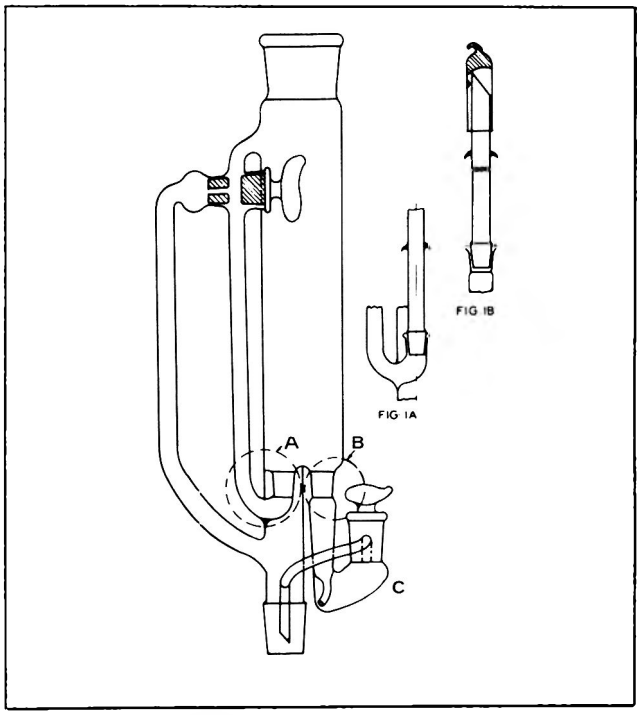


Fig. 1–Sketch of Allpurpose Automatic Solvent Extractor (AASE): (1A) in case of liquid-heavier liquid extraction; (1B) in case of liquid-lighter liquid extraction.

as 'Gwalior Variety.' They analyzed: oil 48%, moisture 5.6%, and proteins 27.4%. Only wholesome kernels were sand-roasted and were de-skinned manually. Initially the roasted peanut kernels contained 1.5% moisture.

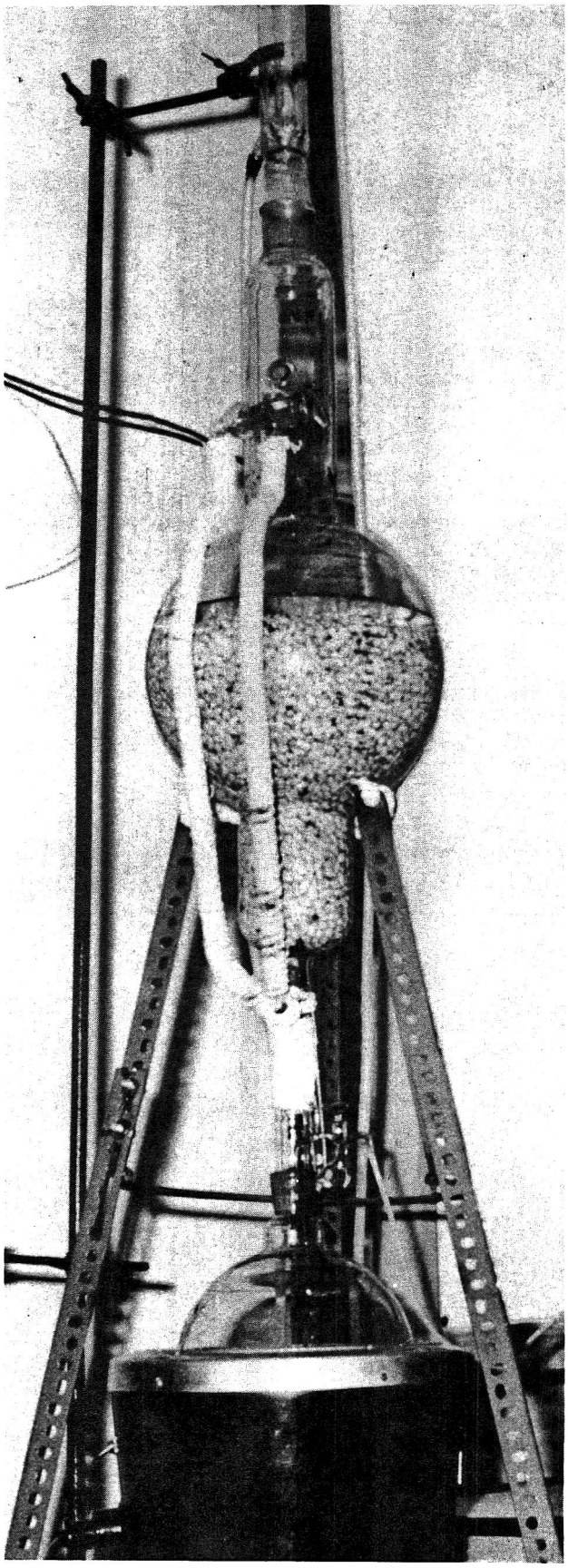


Fig. 2—Photograph of 14 liter spherical Allpurpose Automatic Solvent extractor in actual operation.

The moisture content was determined using a Dean & Stark Apparatus (ASTM, 1971).

The protein content was determined using the Improved ammonia distillation apparatus (Bhuchar and Agrawal, 1975b).

The all-purpose automatic solvent extractor (Bhuchar et al., 1979) of 14 liters or of smaller capacity was utilized for extraction with hexane (boiling range (60-65°C). The progress of extraction was noted (1) by weighing the oil extracted at definite intervals and (2) measured volumetrically by determining bromine absorption by the outflowing oil solution at a particular instance adapting the method of Planck et al. (1953). The concentration of oil in the outflowing oil solution in hexane ranged from a maximum of 0.9% (m/v) after about 3 hr to 0.09% (m/v) towards the end, viz after 16 hr of extraction. The curves obtained by both methods nearly coincide (Bhuchar et al., 1980).

Determination of the residual hexane in the extracted kernels and in the oil was carried out using an Improved Pensky Martens' Closed Tester (Bhuchar et al., 1979).

RESULTS

DRIED PEANUTS were kept immersed in hexane at room temperature (about 30°C). Hexane was renewed after certain intervals, and the extracted oil was weighed. The rate of extraction of the oil is plotted in Figure 3. The extraction could have followed the dotted curve, with an extraction maximum and a near plateau, if our later observations are taken as a guide.

Influence of moisture content

The moisture content of the seed influences the rate of extraction of the oil from the seed (Fan et al., 1948). This aspect was examined for optimum conditions for extraction in the AASE.

Roast-blanched kernels (1-2% moisture content; 200g) were allowed to absorb moisture from the atmosphere for extended periods. The progress of extraction of kernels with increasing moisture content was examined in a 1-liter capacity AAS-extractor. The extraction rates for known time intervals are plotted in Figure 4. These curves give a better idea of the progress of extraction than a plot of the cumulative oil extracted over a period. In the case of kernels with moisture content up to 2%, the extraction curve shows essentially two peaks of extraction rate.

One could compare the kernel to a honeycomb wherein oil takes the place of honey and the proteinous cell walls the place of the wax. The cell walls are impervious to oil but heat makes them pervious to hexane and miscella, just as heat helps to soften the wax to allow the water to reach the honey to dissolve out the latter.

The first peak in the curves in Figure 4 appears to correspond to the permeation of the solvent through the walls

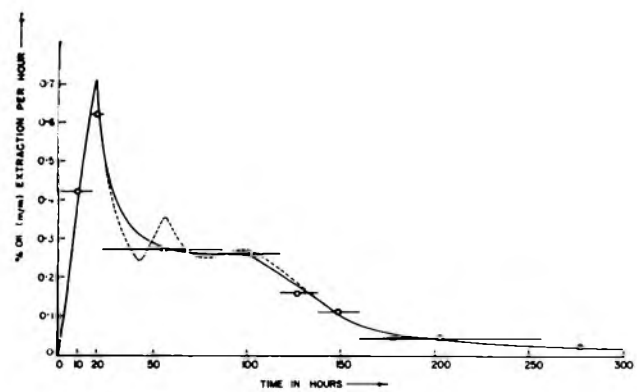


Fig. 3—Curves showing rate of extraction of oil from peanut kernels by immersion in hexane at room temperature (30°C). The broken curve is the possible progress of extraction in the light of experience in Fig. 4.

of the kernels. Permeation distends the cell walls and this is aided by heat. The solubilization of the oil increases with an increase of permeation. When the permeation is complete, the extraction of oil attains a peak value. Then mass transfer of the oil from the cell to the surrounding solution occurs and is controlled by the concentration gradient between the solution and the solvent. Because of continued higher miscella concentration in the cell this remains high and attains a maximum value. It starts falling as the oil content in the seed falls appreciably, viz 10-15% oil) based on the original mass of the kernels) remains.

If kernels with a moisture content of about 2.4% are extracted, a third peak in the extraction rate curve appears. The third peak occurs closer to the second peak as the moisture increases. As the extraction proceeds at the boiling point of extractant (65°C), the moisture vaporises. The vapors perhaps tend to increase the cell volume of the kernels by further distending the cell walls. A better mixing and dissolution of the oil and the mass transfer through the distended walls take place. Consequently the rate of extraction of the remaining oil in the seed becomes faster. The third extraction peak is apparently correlated with the increasing moisture content of the seed and its vaporization.

Two alternative patterns of extracting oil from peanut kernels having the same moisture content are possible, viz. (1) the kernels are dried to a particular moisture content; and (2) the kernels are dried to a lower moisture content; they are then allowed to absorb moisture from the atmosphere gradually, until their moisture content is the same as in alternative (1). With roast-blanched kernels of 4% moisture content, the rate of extraction of kernels by mode (2) is faster than that by mode (1). The moisture absorbed from atmosphere seems to evaporate more easily. These vapors distend the cellular space giving better mixing and dissolution and consequently better mass transfer of oil through the concentration gradient. As a result the third peak in the curve of extraction occurs somewhat earlier.

The difference in the pattern of extraction was not so marked if the absorption of moisture from atmosphere is minimal, as seen in curves (Fig. 5) relating to oil extraction having 2.4% moisture. This observation was kept in view when results of extractions in various extractors were compared.

Comparison of extractionin ASAE, MSE and Soxhlet apparatus

Extraction rate curves for kernels dried to 2% moisture content are given in Figure 6. These were subjected to extraction with hexane within 2 hr of their de-skinning after dry roasting. During this interval there is very little absorption of moisture.

It could be seen that by processing the kernels at a somewhat higher temperature (60-65°C) the rate of permeation is faster than at room temperature with the result that the extraction in the first 5 hr is much higher. The concentration gradient in the two extractions (curves A and B in Fig. 6) is similar and the outflow of the extract to the distillation flask in either case is intermittent. Because of the inflow of fresh solvent into the extractor after each extraction, the concentration gradient between the oil solution in the cell and its surroundings remains high. In the MSE extractor, however, the dissolution of the solute in the solvent is faster because of somewhat higher temperature of extraction. The extraction in MSE in the first 5 hr is 20% greater than in the Soxhlet.

The pattern of extraction in an MSE or in an AASE is essentially similar, with minor difference in the permeation and the dissolution steps. In both these apparatuses, dissolution of oil taken place at the same temperature and the concentration gradient between the oil solution in the cell

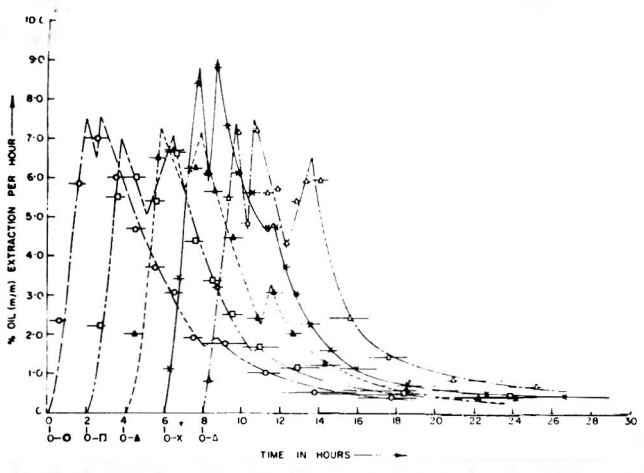


Fig. 4—Curves showing rate of extraction of oil from whole peanut kernels with varying concentrations of moisture viz. ○ —— ○ (1.2%); □ — -- — □ (2.0%); ▲ — — — ▲ (2.8%); x ——— x (4.0%); Δ — · — Δ (4.8%).

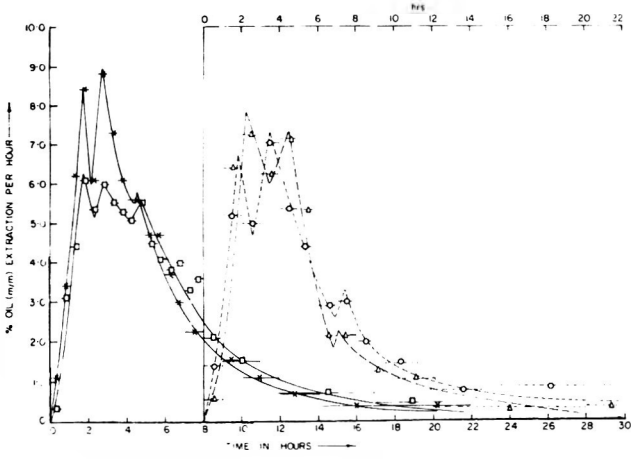


Fig. 5—Curves showing the rate of extraction of oil from whole peanut kernels having □ ——— □ 4% moisture content, having been dried to that extent; x ——— x 4% moisture content; the kernels having been dried to lower moisture content and then having absorbed the moisture; ○ — · — ○ 2.4% moisture content; having been dried to that extent; Δ — — — Δ 2.4% moisture content; the kernels having been dried to lower moisture content and then allowed to absorb moisture from the atmosphere.

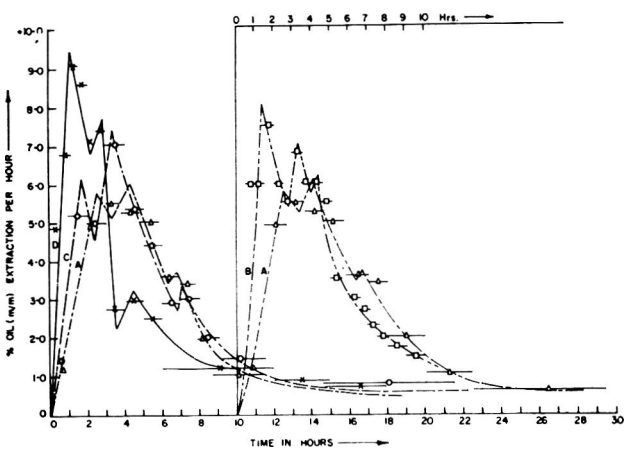


Fig. 6—Curves showing extraction rate of whole peanut kernels (2% moisture content) in: A Δ — · — Δ Soxhlet Extractor; B □ — -- — □ Multipurpose Solvent Extractor (MSE); C ○ — — — ○ Allpurpose Automatic Solvent Extractor (AASE); D x ——— x AASE, the kernels having been finely punctured.

and its surroundings is about equal. The rate of extraction in AASE is enhanced by 20% over the Soxhlet, as is apparent from curve C and A in Figure 6. This occurs because of the continuous maintenance of a high concentration gradient at higher temperature in the AASE extraction.

To simulate the comminution of kernels into flakes, the kernels were punctured with a thin pointed hand-stitching needle (0.7 mm diam) 30-40 times evenly over each kernel on both the cotyledons. The extraction of oil from these kernels is much faster (curve D in Fig. 6), because all the favorable parameters of comminution as recommended by Cater et al. (1974), extraction at higher temperature and continuously high concentration gradient between oil solution in the cell and its surroundings exist in an AASE extraction.

CONCLUSION

THE RATE of extraction over a particular interval has been adopted as a measure for comparing extraction efficiencies and shows the progress of extraction better than the cumulative extraction in a time interval. The results in Figure 3 conform closely to the experience of Willich and Fauge (1957) and Pominski et al. (1964) in that the extraction of oil by immersion is a slow process and is economically nonviable. From the results in Figures 4-6, it may be concluded that the optimal extraction of oil from groundnut kernels is attained at about 2% moisture content. The automatic solvent extractor (AASE) gives about 20% higher extraction compared to the Soxhlet apparatus. Extraction of 70% oil is accomplished in 6-7 hr. This is much more favorable than extraction of 70% of the oil of the whole kernels in 70-80 hr, as reported by Pominski et al. (1964) and also evident from Figure 3. The extraction in the AASE has the advantage of conserving solvent in common with Soxhlet and MSE extraction and conserving energy in common with MSE extraction. The vapors do the stirring and cause intimate contact of the solvent with kernels; also they impart their latent heat for accomplishing extraction at a higher temperature. AASE has the added advantage of maintaining continuously a high concentration gradient. The concentration of the lipids solution during the course of extraction ranges from 0.9% to 0.09% (m/v) in hexane.

The probable mode of extraction of lipids from the kernels appears to be permeation with the distention of the cell wall, dissolution, and solubilization of additional oil after further distention of the cell wall. Mass transfer is assisted by constant maintenance of a high concentration gradient of the miscella vis-a-vis the sorrounding solvent and somewhat higher temperature in the present mode of extraction. The Hagen-Poiseuille law, as postulated by Othmer and Agrawal (1955) and Othmer and Jaatinen (1959), for the extraction of soyabean flakes may at best apply to the last 10% of the lipids after 40% of these have been extracted from the peanut kernel.

Thus by following a single unit-process, in an AASE, the kernels remain primarily in their original form and expanded. However, they do not break. Simultaneous extraction of the oil from the seed kernels takes place with low solvent:seed ratio. The oil obtained is free of fines. These advantages would reduce markedly the cost of production of oil by solvent extraction of peanut kernels as reported by Pominski et al. (1964).

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(pseudoplastic, flow index <1) behavior of EMC system. Results also suggest that the former case (increasing shear rate) exhibits more shear sensitive than the latter (0.28 < 0.39) in our scheme for viscosity measurement. This study therefore discloses the thixotropic shear thinning nature of Miles' enzyme modified cheese system.

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Application of an Improved Experimental Washer for Leafy Greens for Canning

E.M. HOLLEY and W.A. SISTRUNK

ABSTRACT

A study was conducted to compare the quality of the effluent and canned spinach, turnip greens, and mustard when washed by experimental and industrial washing systems. Three 4-hr runs were conducted on spinach and one each on turnips and mustard greens. The effluent from the industrial washing system was significantly higher in total suspended solids, total solids, suspended solids, chemical oxygen demand, and nitrates than that from the experimental washer. Washing system has very little effect on quality of canned spinach. The major differences in quality were caused by cultivar and age of raw product. Total plate counts were not affected by washing system.

INTRODUCTION

THE WASHING of spinach and other leafy greens for processing requires large volumes of water because of the nature of the product and field conditions during harvesting. One of the critical factors in quality of the processed product is the removal of practically all traces of grit. However, an interest has developed among processors in new methods of consolidating and reducing waste loads within the plant in order to comply with the Federal Water Pollution Control Act Amendment of 1972, PL 92-500. In July 1983, the effluent limitation guidelines require that industries use the best available control technology economically achievable; then zero discharge of pollutants into navigable waterways by 1985 (Devit and Forsht, 1977; Wilson and Huang, 1977).

Reduction in fresh water consumption in spinach processing has been achieved by a modified washing system (Frey et al., 1974; Wright et al., 1979). This system is based on recycling the wash water and removal of heavy solids by settling, and large particles of suspended solids by screening. Other methods of reuse water have been successfully applied to reduce volumes of water required to wash vegetables (NCA, 1971; Sistrunk et al., 1977; Crow and Robe, 1977). Other methods of reduction of waste loads in vegetable processing include blanching and cooling operations. Earlier research has shown that steam blanching with minimum or no cooling provided the best method to reduce effluent volumes in canned spinach (Sistrunk et al., 1977). Other comparative studies have been conducted on steam and water blanching for vegetables (Bomben et al., 1975: Guerrant et al., 1947; Lazar et al., 1971; Melnick et al., 1944; Ralls and Maagdenberg, 1972; Sistrunk, 1980). Volumes of water used for washing, blanching and cooling of spinach were shown to be an important factor in retention of nutrients in spinach and leafy greens (Sistrunk et al., 1977; Sistrunk, 1980).

The objectives of this study were to: (1) compare the quality of the effluent from an experimental washer with commercial washers for washing spinach, turnip greens, and mustard; (2) compare the quality and ascorbic acid content of the canned product from the two washers; and (3) determine minimum overflow necessary in the experimental washer to assure a clean raw product.

Author Sistrunk is with the Dept. of Horticultural Food Science, Univ. of Arkansas, Fayetteville, AR 72701. Author Holley, formerly with the Univ. of Arkansas, is currently with the Nebada County Extension Service, Prescott, AR 71857.

MATERIALS & METHODS

THE EXPERIMENTAL (prototype) washing system used in these studies was developed by the Virginia Agricultural Experiment Station and is described in detail by Wright et al. (1970). This system was loaned to the University of Arkansas and Allen Canning Company by the Environmental Protection Agency to conduct research on spinach and other greens. The system was installed adjacent to the commercial processing operation, and fitted with water meters and recycle pumps. The flow of raw product averaged 10 metric tons/hr. The product was unloaded automatically, traveling on a single belt. Then it was evenly distributed to three dry reels, followed by inspection belts. The product from one of the belts was diverted through the experimental washer. The flow of product on the three lines appeared to be evenly distributed from hour to hour at a flow rate of 10 MT/hr.

Water samples were collected at 30-min intervals during a 4-hr continuous run on 5 separate days in the spring of 1978, 3 runs on spinach, and 1 each on turnip and mustard greens. The 4 water samples from the commercial washing system were collected from 2 washing reels and 2 paddle washers, installed in the sequence reel:paddle:paddle:reel. In the experimental washing system, samples were taken from the 2 washers in sequence and 2 adjacent recycle tanks. Raw product samples were taken at the discharge end of the second washing reel in the commercial washing system and at the discharge end of the second experimental washer.

Water and product samples from the 2 washing systems were transported to the laboratory (6 miles) every 2 hours and prepared for analysis and processing respectively. Water samples were either frozen for later analysis, or part of analyses were performed immediately.

The product for canning was blanched 4 min at 80°C, hydrocooled and filled into 303 x 406 J-enamel cans to a fill of 300g. Then a 60 grain salt tablet wass added to each can, and cans were filled with water, exhausted 4 min in steam, closed by an automatic steam-flow closer and processed 50 min at 122°C. The cans were then cooled and stored at 24°C until analyzed.

Water analyses

The analyses for total suspended solids (TSS), total solids (TS), and settleable solids (SS) were performed by standard methods (APHA, 1971). Chemical oxygen demand (COD) was determined by the procedure described by Mercer and Rose (1968). The Nitrate determinations were performed with a nitrate selective electrode (Pfeiffer and Smith, 1975).

Canned product analyses

The following determinations were made on the canned product: drained weight, Color Difference Meter (CDM) L, a, b, shearpress, ascorbic acid, liquor color, nitrates (NO_3) and sand or grit. The CDM measurements were made on both whole and pureed product as described by Sistrunk et al. (1977). Ascorbic acid, liquor color and NO_3 were analyzed on the liquor from the canned product.

The procedures for ascorbic acid and liquor color have been described previously (Sistrunk et al. (1977). The procedure for NO_3 was the same as for water analysis. The chloride was not removed before the NO_3 analysis, but replicated tests on the canned product with and without added salt indicated that NO_3 were not significantly affected by the salt.

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The resistance to shear was determined by weighing 150g of drained spinach and placing it in a standard cell of the shearpress (Food Tech. Corp., Model TP-1A).

The amount of grit was determined by gently floating the pulp from a 300g sample of blended spinach in a 600 ml flask with a stream of water. The grit was quantitatively transferred to tared crucibles, dried in an oven at 80°C and weighed.

The data were analyzed by the analysis of variance, and the means of the main effects recorded in tables. The data for the time of day and six replications were pooled because of the quantity of data. Variation in data with time of day was attributed mainly to differences in the raw product.

Table 1—Main effects of washing system and runs on water quality of the effluent from washing spinach

Variable	Kilograms per Metric Ton				
	Total suspended Solids	Total solids	Settleable solids	COD	Nitrates
Systems					
Ind	6.08	9.82	24.55	2.38	0.65
Exp	2.75	3.62	9.31	0.51	0.17
LSD 0.05[a]	0.22	0.88	2.99	0.25	0.09
Run (days)					
1st	4.83	6.80	23.59	0.77	0.44
2nd	4.01	6.94	16.59	1.42	0.48
3rd	4.40	6.41	10.35	2.16	0.31
LDS 0.05	0.09	NS[b]	3.67	0.30	0.11

[a] LSD 0.05—Least significant difference at 5% level.
[b] NS—Not significant.

Table 2—Water consumption during washing of spinach, turnip greens, and mustard greens

Runs (4 hr)	Washing system			
	Industrial		Experimental	
	L/kg	gal/lb	L/kg	gal/lb
Spinach	10.91	1.31	3.56	0.43
Spinach	11.28	1.36	3.87	0.46
Spinach	10.24	1.23	3.05	0.37
Turnip greens	8.71	1.05	2.76	0.33
Mustard greens	9.41	1.13	2.55	0.31

Table 3—Main effects of washing systems and runs on quality of canned spinach

Variable	Drained wt (g)	Ascorbic acid (mg/100g)	Nitrates (ppm)	a/b ratio whole-leaf	Shearpress (kg/150g)
Systems					
Ind	259	12.7	696	0.136	42
Exp	269	14.3	766	0.136	42
LSD 0.05[a]	5	0.8	5	NS[b]	NS
Runs (days)					
1st	264	8.4	644	0.150	55
2nd	276	14.9	1053	0.148	40
3rd	253	17.3	495	0.112	30
LSD 0.05	6	1.0	6	0.010	6

[a] LSD 0.05—least significant difference at 5% level.
[b] NS—Not significant.

RESULTS & DISCUSSION

WE HAVE FOUND that spinach and other leafy greens pick up as much as 30% water during washing, depending on age, type, holding time after harvest, and time of day of harvesting. This wet weight did not change appreciably during blanching and cooling. In designing and operating washing systems this must be taken into consideration, especially in a water recycling system. It was necessary to provide an overflow of 2500-3500 L/hr to maintain the quality of water in the experimental washer for a raw product flow of 3.34 MT/hr. This varied with cultivar, age of product and field conditions at harvest. Perhaps a larger recycle tank with centrifugal separators for SS would reduce this considerably. Wright et al. (1979) indicated that it was necessary to use a minimum of 4.2 L/kg of product in the experimental washer for spinach, while the conventional washers used 13.27 L/kg. However, water usage in these studies was less in both experimental and commercial washers (Table 2).

There were significant differences in TSS, TS, COD, and NO_3 of the effluent between the industrial and experimental washers (Table 1). Also, there was a difference in quality of the effluent among days of operation except for TS. Part of the difference was caused by the condition of the field and amount of dirt, mud, or dust on the raw product. Water usage was much greater per kilogram (kg) of raw product in the commercial washers (Table 2). Water usage was less on the turnip and mustard greens. There was more physical damage to the raw product by the commercial washers, thus more leaching out of soluble constituents, more TSS, and higher COD. This physical damage from larger volumes of wash water has been shown in earlier studies (Sistrunk et al. 1977), using a small vegetable washer with low volume water sprays.

The quality of the canned product was not affected by washing system as indicated by a/b ratio of the whole-leaf spinach (Table 3). Color differences occurred between runs as measured by the a/b ratio of whole-leaf spinach. An attempt to rate color differences by a semi-trained sensory panel did not reveal any differences that could be consistently detected. The drained weight, ascorbic acid and NO_3 were significantly higher in spinach washed by the experimental washer (Table 3). Differences in quality between runs was attributed to different cultivars and

Table 4—Main effects of washing systems and type of greens on water quality of the effluent from washing turnip and mustard greens

Variable	Kilograms per mitric ton				
	Total suspended solids	Total solids	Settleable solids	COD	Nitrates
Systems					
Ind	1.72	6.05	8.27	2.33	0.60
Exp	0.55	1.80	2.42	0.64	0.19
LSD 0.05[a]	0.07	0.70	1.87	0.38	0.08
Type of greens					
Turnip	0.82	3.68	4.84	1.36	0.47
Mustard	1.43	4.18	5.84	1.62	0.32
LSD 0.05	0.07	0.70	1.87	NS[b]	NS

[a] LSD 0.05—Least significant difference at 5% level.
[b] NS—Not significant.

age of spinach. Grit remaining in the product was not affected by either washing system or runs (data not shown). Wright et al. (1979) indicated that the prototype washer washed slightly more grit from the product than conventional washers. Apparently the product was being washed reasonably clean with only a trace of grit remaining. The CDM values L, a, and b were statistically analyzed on the whole-leaf and puree samples as well as optical density of the liquor from the canned product. However, neither of these determinations revealed any differences in color between washing system, and very little difference among days of operation (data not shown).

In comparing the two washing systems for washing turnip and mustard greens, significantly higher TSS, TS, SS, COD, and NO_3 were obtained in effluent from the industrial washers (Table 4). Although these greens are tougher than spinach, the physical damage by the industrial washers caused more leaching of soluble constituents in the wash water than the experimental washer. Mustard greens were significantly higher in TSS, TS, and SS than turnip greens; however, differences in COD and NO_3 were not significant. Comparison of color by CDM and other quality factors on whole-leaf and puree product between industrial and experimental washers did not reveal any differences, except that the grit content was slightly higher in greens washed in the industrial washers (Table 5). Turnip greens were higher in drained weight and NO_3, while mustard greens were higher in ascorbic acid, grit, and a/b ratio. Visual differences were detected in the washed greens between washing systems, but again these differences could not be discerned by a sensory panel in the canned product.

There were significant interactions between systems and days and systems and time of day on quality of product, but these are not reported. Most of the interactions were caused by changes in the cultivar of spinach and variations in color, dirtiness, and age of product at different sampling times and days of operation. Similar differences in quality parameters due to raw product were reported earlier (Wright et al., 1979). There were significant differences between sampling sites and sampling times; however, these are obvious differences and, therefore, not reported here.

Total plate counts of spinach varied among sampling sites, being significantly higher in water from the first reel of the industrial washer and lower in water from the second reel of the industrial washer and the first recycle of the experimental washer (Table 6). The differences between washing systems were not significant; whereas, there were differences among days of operation. Although far less volumes of water were used in the experimental washer, counts usually did not built up any higher in the recycle

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Table 5—Main effects of washing system and type of greens on quality of canned turnip and mustard greens.

Variable	Drained weight (g)	Ascorbic acid (mg/100g)	Nitrates (ppm)	Gr t (g/150g)	a/b ratio whole-leaf	Shearpress (kg/150g)
Systems						
Industrial	297	8.2	1393	0.064	0.08	206
Experimental	298	7.9	1379	0.049	0.08	214
LSD 0.05[a]	NS[b]	NS	NS	0.011	NS	NS
Type of greens						
Turnip	304	7.1	1900	0.017	0.07	215
Mustard	291	9.0	871	0.048	0.09	206
LSD 0.05	6	1.4	324	0.011	0.01	NS

[a] LSD 0.05—Least significant difference at 5% level.
[b] NS—Not significant.

Table 6—Comparison of bacterial counts of effluent from industrial and experimental washers in washing spinach for canning, 1978

System x sites	Runs (4 hr each day) 1	2	3
Industrial washer			
1st ree	8.4×10^5 a[a]	6.6×10^5 b	2.0×10^6 a
1st paddle	5.3×10^5 b	2.5×10^5 c	2.0×10^6 a
2nd reel	2.1×10^5 c	1.6×10^4 e	3.1×10^5 b
2nd paddle	4.8×10^5 b	2.7×10^4 e	2.2×10^5 b
Average	5.1×10^5	2.4×10^5	1.1×10^6
Experimental washer			
1st washer	3.4×10^5 bc	3.5×10^5 c	1.6×10^6 a
2nd washer	4.5×10^5 b	8.4×10^4 d	7.1×10^4 c
1st recycle	2.0×10^5 c	6.3×10^4 d	5.5×10^5 b
2nd recycle	4.4×10^5 b	8.0×10^5 a	1.4×10^6 a
Average	3.6×10^5	2.0×10^5	9.0×10^5
Grand total	4.4×10^5 c[b]	2.2×10^5 b	1.0×10^6 a

[a] Mean separation within columns for systems x sites by Duncan's Multiple Range Test.
[b] Mean separation in row for runs by Duncan's Multipel Range Test.

Table 7—Comparison of bacterial counts of effluent from industrial and experimental washers in washing turnip greens and mustard for canning, 1978

Systems x sites	Type of greens Turnip	Mustard
Industrial washer		
1st reel	4.6×10^6 a[a]	1.2×10^6 a
2nd reel	1.9×10^6 c	2.5×10^5 bc
1st paddle	3.4×10^6 b	4.9×10^5 b
2nd paddle	2.9×10^6 b	1.2×10^5 c
Average	3.2×10^6	5.3×10^5
Experimental washer		
1st washer	1.7×10^6 c	1.3×10^6 a
2nd washer	1.3×10^6 c	$.9 \times 10^5$ c
1st recycle	2.9×10^6 b	6.4×10^5 b
2nd recycle	3.5×10^6 b	1.4×10^6 a
Average	2.4×10^6	8.6×10^5
Grand total	1.8×10^6 a[b]	6.9×10^5 b

[a] Mean separation within columns for systems x sites by Duncan's Multiple Range Test.
[b] Mean separation in row for type of greens by Duncan's Multiple Range Test.

Role of Flavonoids in the Production of Color in Biscuits Prepared with Wheat and Cottonseed Flours

F. A. BLOUIN, Z. M. ZARINS, and J. P. CHERRY

ABSTRACT

Biscuits prepared with 80% wheat and 20% liquid-cyclone-processed and hexane-defatted glandless cottonseed flours were yellow-brown. To isolate and identify pigments responsible for color, the cottonseed flours were extracted with aqueous isopropyl alcohol. These extracts were fractionated into nonflavonoid and flavonoid components by Sephadex column chromatography. Biscuits containing the extracted cottonseed flours (free of flavonoids) were brown, those prepared with the nonflavonoid fractions were tan, and those to which the flavonoid mixtures were added were yellow. Chromatographic and ultraviolet-visible spectral methods showed that there are seven major flavonoids in both flours, tentatively identified as isoquercitrin, rutin, quercetin 3-0-robinoside, quercetin 3-0-neohesperidoside, kaempferol 3-0-neohesperidoside, quercetin 3-0-glucoglucoside, and kaempherol 3-0-glucoglucoside.

INTRODUCTION

When cottonseed flours are used in certain food products, an undesirable color is observed (Blouin and Cherry, 1980; Kadan et al., 1978; Kim et al., 1971; Lin et al., 1973-1974; Wan et al., 1979). Biscuits prepared with 20% liquid-cyclone-processed (LCP) cottonseed flour and 80% wheat flour were yellow-brown (Blouin and Cherry, 1980). Data obtained by fractionation and by spectral techniques applied to pigments in these fractions showed that the yellow color resulted from flavonoid pigments and that the brown color was caused by gossypol and gossypollike pigments. This manuscript describes further studies on the isolation and identification of the flavonoids in both LCP and hexane-defatted glandless cottonseed flours and the role these pigments play in the production of color in biscuits.

EXPERIMENTAL

Cottonseed flours

LCP cottonseed flour was prepared from glanded seeds at the Southern Regional Research Center (SRRC) by the methods of Gardner et al. (1976). The LCP flour had a proximate composition of 57.3% protein, 32.1% carbohydrate, 1.6% lipid, 7.7% ash, and 2.3% crude fiber.

Hexane-defatted glandless cottonseed flour, also prepared at SRRC, had a proximate composition of 51.8% protein, 38.0% carbohydrate, 1.4% lipid, 7.5% ash, and 1.3% crude fiber.

Analysis of these flours for gossypol indicated 0.04% and 0.01% free gossypol and 0.15% and 0.02% total gossypol for the LCP and glandless flours, respectively.

Flour extractions

Flours were homogenized in an Omni Mixer with petroleum ether (1 g/10 ml for 5 min and then stirred overnight at room temperature to remove residual lipids (0.3-0.6%). After filtration and air drying, the extracted flours were dispersed in 85% aqueous isopropyl alcohol (1 g/10 ml) and stirred overnight at room temperature. This extract was first filtered from the flour, the isopropyl alcohol was removed by rotary evaporation and then the aqueous extract slurry was lyophilized; dried extracts were 7-9% of the original flours. The extracted flours were treated similarly.

Authors Blouin, Zarins, and Cherry are with the USDA-SEA, AR, Southern Regional Research Center, P.O. Box 19687, New Orleans, LA 70179.

Chemical analysis

Flours were analyzed by official methods of the American Oil Chemists' Society (1976) for nitrogen (Ba 4-38), lipid (Ba 3-38), crude fiber (Ba 6-61) ash (Ba 5-49), free gossypol (Ba 7-58) and total gossypol (Ba 8-55). Protein content was estimated by percentage of nitrogen times 5.30 (Watt and Merrill, 1963). Carbohydrate was determined by difference.

Color measurements

A Hunterlab digital color meter D25D2A, calibrated against a white tile standard, was used to measure color. Hunter L (lightness), a (positive a = red; negative a = green) and b (positive b = yellow; negative b = blue) values were measured on biscuits; a values are not reported because they showed no meaningful variation in this study (−4 to +1). Coefficients of variation were L = 4.9% and b = 3.3%.

Preparation of biscuits

Biscuits were prepared with 20.0g wheat or composite (wheat plus cottonseed) flours, 1.0g baking powder, 0.5g salt, 4.5g shortening, and 15.0g liquid milk. The flours, baking powder, and salt were mixed and sifted. Shortening was cut in with a pastry blender and the mixture stirred with milk. When flour extracts, fractions of extracts, or rutin were used in biscuits, uniform color was obtained when these components were mixed with the milk before dough formation. The dough was kneaded lightly and rolled. A 3 in. diameter biscuit was cut out, placed on a greased dish and baked for about 15 min in an oven at 260°C. Composite flours were made to 20.0g by addition of wheat flour to 4.0g of original cottonseed flours, 4.0g of petroleum ether extracted flours, 3.6-3.7g of aqueous isopropyl alcohol extracted flours, 0.30-0.35g of aqeuous isopropyl alcohol extracts, 0.29-0.33g of nonflavonoid fractions of extracts, 0.01-0.02g of flavonoid fractions and 0.01-0.03g of rutin. These quantities were based on the proportion in which these components were present in the original cottonseed flours.

Thin-layer chromatography

Flavonoids in the aqueous isopropyl alcohol extract and in the fractions separated from these extracts were analyzed by two-dimensional thin-layer chromatography (TLC) on precoated cellulose plates (glass, 20 x 20 cm, 0.1 mm thick). Plates were developed in the first dimension for 10 cm in n-butanol:acetic acid:water (4:1:5, upper phase), air dried and developed for 10 cm in a second dimension with 15% acetic acid. After being dried, the plates were sprayed with 4% aluminum chloride in ethanol and allowed to stand at 25°C to develop color with the flavonoids (Stahl, 1965).

Column chromatography

The 85% aqueous isopropyl alcohol extracts of glandless and LCP cottonseed flours were separated into nonflavonoid and flavonoid fractions on a Sephadex G-15 column (26 mm i.d. and 1m long). Elution with water was carried out at flow rates of 44 ml/hr. The eluent was monitored with an ultraviolet detector at 254 and 280 nm and during one run with a refractive index detector. For preliminary qualitative runs 0.08-0.25g of extract was used, whereas for collection of fractions for biscuit preparations, 1.5-1.6g of extracts was used. For purposes of flavonoid isolation for characterization, two runs were made on a column, 10 cm i.d. and 1m long, with 13g of extract at a flow rate of 250 ml/hr. The flavonoid components of the extract were very strongly adsorbed on the Sephadex G-15. Because of this very slow migration of the flavonoids on the column, faster recovery rates and smaller elution volumes were

obtained when the column flow was reversed after elution of the nonflavonoid fraction.

To isolate individual flavonoids, the flavonoid mixture obtained from the Sephadex G-15 column was further fractionated on a Sephadex LH-20 column with 50% methanol as the eluent. The column was 26 mm i.d. and 1m long and had a flow rate of 57 ml/hr. This column separated the flavonoid mixture into two fractions, one containing two major flavonoids and another with the other major components. These mixtures were separated into individual flavonoids by thick layer chromatography. The individual flavonoids isolated from the thick layer plates were further purified by Sephadex LH-20 column chromatography with 50% methanol as the eluent.

Thick-layer chromatography

Cellulose used for thick-layer separations was prewashed with methanol, 50% methanol, and water. Plates spread with a slurry of the washed cellulose at 0.75 mm thickness were allowed to air dry 2-3 days. The plates were streaked with the flavonoid mixtures isolated from the Sephadex LH-20 column and developed in 15% acetic acid for 18.5 cm. Flavonoids, visible under long ultraviolet light as dark purple absorbing bands, were scraped from the plates and extracted from the cellulose with 50% methanol. Flavonoids isolated from these plates were rechromatographed on thick-layer plates with either the same solvent or the n-butanol:acetic acid: water solvent system until two-dimensional TLC plates indicated that they were of satisfactory purity.

Flavonoid characterization

Flavonoid spectra from 500 to 200 nm were determined in spectral grade methanol solutions with a Beckman model 25 spectrophotometer. The methods of Mabry et al. (1970) were used for diagnostic analysis of the flavonoids in methanol solutions. These methods involve spectra determination after addition of (1) three drops of 1.09N sodium methoxide, (2) six drops of 5% aluminum chloride, (3) three drops of 21.3% hydrochloric acid to the $AlCl_3$ containing solution, (4) solid sodium acetate, and (5) solid boric acid to the solution containing sodium acetate.

Purified flavonoids (0.3 mg) were hydrolyzed in 2N hydrochloric acid (3 ml) for 0.5 hr in a boiling water bath. The hydrolyzate was taken to dryness in a rotary evaporator and redissolved in methanol. Aglycones were identified by TLC on silica gel plates with chloroform:acetic acid:water (50:45:5) as the developer. The plates were sprayed with alcoholic aluminum chloride and observed under long ultraviolet light. Commercially available standard aglycones were used for comparisons. Sugar components of the hydrolyzate were identified by TLC on cellulose plates with ethyl acetate: pyridine:water (12:5:4) as the solvent system. The plates were sprayed with aniline phthalate and heated 10 min at 110 °C for color development. Standard sugars were used to identify the unknowns.

RESULTS & DISCUSSION

BISCUITS prepared with 80% wheat and 20% LCP or glandless cottonseed flours were yellow-brown (Fig. 1). The biscuit containing LCP flour was considerably darker (L = 45.7) than that with glandless flour (L = 57.5). Both cottonseed flours caused a yellow color (b = 20-25), which, based on previous studies with LCP flour (Blouin and Cherry, 1980), is believed to be caused by flavonoids. To test this hypothesis, experiments were conducted to extract the flavonoids from the flours with 85% aqueous isopropyl alcohol. Before removal of flavonoids, the flours were extracted with petroleum ether to remove residual lipid that could interfere with the pigment evaluation. Extraction of lipids (0.3-0.6%) in the flours did not significantly alter the color of the biscuits (Fig. 1).

Biscuits prepared with the LCP and glandless cottonseed flours, after extraction with aqueous isopropyl alcohol to remove flavonoids, were brown (Fig. 2). Biscuits prepared with appropriate quantities of the aqueous isopropyl extract (equivalent to that in the original flours) were yellow (Fig. 2). Two-dimensional TLC of the alcoholic extracts of LCP and glandless cottonseed flours showed the presence of at least six major flavonoids (Fig. 3). Other TLC staining techniques showed that these extracts contained considerable amounts of nonflavonoid components, including sugars, free amino acids and polar lipids.

The aqueous isopropyl alcohol extracts of LCP and glandless flours were separated into nonflavonoid (95%) and flavonoid (5%) fractions on a Sephadex G-15 column. The nonflavonoid fraction is eluted first and gives normal gel-permeation separations of components as illustrated by the ultraviolet and refractive index monitored elution curve

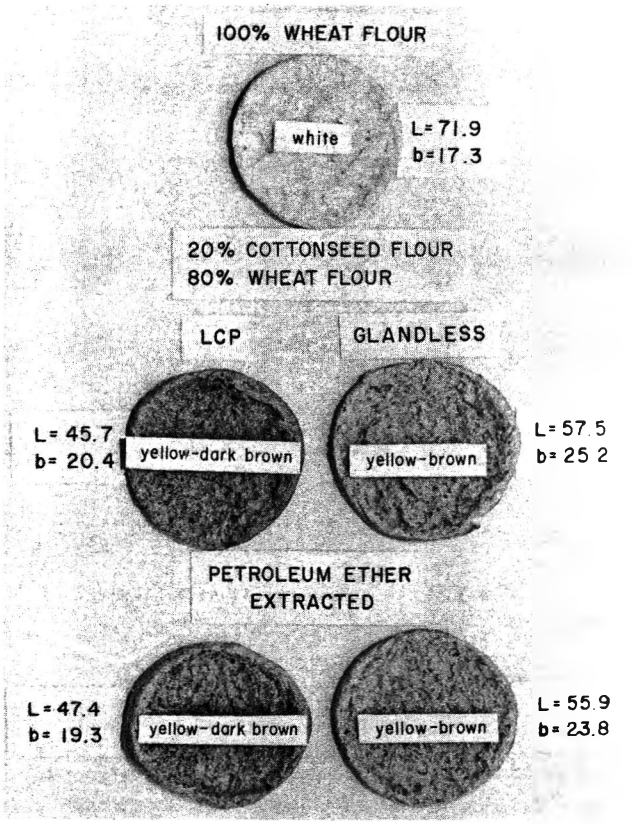


Fig. 1—Biscuits containing 100% wheat flour, 20% LCP and glandless cottonseed flours and 20% LCP and glandless cottonseed flour after extraction with petroleum ether.

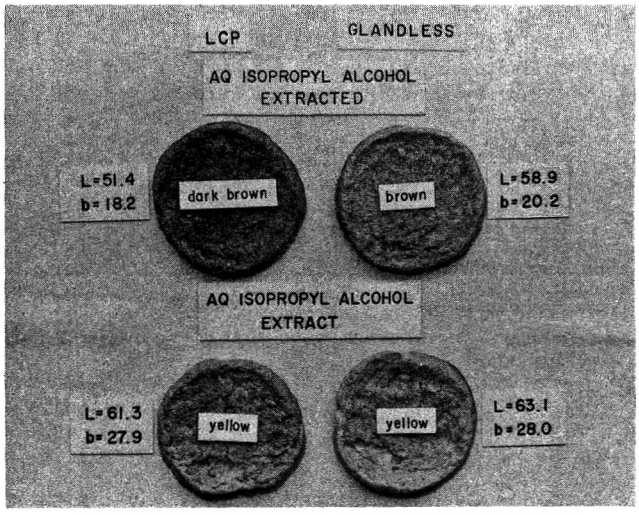


Fig. 2—Biscuits containing 18.0% LCP and 18.5% glandless cottonseed flours after extraction with 85% aqueous isopropyl alcohol and extracts isolated from these flours, 1.8% and 1.5%, respectively.

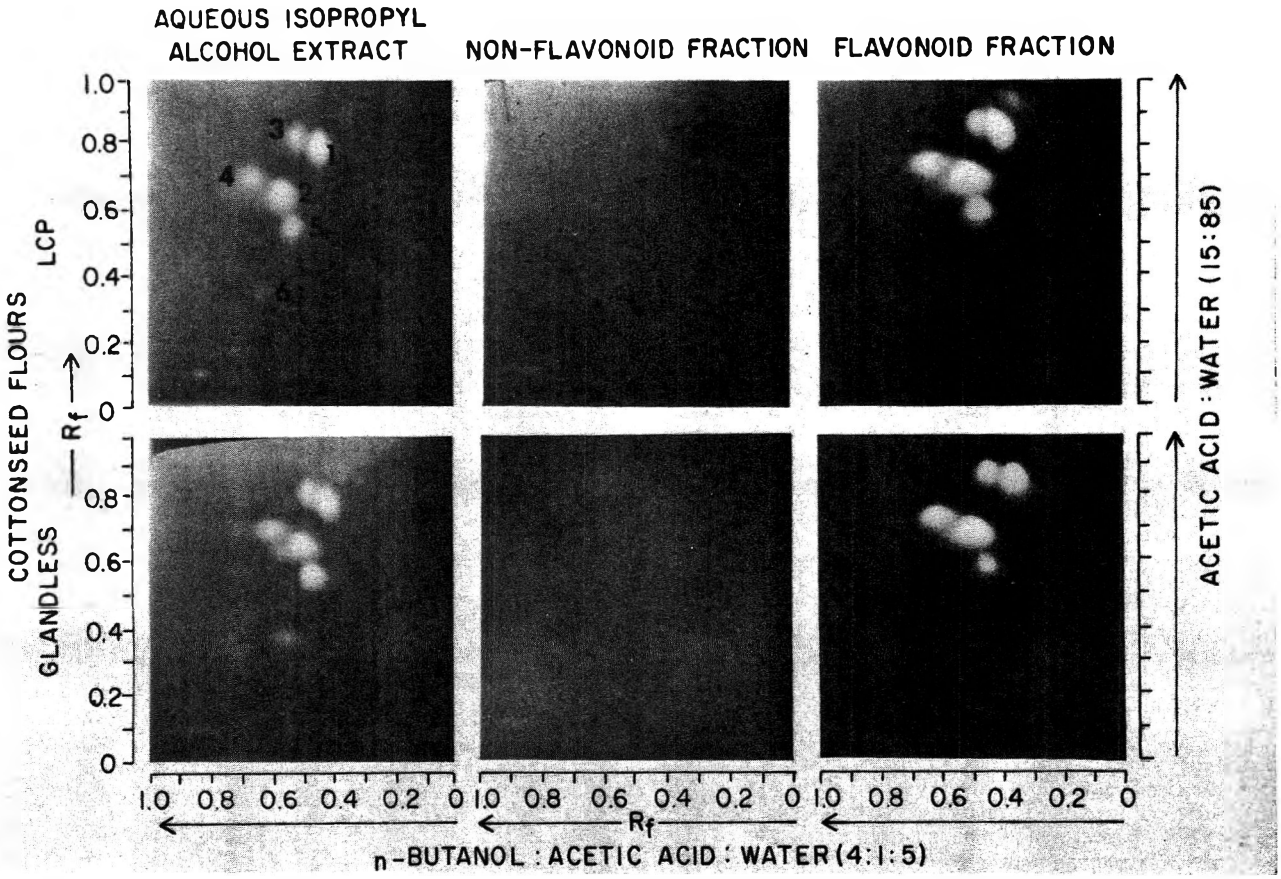


Fig. 3—Two-dimensional cellulose TLC of flavonoids in aqueous isopropyl extracts, nonflavonoid fractions of extracts, and flavonoid fractions of extracts of LCP and glandless cottonseed flours.

(Fig. 4). The first flavonoid components eluted from the column (tube 45) are minor flavonoids. The major flavonoids are strongly adsorbed on the column and elute as broad overlapping bands (starting approximately at tube 132) in a large volume of eluent. In subsequent column runs, the solvent flow was reversed after elution of the nonflavonoid fraction and the flavonoids were eluted as a single peak of smaller total volume. Figure 3 illustrates two-dimensional TLC plates of the nonflavonoid and flavonoid fractions of LCP and glandless flours. These plates can be compared to those obtained with the original extracts, also in Figure 3. Concentrations of the nonflavonoid fractions used on the plates were equivalent to those used on the plates of the total extract and therefore show that removal of flavonoids by the Sephadex G-15 column method was effective. The TLC plates of the flavonoid fractions show more clearly than the plates of the total extract that numerous minor flavonoid components were present in the cottonseed flours.

Biscuits prepared with the nonflavonoid fractions of LCP and glandless flours were tan and near white, respectively (Fig. 5). Those prepared with the flavonoid fractions were yellow. These biscuits and those presented in Figures 1 and 2 clearly demonstrate that the yellow color in biscuits prepared with both types of cottonseed flour is caused by the flavonoids.

Previous studies by Blouin and Cherry (1980) on the flavonoids in LCP cottonseed flour indicated that four of the major flavonoids were 3-0-glycosides of quercetin. Rutin, a comercially available compound, is a 3-0-glucoside of quercetin in which the 6-position of the D-glucose is substituted with an α-L-rhamnosyl residue. Biscuits

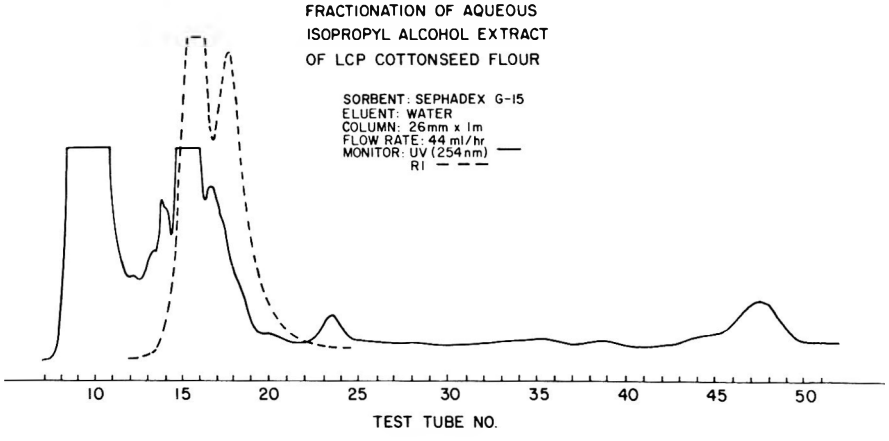


Fig. 4—Sephadex G-15 column fractionation of aqueous isopropyl alcohol extract of LCP cottonseed flour.

prepared with rutin at the 0.05, 0.10 and 0.15% level are yellow (Fig. 6). The color of biscuits caused by rutin is identical in hue and similar in yellow intensity to that of biscuits containing the isolated cottonseed flavonoid fractions.

The flavonoid fraction isolated from LCP flour was separated into two fractions on a Sephadex LH-20 column with 50% methanol as the eluent. The first fraction contained a mixture of flavonoids 1, 3 and some minor flavonoids (see Fig. 3 for numbering designation). The second fraction contained flavonoids 2, 4, 5, and 6. These flavonoids were separated from one another by cellulose thick-layer chromatography. After two or more thick-layer fractionations, the six flavonoids seemed to be pure, based on two-dimensional TLC methods. In a final purification step, the individual flavonoids were passed through the Sephadex LH-20 column with 50% methanol as the eluent. This chromatographic step also indicated that flavonoids 2, 3, 4, and 6 were pure. Flavonoid 5, however, was separated on the column into two components, and designated as 5A and 5B. Flavonoid 1 was fractionated into three components by the Sephadex LH-20 column; only one of these components, the major one of the three (designated 1), was obtained in sufficient purity for further characterization.

The seven purified cottonseed flavonoids exhibited ultraviolet-visible spectra characteristic of flavonols with Band I and II maxima as indicated in Table 1. Diagnostic spectral bandshifts according to the methods of Mabry et al. (1970) and presented in Table 1, indicated that flavonoids 1, 2, 5A, 5B, and 6 are 3-0-substituted quercetin derivatives and that flavonoids 3 and 4 are 3-0-substituted kaempferol derivatives.

TLC of the aglycones after acid hydrolysis of the flavonoids also showed that flavonoids 1, 2, 5A, 5B and 6 are quercetin and 3 and 4 are kaempferol derivatives (Fig. 7). The sugar moieties obtained by acid hydrolysis of the flavonoids were identified by TLC (Fig. 8). This analysis indicated that flavonoids 2, 4, and 6 contained glucose, that flavonoids 1, 3, and 5A contained glucose and rhamnose and that flavonoid 5B contained galactose and rhamnose. Chromatographic mobility suggests that flavonoid 6 is a monosaccharide derivative, whereas the others are disaccharides.

Flavonoid 6 migrates on two-dimensional TLC plates to the same position as isoquercitrin prepared by partial hydrolysis of rutin. The presence of isoquercitrin in cottonseed agrees with the work of Pratt and Wender (1959).

Flavonoids 5A, 5B and rutin migrate on two-dimensional TLC plates to the same position. Because 5A contains glucose and rhamnose, it is identified as rutin, a result that also agrees with the findings of Pratt and Wender (1959). Flavonoid 5B is identified as a quercetin 3-0-robinoside (6-0-α-L-rhamnosyl-D-galactoside).

Flavonoids 1 and 3 are tentatively identified as quercetin and kaempferol 3-0-neohesperidosides (2-0-α-L-rhamnosyl-D-glucosides). This assignment is based on their composition and on the NMR positions of the H-1 rhamnosyl protons at 5.2-5.3 ppm as determined by the methods of Mabry et al. (1970). Pratt and Wender (1961) reported the presence of kaempferol 3-0-rhamnoglucoside in cottonseed.

Flavonoids 2 and 4 are believed to be quercetin and kaempferol 3-0-glucoglucosides, but no information has yet been obtained on the type of disaccharide linkage. Pratt and Wender (1961) reported the presence of a quercetin 3-0-glucoglucoside in cottonseed.

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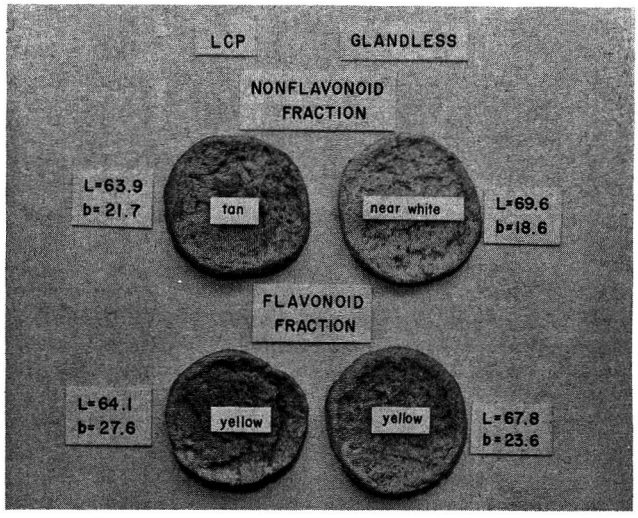


Fig. 5—Biscuits containing the nonflavonoid (1.7% and 1.4%) and flavonoid (0.1% and 0.1%) fractions of the aqueous isopropyl alcohol extracts of LCP and glandless cottonseed flours, respectively.

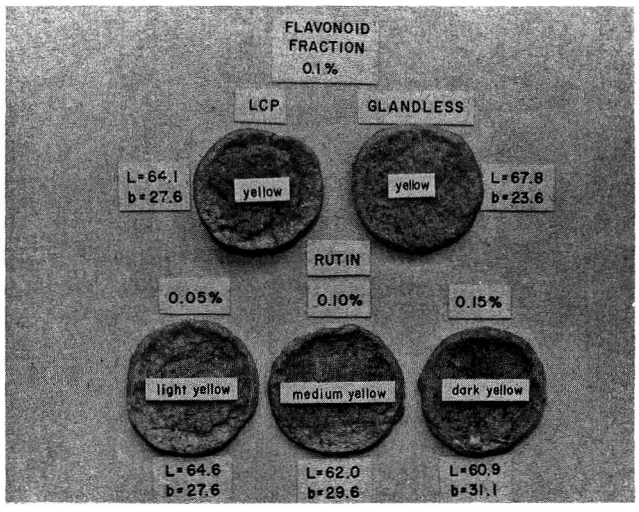


Fig. 6—Biscuits containing flavonoid fractions of LCP and glandless cottonseed flours and rutin (3-0-glycoside of quercetin).

Table 1—Ultraviolet-visible spectral data on flavonoids isolated from LCP cottonseed flour

Flavonoid designation	λ max, nm (MeOH) Ia	Ib (sh)	IIa (sh)	IIb	Diagnostic band shifts, nm Ia (NaOMe −MeOH)	Ia ($AlCl_3$/HCl −MeOH)	Ia ($AlCl_3$/HCl −$AlCl_3$)	IIb (NaOAc −MeOH)	Ia (NaOAc/H_3BO_4 −MeOH)
1	355	297	265	257	48	45	−33	13	16
2	356	296	265	257	48	42	−35	17	17
3	345	322	297	267	48	49	−5	7	3
4	349	318	297	267	45	44	−2	7	0
5A	360	297	266	258	50	38	−33	13	18
5B	363	298	266	258	47	39	−31	14	15
6	358	298	266	258	70	39	−36	15	18

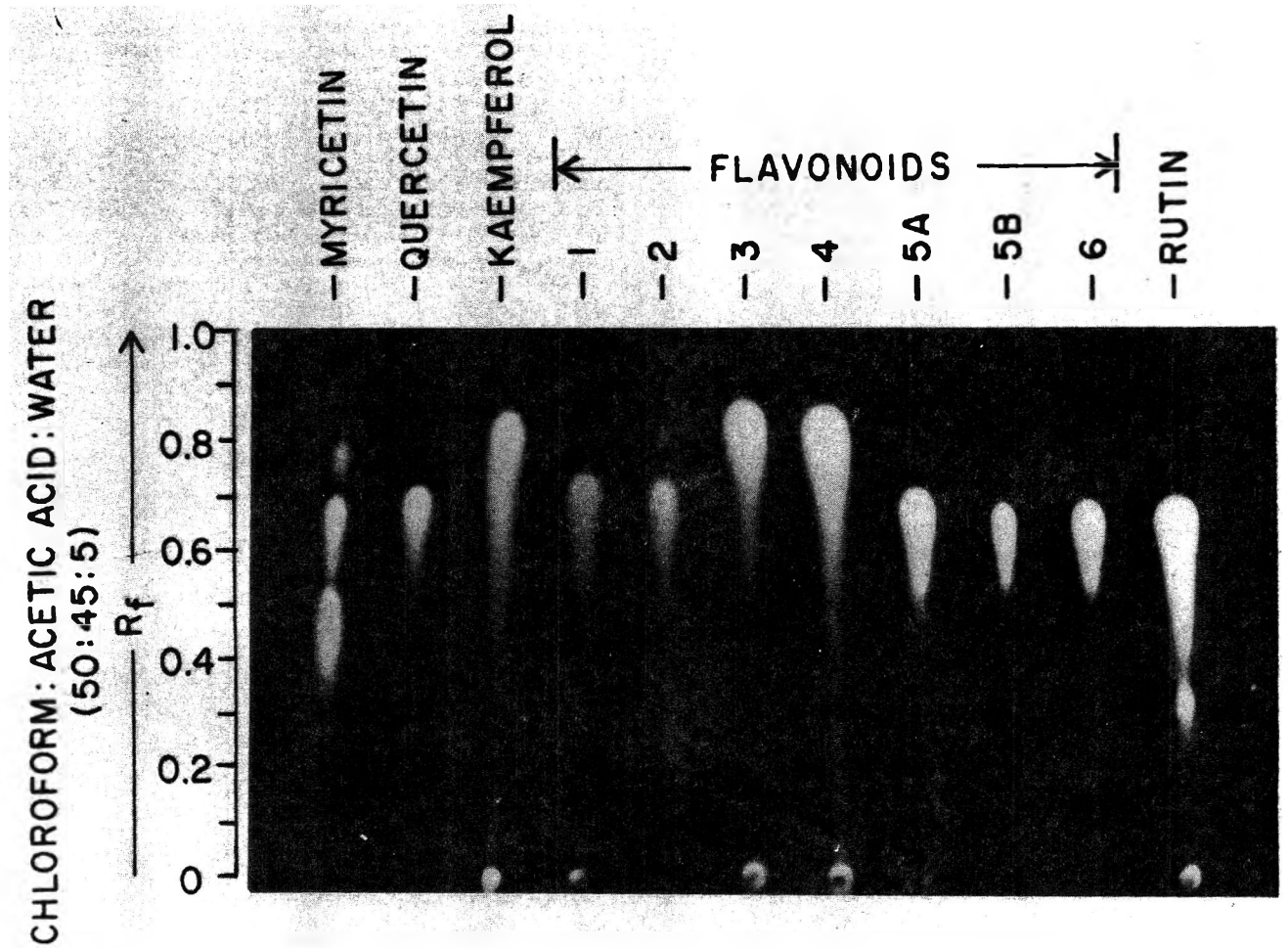


Fig. 7—TLC of aglycones fro acid hydrolyzed cottonseed flavonoids.

CONCLUSIONS

THE YELLOW COLOR in biscuits prepared with wheat and LCP or glandless cottonseed flours is caused by cottonseed flavonoids: (1) Biscuits prepared with flour from which the flavonoids have been removed by extraction with aqueous isopropyl alcohol are brown and biscuits prepared with the alcohol extract are yellow; (2) Biscuits prepared with the nonflavonoid fractions of the aqueous alcohol extracts are tan, whereas those prepared with the flavonoid fractions are yellow; (3) Biscuits prepared with rutin, a flavonoid of the type present in cottonseed flours, have a yellow color identical in hue and similar in intensity (at approximately equal concentrations) to the yellow color obtained with biscuits prepared with cottonseed flavonoids.

The following information on cottonseed flavonoids has thus far been obtained. (1) There are seven major and numerous minor flavonoids present in LCP and glandless

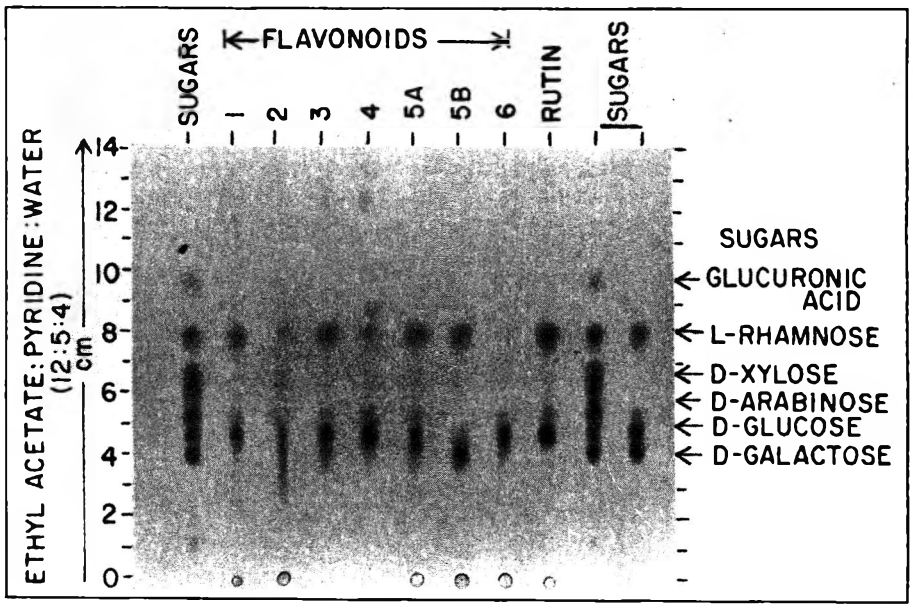


Fig. 8—TLC of sugars from acid hydrolyzed cottonseed flavonoids.

cottonseed flours. (2) The same seven major flavonoids are apparently present in both types of flour. (3) The aglycones of cottonseed flavonoids are quercetin and kaempferol, and the sugar moieties are glucose, galactose and rhamnose. (4) Cottonseed major flavonoids are tentatively identified as isoquercitrin, rutin, quercetin 3-0-robinsoside, quercetin 3-0-neohesperidoside, kaempferol 3-0-neohesperidoside, quercetin 3-0-glucoglucoside, and kaempferol 3-0-glucoglucoside.

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Names of companies or commercial products are given solely for the purpose of providing specific information; their mention does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

EXPERIMENTAL WASHER FOR LEAFY GREENS . . . From page 265

water than occurred in the industrial washers. Similar results were obtained on other greens (Table 7). Again, there were no differences between washing systems. The effluent from washing mustard greens was lower in bacterial count than that from turnip greens. No attempt was made to control the chlorine residual in the wash water, which came from the city water supply.

SUMMARY & CONCLUSIONS

THE EXPERIMENTAL WASHER produced less physical damage to the product as compared to industrial washers. Larger quantities of soluble constituents were leached out by the industrial washers, resulting in higher TSS, TS, SS, COD, and NO_3 in the effluent and lower ascorbic acid and NO_3 in the canned product. There was approximately 70% reduction in water usage by the experimental washer as compared to the industrial washers. Because the experimental washing system produced far less liquid wastes, and the possible advantages of higher nutrient content and quality, food processors should benefit greatly by installing similar washing systems for spinach and leafy greens.

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Effect of Microwave and Conventional Cooking on the Nutritive Value of Colossus Peas (*Vigna uniguiculata*)

S. Y. CHUNG, C. V. MORR, and J. J. JEN

ABSTRACT

The effect of microwave and conventional cooking methods, designed to simulate those used in home meal preparation, upon nutrient retention in Colossus Peas *(Vigna uniguiculata)* was studied. Neither method resulted in significant changes in the fat, protein, β-carotene and ascorbic acid content of the peas. Microwave cooking resulted in significantly greater losses of several amino acids, but resulted in significantly greater retention of thiamin and riboflavin than the conventional treatment. Although each of the mineral components exhibited different magnitudes of loss by the two cooking methods, differences due to method of cooking were not significant. Both Fe and Cu were completely retained in the peas cooked by both methods. Both cooking methods (15-60 min cooking) resulted in 92-97% loss of trypsin inhibitor.

INTRODUCTION

THERE HAS BEEN a widespread increase in the application of microwave processing for both home and institutional meal preparation because of its speed and convenience compared to conventional cooking methods. Microwave cooking results in the simultaneous production of heat throughout the food product when polar molecules, under the influence of rapidly oscillating electromagnetic field (915 or 2,450 MHz) are induced to undergo rapid reorientation, thus, converting electromagnetic energy to thermal energy and resulting in a uniform temperature profile throughout the food. Conventional cooking on the other hand, involves surface absorption of thermal energy from external sources by radiation and conduction, resulting in an uneven temperature profile throughout the product, thus necessitating a longer cooking time to obtain a given internal product temperature.

Theoretically, the heating effect of microwave energy upon various food components could differ significantly from that of conventional cooking. For example, it has been speculated that reactive free radicals might be formed by exposure to microwave energy, especially in those applications that result in abnormally high temperatures, as with frying and toasting. However, Rosen (1972) concluded that microwave radiation does not possess sufficient energy to produce free radicals or to break chemical bonds, as is the case with gamma and X-ray radiations.

Since peas represent a major potential source of protein, mineral and vitamins, it is of interest to determine the relative effect of conventional and microwave cooking on their nutritional quality (Thomas et al., 1949). The present study was also conducted to determine the effects of cooking treatments on Colossus Peas, which are an experimental variety of brown crowder pea and are about twice the size of conventional peas. No attempt was made to distinguish between losses of micronutrients due to leaching and chemical destruction.

Author Morr is with the Dept. of Food Science, Clemson Univ., Clemson, SC 29631. Author Chung, formerly with Clemson Univ., is now with the Dept. of Food Science, North Carolina State University, Raleigh, NC 27650. Author Jen, formerly with Clemson Univ., is now with the Campbell Institute for Research & Technology, Camden, NJ 08101.

MATERIALS & METHODS

Sample treatment

Mature Colossus Peas (*Vigna uniguiculata*) obtained from the South Carolina Agricultural Experiment Station, were hand shelled, blanched and stored in the freezer until studied.

Cooking conditions were selected to simulate those in home meal preparation and to provide comparable doneness, based on chewiness and texture when cut with a knife. One hundred fifty grams of peas were added to 100 ml boiling water in a covered pot and heated to maintain boiling for 12 min. One hundred fifty grams of peas were added to 100 ml water in a covered glass pot, placed in a Model NE 7900 Panasonic Microwave Oven designed to provide 700 watts output and an operational frequency of 2,450 MHz, and subjected to sufficient energy to begin boiling within 30 sec and to maintain boiling for an additional 6 min. Following both cooking treatments, the cooking water was immediately drained and the peas were allowed to cool to room temperature.

Nutrient analyses

Raw and cooked peas were ground in a mortar prior to sampling for chemical and nutrient analyses. Mean analytical values were converted to dry weight basis. These values were obtained from four replicate determinations for all nutrients except protein, amino acids and fat, which were each conducted in duplicate. Comparison of mean values was by Duncan's Multiple Range Test (Steel and Torrie, 1960) at the 5% level for those determinations conducted with 4 replications.

Fat and protein contents were determined by AOAC (1975) methods. Fat content was assayed by the Soxhlet procedure using hexane as the extractant. Protein was determined by macro-Kjeldahl using a nitrogen factor of 6.25.

Vitamins were determined by methods prescribed by the Association of Official Vitamin Chemists (1966) and total ascorbic acid content was determined by the 2,6-dichlorophenolindophenol titration method. Thiamin and riboflavin levels were determined by the thiochrome and fluorometric methods, respectively. β-carotene was recovered on 1.1 x 28 cm columns of 1:1 MgO:supercel and eluted with 1:1 (v/v) acetone: petroleum ether and quantitated spectrophotometrically at 440 nm.

Amino acids were determined after hydrolysis for 22 hr at 110°C in 6N HCl with a Phoenix Model K-8000 automatic amino acid analyzer.

Minerals were determined using a Perkin Elmer Model 303 Atomic Absorption Spectrophotometer after digestion in 2:1 nitric acid:perchloric acid solution.

Trypsin inhibitor levels were determined by the method of Kakade et al. (1974). Fifty ml of 0.01N NaOH were added to 50g raw or cooked peas and blended with a Virtis 45 homogenizer for 3 min. The suspension was centrifuged and filtered and the filtrate diluted sufficiently to inhibit less than 30% of the total trypsin activity of the assay system in order to assure a linear response for quantitating trypsin units inhibited.

RESULTS & DISCUSSION

THE EFFECT of microwave and conventional cooking on fat and protein content of Colossus Peas is shown in Table 1. Conventional cooking produced a slightly greater loss of fat, however, microwave cooking produced a slightly higher protein loss than conventional cooking. However, these differences are probably not statistically significant. Additional experiments are needed to confirm this possibility. Average losses of the 17 individual amino acids (Table 2) were 5.2% for conventional cooking compared to 9.6% for

Table 1—Effect of cooking on nutrients of Colussus Peas[a]

Nutrient	Raw	Cooked	
		Conventional	Microwave
Fat (%)	11.69	10.98	11.52
Protein (%)	22.9	21.8	21.1
Thiamine (μg/g)	2.47[b]	1.52[c](38.5)	1.97[d](20.2)
Riboflavin (μg/g)	4.51[b]	1.89[c](58.0)	2.67[d](40.8)
β-carotene (μg/g)	5.23[b]	5.30[b](+1.3)	5.35[b](+2.3)
Ascorbic acid (mg/g)	124.7[b]	105.1[c](15.7)	110.6[c](11.3)
Ca (mg/g)	3.24[b]	1.72[c](46.9)	1.69[c](47.8)
K (mg/g)	19.56[b]	10.67[c](45.4)	11.27[c](42.3)
Na (mg/g)	2.44[b]	1.23[c](49.6)	1.29[c](47.1)
Mg (mg/g)	2.30[b]	1.69[c](26.5)	1.80[c](21.7)
Zn (mg/g)	0.266[b]	0.166[c](37.6)	0.163[c](39.7)
Cu (mg/g)	0.010[b]	0.009[c](10)	0.010[b](0)
Fe (mg/g)	0.088[b]	0.086[b](2.2)	0.087[b](1.1)
Mn (mg/g)	0.019[b]	0.015[c](21)	0.016[c](15.8)

[a] Dry weight basis. Values in parenthesis are percent losses.
[b,c,d] Means with different superscripts are significantly different at the 5% level.

microwave. Microwave processing was especially destructive of His, Gly, Cys, and Met, which averaged 13.7% in the aggregate compared to only 6.2% destruction by conventional processing. These latter findings suggest that microwave energy may be capable of promoting chemical alteration of the chemical side chains on these particular amino acids. Again, further work is needed to elucidate the details of this possibility.

Microwave processing resulted in significant ($p < 0.05$) losses of thiamin and riboflavin, but these losses were significantly smaller than those obtained with conventional cooking (Table 1). These losses are probably due to a combination of leaching and chemical destruction. The improved retention of these two vitamins by microwave processing may be due to the shorter cooking time compared to conventional cooking Kahn and Livingston, 1970). Both cooking methods produced similar losses of ascorbic acid, but no difference in the β-carotene content of processed peas. This was reported previously by Jonnelle and Sistrunk (1979) for conventional peas and may be due to improved extractability of the β-carotene following cooking. The present findings for ascorbic acid losses are in agreement with those of others (Kylen et al., 1961; Eheart and Gott, 1964), but are at variance with those of Chapman et al. (1960) and Mabesa and Ballwin (1979). It is recognized that relative volumes of water and cooking conditions as well as variations in oxygen content, catalysts and other factors are likely responsible for these apparent differences.

Both conventional and microwave cooking processes resulted in similar (42-50%) losses of the major minerals Ca, K, and Na, and both resulted in similar, but smaller (22-26%) losses of Mg from peas. These findings indicate (Table 1) that Mg is probably bound or otherwise rendered less accessible for leaching into the cooking water than are the other major minerals. The similarity in the extent of major mineral losses for both processes does not agree with those indicated above for proteins and amino acids (Table 2), in which microwave processing resulted in greater losses, and further, they tend to contradict the theory of increased cell membrane disruption by microwave energy compared to conventional cooking.

Among the minor minerals (Table 1), substantial and comparable amounts of Zn and Mn were lost, but with no significant differences in losses due to cooking method employed. Both Cu and Fe were either totally bound to insoluble cellular components or were otherwise rendered unavailable for leaching into the cooking water by both processes.

Both conventional and microwave cooking methods employed in these experiments destroyed or otherwise removed >92% of the trypsin inhibitor content of the peas

Table 2—Effect of cooking on amino acid content of Colossus Peas[a]

Amino acid	Conc, g/100g dry wt		
	Raw	Cooked	
		Conventional	Microwave
Lys	1.82	1.72(5.5)	1.68(7.7)
His	0.86	0.80(7.0)	0.75(12.8)
Arg	1.41	1.35(4.2)	1.28(9.2)
Asp	2.94	2.76(6.1)	2.67(9.2)
Glu	4.46	4.20(5.8)	4.05(9.2)
Thr	1.04	0.98(5.8)	0.94(9.6)
Ser	1.41	1.35(4.2)	1.28(9.2)
Pro	1.09	1.05(3.7)	1.01(7.3)
Gly	0.93	0.89(4.2)	0.83(10.7)
Ala	1.09	1.04(4.6)	1.00(8.2)
Cys	0.11	0.10(9.1)	0.09(18.2)
Val	1.31	1.26(3.8)	1.20(8.4)
Met	0.23	0.22(4.3)	0.20(13.0)
Ilu	1.16	1.09(6.0)	1.07(7.8)
Leu	2.13	2.01(5.6)	1.98(7.0)
Tyr	0.74	0.70(5.4)	0.68(8.1)
Phe	1.53	1.47(3.9)	1.40(8.5)

[a] Values in parenthesis are percent losses.

Table 3—Effect of cooking on trypsin inhibitor content of Colossus Peas

Treatment	Cooking time (min)	Conc, TUI/mg[a]		Loss, %	
		Conventional	Microwave	Conventional	Microwave
Raw	0	11.60	11.60	0	0
Cooked	15	0.84	0.63	92	94
Cooked	30	0.67	0.41	94	96
Cooked	60	0.54	0.34	95	97

[a] Dry weight basis

upon heating for 15 min (Table 3). Microwave processing produced a slightly higher percent of trypsin inhibitor destruction than conventional cooking at each time interval. It would be expected that heating Colossus peas to obtain acceptable doneness, as indicated in the Procedure Section, would destroy ≥90% of the trypsin inhibitor.

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Factors Influencing Softening of Salt-Stock Pickles in Air-Purged Fermentations

KAREN GATES and RALPH N. COSTILOW

ABSTRACT

Cucumber fermentations in 1-gal glass jars were purged with air at high rates to study factors influencing softening of the cucumbers and treatments which might prevent softening. The cucumbers were softened much more rapidly and extensively in fermentations purged at 100 ml air/min than at 20 ml air/min. This could not be prevented by initial acidification (up to 0.6% acetic acid) nor by brining at high brine strength (up to 35° salometer). No soft pickles were found in air-purged lots treated in any of the following ways: (a) acidified and treated with hypochlorite and then buffered and inoculated with *Lactobacillus plantarum* (controlled fermentation process); (b) added 0.035% potassium sorbate; (c) used nitrogen for purging for first two days and then air-purged; and (d) the cucumbers were blanched and inoculated by washing unblanched cucumbers with the brine used to cover them. Pectinolytic enzyme assays were run on most fermentation brines, but enzyme activities were not well correlated with the extent of softening. The results indicate that softening of air-purged cucumbers is caused by the growth of some oxygen-dependent, sorbic acid-sensitive microorganism(s) growing in or on the cucumbers. The first day or two after brining appears to be the most critical in the development of the causative organism(s).

INTRODUCTION

When commercial cucumber fermentations are purged with air to prevent bloater (hollow pickle) formation, some soft pickles are found in an occasional tank (Costilow et al., 1981; Switzer, 1980). The softened salt-stock pickles are consistently found at the top of the tank directly under the purger outlet. The amount of stock affected has consistently been restricted to a few bushels per tank and the softening has not spread through the tank. Fleming et al. (1975) reported that continuous air-purging of cucumber fermentations in 5-gal pails resulted in softening and bleaching of pickles. However, the air flow rates per unit volume of the brine was very high in the pails as compared to rates used for commercial tanks. No reason(s) has been found for the sporadic occurrence of softening in commercial fermentations. Therefore, these experiments were designed to study factors which influenced softening in fermentations purged at high air flow rates, and to investigate possible procedures to prevent such spoilage.

EXPERIMENTAL

Cucumber fermentations

Unless stated otherwise, the cucumbers used were size 3A (1.5-1.75 in. diam) obtained from the Green Bay Food Co., Eaton Rapids, Mich. They were brined in 1-gal glass jars. Each jar contained a new gas sparger (3/4 in. diam x 2.5 in. long) made of ultra high molecular weight polyethylene (10-20 μm pore size) which was weighted on the bottom of the jar. A 1-in. wide strip of semi-rigid polyethylene was wedged under the neck of the jar to keep the cucumbers submerged. Brines of the salometer desired after equilibration with cucumbers were used to cover the cucumbers, and the dry salt required to maintain this salometer added in three equal amounts over a 7-day period. Other additions were as indicated in the Results. Procedures for the controlled fermentations were those of Etchells et al. (1973). Unless otherwise stated, all fermentations were incubated for 2 wk under ultra-violet light at room temperature (25-30°C), and purged with air continuously at ∿20 ml/min. Gas flow rates were controlled with flow meters (Brooks Instrument Division, Emerson Electric Co., Hatfield, Pa.) having maximum flow rates of 146 ml/min.

Authors Gates and Costilow are with the Dept. of Microbiology & Public Health, Michigan State Univ., East Lansing, MI 48824.

Analyses

Yeast populations in brines were estimated by plating serial dilutions on dextrose agar (Difco) acidified with 2 ml of 5% tartaric acid per 100 ml. Acidity was determined by titration with standard base, and pH measured with a Corning pH meter. Dissolved oxygen was determined as described in a companion paper (Costilow et al., 1981).

Pectinolytic enzyme activities were determined on brine samples by the general procedure of Bell et al. (1955). However, viscosity measurements were all made with an Ostwald pipette having an upper bulb capacity of ∿3.8 ml. The flow time for water through these pipettes was about 1 min. A 1 ml dialyzed brine sample and 4 ml sodium polypectate solution were added to each pipette. The percent loss in viscosities were all determined after 20-22 hr incubation at 30°C. Due to variability in flow times, values less than 10% loss of viscosity are of questionable significance.

All of the pickles were removed from each jar and pressure tested with a USDA fruit pressure tester (5/16 in. tip) (Bell and Etchells, 1961) 14 days after brining unless stated otherwise. They were then sliced lengthwise, and the percent cure estimated. The latter was based on the percentage of the tissue which had lost its white color.

RESULTS

Effect of aeration rate and initial acidification

As expected, the rate of aeration had pronounced effects on the development of acid fermentation and the rate and extent of softening of air-purged cucumbers (Table 1). Practically all of the cucumbers purged at 100 ml air/min were very soft after 7 days of fermentation, and there was little or no acid development. Those purged at 20 ml air/min all underwent some acid fermentation, and 47-89% of the cucumbers were still reasonably firm after 14 days fermentation.

It is necessary to acidify cover brines to a pH less than 5.0 in order to remove carbon dioxide rapidly during the first day or two of fermentation. This would also be expected to delay development of some aerobic bacteria which could cause softening. However, it would not be expected to influence the growth of most molds and yeasts. Results of these experiments (Table 1) demonstrate that acidification with up to 0.06% acetic acid will not prevent softening. Acid development was highest and the least softening was encountered in the fermentation which was not acidified and was purged at 20 ml/min. However, less extensive softening occurred in fermentations acidified with 0.06% than in those with 0.02 and 0.04% acetic acid.

Measurements of dissolved oxygen levels (mg O_2/liter brine), microbial activities, and pectinolytic enzyme(s) activities were also made on the fermentations in Table 1. The average dissolved oxygen levels in the four jars purged

at 100 ml/min were much higher during the first 2 days of fermentation than the average levels in those purged at 20 ml/min (Fig. 1A). However, after 3 days, no O_2 was detectable in the brines purged at 100 ml/min. This was undoubtedly due to the development of very high yeast populations in these fermentations (Fig. 1B). The yeast populations were over 10-fold higher in the brines purged at the higher rate. A similar relationship was observed by Potts and Fleming (1979).

Pectinolytic activity was very slow to develop in the nonacidified fermentation purged at 20 ml/min (Fig. 2). None was detected until after 8 days of fermentation. In contrast, enzyme activity developed steadily after the third day in the other three fermentations purged at this rate. The activities in the other two brines were very similar to that shown in Figure 2 for the brine acidified with 0.04% acetic acid. Correlated with the slow development of pectinolytic activity in the nonacidified brine was an initial rapid drop in dissolved oxygen level. After 24 hr, no oxygen was detectable in this brine while the other three fermentations purged at 20 ml/min had levels near 3 mg/liter.

Pectinolytic activity developed rapidly in three of the brines purged at 100 ml/min (Fig. 2). However, no significant level of enzyme was detected in the brine acidified at 0.06% acetic acid during 6 days of fermentation even though extensive softening of the cucumbers occurred (Table 1).

Effect of salt concentration

Cucumbers brined to equilibrate at salt concentrations between 20° and 35° salometer underwent extensive softening during air purging for 2 wk (Table 2). Brines in three of the fermentations developed very high pectinolytic enzyme activities. However, the 35° salometer brine had only a marginal level of activity even though many of the cucumbers in the brine were very soft.

Air purged cucumbers appear to cure (tissue becomes opaque) rapidly. Those in brines aerated at 100 ml/min (Table 1) were cured within 7 days of fermentation, and those in brines up to 25° salometer and aerated at 20 ml/min in Tables 1 and 2 were cured within 14 days. However, high brine strength did delay cure (Table 2).

Effect of initial limitation of oxygen

As noted above in the discussion of the data in Table 1, when the dissolved oxygen level dropped to a low level within 1 day after brining, little or no softening occurred. Many oxygen requiring microbes will die rather quickly in the absence of oxygen. Therefore, experiments were conducted to determine the effects of limiting oxygen early in the fermentation.

Cucumbers from brines purged with nitrogen the first 2, 3, or 4 days followed by air purging until 14 days of fermentation were all firm in texture (Table 3). The extent of softening was reduced by purging with nitrogen for 1 day, but 25% of the cucumbers were seriously affected. In another series of fermentations at different brine strengths (Table 4), there was essentially no softening in fermentations which had been nitrogen purged for even 1 day before purging with air. No soft pickles were found in brines which had been initially purged with nitrogen for 2 days.

It was not possible to correlate pectinolytic enzyme activities in the brines with the extent of softening observed in these experiments (Table 3 and 4). The 35° salometer brine purged continuously with air (Table 4) had only marginal

Table 1—Effect of acidification and of aeration rates on acid development and softening (pressure test) of brined cucumbers[a]

Acid added (% Acetic)	Aeration rate (ml/min)	pH 1 day	pH Final	Maximum acidity (% Lactic)	Pressure test (lb)
None	20	6.2	3.5	0.50	17.2 (11%)[b]
	100	6.7	4.5	0.13	2.6 (93%)
0.02	20	5.8	3.8	0.26	7.2 (58%)
	100	5.8	4.7	0.06	4.1 (93%)
0.04	20	4.9	3.9	0.36	8.4 (53%)
	100	4.9	5.4	0.04	5.5 (86%)
0.06	20	4.7	3.8	0.35	15.8 (25%)
	100	4.5	3.8	0.23	7.8 (67%)

[a] Cucumbers aerated at ∿20 ml/min were evaluated after 13 days fermentation; those aerated at ∿100 ml/min were evaluated after 7 days. All cucumbers had a completely cured appearance when evaluated.
[b] Numbers in parentheses represent the percentage of all cucumbers in the fermentation which were severely soft (<11 lb pressure test).

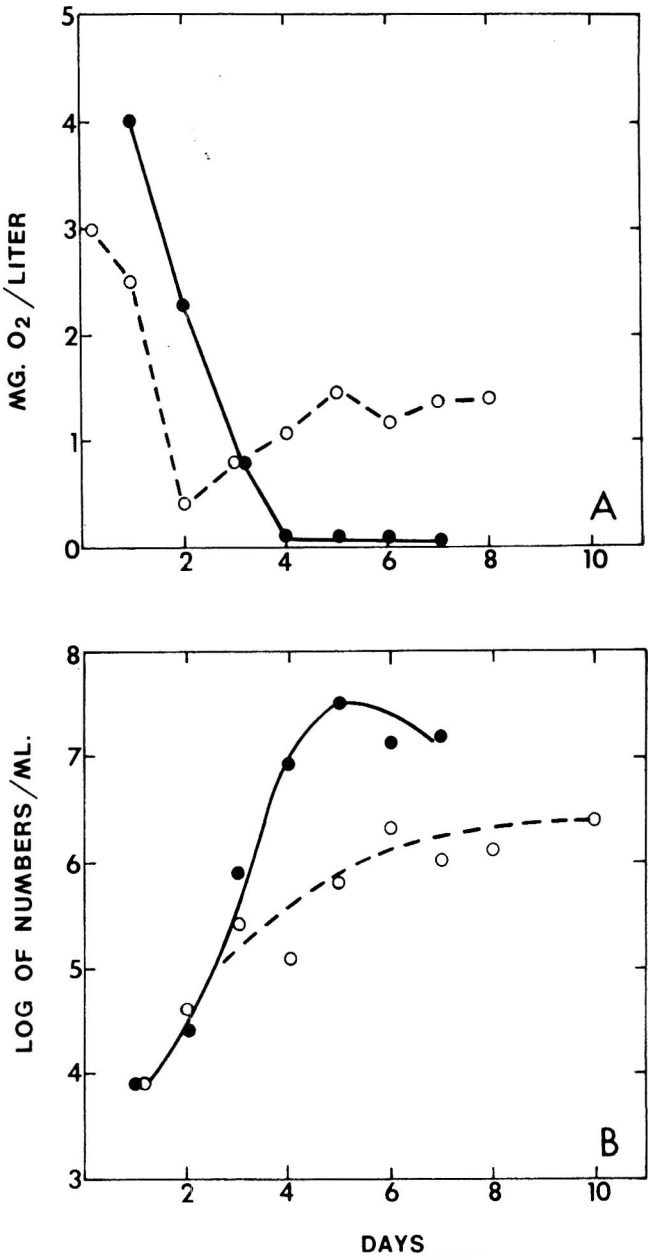


Fig. 1—Average dissolved oxygen levels (A) and average yeast counts (B) in salt-stock pickle brines. See Table 1 for description of the fermentations. Solid lines represent the averages of values for the four fermentations purged at 100 ml air/min, and dashed lines the average values for the four purged at 20 ml air/min.

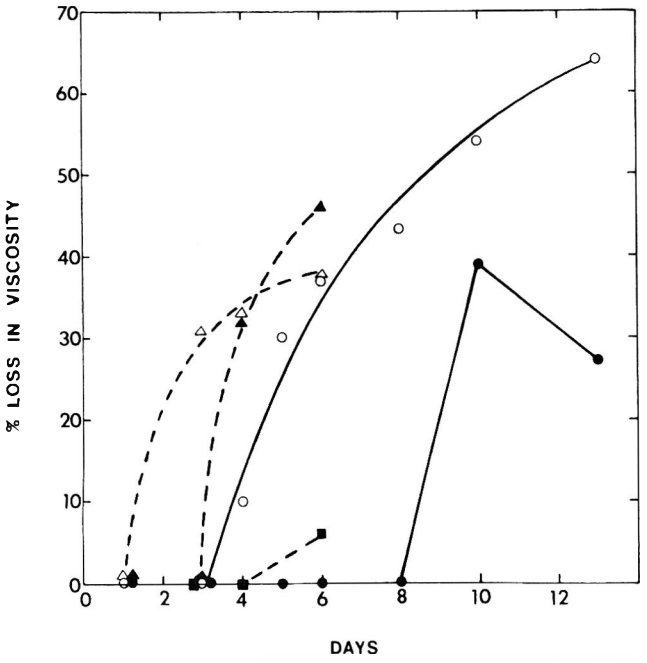


Fig. 2—Effect of aeration rate and of initial acidification on the development of pectinase activities in cucumber brines. Symbols: Dashed lines, 100 ml air/min; solid lines, 20 ml air/min; ●, ▲, no acid added; ○, △, 0.04% acetic acid added; ■, 0.06% acetic acid added.

Table 2. Effect of salt concentration on the development of acid and pectinase activity in the brine, and on softening and extent of cure of brined cucumbers.[a]

Brine-°Salometer	pH		Maximum acidity (% Lactic)	Final pectinase activity (% Δ viscosity	Pressure test (lb)	% cure
	1 day	Final				
20	4.5	3.4	0.55	69	11 (56%)[b]	100
25	4.5	3.5	0.43	53	11 (44%)	100
30	4.5	4.0	0.17	71	5 (76%)	80
35	4.5	3.6	0.26	17	8 (59%)	20

[a] All fermentations were acidified initially with 0.06% acetic acid, and were purged continuously with air at a flow rate of ∿20 ml/min.
[b] Numbers in parentheses represent the percentage of all cucumbers in the fermentation which were severly soft (<11 lb pressure test).

Table 3—Effect of initial nitrogen purging on the development of acid and pectinase activity in the brine, and on softening and curing of brined cucumbers[a]

Treatment	Final pH	Maximum acidity (% Lactic)	Final pectinase (% Δ viscosity)	Pressure test (lb)	% cure
Nonpurged[b]	3.9	0.17	3	16 (6%)[c,d]	15
Continuous air[b]	4.4	0.08	22	6 (86%)	70
N_2 – 1 day	3.6	0.29	16	15 (25%)	99
N_2 – 2 days	3.5	0.27	31	22 (0%)	95
N_2 – 3 days	3.3	0.49	13	18 (0%)	85
N_2 – 4 days	3.4	0.43	19	20 (0%)	85

[a] Cucumbers were brined to equilibrate at 25° salometer and 0.04% acetic acid. All fermentations were aerated continuously at ∿20 ml/min except where indicated.
[b] These two fermentations developed heavy surface scums of yeast because they were not protected by ultraviolet light.
[c] Numbers in parentheses give the percentages of all cucumbers which were seriously soft (<11 lb pressure test).
[d] There was one very soft cucumber near the surface of the brine.

enzyme activity but 20% of the pickles had pressure tests less than 11 lb. In contrast, the 30° salometer brines purged with nitrogen for the first 2 days had reasonably high pectinase levels (46-59%) but none of the pickles was soft.

The effect of air purging on curing is evident in Table 3 when compared to a nonpurged control. Failure of the smaller sized cucumbers used in the experiments reported in Table 4 to cure rapidly is not known.

Effect of controlled fermentation and of potassium sorbate

Pickles from a controlled fermentation which was brined and treated as described by Etchells et al. (1973) but purged constantly with air were all firm (Table 5). In addition, no softening enzyme was detectable in this brine after 2 wk fermentation. The results were exactly the same when 0.035% potassium sorbate was added and a natural fermentation allowed to develop. As the concentration of potassium sorbate was lowered, more extensive softening occurred and higher pectinolytic activities were observed. A fermentation which had not been purged for 1 day (static), and another which had been held static 6 h each day served as controls in this experiment (Table 5). Both of these fermentations contained some soft cucumbers and significant pectinase activities.

Effect of blanching fresh cucumbers

Cucumbers which had been blanched at 65°C for 5 min before brining did not show significant softening during 2 wk air purging (Table 6). This was true even though the cover brine was inoculated by washing unheated cucumbers. Two of three nonblanched cucumbers brined in a jar with blanched fruit all became severely soft. Not one of three blanched cucumbers included in a fermentation of nonblanched cucumbers became soft. These data indicate that either the microbe(s) which cause softening of air purged pickles have infected the cucumber tissue prior to brining and do not readily reinfect after brining or blanching the cucumber prevents the development of the microbe(s) in some way.

DISCUSSION

THE RESULTS of these experiments are consistent with the findings that molds growing in brined cucumber tissues are primarily responsible for softening of pickles from air-purged fermentations (Costilow et al., 1980). Thus, any treatment which would destroy viable molds on the fresh cucumber before brining (hypochlorite treatment as used in the controlled fermentation or blanching) or inhibit their development after brining (potassium sorbate) prevented rapid softening. Initial limitation of oxygen by nitrogen purging for at least 2 days before using air probably killed the fungi in or on the brined cucumbers.

The first 1 or 2 days after brining is probably a very critical period for the development of fungi in the cucumbers. Molds growing in tissue would be protected to some extent from inhibitory effects of the brine during this period. Also, there may be considerable air dissolved in the fresh tissues. Large cucumbers were found to contain 45-50 μl gas per gram which was about 23% oxygen (Jorge, 1978). However, the amount of oxygen in the tissue at brining would vary with the way the fruit had been handled. Those from the central areas of large masses of cucumbers would probably have used all of the oxygen in respiration, while those near the surface would have high amounts. This variable could be important in commercial fermentations. Fresh cucumbers in the top few feet of large tanks are frequently not covered with brine for several hours before the brining operation is completed. It would be much safer to cover them with brine promptly when placed in a tank

Table 4—Effect of brine strength and initial nitrogen purging on acid and pectinase activity development in the brine, and on softening and extent of cure of brined cucumbers[a]

Brine-°Salometer (Initial)	Brine-°Salometer (Final)	N_2 purged (Days)	Final pH	Maximum acidity (% Lactic)	Final pectinase activity (% Δ viscosity)	Pressure test (Lb)	% cure
25	25	0	4.7	0.33	43	18 (10%)[b]	50
	24	1	3.5	0.42	28	21 (0%)	60
	32[c]	1	3.4	0.53	27	21 (0%)	60
	24	2	3.5	0.52	0	21 (0%)	60
	33[c]	2	3.4	0.52	16	22 (0%)	70
30	29	0	4.3	0.39	57	19 (5%)	30
	30	1	3.7	0.42	7	22 (0%)	15
	39[c]	1	3.4	0.50	22	21 (0%)	80
	29	2	3.9	0.43	46	21 (0%)	70
	37[c]	2	3.5	0.50	59	21 (0%)	30
35	35	0	4.0	0.27	7	17 (20%)	50
	34	1	3.9	0.26	4	21 (0%)	50
	39[c]	1	4.0	0.26	21	19 (5%)	40
	33	2	3.5	0.46	12	22 (0%)	20
	40[c]	2	3.7	0.39	18	21 (0%)	10

[a] Size 2B (1¼-1½ in. diameter) cucumbers which had been shipped from Virginia to Michigan (2-3 days post harvest at brining) were brined at the indicated initial brine strengths. All fermentations were acidified with 0.04% acetic acid. All were purged continuously with air (∿20 ml/min) except for the periods indicated for N_2 purging. Heavy scums developed on these brines because they were not protected by ultra-violet light.

[b] Numbers in parenthesis represent the percentage of cucumbers in the fermentation which were severely soft (<11 lb pressure test).

[c] The brine strength was gradually raised to these values during the second week of fermentation by additions of dry salt.

and allow the brine to remain static for a few hours before purging.

The softening of pickles observed in this study was not correlated with pectinolytic enzyme activities in brines. Thus, extensive softening was observed in some fermentations in which negligible enzyme activity was found and vice versa. Therefore, the softening observed after 2 wk of air purging was primarily the result of enzymatic activities produced by the molds in pickle tissues. Further softening would occur if pickles were stored in brines with significant pectinase concentrations (Bell et al., 1950). It is possible that some of the enzyme(s) in the brine was produced by microorganisms growing in the brine on the surfaces of the cucumbers or in the pores of the polyethylene sparger, particularly in those fermentations in which no soft pickles were found. Mold growth on the spargers was observed, and, in some cases, resulted in partial plugging of the pores. However, no pectinase activities developed in pasteurized, aerated cucumber brines inoculated with brines from fermentations in which enzymatic activity was increasing (Costilow et al., 1980).

No comprehensive studies have been reported of softening enzyme(s) levels in commercial fermentations which have been air-purged. No activity was found in the brine from one tank which contained a few bushels of soft pickles in a localized area (Costilow et al., 1981). Also, the

—Continued on page 282

Table 5—Effect of partial air limitation, controlled fermentation, and addition of potassium sorbate on development of acid and pectinase in brines, and on softening of the cucumbers[a]

Treatment	Final pH	Maximum acidity (% Lactic)	Final pectinase (% Δ viscosity)	Pressure test (lb)
Static, first day	3.7	0.56	26	14 (33%)[b]
Static, 6 hr/day[c]	3.7	0.43	53	17 (7%)
Controlled	3.9	0.69	0	19 (0%)
Plus K sorbate, 0.C05%[d]	3.9	—	—	7 (92%)
0.C1%	4.0	0.43	—	10 (77%)
0.C2%	3.7	0.55	—	15 (42%)
0.C35%	3.7	0.68	0	19 (0%)

[a] Cucumbers brined to equilibrate at 25° salometer and 0.04% acetic acid. All fermentations were aerated continuously at ∿20 ml/min except where indicated.

[b] Numbers in parentheses are percentages of all cucumbers which were seriously soft (<11 lb pressure test).

[c] Aeration was started 6 hr after brining, and air was turned off 6 hr each day.

[d] Fermentations with added potassium sorbate were not incubated under ultraviolet light.

Table 6—Effect of blanching cucumbers prior to brining on softening during fermentations with air purging[a]

Treatment	Pressure tests (lb) Individual cucumbers[b]													Average
None — control	0,	0,	0,	5,	7,	0,	13,	0,	0,	4,	0,	5		3
All blanched[c]	16,	16,	19,	16,	18,	16,	19,	17,	19,	19,	20,	17,	19,	18
3 Nonblanched[c,d]	25,	20,	*7*,	14,	18,	9,	17,	19,	17,	18,	*18*,	17		17
3 Blanched[e]	*18*,	14,	18,	20,	*17*,	18,	13,	9,	11,	0,	*18*,	5		13

[a] Size No. 3B (1¾-2 in.) cucumbers were all brined to equilibrate at 25° salometer and 0.04% acetic acid.

[b] Individual cucumbers were removed and pressure tested in order from top to bottom of each jar.

[c] The cover brine was inoculated by washing fresh nonblanched cucumbers before pouring over the blanched cucumbers.

[d] Three nonblanched cucumbers were identified by tying with a string and packed with blanched fruit. One was packed on the bottom, one in the center, and one at the top of the gallon jar. Pressure tests of these three fruits are underlined.

[e] Three blanched cucumbers were packed with nonblanched ones as described in (d). Pressure tests of these three fruits are underlined.

Air Purgining of Commercial Salt-Stock Pickle Fermentations

R.N. COSTILOW, K. GATES, and C.L. BEDFORD

ABSTRACT

A total of 28 commercial salt-stock pickle fermentations were air-purged with a sidearm purger during two brining seasons. Both air flow rates and purging schedules were varied among the fermentations. Air-purging was as efficient as nitrogen in preventing the formation of bloaters (hollow pickles) and there was no significant effects of the various treatments used. The average overall quality of the salt-stock was equivalent to that from nitrogen-purged tanks. Softening of less than 50 bu of salt stock occurred in one tank, and a few pickles in another tank had soft blossom ends. In both instances, these defects were found in a localized area directly under the purger outlet where dissolved oxygen concentrations were highest. No reason(s) for the occurrence of softening in the two isolated tanks was found. Much variation was observed among fermentations in dissolved oxygen levels and in the oxygen demand indicated by the rate of depletion of oxygen when the air supply was turned off. However, the demand was usually higher during the first few days after brining than in older brines.

INTRODUCTION

ETCHELLS et al. (1973) and Fleming et al. (1975) demonstrated that nitrogen purging of controlled fermentations of brined cucumbers greatly reduced the formation of bloaters (hollow pickles). Subsequently, it was found that equally satisfactory results could be obtained with natural fermentations by continuously purging them with nitrogen at about 20 ft^3/hr using an apparatus designated as a sidearm purger (Costilow et al., 1977). This system is now widely used in commercial fermentations.

Use of air instead of nitrogen would greatly reduce the cost of purging. Fleming et al. (1975) reported that continuous air-purging of fermentations in 5-gal pails at air flow rates of 25-100 ml/min resulted in soft and off-colored pickles. However, intermittent air purging at even 425 ml of air/min for 2 hr/day did not result in soft pickles. Purging of pails from the bottom is not comparable to purging large tanks with a sidearm. Even at 25 ml/min, the air flow rate per unit volume of brine in a 5-gal pail is approximately 5× that when 20 ft^3/hr air is used in a 10,000 gal tank. In purging from the bottom, the gas is in direct contact with the cucumbers and gas bubbles may be entrapped in the cucumber mass; this does not occur with the sidearm purger.

This paper presents the results of studies of the use of air for purging commercial cucumber fermentations during two brining seasons. Twenty-eight tanks (∿1,000 bu each) of brined cucumbers were purged with air and the salt-stock compared with that from nitrogen-purged fermentations. Only a minor softening problem was encountered. Data are also presented on dissolved oxygen levels and oxygen demand in air-purged tanks of fermenting pickels.

Authors Costilow and Gates are affiliated with the Department of Microbiology & Public Health, and Author Bedford is with the Dept. of Food Science & Human Nutrition, Michigan State Univ., East Lansing, MI 48824.

EXPERIMENTAL

Fermentations studied

The 20 cucumber fermentations studied during 1976 were brined at the Green Bay Foods Company plant at Eaton Rapids, Mich. The 1978 experiments were conducted at the H.J. Heinz Company station at Lakeview, Mich. All tanks were 8 ft high × 14 ft diameter (∿1,000 bu capacity), and were filled with size 3A (1.5 × 1.75 in. diameter) cucumbers unless noted. They were all brined according to plant procedures. The cucumbers were covered with 45° salometer brine acidified with about 0.05% acetic acid. Sufficient dry salt was added to each tank to equilibrate at 25-28° salometer. After the acidity in the brines had attained about 0.6% lactic acid, the brine strength was slowly increased to 45-50° salometer.

Purging

The standard sidearm purger equipped with a 2 in. × 12 in. ceramic gas sparger (Costilow et al., 1977) was used for sparging all tanks. Gas flow rates were controlled by use of Brooks gas flow meters, Model 1510B, with a capacity of 5-45 standard cubic feet/hr (scfh) (Brooks Instrument Division, Emerson Electric Co., Hatfield, Pa.). In the 1976 studies, each tank purged with air was equipped with a flow meter so rates could be monitored frequently. With all other tanks, the tubes to the spargers were equpped with quick disconnects and needle valves. Gas flow rates were checked and adjusted at least daily by insertion of a flow meter into the line. This procedure results in actual gas flow rates somewhat higher than measured with the flow meters in the line. Therefore, the actual gas flow rates in those tanks were probably 2-3 ft^3/hr higher than recorded.

Two air compressors normally used for spraying paint were used for the 1976 trials. A number of mechanical and electrical problems were encountered in their operation, and considerable moisture accumulated in the lines. During the second year, a high capacity, heavy duty compressor equipped with an efficient air dryer was used and no problems were encountered.

All fermentations during 1976 were purged for 14 days. In 1978, purging was discontinued when the brine samples were found to have less than 0.05% reducing sugar as determined by the quality control personnel of H.J. Heinz Company. On an average, these tanks were purged for 9 days.

Analyses.

Brine samples from the air-purged and from two nitrogen-purged tanks studied in 1978, were plated on dextrose agar (Difco) acidified with 2 ml of 5% tartaric per 100 ml in attempts to isolate molds or unusual yeasts. Aliquots (0.1 ml) of serial dilutions of the brines were spread on pre-poured plates within 4 hr after removing from the tanks.

Dissolved oxygen concentrations were determined with a dissolved oxygen meter YSI Model 57 (Yellowsprings Instrument Co., Inc., Yellow Springs, Oh.) equipped with oxygen probe, YSI 5739, and submersible stirrer, YSI 5791A. The instrument was calibrated daily in air saturated brine at the same salometer (±2°) and temperature as the fermenting brines which were to be measured. Consistent meter readings of 0.1-0.3 ppm oxygen were obtained with brines in nitrogen-purged tanks which should have no dissolved oxygen in them. Therefore, all readings were corrected by −0.2 ppm. Measurements made in brine near the surface of tanks (above the headboards) were made by submerging the probe and stirrer in the brine in the tank. Center of the tank determinations were made by insertion of a stainless steel tube to the approximate geometric center of a tank and siphoning brine into the bottom of a tall 1-liter beaker containing the probe and stirrer. The beaker was al-

lowed to overflow until at least 1 liter of brine had passed through it before the dissolved oxygen reading was taken.

Brines were analyzed daily for titratable acidity, salometer, and CO_2 concentrations. The CO_2 was measured as described previously (Costilow et al., 1977).

The salt stock was evaluated 2-3 months after brining. Routinely, samples were taken from near the surface of the brine directly under the purger outlet. Samples of 100 pickles were tested for firmness by puncturing with a USDA fruit pressure tester equipped with a 5/16 in. tip (Bell and Etchells, 1961). The authors performed the evaluation in the 1976 studies and rated bloaters as described previously (Costilow et al., 1977). Personnel of the H.J. Heinz Co. conducted the evaluation of the pickles in 1978, and used the evaluation procedure of Shoup et al., 1976.

RESULTS

Effect of air as compared to N_2 for purging at different gas flow rates

The 1976 experiments were designed to purge commercial cucumber fermentations at 20, 30, and 40 ft^3/hr with nitrogen and air as described in Table 1. However, due to difficulties with the air compressors and with the electrical supply, air purging frequently stopped for variable and unknown periods of time during the nights. The number of times this occurred varied among the tanks from zero to nine nights. This resulted in large variations in the concentrations of CO_2 which were found in the brines. The CO_2 concentrations exceeded 90 mg/100 ml brine in four of the ten air-purged tanks. An example of the difference in paired tanks is shown in Figure 1. In contrast, the CO_2 control achieved was equivalent in two tanks purged continuously with both gases at 20 ft^3/hr.

The variable CO_2 control was not reflected in control of bloaters. There were very few pickles with defects classed as balloon or moderate bloaters in any of the tanks (Table 1). Neither was the occurrence of honeycomb damage correlated with CO_2 concentrations in the brine. The salt-stock pickles from the air-purged tank represented in Figure 1 had only 4% honeycombs, while 41% honeycombs were found in the stock from the N_2-purged tank represented. No balloon bloaters were found in either tank.

There was no evidence of softening of the pickles from any of the fermentations (Table 1). Neither was there any evidence of bleaching or off-colors in the pickles as a result of air purging. The lactic acid fermentation developed normally in all tanks.

Effect of various schedules for air purging on quality of salt stock

The purging treatments used, the quality of the salt-stock pickles, and the highest and lowest concentrations of CO_2 observed in each tank during 1978 are recorded in Table 2. Bloater control was excellent in all tanks even through maximal CO_2 concentrations greater than 70 mg/100 ml were observed in three of the tanks (Nos. 9, 17, and 18) during the fermentation.

Some softening was observed in two tanks. In one of these tanks, No. 13, a number of pickles in the first sample taken were found to have soft blossom ends. However, this was obviously restricted to a very small area of the tank since no evidence of softening was found in three additional samples from the same general area. Softening was more extensive in tank No. 19, but was restricted to pickles in the purger outlet side of the tank (Table 3). When this tank was emptied the number of pickles affected was estimated to amount to less than 50 bu (Rogers, 1979). Of particular interest is the fact that the softening was not uniform from pickle to pickle even in a very restricted area of the tank. Firm pickles were found adjacent to mushy ones, and a part of a single pickle was frequently soft and the rest firm. There was no evidence that the softening would spread through the tank. No pectinolytic enzyme activity was detectable in the brines (assay conducted by H.J. Heinz Co.). All of these facts suggest that the softening enzyme(s) is produced by an oxygen requiring organism growing on or in the surface tissue of individual cucumbers.

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Table 1—Comparison of salt stock pickles from nitrogen- and air-purged fermentations with respect to firmness (pressure tests) and bloater-type defects (1976)

	Purging gas	
	Nitrogen	Air
No. of tanks purged:[a]		
20 ft^3/hr	4	4
30 ft^3/hr[b]	4	4
40 ft^3/hr	2	2
Pressure test (lb):		
Average	20.7	20.7
Range	17-25	18-23
Percentage with devects:		
Balloons	1.3 (0-4)[c]	1.5 (0-4)
Honeycombs	17.6 (5-41)	13.1 (0-22)
Moderate bloat	5.3 (1-16)	6.7 (2-11)
Carpel separation	1.7 (0-4)	3.7 (0-10)
Lens	14.2 (9-22)	15.3 (8-29)
Total defects	40.1 (20-67)	39.0 (18-67)

[a] Due to mechanical problems, air-purging was discontinuous in most tanks. All other tanks were purged continuously for 14 days except those purged at 40 ft^3/hr which were allowed to remain static 8 hr per day when CO_2 levels were under 30 mg/100 ml of brine.

[b] The flow rates of nitrogen in one tank and of air in one tank were reduced to 10 ft^3/hr 5 days after brining.

[c] Numbers in parentheses are the ranges of values for cucumbers from individual tanks.

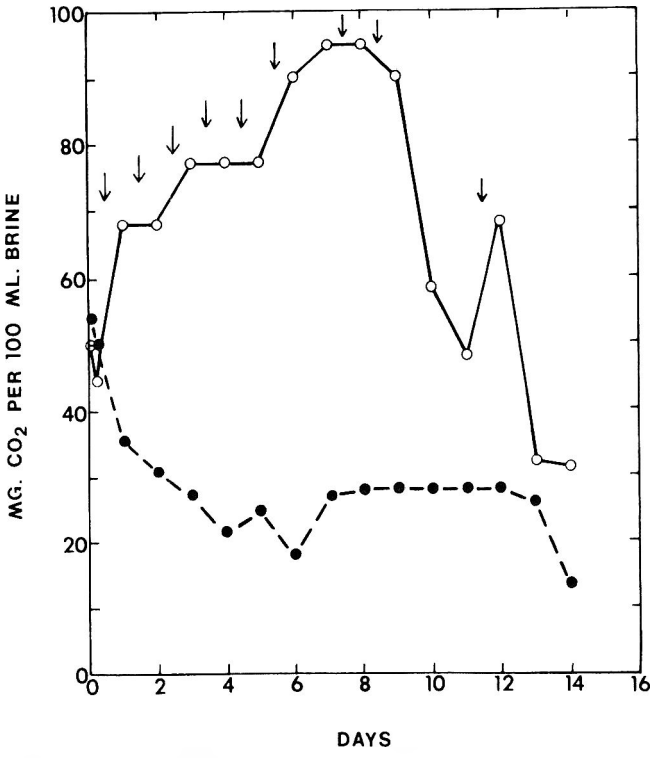


Fig. 1—Carbon dioxide concentrations in a fermentation purged at 40 ft^3/hr with air (○———○) and one purged with nitrogen (●———●). The air purging was stopped for unknown periods at points indicated by the arrows. Nitrogen purging was stopped for 8 hr per day starting 4 days after brining.

Table 2—Influence of various purging treatments on the quality of salt-stock and the maximum and minimum CO_2 levels in the brines (1978)[a]

Treatments	Tank no.	% Defect-free	% Recovery	No. pressure test	mg CO_2/100 ml High	mg CO_2/100 ml Low
Continuous nitrogen	1	60	80	13	40	15
	2	91	95	15	28	10
Continuous air[b]	3	96	96	–	62	13
	4	91	91	15	43	16
Air turned off	5	93	97	14	42	28
1 hr/day	6	74	82	15	53	12
Air turned off	7	81	92	15	45	25
3 hr/day	8	84	88	14	68	34
Air turned off	9	88	94	16	77	15
6 hr/day	10	98	99	18	52	14
Air replaced with N_2	11	89	94	14	45	18
3 hr/day	12	96	98	15	43	20
Air replaced with N_2	13	75[c]	94	19	48	18
6 hr/day	14	94	97	18	53	14
N_2 first day, then	15	92	98	19	58	13
air off 3 hr/day	16	90	95	17	48	23
25° salometer, air	17	65 (94)[d]	87 (94)	15 (19)	70	26
off 3 hr/day	18	80	92	12 (17)	95	18
Air turned off	19	77	88	9[e]	43	15
3 hr/day[f]	20	98	98	18	53	18

[a] Salt-stock was evaluated by personnel of the H.J. Heinz Company by the procedure of Shoup et al. (1976). The expected percent recovery represents the percentage of all of the salt-stock which was free of gas pockets or other separations and which would yield good quality cross-cut slices. Samples of 100 pickles were taken from the top of the tank on the purger outlet side.

[b] The cucumbers in these two tanks were 1.25-1.5 in. diameter.

[c] Several pickles with softening at the blossom end were found in the first sample, but none in a second sample.

[d] Numbers in parenthesis represent results of a second sample.

[e] Sample from soft area (See Table 3).

[f] Planned to brine at 30° salometer, but neither tank reached that level during first 5 days.

Analysis of all the data failed to reveal any difference between tank No. 19 and other air purged tanks. The dissolved oxygen levels were not higher than normal, the acid fermentation developed normally, the salt concentration was not unusually low, and no molds or unusual appearing yeasts were detected by plating of five brine samples during the active fermentation period. Unfortunately, pH of the brine was not measured and we cannot be sure that the initial pH was not unusually high.

As evident in Table 2, no significant differences in salt-stock quality resulting from the different purging treatments was noted. The salt-stock from the two tanks which were continuously purged with air was excellent, and the soft pickles were from a tank in which the air was turned off 3 hr per day. The average quality of the pickles from all 18 air-purged tanks was as good as that from 23 tanks at the same location purged continuously with nitrogen (Table 4).

Dissolved oxygen in brines

The air flow rate has a pronounced effect on the dissolved oxygen levels in brines (Table 5). In some instances, the oxygen concentrations were doubled by increasing the flow rate from 10 ft^3/hr to 30 or 40 ft^3/hr (see data for fermentations, 2, 4, and 5 days old). As expected, the highest levels were found in brines directly under the purger outlet. However, significant amounts of oxygen were consistently found on the opposite side (purger side), and the concentrations found were also influenced by air flow rate. In most instances, no dissolved oxygen was detectable in samples taken from the center of the tank.

There was a trend for the oxygen concentrations to increase as the fermentations progressed in age. This undoubtedly results from differences in the oxygen demand in the brines. The actual demand varied widely from tank to tank, but brines in older tanks (7-10 days) usually had a lower demand than those in young ones (Fig. 2). The cucumbers will take up oxygen rapidly immediately after brining, and the lactic acid bacteria will also use oxygen during their most active fermentation period. Most of the demand in the older fermentations probably results from the development of yeasts. In most instances, the oxygen concentrations in brines from tanks of all ages studied were reduced to essentially zero (less than 0.2 ppm) within 1 hr after turning off the air. When nitrogen was used to replace air through the purger, the oxygen levels declined very rapidly.

The dissolved oxygen values obtained for brine from one tank (No. 7, Table 1) at 2, 3, and 4 days after brining were very different from those observed in any other tank. The brine did not appear to have any oxygen demand. In fact, readings of samples taken from the center of the tank after 3 days appeared to increase in dissolved oxygen from 2.2 ppm when the air was turned off to 2.95 ppm 3 hr later. Samples taken 1 day and 5-9 days after brining were normal with respect to oxygen demand. The oxygen meter appeared to be functioning normally, since brines in other tanks were found to respond in a normal manner. No reason for this anomalous response was apparent, and it was of much concern. However, as indicated in Table 1 (Tank No. 7), no effect on the salt stock was evident. It is probable that there was something in that particular brine which was interfering with the meter readings.

DISCUSSION

THESE RESULTS demonstrate that air purging can be used to prevent bloater formation in commercial salt-stock pickle fermentations. However, there is some element of risk in using air instead of nitrogen. Of ~28,000 bu of cucumbers purged with air during two seasons in these experi-

Table 3—Pressure tests of salt-stock removed from different areas of tank no. 19 which had been purged with air

Area of tank sampled[a]	Individual pressure tests										Mean test
	1	2	3	4	5	6	7	8	9	10	
Purger outlet side:											
Top of tank	3	3	4.5	8	11	11	11	13	14	16	9.5
Lower half	5	6.5	7.5	11	12.5	13.5	13.5	15.5	15.5	17	11.6
Purger side:											
Top of tank	16	16	16.5	17	17.5	17.5	18	18.5	19	19	17.5
Lower half	13.5	15	15.5	16	16.5	16.5	17	17	18	21	16.6

[a] Samples taken with a net. Therefore, samples from lower half of tank may have contained some pickles from near the surface.

ments, a few bushels (<50) in one tank were soft and a few cucumbers (<5 bu) in another tank had soft ends. During the last 3 yr, industry representatives attending meetings at MIchigan State University have reported experiences with air purging about seven million bushels of salt-stock pickles. As observed in these experiments, there were reports of finding an occasional tank with a few bushels of soft pickles directly under the purger outlet. However, no one has reported a serious loss, and a few companies are now using air for purging all fermentations of large sized cucumbers. One company has air-purged all tanks of cucumbers regardless of size during the last 3 yr (Switzer, 1980).

Molds growing in the brined cucumbers are the primary cause of softening in air-purged fermentations (Costilow et al., 1980). While high pectinolytic enzyme activities were found in some brines from laboratory fermentations purged at high air flow rates (Gates and Costilow, 1981), no such activity was detected in the brine of the one commercial tank in which soft pickles were found. Furthermore, finding of firm and soft pickles in the same area of the tank and of individual pickles with soft areas strongly indicate that brine enzymes were not involved.

Data in the accompanying report (Gates and Costilow, 1981) indicate that the first day or two after brining is a critical period when air is used for brining. No soft pickles were found when nitrogen was used for purging for 2 or more days after brining before starting continuous aeration. It is quite possible that careful limitation of the air flow rate and purging on an intermittent schedule would eliminate the sporadic softening problem. These results and those of Potts and Fleming (1979) show that dissolved oxygen usually disappears from brines rapidly when the air supply is turned off. Although the oxygen demand is variable, it was usually maximal during the first 3 days of fermentation. Fleming et al. (1975) found no softening of pickles fermented in 5-gal pails and air purged on an intermittent schedule (2 hr/day). However, purging schedules should be tested under commercial conditions to ensure that they are adequate to prevent bloater damage.

The real relationship between levels of CO_2 in the brines and bloater control is still obscure. The original recommendation for controlled fermentations was to maintain CO_2 concentrations below 20 mg/100 ml of brine (Etchells et al., 1973). Fleming et al. (1978) reported that maintenance of CO_2 concentrations below 50% of saturation was necessary to ensure against bloating. However, essentially no bloaters were found in some of the commercial tanks in which CO_2 concentrations in excess of 90 mg/100 ml of brine were observed. The temperature of these brines was 70±2°F (∿21°C), and most brine strengths were near 25° salometer so there would be about 120 mg CO_2/100 ml of brine at saturation. Therefore, percent CO_2 saturation levels in excess of 75% occurred in some of these tanks without a noticeable effect on bloater control.

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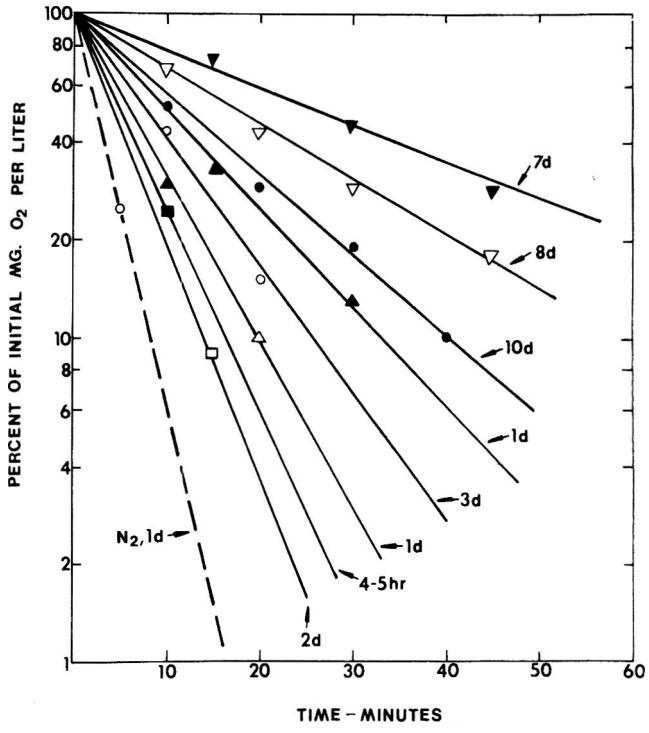


Fig. 2—Rates of depletion of dissolved oxygen from air-purged cucumber brines at various fermentation ages [hr or days (d) after brining]. All measurements were made with the oxygen electrode submersed in brine on top of the tank directly under the purger outlet. The air was turned off at time zero and measurements made at the points indicated. The oxygen concentrations at time zero were variable (see Table 5), so the actual concentration in each brine represented the 100% initial value used in the calculations. The dashed line (N_2, 1 d) represents the depletion rate when air was replaced with N_2 for purging.

Table 4—Comparison of average salt-stock quality from tanks purged with nitrogen and with air

Purging gas	No. of tanks	% with defects		Expected % recovery	Pressure test	
		Severe[a]	Total		Mean	Range
Nitrogen	23	3.0	15	92.0	15.0	13-17
Air	18	3.4	13	93.2	15.5	9-19

[a] These pickles would yield no cross-cut slices.

Table 5—Effect of air flow rate on dissolved oxygen concentrations in brine from different areas in the tank[a]

Location in tank	ft^3/hr air	Fermentation age (days) 1	2	4	5	6	7	8	9	10	Mean
		ppm O_2									
Top, purger outlet[b]	10	1.85	0.95	1.20	0.55	3.0	2.45	1.60	2.00	1.70	1.70
	20	2.25	1.65	–	1.05	3.2	2.55	1.95	2.60	2.30	1.95
	30	–	–	2.70	2.15	–	–	–	2.85	–	2.57
	40	2.50	2.05	–	–	3.45	2.80	2.65	–	2.35	2.63
Top, purger side[b]	10	0.75	0.40	–	–	–	2.05	0.60	–	–	0.96
	20	0.95	0.95	–	–	–	2.15	1.15	–	–	1.30
	30	–	–	–	–	–	–	–	–	–	–
	40	1.65	1.05	–	–	–	2.45	2.20	–	–	1.84
Center of tank	10	0	0	0	0	0	0	0	0	0.05	–
	20	0	0	–	0	0.05	0	0	0	0.10	–
	30	–	–	0	0	–	–	–	0.05	–	–
	40	0	0	–	–	0	0	0	–	0.25	–

[a] A single tank of a given fermentation age was purged at each air flow rate until the dissolved oxygen concentration remained constant at each location.

[b] The oxygen probe was resting on the headboard, 4-6 in. under the brine surface for these readings.

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soft pickles observed in air purged commercial tanks have remained localized in a small area of the tank during several months storage (Switzer, 1980). In addition, not all pickles in a given area are soft and some pickles have only soft spots.

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Sweet Potato Quality After Baking

JUNE M. LOSH, JEAN A. PHILLIPS, JULEIN M. AXELSON, and ROBERT S. SCHULMAN

ABSTRACT

Painter and Jewel sweet potatoes baked at oven temperatures of 150, 180, 200, and 230°C were judged for sensory qualities and were evaluated for resistance to pressure (softness) and content of sugars. Roots baked at 200 and 230°C were scored significantly higher for sweetness, mouthfeel, moistness, overall flavor, and overall quality; contained a greater percentage of reducing sugars; and were measured as softer than roots baked at 150 and 180°C. Painter roots were scored significantly higher for sweetness, mouthfeel, moistness, overall flavor, and overall quality than the Jewel variety. Positive correlations were observed between the concentration of reducing sugars, the resistance to pressure, and the sensory evaluation; and negative correlations were noted between the moisture content and the subjective measurements.

INTRODUCTION

THE SWEET POTATO is a relatively cheap source of food energy and of provitamin A, and is moderately high in ascorbic acid. Sweet potato breeders, biochemists, and plant physiologists have studied the effects of variety and environmental factors on the quality of the baked flesh because sweet potatoes are often baked whole when prepared for the table. Edmond (1971) stated that the changes that occur in the baked flesh involve enzymatic hydrolysis of the starch to sugars, and that the conditions of baking, of curing, and the variety all affect the amount of starch hydrolysis that occurs. The greater the starch hydrolysis, the more desirable the final product.

One of the enzymes involved in starch hydrolysis, α-amylase, has optimum activity at 70-75°C, and has a low activity at cooler temperatures (Walter et al., 1976). Evidence indicates that α-amylase functions during the processing of sweet potatoes to hydrolyze starches to sugars and to produce the desired changes in the cooked root (Walter et al., 1975). Jenkins and Gieger (1957a) found the major conversion of starch to reducing sugars, dextrins, and maltose occurred during the first 30 min of baking and at a temperature below 70°C. Sarhan et al. (1976) reported that total solids decreased during boiling, but increased during baking. Twenty minutes of boiling were necessary to produce an acceptable product, as compared to 30 min of baking.

The purpose of this study was to compare the effects of variety, storage time, and temperature/time combinations for baking on the perceived qualities of baked sweet potatoes; and on the sugar content, the resistance to compression and the moisture content.

MATERIALS & METHODS

Procurement and storage

Freshly harvested and cured sweet potatoes of Painter and Jewel variety were obtained from the Eastern Shore Research Station in Painter, Va. Medium sized potatoes (mean weight 204g, length 12.5 cm, and circumference 17.5 cm) were selected and were stored in the dark in covered crates until needed.

Preliminary preparations

Eleven roots of each variety were removed from storage for each of five consecutive weeks, washed, scrubbed, weighed on a Metler P1000 balance, and measured with a flexible ruler calibrated in metric units. One root of each variety was pared by hand with a carbon steel paring knife, cut into pieces, and blended at high speed with a solid-state blender (Sunbeam model no. B1-Y) until the potato appeared finely chopped. The pulp was stored in a beaker covered with parafilm at room temperature until analysis later the same day.

The remaining ten roots of each variety were divided into two groups for the baking tests. Roots were baked on 2 days each week, and two temperature/time combinations were used each day. The temperature/time combinations for the five repetitions were identified by random drawing and were selected from the following:

Temperature (°C)	Time (min)
150	120
180	90
200	75
230	75

Baking of potatoes

The roots were baked in homestyle ranges with "self-cleaning" ovens (Frigidaire model no. RSE 3-36W). The internal temperature of roots (Fig. 1) and the oven heating cycles, measured by Ecklund copper constantan thermocouples, were recorded with a Honeywell Potentiometer once each minute. The baked roots were removed promptly from the oven at the end of the baking period. One root from each variety was cooled for 5 min then used for sensory evaluation, the remaining roots were allowed to cool to room temperature.

Sensory evaluation

One-half of a crosswise slice from one baked root of each variety was presented to each of eight judges who were asked to score the samples for mouthfeel, sweetness, overall flavor, moistness, and overall quality according to a five-point hedonic scale: a five indicating the highest quality.

Measure of softness

One baked root from each temperature/time combination was used to measure the resistance to compression. Three slices, one centimetre thick, were cut crosswise from the root with a stainless steel serrated knife, one from the center of the root, the other two from each end of the root, after a 1 cm end piece was removed and discarded. The slice was placed on a square of waxed paper, then placed on the base of a penetrometer (model no. 4100T) equipped with a flat metal disk, 3.3 cm in diameter and 6.5 mm thick, attached to a spindle, 5.3 cm long. The disk and spindle weighed 14.7g, equivalent to the weight of a plastic cone attachment for a penetrometer. The disk attachment was centered directly over the potato slice, just touching the surface of the potato, with the plane of the disk perpendicular to the longitudinal potato fibers. The disk was released for 1 sec and allowed to fall for 5 sec. The resistance to compression was recorded as mm of compression.

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Authors Phillips, Axelson, and Schulman are affiliated with the Dept. of Human Nutrition & Foods and Dept. of Statistics, Virginia Polytechnic Institute & State Univ., Blacksburg, VA 24061. Author Losh, formerly with VPI&SU, is now affiliated with James Madison Univ., Harrisonburg, Va.

Moisture determination

The three remaining roots were peeled with a carbon steel paring knife, then the flesh was blended and mashed using a wooden spoon in a ceramic bowl. A 10-g sample of mashed root was placed in a Brabender moisture tester to determine the percentage moisture content.

Sugar identification

Alcohol soluble solids were extracted from a 10-g sample of blended mashed root from each temperature/time combination and from the chopped raw root of each variety as described by Jenkins and Gieger (1957a). The sugars present in the alcohol extract were separated and identified using thin-layer chromatography (TLC) by the method of Raadsveld and Klomp (1971) modified as follows:

The plates were spotted with 15 μl of each of the following: 1% aqueous solution of the reference sugar mixture containing fructose, glucose, maltose, sucrose, and xylose; 1% aqueous solution of each of the individual reference sugars; and the alcohol extract from the raw and cooked roots. Then a line, 1 cm from the edge, was drawn with a lead pencil across the plate, opposite from the spotted end, to prevent the solvent from migrating beyond this point. The plates were developed for 2 hr, and R_f values were calculated using the following equation:

$$R_f = \frac{\text{distance from starting point to center of spot}}{\text{distance from starting point to solvent front}}$$

Quantification of sugars

Reducing sugars before and after acid hydrolysis were analyzed by the dinitrosalicyclic acid method (DNA) of Miller (1959), modified by using 0.2 ml aliquot of the alcohol extract from the raw sample instead of 1.0 ml and glucose as the standard.

Statistical analysis

The data were treated as a three factor design with storage time considered as a blocking factor. Analysis of variance, correlations and multiple regressions were performed by ANOVA, CORR, and GLM procedures of the Statistical Analysis System and Duncan's Multiple Range Test.

RESULTS & DUSCUSSION

THE INTERNAL TEMPERATURE of all roots (Fig. 1) increased sharply during the first 30 min of baking time with the roots baked at 200 and 230°C heating more rapidly than those baked at 150 and 180°C. Contrary to Jenkins and Gieger (1957b), a maximum internal temperature greater than 100°C was recorded. Roots baked at the four oven temperatures, 150, 180, 200, 230°C reached internal temperatures of 101, 102, 106, and 115°C, respectively. Increased concentration of solutes in the potatoes may have been reasons for the greater maximum internal temperature recorded.

Concentration of sugars

There was an increase in the concentration of sugars during baking (Table 1). The concentration of reducing sugars increased from 6 to 12%. Sistrunk et al. (1954) reported that reducing sugars increased from 1% in the raw root to 14% in the baked root. The length of storage did not contribute to significant differences in the concentration of reducing sugars.

There was no difference in the concentration of reducing sugars between the two varieties (Table 2). Variety was, however, a significant ($P \leq 0.05$) factor contributing to the differences in the concentration of reducing sugars after acid hydrolysis (Table 2). The Painter variety contained a greater percentage of reducing sugars after acid hydrolysis in the cooked roots than did the Jewel variety (Table 2).

There was a significant ($P \leq 0.05$) difference in the reducing sugar content in the roots baked at the four baking temperatures (Table 1). The concentration of reducing sugars was greatest in those roots baked at 200 and 230°C (Table 1).

The factors variety and baking temperature contributed simultaneously to observed significant differences ($P \leq 0.05$) in the mean concentration of reducing sugars in the roots baked in these five repetitions (Table 2). Thus, the results obtained for roots of one variety baked at one oven temperature would not necessarily be the same as those of another variety baked at the same oven temperature.

For the roots used in this study, a higher concentration of sugar occurred when the roots were baked at 200 and 230°C than at 150 and 180°C. It is possible that there was more enzyme activity at these temperatures for, in this study, the internal temperature of the roots reached optimum temperature range of the amylase enzyme in less

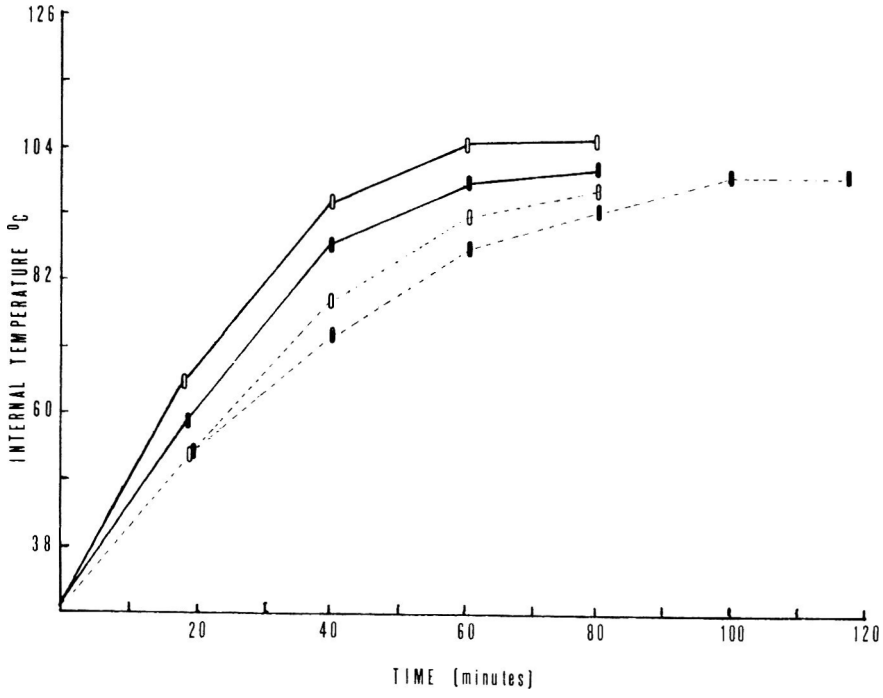


Fig. 1—Internal temperature of sweet potatoes baked at four temperatures: ● — — — ●, 150°C; ○ — — — ○, 180°C; ● —— ●, 200°C; ○ —— ○, 230°C.

Table 1—Mean objective measures[a] of quality factors of sweet potatoes baked at four temperatures

Baking temperature °C	Reducing sugars %	Reducing sugars after hydrolysis %	Moisture %	Dry[b] matter %	Softness[c] mm
Raw	1.29	4.69	69.93	30.07	–
150	8.03[d]	14.85[d]	65.61[d]	34.29[d]	0.910[d]
180	10.72[de]	17.19[d]	64.35[d]	35.65[d]	1.757[e]
200	12.32[e]	19.22[d]	61.95[d]	38.05[d]	1.544[de]
230	12.41[e]	20.39[e]	65.07[c]	34.93[d]	2.096[e]

[a] Mean value of five repetitions
[b] Calculated as equal to 100 minus % moisture as determined
[c] Softness is based on compression. A higher number indicates greater softness.
[d,e] Values in one column with same superscript are not significantly different ($P \leq 0.05$), using ANOVA and Duncan's Multiple Range Test.

time when the roots were baked at 200 and 230°C than when baked at 150 and 180°C (Fig. 1) and the enzyme began to work earlier in the baking process.

Identification of sugars

Sugars identified from samples of the raw sweet potatoes included sucrose, fructose, and glucose (Fig. 2). There was no maltose identified in the raw roots. Sucrose, maltose, fructose, and glucose were identified in the baked samples. Since there was no maltose identified in the raw samples, the increase during baking in the concentration of reducing sugars was probably due to hydrolysis of some of the starch in the roots to maltose. Lambou (1958) reported that of the total sugars present, the raw root contained mostly sucrose (66-75%), some glucose (7-11%) and fructose (6-11%), and small amounts of maltose (2.7-4%). After heating, the concentration of maltose increased to 51-60% of the total sugars present. He attributed the large increase in the concentration of maltose to starch hydrolysis.

Moisture

Determination of the moisture content for samples of sweet potatoes at the four temperatures indicated that the moisture content was similar regardless of the baking temperature used. There was approximately 4-8% moisture lost during the baking process (Table 1). This was due to evaporation from the roots, and is comparable to the 1-9% loss reported by Sistrunk et al. (1954). Dry matter content was found to be 28-40% of the total weight of the root. These values are slightly higher than the 25-30% dry matter content reported by Constantin et al. (1966), in Louisiana. Possible differences may be due to differences in the relative humidity in Louisiana compared to Blacksburg, Va,; differences in the measuring device; or differences in storage conditions.

Storage was found to be a significant factor contributing to the differences in moisture content. The longer the samples were stored, the greater the moisture loss; perhaps a result of evaporation.

Resistance to compression

Baking temperature and the length of storage contributed significantly ($P \leq 0.05$) to the differences observed in the resistance to compression of baked sweet potato slices (Table 1). As the baking temperature was increased, there was more softening of the fibers of the potatoes. Thus, potatoes baked at 180, 200, and 230°C had less resistance to compression. Softness also was increased with the length of storage until about the third week, then was decreased. It is possible that softness is related also to the concentration of sugars, for they too, increased in concentration to the third week of storage and then decreased. Ali and Jones (1967) reported no significant correlation between the softness of baked tissues and the presence of reducing sugars, maltose, or dextrins.

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Table 2—Mean objective measures[a] of quality factors of sweet potatoes baked at four temperatures

Baking temp °C	Reducing sugars total %	Reducing sugars after hydrolysis[c] %	Softness[bc] mm
	Painter		
Raw	0.44	4.85	–
150	6.69[d]	15.47[d]	0.952[d]
180	9.75[de]	20.00[e]	1.902[d]
200	12.45[e]	20.63[e]	1.634[d]
230	10.82[e]	19.85[e]	2.006[d]
	Jewel		
Raw	2.14	4.53	–
150	9.38[d]	14.22[d]	0.863[d]
180	11.69[e]	14.37[d]	1.612[d]
200	12.19[e]	17.80[e]	1.454[d]
230	14.00[e]	20.93[f]	2.186[d]

[a] Mean value of five repetitions
[b] Softness is based on compression. A higher number indicates greater softness.
[c] Values for reducing sugars after hydrolysis and softness were significantly different ($P \leq 0.05$) between varieties, using ANOVA.
[d,e,f] Values in one column with same superscript for a specific variety (baked) are not significantly different ($P \leq 0.05$), using ANOVA and Duncan's Multiple Range Test.

Table 3—Mean scores for sensory evaluation[a] of two varieties of sweet potatoes baked at four temperatures

Baking temp °C	Mouth-feel	Sweet-ness	Overall flavor	Moist-ness	Overall quality
		Painter[b]			
150	2.98[c]	3.30[c]	3.34[c]	3.26[c]	3.20[c]
180	3.72[cd]	4.16[cd]	3.84[c]	3.92[c]	3.68[c]
200	4.18[cd]	4.40[cd]	4.32[c]	4.16[c]	4.00[c]
230	4.00[d]	4.30[d]	4.28[c]	4.10[c]	4.12[c]
		Jewel[b]			
150	2.80[c]	3.28[c]	3.18[c]	2.92[c]	2.90[c]
180	3.20[c]	3.84[c]	3.60[c]	3.66[c]	3.22[cd]
200	3.30[c]	3.50[c]	3.52[c]	3.66[c]	3.30[cd]
230	3.68[c]	3.98[c]	3.86[c]	3.76[c]	3.82[d]

[a] Mean value of five repetitions
[b] Values for all characteristics were significantly different ($p \leq 0.05$) between varieties, using ANOVA.
[c,d] Values within a column for a specific variety identified with same superscripts were not significantly different ($p \leq 0.05$), using ANOVA and Duncan's Multiple Range Test.

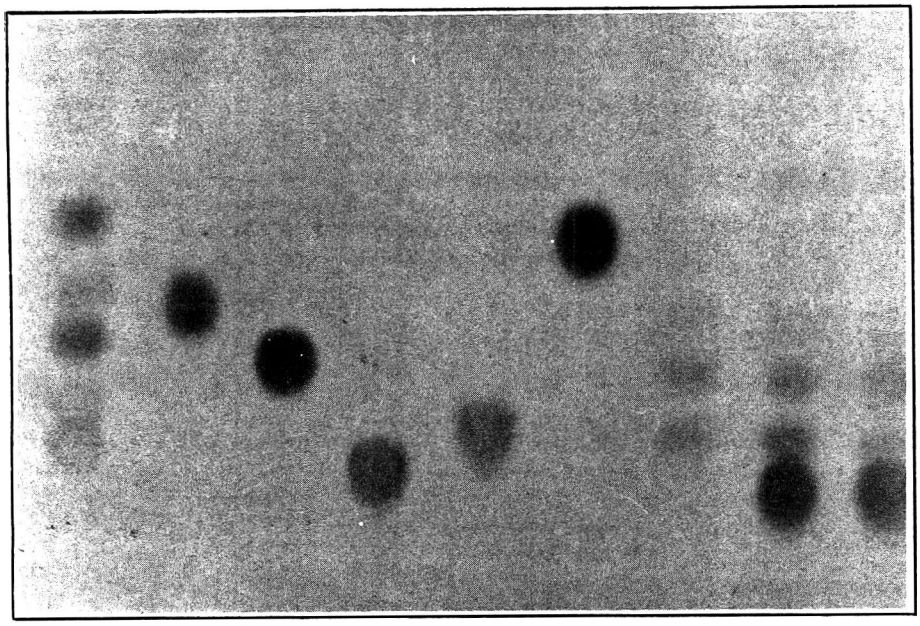


Fig. 2—A sample plate from thin-layer chromatography. From left to right: reference sugar mixture, aqueous fructose solution, aqueous glucose solution, aqueous maltose solution, aqueous sucrose solution, aqueous xylose solution, alcohol extract from raw potato, alcohol extract from baked root, alcohol extract from a second baked sample.

Sensory evaluation

Responses from the sensory evaluation panel indicated that the variety of sweet potato and the baking temperature used contributed significantly ($P \leq 0.05$) to differences in the mouthfeel, sweetness, overall flavor, moistness, and overall quality of the baked roots (Tables 3 and 4). In general, the Painter variety was ranked higher in all five areas (Table 3). Also, those roots baked at the higher oven temperatures (200 and 230°C) were ranked significantly higher ($P \leq 0.05$) than those baked at the lower oven temperatures (100 and 150°C) (Table 4).

The scores for sweetness, mouthfeel, moistness, overall quality, and overall flavor were positively correlated (Table 5) with the reducing sugar content ($P \leq 0.05$). As the percentage of reducing sugars increased, the scores for sweetness, mouthfeel, moistness, overall quality, and overall flavor were increased. Thus, it was concluded that the reducing sugars contributed to the desirable characteristics of these baked roots.

Constantin et al. (1966) reported a positive correlation between sweetness and moisture content; as the moisture content increased, so did the perception of sweetness. A negative correlation ($r = -0.591$) between moisture and sweetness was observed in this study; as moisture content decreased, sweetness perception increased (Table 5). This is possibly due to concentration of the sugar and other dry materials as moisture was lost.

The scores from the sensory evaluation tests were negatively correlated ($P \leq 0.05$) with the moisture content, and positively correlated ($P \leq 0.05$) with softness as measured by the resistance to compression (Table 5). As the moisture content of the samples decreased and the distance to compression increased the scores of sweetness, mouthfeel, moistness, overall quality, and overall flavor were increased. Apparently, moisture content had little to do with the perception of moistness. According to Walter et al. (1975), the degradation of large molecules, such as starch, lowers the viscosity of the substance and the water-binding ability, thus contributing to a moist mouthfeel. Thus, the sample would appear to be moist although the water content was decreased. Also, a lower viscosity would result in less resistance to compression and a greater perceived softness. The degradation of starch, as indicated by less resistance to compression, would result in an increase in sugar content and perceived sweetness.

Table 4—Mean scores[a] for sensory evaluation of sweet potatoes baked at four temperatures

Baking temp °C	Mouth-feel	Sweet-ness	Overall flavor	Moist-ness	Overall quality
150	2.91[b]	3.29[b]	3.26[b]	3.10[b]	3.05[b]
180	3.46[bc]	4.00[bc]	3.72[bc]	3.79[c]	3.45[bc]
200	3.74[c]	3.95[bc]	3.92[bc]	3.91[c]	3.65[bc]
230	3.84[c]	4.14[c]	4.07[bc]	3.93[c]	3.97[c]

[a]Five repetitions
[b,c] Values in one column with same superscript are not significantly different ($P \leq .05$), using ANOVA and Duncan's Multiple Range Test.

Table 5—Correlation coefficients: Objective measurement data versus sensory evaluation data

	Objective measurements			
Sensory evaluation	Reducing sugars	Alcohol soluble solids	Moisture	Softness
Sweetness	0.401[a]	0.064	−0.591[a]	0.480[a]
Mouthfeel	0.348[a]	0.109	−0.591[a]	0.485[a]
Moistness	0.309[a]	0.120	−0.601[a]	0.401[a]
Overall Quality	0.315[a]	0.311	−0.506[a]	0.494[a]
Overall Flavor	0.358[a]	−0.003	−0.625[a]	0.492[a]

[a] Significant at $P \leq 0.05$.

In summary, the oven temperature at which sweet potatoes were baked affected the quality of the baked flesh. Roots baked at 200 and 230°C contained a greater percentage of sugars, were less resistant to compression, and were scored higher by the sensory evaluation panel than roots baked at 150 and 180°C.

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Stability of Proteinase Inhibitors in Potato Tubers During Cooking

D. Y. HUANG, B. G. SWANSON, and C. A. RYAN

ABSTRACT

Three proteinase inhibitors, Inhibitor I (a chymotrypsin inhibitor), Inhibitor II (a chymotrypsin and trypsin inhibitor), and Carboxypeptidase Inhibitor were quantified immunologically in extracts of various tissues of Russet Burbank potato tubers and were monitored over various intervals during boiling (30 min), oven baking (80 min, 191°C) and microwave baking (7 min) to assess the effectiveness of cooking in denaturing the potentially toxic proteins. In cooked tubers, Inhibitor II was completely inactivated during all cooking treatments and Inhibitor I was partially inactivated, depending upon the cooking method. The Carboxypeptidase Inhibitor was extraordinarily stable during all of the cooking conditions. Overall, microwave baking was most effective for cumulatively inactivating Inhibitor I and Inhibitor II. The unusual stability of CPI to all types of cooking raises questions concerning the effect of this inhibitor on the total digestibility of cooked potato proteins.

INTRODUCTION

POTATO TUBERS (*Solanum tuberosum*) are a major source of high quality protein (Schuphan, 1970) which ranks high in nutritional value among vegetable proteins available (Kaldy, 1972). Although the potato contains only about 5-6% protein on a dry weight basis, potato protein yields per acre are presently rivaled only by soybeans (Kaldy, 1972). The potential for genetically increasing this percentage of potato protein in a significant way exceeds that of any other major crop.

About 10% or more of potato proteins consist of powerful inhibitors of the major pancreatic proteinases of higher animals (Ryan, 1973). Several species of heat stable proteinase inhibitors have been isolated and studied and are of three major classes, called Inhibitor I (Melville and Ryan, 1972), Inhibitor II (Bryant et al., 1976; Iwasaki et al., 1972), and Carboxypeptidate Inhibitor (CPI) (Ryan et al., 1974). Inhibitor I (MW about 40,000) is a potent chymotrypsin inhibitor and a weak trypsin inhibitor. Inhibitor II (MW 21,000) inhibits both chymotrypsin and trypsin very strongly. CPI (Ryan et al., 1974) is a polypeptide (MW 4200) that strongly inhibits the pancreatic carboxypeptidases A and B.

Inhibitor I and Inhibitor II concentrations in potato tubers have been found to correlate fairly well with the total soluble proteins and a rapid quantitative immunological assay for the two inhibitors has been utilized to rapidly and successfully select for high protein potato lines (Ryan et al., 1976; Ryan and Pearce, 1978). However, the selection of high protein potatoes also having high concentrations of proteinase inhibitors with potentially toxic properties toward animals raises questions concerning the fate of proteinase inhibitors in potato tubers during cooking and the possible effects such antinutrients might have on the digestibility of cooked potatoes.

Authors Huang and Swanson are with the Dept. of Food Science & Technology, Washington State Univ., Pullman, WA 99164. Author Ryan is with the Institute of Biological Chemistry and Program in Biochemistry & Biophysics, Washington State Univ., Pullman, WA 99164.

Pearce et al. (1979) reported that the Inhibitor I and II present in a paritally purified inhibitor preparation were destroyed upon autoclaving (121°C) for 2 hr. Carboxypeptidase inhibitory activity was not destroyed by this treatment but was reduced significantly. Chicks that were fed a diet supplemented with the uncooked, partially purified, inhibitor-enrichced potato proteinase inhibitor fraction exhibited severe growth depression. However, when autoclaved, the protein preparation was readily digested and provided for normal growth of the newly hatched chicks. The objectives of the present investigation are to further investigate the stability of the heat stable proteinase inhibitors—Inhibitor I, II and CPI—in potato tuber tissues during cooking and to compare the rate and extent of denaturation of the inhibitors during three cooking methods: boiling, oven baking, and microwave baking.

MATERIALS & METHODS

RUSSET BURBANK potatoes were a gift from the Dept. of Horticulture, Washington State Univ. The tubers had been in storage at 7°C for 10-12 months prior to use. Uniform medium sized tubers 175-185g were selected for the experiments. Tosyl-L-agrinine methyl ester, benzoyl-L-tyrosine ethyl ester, hippuryl-L-phenylalanine, chymotrypsin (2 x crystallized), trypsin (2 x crystallized), and carboxypeptidase A (Crystilline) were purchased from Sigma Chemical Co. Inhibitor I, Inhibitor II, and CPI were prepared as described previously (Pearce et al., 1979). CPI was prepared by the method of Ryan et al. (1974). Rabbit antiserum was prepared against the individual inhibitors as previously described (Pearce et al., 1979).

Inhibitors were quantified immunologically by radical diffusion (Ryan, 1967; Trautman et al., 1971). One gram of potato tuber tissue was mascerated with a mortar and pestle and the concentration of inhibitors in the expressed juice was determined. In some tissues, after cooking, it was necessary to incubate the mascerated tissue with 0.02 mg α-amylase per gram potato tissue for 10 min at 21°C to destroy the starch gels. Using known standards this method was demonstrated to be satisfactory in quantifying the inhibitors present in the tissues.

Concentrations of Inhibitor I, Inhibitor II, and CPI were determined in tissues from six locations within raw Russet Burbank potato tubers (Fig. 1). Cooking treatments for whole potato tubers without peeling were as follows: Boiling—potato tubers were heated in boiling water for 1, 3, 5, 10, 20, and 30 min; Oven baking—potato tubers were baked in the 375°F preheated oven for 1, 3, 10,

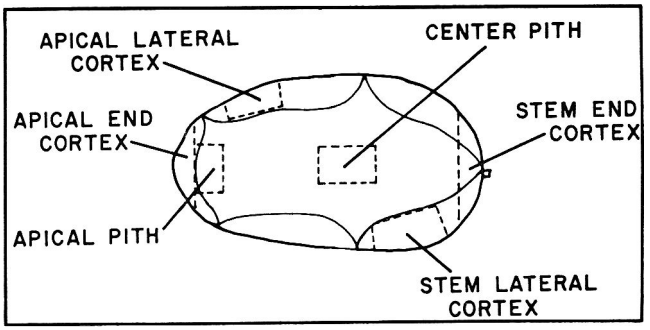


Fig. 1—Position of samples employed to study the stabiity of proteinase inhibitors during cooking.

20, 40, 60 and 80 min; and Microwave baking—the potato tubers, in sets of two, were baked in a microwave oven (Litton Industry Model 500, 2450 MHz) for 1, 3, 5, and 7 min. The internal temperatures of the potato tubers during cooking is illustrated in Figure 2. Triplicate cooking treatments of three potatoes each were assayed in duplicate.

RESULTS & DISCUSSION

Proteinase inhibitor concentrations in raw potato tubers

The concentrations of Inhibitor I, Inhibitor II, and CPI in the six locations of a typical tuber are presented in Table 1. Apical end cortical tissues exhibited a significantly higher concentration of all inhibitors than the other five locations and the levels of the inhibitor decreased in other tissues proceeding from the apical end of the raw tubers toward the stem end. Ryan et al. (1968) previously reported that Inhibitor I in the cortex tissue of freshly harvested potatoes were uniformly distributed throughout the tubers but it decreased significantly, primarily in stem end cortex, during storage for 1 yr. The tubers studied here were stored for 10-12 months and the Inhibitor I concentration was low in the stem end as expected (Ryan et al., 1968). The center pith exhibited the lowest concentration of all inhibitors among the six selected locations (Table 1). Inhibitor II exhibited the highest concentration among the three proteinase inhibitors in raw potato tubers. The ratio of Inhibitor I/Inhibitor II was about 0.4 which also agrees with previous data reported by Ryan et al. (1976).

Thus, with the data in Table 1 serving as a general guide for the levels of the four inhibitors in raw Russet Burbank potatoes, a study was begun to monitor levels of each of the inhibitors during baking, boiling and microwave cooking. For this study only four locations were chosen as representative tissues; apical cortex, apical lateral cortex, stem end cortex, and center pith.

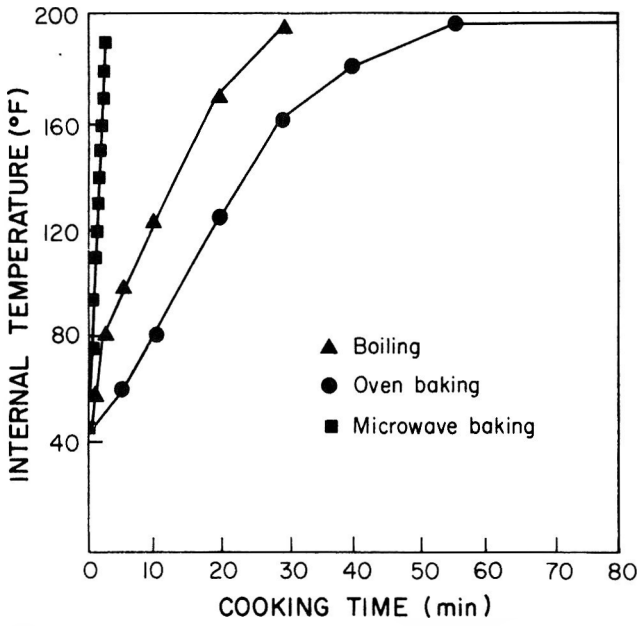


Fig. 2—Internal temperature of potato tubers during cooking.

Proteinase inhibitor concentrations in cooked potatoes

The concentration of Inhibitor I and Inhibitor II in the four selected tissue locations during the three methods of cooking is shown in Figure 3. Inhibitor I was completely denatured by boiling and microwave baking. When cooked by microwave baking, Inhibitor I was almost completely denatured within 5 min. Boiling the tubers for 30 min was as totally efficient in denaturing this inhibitor as microwave baking. On the other hand, even after 80 min of oven baking a high percentage of Inhibitor I remained in the cortical tissues of the tubers.

All three cooking methods completely denatured Inhibitor II (Fig. 3). During the first 5-10 min of boiling or oven baking the immunologically reactive Inhibitor II increased dramatically indicating that some Inhibitor II

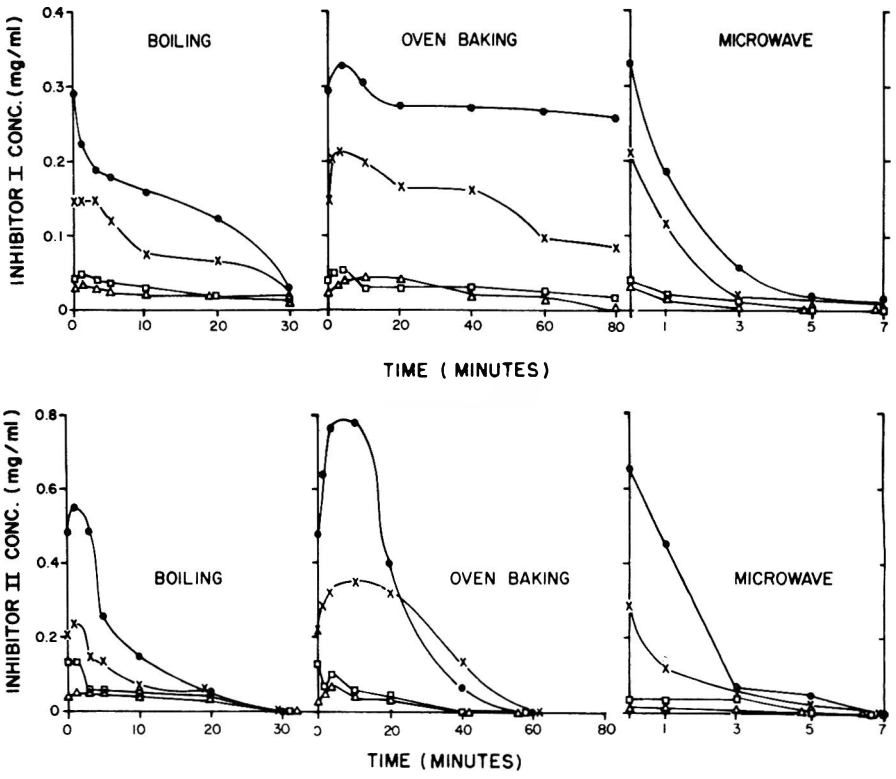


Fig. 3—Time course of Inhibitor I (top) and Inhibitor II (bottom) stability during various cooking methods. Apical end cortex — ○ —; apical lateral cortex — x —; stem end cortex — □ —; and center pith — Δ —.

protein must be solubilized during heating. This suggests that some of the inhibitor is bound in uncooked tubers and is unavailable to the immunological assay; perhaps loosely bound to membranes or ionically bound to the cell walls. It is of interest that no such increase was observed during microwave cooking. However, denaturation may have been so rapid that release of the inhibitor may not have been detectable. Further studies of this phenomena are in progress.

In order to confirm, in a general way, the immunological data, water extracts of cooked and uncooked potatoes were assayed for inhibitory activity against trypsin and chymotrypsin, enzymes that are potently inhibited by Inhibitors I and II. In Figure 4 the inhibitory activity against trypsin and chymotrypsin in potato powder prepared from uncooked potatoes and potatoes cooked by boiling, oven baking and microwave baking is shown. It can be seen that in all three methods the trypsin inhibitory activity was abolished. On the other hand chymotrypsin inhibitory activity was entirely abolished by microwave baking but was less destroyed by oven baking or boiling. These results confirm that microwave baking very efficiently destroys proteinase inhibitor activity toward both trypsin and chymotrypsin, but that significant chymotrypsin inhibitor activity survived boiling and oven baking.

The carboxypeptidase inhibitor is the most heat stable of the four inhibitors assayed. As shown in Figure 5 this inhibitor was stalbe to all three cooking methods. Boiling for 30 min or oven baking for an hour had little effect on the inhibitor. Microwave baking for 7 min denatured only about 25% of the polypeptide.

The stability of CPI was confirmed using enzymic techniques (Figure 6). The inhibitory activity in extracts of dried, powdered potato tissues against carboxypeptidase A was assayed with extracts of both fresh potato tubers and of potato tissues cooked by the three methods. It is clear that significant carboxypeptidase inhibitory activity survived all three cooking methods, confirming the immunological data.

Among the three cooking methods employed to study the denaturation of these proteinase inhibitors in tissues of Russet Burbank potato tubers, the most effective method for denaturing Inhibitor I and II was microwave baking which completely denatured the two inhibitors within 7 min (Fig. 3 and 4). Boiling and oven baking were as effective as microwave baking in denaturing Inhibitor II but boiling was considerably more effective in destroying Inhibitor I than oven baking. Neither boiling, oven baking nor microwave baking effectively denatured the small molecular weight inhibitor of carboxypeptidase, CPI (Fig. 5 and 6).

The unusual stability of CPI deserves attention in assessing the potential nutritive value of cooked potatoes. This inhibitor can constitute several percent of potato proteins and is a powerful inhibitor of two important digestive proteinases, carboxypeptidase A and B. A 1-lb raw potato can contain as much as 100 mg of the inhibitor which, with a MW of 4200, could theoretically inactivate about 8 times its weight of carboxypeptidases A and B, (MW 35,000) or over 1/2 gram of enzyme.

We do not yet know if CPI can inhibit human carboxypeptidases, or if the inhibitor can survive digestion of the stomach. However, if the inhibitor is still active in the intestinal tract and can significantly reduce proteolysis, then its removal by genetic selection may significantly help to improve the nutritional quality of cooked potatoes. We have already determined that many experimental potato clones exhibit very low concentrations of the inhibitors. Such clones may provide valuable genetic material for future research.

—Continued on next page

Table 1—Proteinase inhibitor concentrations of juice expressed from raw Russet Burbank potatoes

Location	Inhibitor concentration (mg/ml)		
	Inhibitor I	Inhibitor II	CPI
Apical end cortex	0.25	0.61	0.25
Apical lateral cortex	0.13	0.22	0.17
Stem lateral cortex	0.08	0.13	0.16
Stem end cortex	0.04	0.08	0.10
Apical pith	0.06	0.22	0.12
Center pith	0.03	0.03	0.09

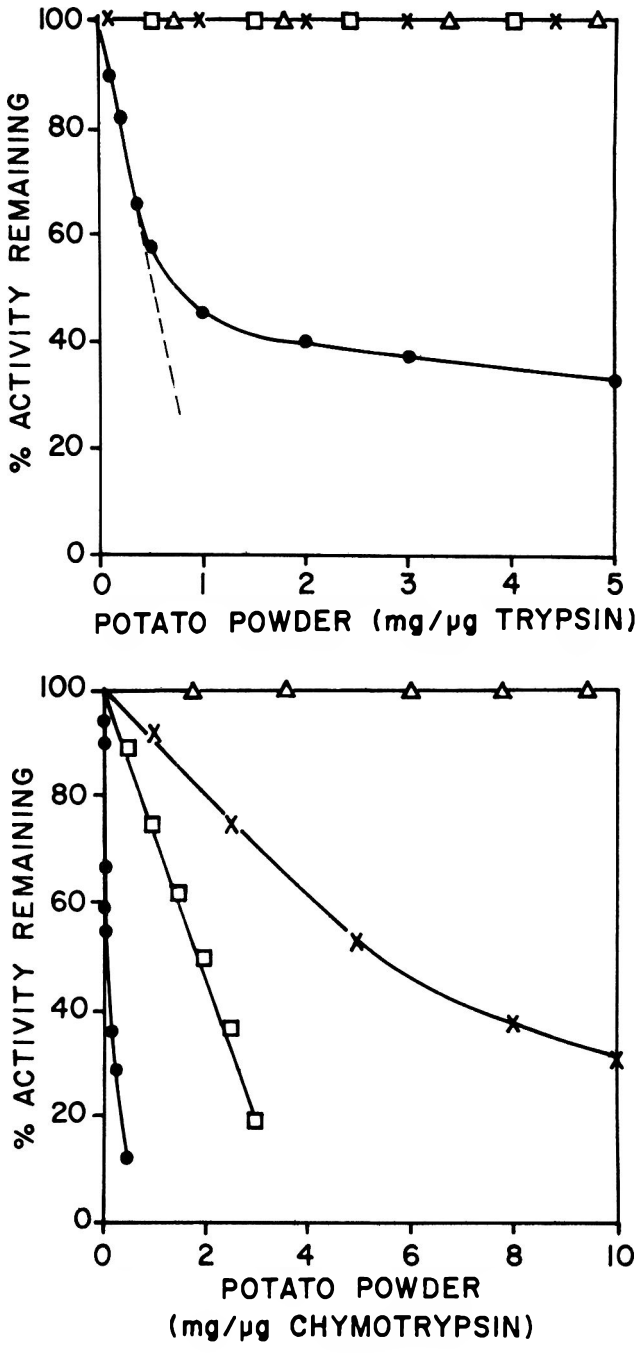


Fig. 4—Inhibition of trypsin (top) and chymotrypsin (bottom) esterase activities by extracts from raw and cooked potato tubers. Raw — • —; boiled — □ —; oven baked — x —; and microwave baked — Δ —.

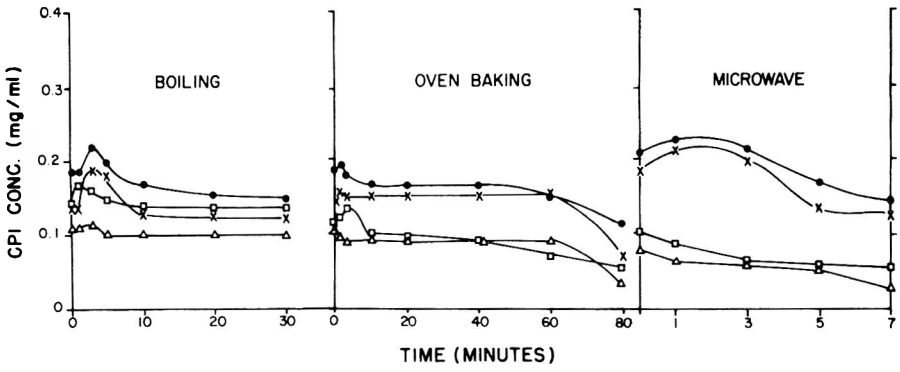


Fig. 5—Time course of Carboxypeptidase Inhibitor stability during various cooking methods; Apical end cortex — ● —; apical lateral cortex — x —; stem end cortex — □ —; and center pith — Δ —.

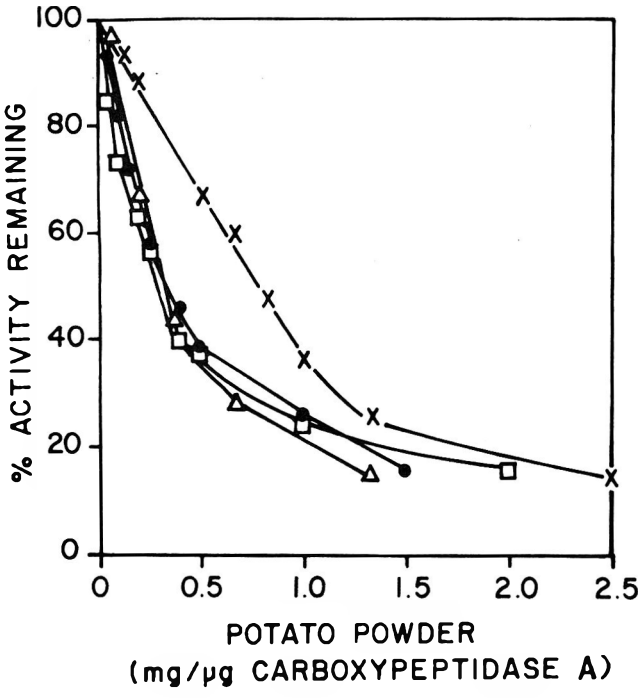


Fig. 6—Inhibition of Carboxypeptidase A with extracts from raw and cooked potato tubers. Raw — ● —; boiled — □ —; oven baked — x —; and microwave baked — Δ —.

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A Research Note

Cholesterol Content of Chicken Skin

PETER van de BOVENKAMP and MARTIJN B. KATAN

ABSTRACT

In order to resolve conflicting reports on the cholesterol concentration in chicken skin, we have assayed cholesterol in skin by gas-liquid chromatography. The mean content for six chickens was 71 mg/100g raw skin. Thus the suggestion that chicken skin is particularly high in cholesterol is probably not correct.

INTRODUCTION

WIDELY DIVERGENT FIGURES have been published for the cholesterol content of chicken skin (Sweeny and Weihrauch, 1976). Mickelberry et al. (1966) reported values of 106.7-111.4 mg/100g, similar to that in other chicken tissues and in beef or pork. From the data of Marion and Woodroof (1965), however, a value of 428 mg/100g can be deduced, which would put chicken skin on a par with high-cholesterol foodstuffs such as eggs and liver as far as cholesterol content is concerned. As this question is of importance to nutritionists, we have redetermined the cholesterol content of chicken skin by gas-liquid chromatography.

MATERIALS & METHODS

SIX CHICKENS (five deep-frozen and one fresh) were obtained locally from five different shops. Weights ranged from 1014-1248g. The total skin was separated from half of each carcass and homogenized. After homogenization, tissue samples of 10g were refluxed for 30 min with 100 ml 2M NaOH in 96% ethanol. The hydrolysate was first mixed with 100 ml benzene and then with 100 ml 2M aqueous KOH. The benzene layer was separated and washed free from alkali with water. One ml was combined with internal standard, dried down and silylated with 1.25M N,O-bis-silylacetamide in dimethylformamide (Trisyl-BSA, Pierce).

The silyl ethers were analysed on a Varian 2100 gas chromatograph with flame ionization detector. The 6 ft column was packed with 1.5% XE-60 on Varaport 30. Oven temperature was 230°C. Pure cholesterol (Merck 24622) and 5α-cholestane (Serva 17060) were used as calibration and internal standard, respectively.

Authors van de Bovenkamp and Katan are with the Dept. of Human Nutrition, Agricultural Univ., De Dreijen 11, 6703 BC Wageningen, The Netherlands.

RESULTS AND DISCUSSION

AS THE VALUES for the cholesterol content in the fresh broiler tissues were very close to the average of the deep-frozen samples only the combined data are presented here.

The average cholesterol content of total skin was 71 mg/100g wet tissue (range, 58-95). If we take into account the many possible sources of biological variation, then our results appear to be in reasonable agreement with those of Mickelberry et al. (1966), who reported a value of 106.7-111.4 mg/100g. Marion and Woodroof (1965), however, reported a concentration per 100g of broiler skin tissue of 361 mg cholesterol and 111 mg cholesterol esters. As the lipids were quantitated by weighing, the latter figure should be corrected for the weight of fatty acids in the esters. According to the data of Marion and Woodroof these accounted for about 44 mg. This leaves a total content of 428 mg/100 g, expressed as unesterified cholesterol. It seems unlikely that such a high value could be due to biological variation alone. Marion and Woodroof already pointed out, that it was possible that materials other than cholesterol were included in the cholesterol fraction in their study: it was noted that yellow pigments were eluted at the interface of the triglyceride and cholesterol fractions, and these pigments were weighed with these fractions. In the light of the findings of Mickelberry et al. (1966) and the figures reported here it appears likely, that the high value reported by Marion and Woodroof was erroneous.

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A Research Note

Effect of Electrical Stimulation and Postmortem Boning Time on Sensory and Cookery Properties of Ground Beef

H. R. CROSS and IVONNE TENNET

ABSTRACT

Ground beef was prepared from electrically stimulated and nonstimulated sides of 24 U.S. Utility grade carcasses that were boned 1, 3 or 24 hr postmortem. Postmortem electrical stimulation had no negative effects on the physical, sensory or cooking properties of ground beef patties. Time of boning had significant effects on percent height change during cooking, total cooking loss, tenderness, and juiciness. As boning time increased, the effects on the traits were negative.

INTRODUCTION

CROSS et al. (1979) successfully demonstrated that ground beef prepared from unchilled beef muscle is equal or superior to ground beef from chilled muscle in palatability and cooking properties. However, if processing of ground beef from hot-boned beef is to be practical, the hot processing of selected cuts for steaks and roasts from the same carcass must also be feasible. Gilbert and Davey (1976) and Gilbert et al. (1976) have demonstrated that hot-boned primal cuts from young beef (<30 months) should be electrically stimulated before boning to avoid the toughening effects of cold shortening. Berry et al. (1979) reported borderline tenderness ratings for hot-boned mature beef primal cuts that had been mechanically and enzymatically but not electrically tenderized. Thus, it may be necessary to electrically stimulate mature beef carcasses before hot-boning if the primal cuts are to be utilized as steak and roast cuts.

No data have been published on the possible negative effects of electrical stimulation on the sensory and cookery properties of hot-boned ground beef. The rapid pH decline resulting from electrical stimulation could cause an increase in cooking loss and subsequent decreases in juiciness and tenderness. The objective of our study was to evaluate the effect of electrical stimulation and postmortem boning time on sensory and cooking properties of ground beef.

EXPERIMENTAL

TWENTY-FOUR U.S. Utility grade (C to E maturity) carcasses were used to prepare the ground beef. The top round (semimembranosus), strip loin (longissimus) and ribeye (longissimus) cuts were removed and set aside for a different study. The remainder of the carcass was used for ground beef.

Ground beef was formulated into one of six treatments representing three postmortem boning times (1, 3 or 24 hr) and electrical or no electrical stimulation. Two 250-kg batches were prepared for each treatment. Eight carcasses were used for each postmortem boning time; one side of each carcass was stimulated and the opposite side was not. Sides were stimulated within 45 min postmortem by placing metal pins in the round muscle near the Achilles tendon and in the muscles between the scapula and the thoracic vertebrae. Each treated side received 1.5 amp of AC = alternating current (60 Hz) through the carcass for 3 min with four 10-sec shocks per minute.

Author Cross is with the Meats Research Unit, Roman L. Hruska U.S. Meat Animal Research Center, Clay Center, NE 68933. Author Tennent is with the Meat Science Research Laboratory, USDA, Agricultural Research, Beltsville, MD 20705.

Grinding

The raw material was ground through a large kidney breaking plate, mixed for 3 min, ground through a 1.3-cm plate, mixed for 2 min, and then ground through a 0.3-cm plate for the final grind. Fat content was adjusted following the first mix to 20 ± 2% using the Anyl Ray instrument. For those treatments involving 1 or 4 hr boning, CO_2 snow was added at a ratio of 1 part CO_2 to 10 parts beef to reduce the internal muscle temperature from 25-35°C down to 0-2°C. Two-thirds of the CO_2 was added during the first mix and the remainder during the last.

Patty formulation

Ground beef from all formulations was formed into 113-g (4-oz) patties with a Formax model 26 patty machine. Patties were stacked (10 per stack), boxed and frozen in a blast freezer, then stored at −10°C for 5 days before shipment to Beltsville, MD, for analysis.

Trained panel

In a total of five sessions, a 10-member descriptive attribute panel, trained by the procedures of Cross et al. (1978a) and the American Meat Science Association (1978), evaluated samples from each treatment. Six samples were evaluated per session, and each treatment was replicated five times. The panel rated each sample for differences in tenderness, juiciness, amount of connective tissue, and ground beef flavor intensity with 8 = extremely tender, juicy, no detectable connective tissue, or intense flavor and 1 = extremely tough, dry, abundant connective tissue, or bland in ground beef flavor.

Cookery and presentation to panel

Frozen patties were broiled on electric Farberware broilers (model 450-A– to an internal temperature of 60°C. Temperature was monitored during cooking with Teflon-coated iron/constantan thermocouples. Total cooking losses were calculated from frozen and cooked weights. Four patties from each treatment were prepared for each session. Each patty was sectioned into six pieces and two of the 24 pieces (4 patties) were randomly assigned to each panelist. The samples were served as warm as possible to the panelist, as described in AMSA (1978). Sections also were pictorially scored for degree of doneness from color photographs, with 1 = well done and 8 = rare, by a trained laboratory technician.

Shear force

Ten patties from each treatment were used for determination of Instron shear force by the single-blade procedure of Cross et al. (1978b). Four 2.5-cm squares were cut from each patty, thus each mean per treatment represents 40 observations.

Physical and chemical

Height and diameter were measured on the ten frozen and cooked patties used for the Instron in order to evaluate dimension changes during cooking. The pH was determined initially, after freezing (48 hr), after thawing, and after cooking according to the procedure outlined by Carse (1973).

Statistical analysis

Data from all six treatments were treated by analysis of variance (Snedecor and Cochran, 1967) and mean separation determined by Scheffes' procedure (Guenther, 1964).

Table 1—Chemical and cooking properties of beef patties as influenced by electrical postmortem stimulation and boning time[a]

	Postmortem boning time					
	1 hr		3 hr		24 hr	
Trait	ES	NS	ES	NS	ES	NS
pH						
Initial	6.21[c]	6.61[b]	5.76[d]	6.22[c]	5.54[d]	5.66[d]
After freezing	5.63[b]	5.71[b]	5.54[b]	5.64[b]	5.43[b]	5.45[b]
After thawing	5.64[b]	5.64[b]	5.61[b]	5.56[b]	5.54[b]	5.52[b]
After cooking	5.68[b]	5.71[b]	5.65[b]	5.53[b]	5.51[b]	5.58[b]
Diameter change, %	21.4[bc]	18.7[cd]	17.1[d]	19.4[c]	20.2[c]	22.1[b]
Height change, %	9.0[c]	8.7[c]	16.4[b]	14.5[b]	13.7[b]	13.4[b]
Cooking loss, %	40.5[c]	36.0[c]	41.7[c]	40.2[c]	43.9[b]	44.4[b]

[a]ES = electrically stimulated; NS = not stimulated.
[bcd]Means in the same row with different superscripts differ significantly ($P<0.05$).

Table 2—Sensory and shear properties of beef patties as influenced by electical stimulation and postmortem boning time[a]

	Postmortem boning time					
	1 hr		3 hr		24 hr	
Trait	ES	NS	ES	NS	ES	NS
Tenderness[b]	5.2[fg]	5.5[f]	5.1[gh]	5.1[gh]	4.8[hi]	4.6[i]
Connective tissue amount[c]	3.7[g]	3.9[fg]	3.9[fg]	4.1[f]	3.8[fg]	3.9[fg]
Flavor intensity[d]	4.8[f]	5.0[f]	5.1[f]	5.1[f]	5.1[f]	5.0[f]
Juiciness[e]	5.7[f]	5.7[f]	5.4[f]	5.9[f]	4.5[g]	4.1[g]
Instron work (area under max curve)	65.3[f]	61.9[fg]	66.1[f]	61.7[fg]	60.7[fg]	57.4[g]

[a] ES = electrically stimulated; NS = not stimulated.
[b] 8 = extremely tender and 1 = extremely tough.
[c] 8 = no connective tissue and 1 = abundant.
[d] 8 = extremely intense and 1 = extremely bland.
[e] 8 = extremely juicy and 1= extremely dry.
[fgi] Means in the same row with different superscripts differ significantly ($P<0.05$).

RESULTS AND DISCUSSION

MEANS for pH and cooking properties are presented in Table 1. Differences in pH were evident only among the initial values. As expected, electrical stimulation significantly increased the rate of pH decline in the muscle postmortem. These differences were most pronounced in the 1 and 3 hr treatments. By 24 hr postmortem, all samples had reached their ultimate pH. The pH values reported here are consistent with those reported by Chrystall and Hagyard (1976), Sorinmade et al. (1978), and Cross et al. (1980).

Differences in percentage of diameter change during cooking were significant, but it was difficult to establish a pattern with regard to boning time and electrical stimulation (Table 1). Percentage of height change was significantly affected by time of boning. Ground beef patties prepared from lean and fat boned at 1 hr postmortem shrank significantly less than those prepared at 3 and 24 hr. Percentage of total cooking loss was also affected by boning time ($P<0.05$). Differences were not statistically significant between the 1 and 3 hr, but these two differed ($P<0.05$) from 24 hr (Table 1). Cross et al. (1979) and Cuthbertson (1977) reported that weight loss of boneless beef primal cuts during storage was directly related to the time of postmortem boning. It is likely that this could also affect cooking losses.

Electrical stimulation had nonsignificant effects on all palatability and Instron traits (Table 2). It appears that grinding the lean and fat into ground beef offsets any potential tenderness advantage derived from electrical stimulation. It is also evident that electrical stimulation does not adversely affect the juiciness of ground beef patties. However, time of boning had significant effects on tenderness and juiciness. As boning time increased from 1 to 24 hr, sensory panel ratings for tenderness and juiciness decreased significantly. Treatments 1 and 3 did not differ statistically but both of these differed ($P<0.05$) from the 24-hr treatment. These results are similar to those reported by Cross et al. (1979), who found significant decreases in tenderness and juiciness and increases in cooking losses when comparing hot-boned and cold-boned ground beef.

In conclusion, these data indicate that postmortem electrical stimulation has no negative ffects on physical, sensory or cooking properties of ground beef patties. Postmortem boning time had significant effects on percentage of height change during cooking, total cooking loss, tenderness and juiciness. The shorter or earlier boning time resulted in higher tenderness and juiciness ratings, less height shrinkage and less cooking loss.

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Measuring Skeletal Muscle Fiber Width or Diameter: Variance Within and Among Methods

HANIF A. KHAN, DOROTHY L. HARRISON, and ARTHUR D. DAYTON

ABSTRACT

A bovine longissimus dorsi muscle was divided into anterior and posterior pieces, half of each piece was roasted at 140°C to 70°C. "Widths" or "diameters" of fibers from each position (anterior, posterior) and treatment (raw, cooked) were measured by three methods: A, width of separated fibers on a longitudinal plane; B, width of fibers in longitudinal sections; and C, fiber diameter calculated from the area of fibers in cross sections. Bartlett's test for homogeneity of variance indicated greater ($P<0.05$) variance among observations by method A than by methods B or C; position-treatment variances were larger ($P<0.05$) for method A than for B or C.

INTRODUCTION

SKELETAL MUSCLE FIBER width and diameter have been measured on fibers on longitudinal and cross sections of muscle tissue. Values in the literature for mean fiber "width" or "diameter" vary greatly. Joubert (1955) found a range in fiber diameter of 28-73μ for raw bovine longissumus dorsi muscle. Bourne (1960) reported a range of 10-100μ in the diameter of fibers in different voluntary muscles.

Part of the variation among data for a given muscle may be attributed to the method of measuring. Because fibers occur in many shapes, values for "width" measured across fibers from a longitudinal plane will depend on the plane on which the muscle tissue was sectioned. Deviation from a true cross section will increase the area exposed for measurement, and because fibers are not uniformly round, it is difficult to select an area to measure (Swanson et al., 1965). We estimated variance within each of three methods of measuring "width" or "diameter" of fibers in both raw and cooked tissue and variance among those methods.

MATERIALS & METHODS

TO ELIMINATE animal variation, we planned the experimental design so all samples were from one bovine longissimus dorsi (LD) muscle. The plan for sampling a left bovine LD rib section (cut between the 4th and 5th ribs and posterior to the 12th rib) is presented in Figure 1. The muscle was divided into posterior (X) and anterior (Y) pieces. Each of those two pieces was divided again; the posterior portion of both X and Y pieces was used for raw tissue (r) samples. The anterior portions were roasted at 149°C to an end point of 70°C (c), and sampled. Samples used to measure fiber "width" or "diameter" by three methods (A,B,C) were selected randomly (Fig. 1).

Method A–Width of separated fibers measured on a longitudinal plane

Samples (ca 1.5 x 1.5 x 2.0 cm) were fixed in 10% formalin and physiological saline solution 48 hr, then using a razor blade, thin slices were cut about 0.5 cm from each end of the sample. Slices were placed in a Waring Blendor with blades reversed and blended with 50 ml physiological saline solution at low speed (50 on a rheostat) for 30 sec. Part of the blended material was poured into a clean petri dish and observed through a phase contrast microscope at 100x magnification. The "width" of 200 (100 raw, 100 cooked) randomly selected fibers, 25 from each of eight sampling areas (Fig. 1), was measured with an ocular micrometer in the eye piece of the microscope.

Method B–Width of fibers measured on longitudinal sections

Samples (ca 1.5 x 1.5 x 2.0 cm) were fixed in 10% formalin and physiological saline solution for 2-3 wk. Specimens (approx. 1.0 x 1.0 x 0.5 cm) were cut parallel to the muscle fibers. A small amount of Cryoform, an embedding matrix, was placed on the tissue holder of a cryostat microtome, and the tissue was placed on the Cryoform. The tissue holder was placed in the microtome, and the specimen was frozen rapidly with Cryokwik, a commercial preparation of Freon gas. Sections were cut 19μ thick (microtome set at −20°C), and transferred immediately, with a small camel hair brush, to a microscopic slide containing a drop of albumin-water mixture. At least 10 sections from each specimen were mounted. Slides were warmed (32-38°C) to evaporate the albumin-water mixture. Sections were stained with picric acid and picro-ponceau stain, cleared in xylene, and mounted with permount. Muscle fibers stained yellow and connective tissue stained pinkish red. Each member of a three-member panel used an ocular micrometer in the eyepiece of a Bausch & Lomb Dynazoom microscope and a magnification of 430x to measure the "width" of 50 randomly selected fibers in each specimen. That procedure provided measurements of 600 fibers in raw tissue and 600 fibers in cooked tissue.

Method C–Diameter of fibers in cross sections

Specimens (ca 0.5 x 0.5 x 0.5 cm) of unfixed muscle tissue were cut with a sharp razor blade and placed on a corkboard (ca 1.5 x 1.5 cm) that had been soaked in 10% formalin and physiological saline solution. Specimens were frozen by holding the square of a corkboard containing the specimen in 2-methylbutane cooled to −25°C in liquid nitrogen. The frozen specimen was heat sealed in plastic tubing and kept on dry ice until sectioned (ca 3 wk).

When sectioned, the specimen on the corkboard was secured to the platform of a cryostat microtome with Cryoform. The specimen was trimmed so the top was slightly smaller than the bottom;

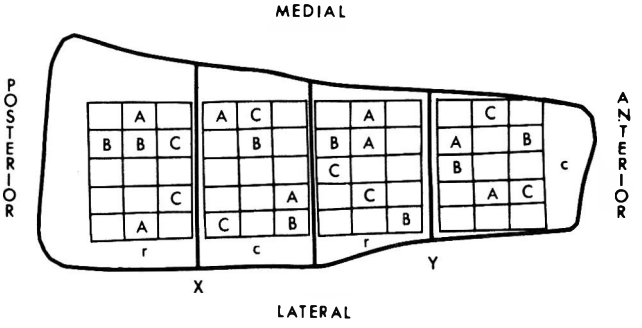


Fig. 1–Sampling of the longissimus dorsi muscle, between 4th and 5th rib and posterior to the 12th rib: A, samples used to measure "width" of separated fibers measured on a longitudinal plane; B, samples used to measure "width" of fibers measured on longitudinal sections; C, samples used to measure "diameter" of fibers calculated from the area of fibers measured on cross sections; X, posterior half of the rib section; Y, anterior half of the rib section; r, raw tissue; c, cooked tissue.

Authors Harrison and Dayton are affiliated with the Departments of Foods & Nutrition and Statistics, Kansas Agricultural Experiment Station, Kansas State Univ., Manhattan, KS 66506. Author Khan, formerly with Kansas State is now affiliated with Mansfield State College, Mansfield, Pa.

it was oriented on the platform so the cross section could be cut with a sharp razor blade in the microtome knife holder. Sections, cut 10μ thick using a cryostat microtome set at $-20°C$, were transferred to microscopic slides (one section per slide) by lightly touching the slide to the section while the section was still on the razor blade. At least 10 sections from each specimen were stained with hematoxylin and eosin stains, and mounted with permount.

A microprojector was calibrated to determine magnification by projecting a 2-mm micrometer on a wall in the laboratory and measuring the projected line. Randomly selected sections were projected on the wall, and the areas of 200 randomly selected muscle fibers from eight sampling areas (100 cooked, 100 raw fibers), Figure 1, were traced onto plain white paper. The area (sq cm) of each fiber was determined with a polar planimeter. With fibers assumed to be round, the area (A) was converted to diameter (D) by the formula, $D=2\sqrt{A/\pi}$. Diameter in centimeters was converted to microns, and correction for magnification was made by dividing the diameter by its magnification.

Statistical analyses

To study the precision of the three methods used to measure fiber "width" or "diameter", we applied Bartlett's test for homogeneity of variance (Snedecor, 1956) to the data to test the significance of differences among observations for each method, and to estimate variance among the three methods.

RESULTS & DISCUSSION

Variance within methods

Bartlett's test for homogeneity of variance indicated that differences among observations by methods B or C were not significant (chi-square = 27.3 with 23 degrees of freedom and chi-square = 9.26 with 7 degrees of freedom, respectively); variance among observations by method A was significant ($P<0.05$; chi-square, 16.59 with 7 degrees of freedom). The nonsignificant differences among observations by methods B and C indicate that data obtained by those methods were precise, so either method is acceptable to study effect of treatment on fiber "width" or "diameter." Either method B or C is preferable to the less precise method A. Also, variance among observations by the three persons who measured fibers by method B was not significant, which suggests that precise data can be obtained by one person using method B.

Table 1—Variance among methods of measuring bovine longissimus dorsi fiber "width" or "diameter" by position and treatment of LD muscle

Group	Variance[a]	Method	Position	Treatment
I	191.1	A	Posterior	Raw
II	127.1	A	Anterior	Cooked
	121.1	A	Anterior	Raw
III	86.7	A	Posterior	Cooked
	80.8	C	Anterior	Raw
	73.5	C	Posterior	Raw
IV	55.3	B	Posterior	Raw
	50.8	C	Anterior	Cooked
	47.6	B	Anterior	Raw
	42.8	B	Anterior	Cooked
	42.2	B	Posterior	Cooked
	38.7	C	Posterior	Cooked

[a] Variances within a group do not differ significantly from one another. Variances differ ($P<0.05$) between groups A, "width" of separated fibers measured on a longitudinal plane, n = 50; B, "width" of fibers measured on longitudinal section, n = 300; C, "diameter" of fibers calculated from the area of fibers measured on cross sections, n = 50.

Variance among methods

Estimates of variance (S^2) for 12 treatment combinations (2 positions; anterior, posterior × 2 treatments; raw, cooked × 3 methods) were placed in four groups so that within a group variances were homogeneous (statistically equal), but differed between groups (Table 1). All position-treatment variances for method A were larger ($P<0.05$) than all variances for method B. All but one of the position-treatment variances for method A (86.7, posterior-cooked) were larger ($P<0.05$) than those for method C; that one variance for method A was larger than two of the variances for method C (80.8, anterior-raw; 73.5, posterior-raw), but it did not differ significantly from them.

CONCLUSIONS

UNDER THE CONDITIONS of our study, we concluded: (1) methods B and C are more precise than method A, so either B or C is acceptable to study treatment effects on "width" or "diameter" of fibers in skeletal muscle tissue, and (2) methods B and C are superior to A for studying treatment effects on skeletal muscle fibers.

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Contribution 80-372J Dept. of Foods & Nutrition and Dept. of Statistics, Kansas Agricultural Experiment Station, Kansas State Univ., Manhattan, KS 66506.

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A Research Note

Comparison of Spectrophotometric and HPLC Methods to Quantify Betacyanins

S. J. SCHWARTZ, B. E. HILDENBRAND and J. H. VON ELBE

ABSTRACT

Quantitative data for betacyanins determined by a spectrophotometric method and a high performance liquid chromatography (HPLC) method were compared. Pigment solutions at pH 4.0, 5.0 and 6.0 were heat treated under a nitrogen atmosphere and analyzed for pigment losses at appropriate intervals. Fresh, blanched and canned beets were measured for pigment content by the two methods. Quantitative determinations of pigment in all purified or undegraded samples compared well when analyzed by either method. Discrepancies between results of the two procedures occurred and increased up to 15% with extended heat treatment of the pigment. The differences were attributed to formation of degradation products or interfering substances. Of the two, the HPLC method is preferred when interfering substances are present.

INTRODUCTION

NATURAL PIGMENTS of the beet root (*Beta vulgaris var rubia*), collectively known as the betalains, are useful as a red food colorant. Extensive research has been carried out to identify factors affecting the stability of the pigments in food and model systems. Published quantitative data indicate variability of results among studies. Methods used to quantify betalains may be responsible for these differences.

Nilsson (1970) developed a method to spectrophotometrically measure both red and yellow pigments in fresh beet juice without prior separation of pigments. von Elbe et al. (1972) separated the betacyanins using electrophoresis. Subsequent quantification of betanin and total betacyanins was obtained by densitometry. A computer aided determination of beet pigments, based on previously reported absorptivity values has been developed by Saguy et al. (1978a). Quantification by high performance liquid chromatography (HPLC), which removes interfering substances, was employed by Vincent and Scholz (1978). Schwartz and von Elbe (1980) recently described an HPLC method which separates individual betacyanin pigments and quantifies each component on the basis of their appropriate detector response and molar absorptivity values.

The purpose of this study was to compare quantitative data from the method developed by Nilsson (1970) and employed by others, with data obtained by the HPLC method of Schwartz and von Elbe (1980). Pigment solutions at pH 4.0, 5.0, and 6.0 were heat treated under a nitrogen atmosphere and analyzed for pigment loss at appropriate intervals. Furthermore, the pigment content of fresh, blanched and canned beets was measured by the two methods.

MATERIALS & METHODS

Preparation of pigment samples for model system studies

A purified pigment concentrate, obtained from chromatography on polyvinylpolyrrolidone (von Elbe et al., 1972) containing betanin and isobetanin was diluted with 0.05M phosphate-acetate buffers, pH 4.0, 5.0, and 6.0 to produce a solution with absorbance (Beckman Spectrophotometer Model 24, Beckman Inst. Inc., Fullerton, Calif.) of approximately 1.6-1.7 at 535 nm. A minimum of seven vials (0.5 dram) (Sargent-Welsh Scientific Co., Skokie, Ill.) at each pH value were filled with 2.0ml of the pigment solution, capped with a 5 x 9 mm airtight septum (Arthur H. Thomas Co., Philadelphia, Pa.) and flushed for 5 min with high purity N_2. The N_2 was bubbled at a flow rate of 100 ml/min through a solution of 35% KOH w/w plus 7.5% w/w pyrogallic acid to remove residual O_2. One vial from each lot was removed (time = zero) and analyzed for pigment content by the methods of Nilsson (1970) and Schwartz and von Elbe (1980). Vials containing the solutions at pH 4.0 and 6.0 were immersed in a water bath at 35°C. Vials containing the solution at pH 5.0 were heated at 65°C. Each experiment was carried out in duplicate. Samples were covered to prevent light exposure. At appropriate intervals, one vial from each lot was removed for analysis of pigment content by the methods of Nilsson (1970) and Schwartz and von Elbe (1980).

Processing of beets for comparative quantitative pigment analyses

Fresh beets, Ruby Queen variety, were obtained from a commercial source and analyzed for pigment content by the methods of Nilsson (1970) and Schwartz and von Elbe (1980). Three sample lots of beets, A, B, and C, were washed, peeled, and blanched to an internal temperature of 66 ± 1°C, as measured by a thermocouple. Heating times ranged from 15-17 min. The beets were sliced (Slicer; Urschel Labs, Inc., Valparaiso, Ind.) to a thickness of 0.6 cm. A randomly selected sample from each blanched lot was removed, cooled and analyzed immediately for pigment content. Three hundred grams of blanched beets were placed in 303 size cans with 180g of hot H_2O, sealed, and processed with agitation at 126 ± 1°C for 10 min in a Steritort (FMC Corp., San Jose, Calif.). In addition, two samples from lot A were processed in a still retort at 116 ± 1°C for 31 min. Two cans from each lot were stored at room temperature for 1 wk and analyzed by the methods of Schwartz and von Elbe (1980) and Nilsson (1970).

Preparation of beet samples for pigment analyses

One hundred grams of fresh beets from five randomly selected roots were used for analysis. The tissue was blended for 1 min with 100g of EtOH:H_2O (50:50 v/v) under a stream of N_2 to lessen oxidative enzymatic reactions. The blender walls were washed with 100g of H_2O, and the mixture was blended for an additional 5 min under N_2. Fifty grams of the homogenate were removed, filtered through a bed of celite, and washed with 300-400 ml of H_2O until the tissue-celite mixture was colorless. The filtrate was quantitatively transferred to a 500 ml volumetric flask and brought to volume. A 1:5 dilution of this sample was analyzed by the two methods. In the spectrophotometric method, light absorbance values at 538, 476, and 600 nm were read on a spectrophotometer (Model 24, Beckman Instruments, Fullerton, Calif.) using 1.0 cm quartz cuvettes.

Blanched samples were prepared for analysis as just described but using H_2O rather than EtOH:H_2O as the initial solvent. For processed samples, the entire contents of a can were transferred quantitatively into the blender jar and blended for 2 min. Fifty grams of the homogenate were filtered through 20g of celite and washed with H_2O (300-400 ml) until the tissue-celite mixture was colorless. The filtrate was transferred to a 500 ml volumetric flask and made up to volume. A 1:10 dilution was used for HPLC analyses.

RESULTS AND DISCUSSION

Analyses of betacyanin content in heated pigment solutions

Data in Table 1 compare quantitative results of betanin-isobetanin content in solutions of pigments when exposed to different temperatures and times. In all instances, excellent agreement between results from the spectrophotometric method and from the HPLC method were observed

Authors Schwartz, Hildenbrand, and von Elbe are affiliated with the Dept. of Food Science, Univ. of Wisconsin-Madison, Madison, WI 53706.

at zero time. This is to be expected since both methods are based on measuring pure undergraded pigment. Discrepancies in results between these methods increased as the heating time of samples increased.

The reason for the differences can be attributed to the formation of breakdown products which absorb light at the chosen wavelengths. If separation of degradation products and pigments is not accomplished before analysis, as in the spectrophotometric method, a larger value will be obtained when compared to the HPLC method.

Chromatogram analysis of betanin-isobetanin solutions throughout heating indicate the appearance of a major degradation product. This product does not correspond to any chromatogram peaks separated from a mixture of betacyanins identified by Schwartz and von Elbe (1980). The degradation product increased with an increase in time of heating. Its strong light absorption at 535 nm accounts for some differences in concentration as measured by the two methods. The presence of this product seemed to be pH dependent and amounts were less at pH 6.0 than at pH 4.0 and 5.0. Other compounds were formed at all pH values and further contributed to the differences but were not eluted under the conditions used; however, they were washed from the column with a solution of methanol: water (70:30 v/v) during column cleaning.

The chemical nature of the degradation product is unknown and conjectures as to its identity would be speculation. Because of the relative retention time of the peak and its absorption at 535 nm, the compound must be less polar than betanin and isobetanin, and possess a highly conjugated structure. Further work is now in progress to elucidate the chemical structure of the formed product.

Table 1—Betanin-isobetanin content in solutions of pigments at pH 4.0, 5.0, and 6.0 heated at 35 or 65°C for various time intervals when analyzed by the spectrophotometric method and HPLC

Reaction conditions	Reaction time (hr)	Betacyanin conc μg/ml Spec[a]	HPLC	△[b]
pH 6.0	0	15.5	15.4	0.1
35°C	45	7.4	6.8	0.6
	80	5.6	5.2	0.4
	127	4.6	3.6	1.0
	175	4.2	3.5	0.7
	227	3.1	1.6	1.5
pH 4.0	0	14.1	14.1	–
35°C	51	10.7	10.1	0.6
	75	10.0	8.6	1.4
	127	9.5	6.9	2.6
	174	7.2	2.4	4.8
	225	6.4	1.2	5.2
pH 5.0	0	15.0	15.0	–
65°C	45	10.7	10.7	–
	75	9.0	8.8	0.2
	115	7.4	7.0	0.4
	165	6.7	6.2	0.5
	220	6.2	5.1	1.1

[a] Spectrophotometric method
[b] △ = difference in μg/ml between methods

Table 2—Betacyanin content of fresh and processed beets as determined by the spectrophotometric method and HPLC

Treatment	Replicate[a]	Betacyanin conc in mg/100 Product Spec[b]	HPLC	△[c]	%△[d]
Fresh		89.3	89.0	0.3	0.3
Blanched	A	76.3	73.5	1.9	2.6
	B	74.0	72.2	1.8	2.5
	C	60.3	57.4	2.9	5.1
Canned	A	58.8	53.4	5.5	10.3
	A^1	43.5	37.7	5.8	15.4
	B	56.1	50.9	5.2	10.2
	C	52.8	47.6	5.2	10.9

[a] Blanching: (samples A, B, C) 100°C to reach an internal temperature of 66°C; canning: (samples A, B, C) processed at 126°C, 10 min, sample A^1 processed at 116°C, 31 min.
[b] Spectrophotometric method.
[c] △ = difference in mg/100g product between methods.
[d] % of HPLC method.

Analyses of betacyanin content of fresh and processed beets

Data in Table 2 are the results of the two analytical methods when amounts of pigment were measured in fresh, blanched and canned beets. Excellent agreement in results from both methods for pigment content was noted for the fresh sample. As observed in experiments with the solution of pigment, a difference in amount of pigment as measured by the two methods occurred when beets were heated and the difference increased with severity of the heat treatment. The differences were up to 5% for blanched beets and 10% for canned beets processed at 126°C for 10 min and 15% when the beets were processed at 116°C for 31 min. In all analyses, the results of the spectrophotometric method suggested a higher concentration of pigment than did the results of the HPLC method.

The spectrophotometric method developed by Nilsson has been applied by Wiley and Lee (1978). The computer-aided determination developed by Saguy et al. (1978a) increased the precision of the method developed by Nilsson by measuring pigment content based on ten wavelength readings rather than three. This method was utilized by Kopelman and Saguy (1977) and Saguy (1979) to determine thermal stability of beet pigments and by Sapers and Hornstein (1979) to determine varietal differences in pigment content. The data obtained in our study suggest that quantitative results reported in the other studies just mentioned are higher than the true values. Spectrophotometric methods may be applicable when a comparison between samples is made having similar errors. The spectrophotometric method by Nilsson was developed solely for the purpose of studying quantitative pigment differences among beet varieties and, as results of this study indicate, is applicable and accurate for such purposes. If degradation products are believed to be present in a sample, separation of these from the pigment by HPLC should be the method of choice.

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A Research Note

Thin-Layer Chromatographic Separation of Beet Pigments

ALEXANDER BILYK

ABSTRACT

A new simple method for the thin-layer chromatographic (TLC) separation of betalaines has been developed and is described in this paper. The individual components were separated by preparative TLC on cellulose-coated plates and analyzed by a multiple development TLC technique with solvent mixtures consisting of ethanol, isopropanol, water, and acetic acid. Separated components of various hues were clearly visible after development without application of an indicator. Spectral analyses of fractions also were conducted. By this TLC procedure, analytical data were obtained more rapidly and conveniently than could be obtained by other methods such as electrophoresis.

INTRODUCTION

AMONG VARIOUS PLANTS that produce natural colorants, the red table beet (*Beta vulgaris*) is a potential source of valuable water-soluble pigments, the betalaines, which are comprised of two main groups: betacyanines (red) and betaxanthines (yellow) pigments. However, efficient and economical methods for isolation and identification of these components still present a problem. Commonly, the separation of individual pigments is carried out by column chromatography (von Elbe et al., 1972) or paper electrophoresis (Powrie and Fennema, 1963). Since these methods are costly and time consuming, multiple development TLC was investigated as a rapid and more convenient method of separating and quantitating betalaines.

Preparative TLC

The multiple development technique of TLC is used when one solvent is incapable of separating some of the substances (Stahl and Kaltenbach, 1961). It is often possible to separate the components first with one solvent mixture and then with another, each component being developed to a different length on the plate. Separation of betalaine pigments was accomplished by the preparative TLC procedure with the sequential use of two distinct solvent mixtures consisting of various proportions of isopropanol, ethanol, and water containing 5% acetic acid. 500 μm thick cellulose-coated TLC plates provided by Brinkmann Instruments, Inc., Westbury, NY 11590 (Cat. No. 66-14-100-4), were used. Each sample was applied on preparative TLC plates by means of a streaker made by Applied Science Laboratories Inc., State College, PA 16801 (Cat. No. 17700). The solvent mixtures, containing acetic acid, denoted by Roman Numerals I, and II, listed in Table 1 were used for development.

Two hundred mg of beet juice powder, obtained by the procedure previously described (Bilyk. 1979), were dissolved in 4 ml of distilled water and applied on the cellulose-coated plate. The sample was developed first with the more polar System II which separates the pigments from insoluble substances. After the solvent moved 10 cm, the plate was dried under a nitrogen atmosphere, and two successive developments were made with the less polar System I to a height of 15 cm each time.

Table 1—Solvents used for TLC separation of betalaine components

Solvents	Solvent mixtures, ml I	II
Isopropanol	55	30
Ethanol	20	35
Distilled water	20	30
Acetic acid	5	5

RESULTS & DISCUSSION

BETALAINE PIGMENTS, being compounds of ionic nature, are difficult to separate. Previously, the most reliable methods for separating, isolating, and quantifying the betacyanines (Piatelli and Minale, 1964) and betaxanthines (Piatelli et al., 1965) in plant material were column chromatography and paper electrophoresis (Powrie and Fennema, 1963; Nilsson, 1970). Thin-layer chromatography with unacidified solvents gives poor results because the migration of unprotonated betalaine is irregular and separation of individual components incomplete. However, when acid is incorporated in the developing solvent, the movement of betalaine on the TLC plate is facilitated, due to the protonation of the carboxyl group of the betacyanine molecule. The acid anion provides an electrically neutral system with the quaternary nitrogen. The same phenomenon occurs with betaxanthine. Such a protonated pigment acquires a chromatographic mobility which facilitates the preparative TLC development.

No indicator is needed for visualization of the separated pigments; they appear clearly on the TLC plate in their natural colors. During the development procedure, the TLC tanks should be covered with aluminum foil to shield the plate from light, which may cause pigment degradation.

The success of TLC separation is greatly influenced by the proper application of water-soluble pigment. For preparative TLC a special sample streaker is recommended. The streak applied on the plate should be narrow, and solvent (water) should be evaporated immediately at room temperature under a nitrogen stream. In order to avoid band irregularities during the development, the plate must be dried thoroughly before it is placed in new developing mixture. Material extracted from the TLC bands should be stored at 5°C after solvent removal to prevent degradation.

The separation of individual components on the TLC plate is shown in Figure 1. The band denoted by B represents the red betacyanines; band C, the yellow betaxanthines, which consist of Vulgaxanthine-I and Vulgaxanthine-II. The top band D was a very light-yellow pigment which had no measureable absorption maximum in the 375-650 nm range (color picture can be obtained from the author).

Figure 2 illustrates the spectrophotometric evaluation of the individual fractions obtained by preparative TLC with the multiple development technique. Absorbance

Author Bilyk is with the USDA-SEA-AR, Eastern Regional Research Center, 600 E. Mermaid Lane, Philadelphia, PA 19118.

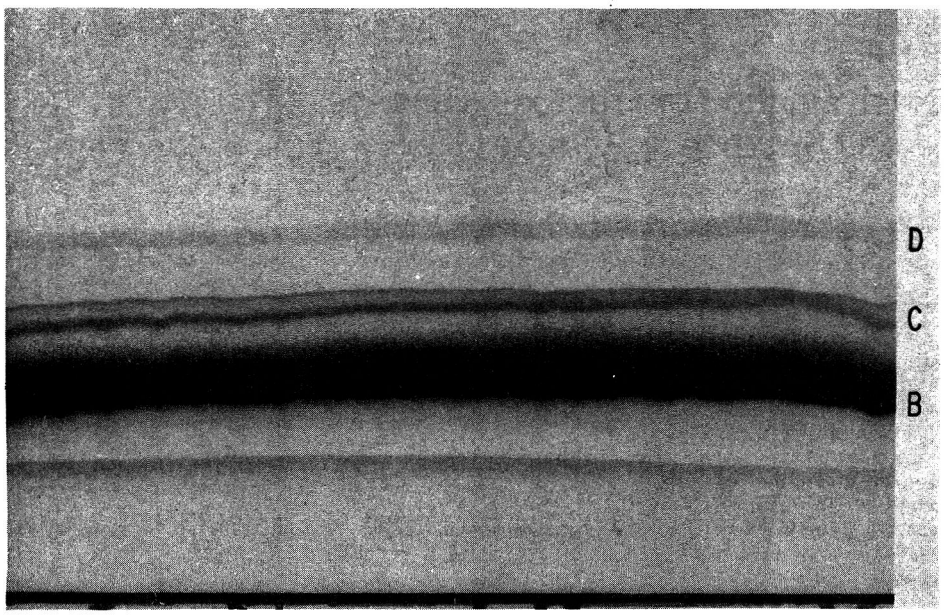


Fig. 1—Preparative thin-layer chromatography: (B) betacyanine fraction (red); (C) betaxanthine fraction (dark yellow); (D) light yellow fraction, no peak in the visible spectrum.

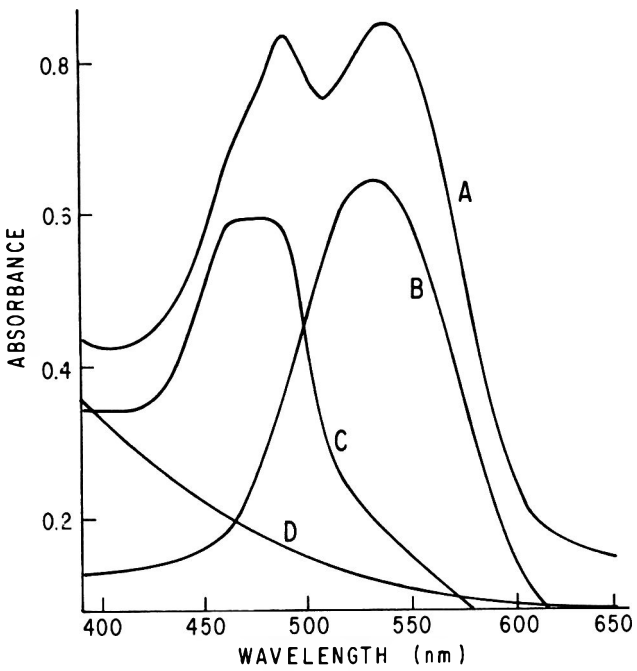


Fig. 2—Visible spectra of betacyanines and betaxanthines obtained from the preparative thin-layer chromatography: (A) beet juice pigments; (B) betacyanine fraction; (C) betaxanthine fraction; (D) top layer light yellow fraction, no peak in the visible spectrum.

measurements were made between 375 and 650 nm with a Bausch and Lomb spectronic 505 recording spectrophotometer. The absorption spectrum of crude beet juice powder, denoted by A, shows two absorption peaks, one at 537 nm, which is typical of the red betacyanines, and the other around 480 nm, characteristic of the yellow betaxanthines. The fraction of red pigment, denoted as spectrum B, exhibited a single peak at 537 nm. The yellow pigment fraction, spectrum C, gave a single peak at 480 nm. Spectrum D represents the topmost light yellow TLC band; no peak was evident for this pigment in the 375-650 nm range spectrum.

This method provides a rapid and satisfactory separation of the red and yellow pigments of beet, in quantities suitable for studies of their chemical and physical properties.

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Reference to brand or firm name does not constitute endorsement by the U.S. Dept. of Agricluture over others of a similar nature not mentioned.

A Research Note

HPLC Determination of Fructose, Glucose, and Sucrose in Potatoes

A. M. WILSON, T. M. WORK, A. A. BUSHWAY, and R. J. BUSHWAY

ABSTRACT

A high-performance liquid chromatographic (HPLC) method has been developed to determine fructose, glucose, and sucrose content of potatoes. The HPLC system consisted of a μBondapak/carbohydrate column, a solvent system of acetonitrile-water (75:25), a flowrate of 1.8 ml/min, and a refractive index detector. Analysis, including sample preparation, was complete in 30 min. With the exception of high concentrations of sucrose (8 mg/g or above), the method recovered 93% or more of all sugars. The coefficients of variation for the procedure ranged from 1.39-13.31%. TLC indicated that there were no interfering compounds eluting with any of the three sugars.

INTRODUCTION

INDIVIDUAL DETERMINATION of glucose, fructose, and sucrose, the major sugars in potatoes, is becoming essential as more studies are being conducted on genetic and biochemical mechanisms of carbohydrate formation and degradation in potato tubers. There is also an interest in predicting the storage potential of tubers based on their sucrose content at harvest (Sowokinos, 1978) and in analyzing sugar content of potatoes stored at various conditions.

Previous methods include colorimetric procedures which do not distinguish between the monosaccharides, glucose and fructose, directly. Furuholmen et al. (1964), using a modification of Ting's method (1956), determined glucose concentration as the difference between total reducing sugars and fructose. Della Monica et al. (1974), using Furuholmen's procedure and a glucose oxidase method, quantified fructose by subtracting glucose determined enzymatically from total reducing sugars. Sucrose was determined as the increase in glucose content after acid hydrolysis of sucrose.

Chromatographic procedures include gas chromatography which differentiates between the three major sugars but requires a time-consuming derivatization step (Kimura et al., 1969; Shaw, 1969; Varns and Shaw, 1973). HPLC methods determine the individual sugars and do not require derivatization. Currently there are HPLC methods available to analyze sugars in boiled sweets and jelly (Hunt et al., 1977), milk and ice cream (Warthesen and Kramer, 1979), milk chocolate (Hurst and Martin, 1977), and confectionary products (Timbe and Keeney, 1977). This paper describes and HPLC procedure for analyzing fructose, glucose and sucrose in potatoes.

MATERIALS & METHODS

Liquid chromatography

A Waters Associates liquid chromatograph, model ALC/GPC 244 (Milford, MA), equipped with a 6000A pump, a U6K injector, and a differential refractometer, was used. The detector signal was recorded on a Houston Instruments (Austin, TX) dual-pen recorder. The column system consisted of a 3.2 mm i.d. x 4.0 cm precolumn packed with C_{18} Porasil B (Waters Associates) and a 3.9 mm i.d. x 30 cm column packed with μBondapak/carbohydrate packing. The mobile phase was acetonitrile-water (75:25). Operating conditions were flowrate: 1.8 ml/min, detector sensitivity: 8X, and chart speed: 0.4 in/min.

Standard preparation

A standard containing the three sugars (fructose, glucose, and sucrose) was prepared by placing 250 mg of each sugar in a 25 ml volumetric flask and dissolving them in water. A 10 ml aliquot was removed and mixed with 10 ml of methanol followed by centrifugation at 43,000 x G for 5 min (Sorvall RC2B, Dupont Instruments, Newtown, Conn). After passing the supernatant through a C_{18} Sep Pak (Waters Associates), the first milliliter was discarded and the next 2.5 ml was collected. A standard curve was prepared each day by injecting 2, 6, 10, 14, and 18 μl of the standard solution. Quantification of each of the sugars was accomplished by comparing peak height of the samples to peak height of the standard since peak height was directly proportional to concentration of the standard throughout the concentration range used.

Sample preparation

Cultivars analyzed were Kennebec, Katahdin, and Russet Burbank, all stored at 3.3 and 7.2°C, and Monona and Norchip, which were stored at 12.8°C. A center cut from each of six tubers of uniform size was removed, peeled, and diced. A 200g random sample was

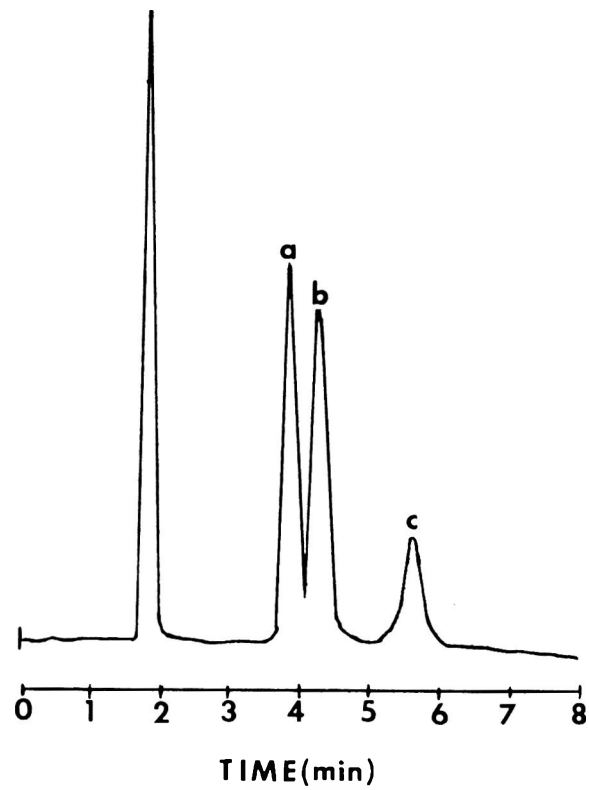


Fig. 1—Chromatogram of fructose, glucose and sucrose present in Russet Burbank tubers stored at 3.3°C. Peaks: (a) fructose, (b) glucose, (c) sucrose.

All authors are affiliated with the Food Science Dept., Univ. of Maine, Orono, ME 04469.

Table 1—Reproducibility study of the HPLC method for sugars in three cultivars at two storage temperatures[a]

Cultivar	Storage temp (°C)	Fructose		Glucose		Sucrose	
		Mean (mg/g)	SD	Mean (mg/g)	SD	Mean (mg/g)	SD
Kennebec	3.3	5.91	0.55	6.76	0.69	2.26	0.24
	7.2	0.78	0.06	1.56	0.10	0.65	0.06
Katahdin	3.3	8.01	0.11	8.87	0.29	2.51	0.12
	7.2	1.06	0.04	1.73	0.14	0.78	0.05
Russet Burbank	3.3	6.75	0.34	8.02	0.36	2.57	0.34
	7.2	0.77	0.06	1.55	0.10	0.65	0.06

[a] Data are based on the analysis of five samples for Katahdin potatoes stored at 3.3° and 7.2°C and for Russet Burbank and Kennebec tubers stored at 7.2°C. For the latter two varieties stored at 3.3°C, six samples were analyzed.

extracted in an Acme Juicerator (Lemoyne, Pa). A 10 ml aliquot of the juice was prepared as was the 10 ml aliquot of the standard described in the Standard Preparation section. (The methanol was used to precipitate the protein in the samples.) The amount of sample injected into the HPLC ranged from 6-20 μl depending upon the concentration of the sugars in the sample.

Recovery studies

The recovery of added fructose, glucose, and sucrose at two levels (2 mg/g and 8 mg/g) was determined by dividing the juice of 200g of Kennebec potatoes into two equal portions with one portion having no sugar added while the other was spiked at the appropriate level. These samples were prepared as described in the Sample Preparation section. The recoveries were calculated based on the difference between the total amount determined in the spiked samples and the amount observed in the non-spiked samples.

RESULTS & DISCUSSION

FIGURE 1 shows a typical separation of fructose, glucose, and sucrose in potatoes. With a new column, a baseline separation between the two monosaccharides was possible. After 1 month of continuous use, the column lost activity and the resolution between fructose and glucose decreased. The column's condition remained at this point for several months even after periods of prolonged storage. To compensate for this change, the water composition of the mobile phase may be lowered and/or the flowrate decreased to maintain the desired separation.

For fructose, glucose, and sucrose, percent recovery of the added 2 mg/g was 101, 98, and 93%, respectively, while recoveries were 105, 101, and 83% for the added 8 mg/g. The 83% recovery for sucrose at the 8 mg/g level may be due to the insolubility of the disaccharide at that high a concentration in methanol.

Analysis of three varieties of potatoes, Kennebec, Katahdin, and Russet Burbank, stored at 3.3 and 7.2°C, demonstrated that the method is reproducible (Table 1). Coefficients of variation ranged from 1.39-13.31% with all but three coefficients less than 10%.

To determine if interfering peaks might be eluting at the retention times of any of the three sugars, peaks from the HPLC were collected and spotted on TLC plates. One spot for each collected peak was observed on the TLC plate after it was sprayed with sulfuric acid (50% v/v) and the R_f value of each spot corresponded to the correct sugar, thus indicating that the chromatographic peaks represented only one sugar (Stahl, 1965).

Other applications of this method include determination of sugars in the blanch water from a processing operation. As the method is sensitive to the 0.01% level, it is useful for sugar determination in such situations. Also the method can be used for determination of sucrose content alone, an advantage for some growers of chipping potatoes (Sowokinos, 1978). Increasing the water content in the solvent system to 35% decreases the analysis time to about 15 min but does not provide separation between the monosaccharides. With the modification in the procedure, the mean sucrose content of Norchip and Monona chipping potatoes was determined to be 2.95 and 3.25 mg/g, respectively.

The results of this study show that this method is a rapid and quantitative analytical technique, which is potentially useful in both basic research and in industrial operations.

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A Research Note

Comparison of Sodium and Sodium/Potassium Salt Mixtures in Processed Vegetables

C. JANE WYATT

ABSTRACT

Green beans and corn were heat processed with different concentrations of a salt mixture as a sodium chloride (NaCl) substitute. The sodium (Na) and potassium (K) concentrations of the finished product were determined as well as the flavor desirability by an untrained panel. Certain levels of the salt mixture were judged not significantly different in desirability from the reference which contained 1.5% NaCl. The use of an equal mixture of NaCl and KCl was effective in improving the Na/K ratio.

INTRODUCTION

CONCERN by consumers over excessive intake of salt in their diets has exerted pressure upon the food processing industry to voluntarily decrease the amount of Na added to foods. The U.S. Select Committee on Nutrition has recommended lowering the level of salt in processed foods. The proposed dietary goals have called for reducing the salt intake to 5g added salt per day (Dietary Goals, U.S. Senate, 1977). Based upon the Na content of processed foods presently available, it may be difficult to select a diet containing processed foods which meets this goal.

Furthermore, Na content labeling on foods is emerging. The Food and Drug Administration has proposed regulations to require declaration of Na and K content on food labels (Federal Register, 1979).

The objective of this research was to evaluate different combinations of NaCl and KCl in canned green beans and corn. The salt mixtures studied were designed to lower the Na content and improve the Na:K ratio in these products.

EXPERIMENTAL

GREEN BEANS and corn were heat processed according to recommended procedures (NCA, 1976). The following salt mixtures were used:

	% by wt added at processing		
NaCl	0.75	1.0	1.5
Salt blend (1:1, NaCl:KCl)	1.7	2.3	3.4

Na and K were determined by atomic absorption (PE 303) techniques. Samples were drained on a No. 8 sieve, 2 min, homogenized 2 min in a Waring Blendor, dried at 100°C for 24 hr, and ashed at 550°C overnight. All glassware and equipment were treated with 10% HNO_3, rinsed with deionized water and dried prior to each use. Blanks were run through all procedures for each sample. The ash was taken directly for mineral determination by dissolving in 50% HCl, evaporating to near dryness and redissolving in 0.1N HCl. Samples were brought to volume with 1500 ppm $LiCl_2$ (Noller and Bloom, 1978).

All flavor panels were conducted using the same procedures. The samples were heated to 100°C then placed in the top of a double boiler over hot water to keep hot for serving. All samples served at a given time were at the same temperature; the samples were served in coded paper portion cups to the judges seated in individual testing booths. The judges were not pre-selected or trained but selected only on the basis of availability. Each judge was asked to score the samples for the degree of saltiness and overall desirability. The data were analyzed and the significance of differences were determined at the 5% and 1% levels (ASTM, 1968).

RESULTS & DISCUSSION

TABLE 1 shows the results of the reference-difference flavor panels for canned green beans and corn. The 3.4% salt blend scored significantly more "salty" than the reference for both green beans and corn even though the Na content was similar to the reference. The enhancement effect of the KCl was apparent. When the higher and lower treatments were eliminated from the flavor tests the results for green beans show the 2.3% salt blend treatment had a more intense salt flavor than the reference and was not as desirable. The lowest level of the salt blend (1.7%) was perceived as being of the same salt intensity as the reference, but it was not as desirable.

For corn, the 2.3% salt blend sample was not significantly different from the reference in intensity yet it was judged less desirable, which indicates a flavor note other than "salty" probably influenced the desirability rating. The other treatments, 1.0% NaCl and 1.7% salt blend were not significantly different in desirability from the reference.

The overall desirability scores show that for green beans the 1.5% NaCl, 1.0% NaCl, and 1.7% salt blend samples were rated equally desirable. The 2.3% salt blend was rated less desirable as it was too salty. For the corn samples, there was no significant difference between the overall desirability scores of 1.5% NaCl and 1.0% NaCl. The 2.3% and 1.7% salt blend treatments were rated less desirable due to the enhancement effect of the KCl.

The results of the Na and K analysis for green beans and corn are shown in Table 2. The Na:K ratio of unprocessed green beans is 0.08. During processing it appears K is lost into the liquor to a larger extent than the Na present in the raw green bean. The Na: K ratio for the processed item (without added salt) is 0.16. When NaCl is added at 1.5% the Na:K ratio is 3.41. The Na:K ratio of a product processed with an equal mixture of NaCl/KCl is 0.65. Products processed with this salt blend approaches the Na:K ratio of the unprocessed green bean.

The Na:K ratio of unprocessed corn is 0.20 and 0.06 when processed without the addition of NaCl. When 1.5% NaCl is added the Na:K ratio is 1.43. By using an equal mixture of NaCl/KCl, the ratio is 0.66.

Vegetables could be processed with lower Na content by using mixtures of NaCl and KCl and still be perceived as tasting as "salty" as the higher NaCl level normally used in canned green beans and corn.

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Author Wyatt is with the Dept. of Food Science & Technology, Oregon State Univ., Corvallis, OR 97331.

Table 1—Results of reference-difference and desirability flavor panels on canned vegetables with different Na and Na/K salts

Treatment (%) by weight	Mean Flavor Panel Scores[c] N = 40 Judgements									
	Green Beans					Corn				
	R-D[a]	D[b]	R-D[a]	D[b]	Overall[b] desirability	R-D[a]	D[b]	R-D[a]	D[b]	Overall[b] desirability
1.5 NaCl (Reference)	4.05	5.10	4.08a	5.46a	6.15a	3.93	5.13	4.03a	5.07a	6.68a
1.0 NaCl	2.30	3.73	2.33b	4.26b	5.55a	3.38	5.05	3.70b	4.75a	6.60a
0.75 NaCl	1.83	3.50				2.28	4.43			
3.4 salt blend	6.05	2.88				6.25	2.63			
2.3 salt blend	4.60	4.30	2.80b	4.48b	4.98b	4.78	3.98	4,38a	3.55b	4.80b
1.7 salt blend	2.85	3.55	4.80a	3.75b	5.75a	3.65	4.60	3.63b	4.31a	5.33b

[a] Score range from 7, extremely less salty, to 1, extremely more salty, with 4 same as reference.
[b] Score range from 7, very desirable, to 1, very undesirable, with 4 as neither.
[c] Mean scores with the same letter are not significantly different at the 5% probability level.

Table 2—Na and K in canned vegetables

Treatment (%) by weight	Na mg/100g		K mg/100g		N:K		Na mg/100g		K mg/100g		N:K	
	Bean	Liquor	Bean	Liquor	Bean	Liquor	Corn	Liquor	Corn	Liquor	Corn	Liquor
1.5 NaCl	334	355	98	100	3.41	3.55	230	271	161	179	1.43	1.51
1.0 NaCl	167	162	90	89	1.86	1.82	129	158	163	182	0.79	0.87
0.75 NaCl	137	135	102	99	1.34	1.36	96	107	138	177	0.70	0.61
3.4 salt blend	400	416	614	630	0.65	0.66	348	353	526	584	0.66	0.60
2.3 salt blend	188	185	340	344	0.55	0.54	132	151	354	412	0.37	0.37
1.7 salt blend	137	136	272	251	0.50	0.54	110	119	341	392	0.32	0.30
Raw	16		195		0.08		35		193		0.20	
Processed (no salt added)	15		90		0.16		10	14	161	165	0.06	0.08

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A Research Note

Protein Concentrate from Air Classification of Flour and Horny Endosperm from High-Lysine Sorghum

Y. VICTOR WU and ARTHUR C. STRINGFELLOW

ABSTRACT

Flour and shorts from a high-lysine sorghum were finely ground and air classified. The protein contents of the original flour and horny endosperm were 9.9 and 18.6%, respectively, and the protein contents of the combined high-protein fractions after air classification were 18.6 and 23.6%. These fractions accounted for 18 and 15% of the flour and horny endosperm weights and for 31 and 19% of the total protein. Protein shifts were 39% for flour and 23% for horny endosperm.

INTRODUCTION

ALTHOUGH PROTEIN from normal sorghum is deficient in lysine (Deyoe and Shellenberger, 1965), two floury lines of Ethiopian origin were exceptionally high in lysine at relatively high levels of protein (Singh and Axtell, 1973). Another high-lysine sorghum, P-721 opaque, also has been reported (Mohan and Axtell, 1975). The availability of high-lysine sorghum appears particularly attractive for preparing protein concentrates by air classification. Flour and horny endosperm from a high-lysine sorghum were air classified, and the protein, fat, and ash contents, as well as amino acid composition of the fractions, were determined.

MATERIALS & METHODS

P-721 OPAQUE is a high-lysine soft-endosperm sorghum produced by chemical mutagen treatment of normal grain (Mohan and Axtell, 1975). The sorghum grain was debranned and degermed with a Cecoco Abrasive cone mill. The yield of grits was 85% of the weight of grain. The resulting grits were Buhler milled, and flour fractions were collected in bins 1-6. The next coarser fraction after the 6 flour bins is horny endosperm. Horny endosperm from Buhler milling was defatted three times with hexane to reduce the fat content before air classification. The yields of flour and horny endosperm were 61 and 27%, respectively, of the weight of sorghum grits or 52 and 23%, respectively, of the weight of grain.

The flour from the Buhler mill was air classified by the same flow sheet for wheat flour (Wu and Stringfellow, 1979) into fine and coarse fractions with a Pillsbury model number 1 laboratory air classifier. The defatted horny endosperm was pin-milled three times at 14,000 rpm and air classified with cut points of 15, 18, 24, and 30 microns in successive passes to give four fine fractions and a coarse residue in addition to an ultrafine fraction collected in and air filter bag of the classifier.

Protein content (nitrogen x 6.25) was determined by duplicate micro-Kjeldahl nitrogen analysis. Moisture was determined by heating duplicate samples at 105°C to constant weight. Protein, fat, and ash determinations were described by procedure numbers 46-13, 30-26, and 08-16 of AACC Approved Methods (1971), respectively. Samples for amino acid analyses were hydrolyzed for 24 hr by refluxing in constant-boiling hydrochloric acid. A portion of the acid hydrolyzate was analyzed on a Beckman Spinco Model 121 amino acid analyzer, and the amino acid data were calculated with the aid of a computer (Cavins and Fridman, 1968).

Authors Wu and Stringfellow are with the USDA-SEA-AR, 1815 N. University St., Peoria, IL 61604.

RESULTS AND DISCUSSION

Air classification

The fat and ash contents of the air-classified flour fractions generally fractionate in the same pattern and correlate with the protein content of the fraction. Table 1 compares air-classification response of various combined fractions of P-721 opaque sorghum flour and horny endosperm. The combined high-protein fractions from flour had a protein content of 18.6% and accounted for 18% of the flour weight and 31% of the total protein. The corresponding fractions from horny endosperm had a protein content of 23.6% and accounted for 15% of the horny endosperm weight and 19% of the total protein. The higher protein content for horny endosperm compared with flour is reflected in higher protein contents of the air-classified horny endosperm fractions. The coarse residue fraction 5 had higher protein content than the starting material for both flour and horny endosperm. However, the coarse residue fraction from horny endosperm accounted for a much higher percentage by weight of the starting material compared with that from flour.

Protein shift is a calculated value to compare protein displacement and equals the sum of the protein shifted into the high-protein fractions and out of the low-protein fractions as a percentage of the total protein present in the starting flour (Gracza, 1959). Protein shift for P-721 opaque flour is 39%; this value is not much different from the 36 and 38% values reported for two commercial sorthum flours (Peplinski et al., 1963) and close to 40 and 41% for floury endosperm from two soft sorghums (Stringfellow and Peplinski, 1966). The protein shift for horny endosperm at 23% is considerably below that for flour, but the higher protein content of horny endosperm com-

Table 1—Comparison of air-classification response of P-721 opaque sorghum

	% Dry basis	
	Flour	Horny endosperm
Starting material		
Yield	100	100
Protein content	9.9	18.6
Maximum range of protein contents	6.6-50.1	12.1-48.4
Combined high-protein fractions 1		
Yield	18	15
Protein content	18.6	23.6
Protein, total	31	19
Combined low-protein fractions[a]		
Yield	58	30
Protein content	7.0	12.8
Protein, total	40	21
Coarse residue fraction 5		
Yield	16	40
Protein content	13.8	22.3
Protein, total	21	47
Protein shifted, total	39	23

[a]Fractions 3 and 4 for flour, fractions 2 and 3 for horny endosperm

Table 2—Essential amino acid composition of P-721 opaque sorghum, flour, horny endosperm, and their air-classified fractions (g/16 g nitrogen recovered)

Amino acid	Ground sorghum	Flour	Flour fraction[a] 1A	1B	1C	1D	1E	Horny endosperm	Horny endosperm 1A exhaust bag	1B fine	NAS[b] (1974)
Isoleucine	4.2	3.7	3.8	3.9	4.0	4.0	4.1	4.1	3.4	3.2	4.2
Leucine	13.8	12.9	8.6	11.5	12.6	13.0	13.6	12.9	8.6	8.2	7.0
Lysine	2.9	2.0	2.0	2.2	2.2	2.1	2.0	2.5	3.5	3.7	5.1
Methionine + cystine	3.1	3.9	4.6	4.7	4.1	4.2	4.2	2.6	1.7	2.4	2.6
Phenylalanine + tyrosine	10.2	9.8	9.7	10.2	10.5	10.5	10.8	7.4	6.4	7.4	7.3
Threonine	3.8	3.4	3.2	3.5	3.6	3.7	3.7	3.0	3.1	2.8	3.5
Valine	5.6	5.1	4.9	5.1	5.3	5.3	5.5	5.8	5.8	4.9	4.8

[a]1A, exhaust bag; 1B, as-milled; 1C, ground 1 x 9,000; 1D, ground 1 x 14,000; 1E, ground 3 x 14,000
[b]The National Academy of Sciences (1974) values for high-quality protein

pensates for the lower protein shift and still results in a combined high-protein fraction with higher protein content but lower yield compared with that for flour. The horny endosperm is harder to grind than flour and results in lower protein shift than flour in air classification.

Amino acid composition

The essential amino acid compositions of P-721 opaque sorghum flour, horny endosperm, and their air-classified fractions are compared with the amino acid pattern for high-quality protein (National Academy of Sciences, 1974) in Table 2. This high-lysine sorghum had 2.9g lysine per 16g nitrogen compared with around 2.0g lysine for normal sorghum. The air-classified flour fractions 1A-1E had similar amino acid composition compared with the parent flour, except fraction 1A had lower leucine and fractions 1A and 1B had higher methionine + cystine. The horny endosperm had higher lysine but lower methionine + cystine and phenylalanine + tyrosine compared with flour. The air-classified horny endosperm fractions 1A and 1B had lower isoleucine and leucine but higher lysine compared with horny endosperm. In addition, fraction 1A had lower methionine + cystine and phenylalanine + tyrosine and fraction 1B had lower valine than horny endosperm. In general, sorghum, flour, horny endosperm, and air-classified fractions meet or exceed the National Academy of Sciences pattern for all amino acids except lysine. However, the increase in lysine content of P-721 opaque sorghum over normal variety is sufficient to approximately double the protein efficiency ratio compared with normal lines (Mohan and Axtell, 1975). Horny endosperm fractions 1A and 1B were also low in isoleucine; horny endosperm fraction 1A was low in methionine + cystine, phenylalanine + tyrosine; and horny endosperm, horny endosperm fractions 1A and 1B were low in threonine compared with the National Academy of Sciences pattern.

CONCLUSION

THE AIR-CLASSIFICATION result of high-lysine P-721 opaque sorghum flour was comparable to the normal sorghums in terms of protein shift. Horny endosperm of higher protein content but lower protein shift compared with flour also resulted in significant increase in protein content for the combined high-protein fine fraction over the starting material. In addition, the coarse residue fraction from flour and horny endosperm also had enhanced protein content compared with the original material. Fractions of high-lysine sorghum flour and horny endosperm may be used to create foods with better nutritional quality or used in sorghum foods to improve their nutritional quality.

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A Research Note

Effect of Japanese Green Tea on Nitrosamine Formation in Vitro

MASAMICHI NAKAMURA and TOSHIHARU KAWABATA

ABSTRACT

The effect of Japanese green tea extract and various phenolic components of green tea on nitrosamine formation in vitro were investigated. We found that green tea extract seems to enhance the nitrosation of four secondary amines under acidic conditions; however, there exists an optimum concentration for the catalytic effect on nitrosamine formation. Among various polyphenols in Japanese green tea, only catechins catalyze nitrosamine formation, while pyrocatechol, pyrogallol, and gallic acid inhibit the reaction. No significant effect on the nitrosation reaction could be observed in the presence of either flavonols or flavones.

INTRODUCTION

SEVERAL PAPERS have been puslished on the effect of tannis on the nitrosation reaction. Bogovski et al. (1972) demonstrated an inhibiting effect of tannins on nitrosamine formation due to their competition for the available nitrite. They also found that apple juice and black tea extract show similar inhibiting effects on nitrosamine formation. Mirvish et al. (1974) found that gallic acid acted in the same way, inhibiting the nitrosation of morpholine and piperazine over a pH range 1-4. Gray and Dugan (1975) reported the inhibition effect of tannic acid on nitrosation. In contrast, Challis and Bartlett (1975) observed a catalytic effect by chlorogenic acid, a naturally occurring polyphenol, on the formation of nitrosopiperidine from piperidine. Pignatelli et al. (1976) showed that the catalytic effect of gallic acid on nitrosodiethylamine formation depends on the pH and relative concentrations of the reactions. Green tea has long been the most popular beverage of the Japanese. Therefore, we examined the effect of Japanese green tea on nitrosamine formation in vitro.

EXPERIMENTAL

Green tea sample

Japanese green tea (*Thea sinensis* L.) harvested in Kanagawa Prefecture was used.

Reagent

Four catechins isolated from plants were kindly provided by Dr. M. Nakagawa of Tea Research Station, Ministry of Agriculture, Forestry and Fisheries: (-)-Epigallocathechin and (-)-epicatechin gallate (*Thea sinensis* L.), (-)-epicatechin (*Camellia japonica* L.), and (+)-catechin (*Catechu*).

Eight flavonoids isolated from plants were kindly provided by Dr. H. Matsuda of The Dept. of Botany, Faculty of Science, Univ. of Tokyo: Kaempferol, quercetin, and genistein (*Sophora japonica* L.); fisetin (*Rhus succedanea* L.), quercitrin (*Tanakaea radicans*), myricetin (*Rhus javanica* L.), flavone (*Primula modesta*), luteolin-7-glucoside (*Humulus japonicus*).

The other polyphenolic compounds were of analytical reagent grade. Pyrocatechol, pyrogallol, and gallic acid were purchased from Wako Pure Chemical Co., and 4-methylcatechol, 3-methylcatechol, and chlorogenic acid were from Tokyo Kasei Co. The other reagents used in the present study were analytical grade from Wako Pure Chemicals Company, used without further purification.

Reaction conditions

Five grams of dried tea leaves were extracted with 50 ml of boiling water for 5 min. The filtrate obtained by filtering the liquid through a filter paper was employed as the test tea extract. The concentrations of nitrite (10 mM) and amines (1 mM) were fixed and the final volume of the reaction mixture made up to 10 ml with Clark and Lubs solution. For experiments on the effect of the concentration of the tea extract on the nitrosation of four secondary amines [i.e., dimethylamine (DMA), diethylamine (DEA), pyrrolidine (PYR) and piperidine (PIP)], the pH of the reaction mixture was fixed at 3.4 for DMA and DEA, and 3.0 for PYR and PIP. The volume of tea extract added to these reaction mixtures varied from 1-8 ml. For studies on the effect of pH on nitrosamine formation, 1 ml of tea extract was added to the reaction mixture and the pH was varied from 1-5. The reaction was allowed to proceed at 37°C for 30 min and was arrested by adding 20% ammonium sulfamate in 2N H_2SO_4. Then 2g of NaCl were added, followed by extraction twice with 15 ml of CH_2Cl_2. Aliquots of the extract, without concentration, were used for gas chromatographic analysis. For gas chromatographic analysis of nitrosamines, a Shimadzu GC-5APF gas chromatograph equipped with an alkali flame ionization detector (AFID) was employed (Kawabata et al., 1972). A glass column (3 mm i.d. x 3m) packed with 20% PEG-20M on Chromosorb W (60-80 mesh) was employed. The carrier gas was nitrogen used at a flow rate of 40 ml/min. The temperatures of the column and injection port were 170° and 200°C, respectively.

RESULTS & DISCUSSION

FIGURE 1 illustrates the catalytic effect of the volume of tea extract added to the reaction mixture on the nitrosation of secondary amines. The maximum catalytic effect on nitrosamine formation occurred when 1 ml of tea extract was added to reaction mixtures having a pH of 3.4 or 3.0. On the contrary, when the volume of tea extract was increased, nitrosamine formation rate decreased markedly. This implies there is an optimum concentration of tea extract which gives maximum nitrosamine formation.

The inhibition of nitrosation of secondary amines with higher levels of tea extract seems to depend on the inhibiting effect on the nitrosamine formation of various components in the tea extract, and on the decrease of sodium nitrite due to reaction of tea extract and nitrite (Nakamura et al., 1977).

The polyphenol content of Japanese green tea (dry leaves) is known to be 12.5-14.6%; 80% of these are catechins, while the remaining 20% consist of leucoanthocyans, flavonols, phenolic acids, theogallin, chlorogenic acid, etc. (Fujita, 1963). The following is an example of the catechin components: (-)-epigallocatechin gallate, 6.1%; (-)-epicatechin gallate, 2.0%; (-)-epigallocatechin, 2.3% (-)-epicatechin, 0.7%; (-)-gallocatechin, 0.5%, and (-)catechin, 0.1% (Nakagawa, 1970).

We examined the effect of some of these green tea components, including four catechins, five flavonols, three flavoens and eight other types of polyphenols, on the nitrosation of four secondary amines. The results are shown in

Author Nakamura is with the Dept. of Food & Drug Science, Kanagawa Prefectural Public Health Laboratory, 52-2 Nakao-cho, Asahi-ku, Yokohama, Kanagawa-ken 241, Japan. Author Kawabata is with the Dept. of Biomedical Research on Food, National Institute of Health of Japan, 2-10-35 Kamiosaki, Shinagawa-ku, Tokyo 141, Japan.

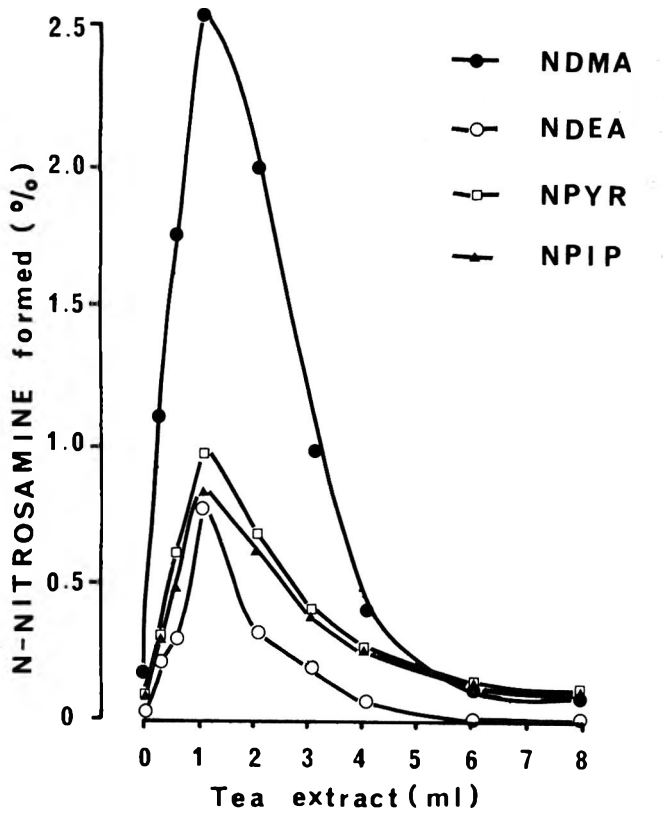


Fig. 1—Effect of volume of tea extract on the nitrosation of secondary amines: NDMA, nitrosodimethylamine; NDEA, nitrosodiethylamine; NPYR, nitrosopyrrolidine; and NPIP, nitrosopiperidine. Test amines: dimethylamine (DMA), diethylamine (DEA), pyrrolidine (PYR), and piperidine (PIP). Reaction conditions: amine (0.1 mM), at pH 3.4 for DMA and DEA, and at pH 3.0 for PYR and PIP; total volume of reaction mixture, 10 ml; reacted at 37°C for 30 min.

Table 1. It was found that the presence of polyphenols such as pyrocatechol, gallic acid, and pyrogallol markedly inhibited nitrosamine formation in reaction mixture under acidic conditions. In contrast, catechins seemed to enhance the nitrosation reaction. It was also found that neither flavoens nor flavonols significantly affected nitrosamine formation. As described above, phenolic or polyphenolic compounds in the green tea have various effects on nitrosation of secondary amines. However, in this study we did not perform quantitative analyses of these compounds in the tea extracts.

Table 1—Effect of polyphenols on N-nitrosamine formation from secondary amines and sodium nitrite[a]

Polyphenol	Yield of nitrosamine (%)			
	NDMA	NDEA	NPIP	NPYR
Control				
(without polyphenol)	0.18	0.04	0.07	0.07
Catechin				
(+)catechin	0.73	0.16	0.23	0.24
(-)epicatechin	0.63	0.14	0.21	0.22
(-)epigallocatechin gallate	0.84	0.19	0.31	0.32
Flavonol				
kaempferol	0.17	0.03	0.05	0.06
fisetin	0.18	0.04	0.06	0.06
quercetin	0.15	0.03	0.06	0.07
quercitrin	0.19	0.04	0.06	0.07
myricetin	0.18	0.04	0.06	0.06
Flavon				
flavon	0.12	0.03	0.05	0.05
luteolin-7-glucoside	0.15	0.04	0.06	0.06
Isoflavon				
genistein	0.18	0.04	0.06	0.06
Pyrocatechol	0.04	0.01	0.01	0.02
4-Methylcatechol	0.01	0.01	0.01	0.01
3-Methylcatechol	0.04	0.01	0.01	0.01
Pyrogallol	0.11	0.01	0.01	0.01
Gallic acid	0.05	0.01	0.03	0.03
Chlorogenic acid	0.19	0.03	0.04	0.05

[a] Reaction conditions: amine (1 mM) + $NaNO_2$ (10 mM) + polyphenol (1 mM), at pH 3.0; reacted at 37°C for 30 min.

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A Research Note

CAF Activity, Calcium Concentration, and the 30,000-Dalton Component of Tough and Tender Bovine Longissimus Muscle

F.C. PARRISH, JR., C.J. SELVIG, R.D. CULLER, and M.G. ZEECE

ABSTRACT

Four tender and four tough bovine longissimus samples from A-maturity carcasses and four tender and four tough bovine longissimus samples from E-maturity carcasses postmortem aged 10-14 days were used to study calcium-activated factor activity (CAF), calcium concentration, and the 30,000-dalton component. Significant differences in CAF and free calcium were not found between tender and tough for CAF and free calcium, although the tough samples had more free calcium. Tender bovine longissimus muscle from A-maturity carcasses had a 30,000-dalton component and a high ratio of 30,000-dalton component to actin, whereas tough did not. In E-maturity, however, both tender and tough longissimus had a 30,000-dalton component.

INTRODUCTION

IN AN EARLIER PAPER, we reported that a 30,000-dalton component was present in tender, but it was absent in tough, bovine longissimus muscle from A-maturity carcasses postmortem aged for 1 day (McBride and Parrish, 1977). Coupled with our previous studies (Olson et al., 1976, 1977; Parrish et al., 1973), we also introduced the term "myofibril fragmentation tenderness" to describe a state of tenderness of conventionally handled bovine longissimus muscle. Several questions, however, were prompted from this discovery of a 30,000-dalton component in tender, but not in tough, longissimus concerning calcium activated factor (CAF) activity and concentration of calcium. CAF seems to be responsible for the degradation of troponin T to a 30,000-dalton component (Olson et al., 1977), and CAF requires calcium for optimum activity (Dayton et al., 1976). Furthermore, addition of calcium to bovine muscle minces accelerates the appearance of the 30,000-dalton component (Cheng and Parrish, 1977).

The purpose of this paper is to report the relationship among CAF, calcium, the 30,000-dalton component, and tenderness of postmortem-aged bovine longissimus muscle.

MATERIALS & METHODS

LONGISSIMUS MUSCLE of loin steaks from four A-maturity tender, four A-maturity tough, four E-maturity tender, and four E-maturity tough, which had been postmortem aged for 10-14 days, was used in this study. Loin steaks were characterized as tender or tough based on significant differences in sensory tenderness score, Warner-Bratzler shear force value and myofibril fragmentation index (Culler et al., 1978).

Procedures for sensory panel scores, Warner-Bratzler (W-B) shear force values and myofibril fragmentation index were carried out as described by Culler et al. (1978). CAF was prepared and assayed as described by Olson et al., (1977).

Free calcium was determined by placing 7g of scissor-minced muscle in tubes and centrifuging for 1 hr at 183,000 x G. The supernatant was decanted, and then 1 ml of supernatant was removed and treated with 0.1 ml of 27.5% TCA. This mixture was centrifuged for 20 min at 1000 x G. Then, 0.2 ml of the supernatant was pipetted into a tube, and 5% lanthinium chloride was added to it. Appropriate standards of 2, 4, and 8 ppm were prepared along with experimental samples. Calcium was determined by using a Cary Techtron Atomic Absorption Spectrophotometer.

Sarcomere lengths of myofibrils were measured according to the procedure described by Parrish et al. (1973). SDS-polyacrylamide gel electrophoresis was done as earlier cited (Olson et al., 1977).

Quantitation of the 30,000-dalton component was accomplished by using a Zeiss M4Q III spectrophotometer equipped with a gel scanner and a spectra-physics auto-lab system I computing integrator. Scanning was done on three gels of each sample, of which one contained 10 μg and the other two each contained 15 μg of protein. The value for the amount of 30,000-dalton band was reported as a ratio of 30,000 to actin.

Data were analyzed for means, standard errors, simple correlations and significant mean differences by analysis of variance tests by the Statistical Analysis System of the Iowa State University Computation Center.

RESULTS & DISCUSSION

SENSORY TENDERNESS SCORES (STS), W-B shear force values and MFI were significantly different between tender and tough A- and E-maturity bovine longissimus muscle (Table 1). Juiciness scores (J), sarcomere length (SL), CAF activity, and free calcium, however, were not significantly different among different maturities and tenderness groups. Connective tissue score (CTS) was significantly higher for tender A-maturity than for tough A-maturity, but there was no significant difference between tender and tough E-maturity. In addition, MFI values of tender E were very similar to tender A. These results indicate that differences in tenderness between tender A and E maturities (STS of 7.4 for tender A and 5.4 for tender E) seem to be due then to differences in connective tissue (CTS of 7.1 for tender A and 4.0 for Tender E) and not due to the myofibrillar component. Results of the values among sensory tenderness score (STS), W-B shear force, MFI and sarcomere length substantiate our earlier observations (MacBride and Parrish, 1977; Culler et al., 1978).

Our primary objective, however, was to determine if amount of CAF activity and free calcium was related to tenderness, myofibril fragmentation and the 30,000-dalton component. Olson et al. (1977) have previously shown that CAF activity of bovine longissimus muscle declined precipitously with postmortem aging time, but their results on CAF activity were obtained using samples not characterized according to tenderness differences in bovine longissimus muscle (Culler et al., 1978). Furthermore, about half of the CAF activity remained in postmortem muscle and CAF was shown to degrade troponin T to a 30,000-dalton component (Olson et al., 1977). Therefore, it was of interest to determine if CAF activity would be different in tough and tender bovine longissimus muscle postmortem aged 10-14 days. Little difference, however, was found in CAF activity among tenderness and maturity groups. A tendency for more free calcium was found in the tough groups; however, these values were not significantly different from the tender groups. Hence, our hypothesis that more tender bovine longissimus muscle from 10-14 days postmortem aged carcasses with a higher myofibril fragmentation index and the presence of a 30,000-dalton component would have greater CAF activity is not supported. A possible reason for lack of

All authors are affiliated with the Muscle Biology Group, Departments of Animal Science and Food Technology, Iowa State Univ., Ames, IA 50011.

Table 1—Sensory, chemical and physical properties of tender and tough bovine longissimus muscle from A- and E-maturity carcasses[a]

	STS[b]	CTS[b]	J[b]	WB kg/cm^2	MFI[d] O.D. 540 nm x 200	SL[e] μm	CAF[f] O.D. 278 nm/100 g	Calcium[g] μM
Tender-A	7.4a ±0.1a	7.1a ±0.2	5.3a ±0.3	1.70a ±0.05	65.2a ±2.0	1.80a ±0.06	12.1a ±1.9	638.0a ±43.3
Tough-A	3.5b ±0.4	5.9b ±0.3	5.9a ±0.2	3.94bc ±0.44	25.5b ±7.0	1.73a ±0.08	13.6a ±3.2	970.6a ±146.5
Tender-E	5.4c ±0.2	4.0c ±0.3	5.7a ±0.2	2.79b ±0.22	65.0a ±0.4	1.79a ±0.13	10.6a ±1.0	668.8a ±97.8
Tough-E	2.5b ±0.3	4.3c ±0.3	5.2a ±0.2	5.02c ±0.26	32.8b ±2.3	1.76a ±0.07	10.8a ±1.2	833.2a ±69.6

[a] Means and standard errors of the mean of four samples within each group not followed by the same letter within a column are significantly different at the 5% level of probability.

[b] Eight point descriptive scale (8 = extremely tender or juicy or no detectable connective tissues; 1 = extremely tough or dry or abundant connective tissue).

Table 2—Correlation coefficients among sensory, chemical and physical properties of tender and tough bovine longissimus muscle from A- and E-maturity carcasses

	CA	MFI	STS	CTS	J	WB	Sarcomere length
Calcium activated factor (CAF)	0.49*	−0.19	0.08	0.24	0.39	−0.03	−0.14
Free calcium (CA)		−0.42	−0.32	0.16	0.38	0.29	−0.34
Myofibril fragmentation index (MFI)			0.82**	0.14	0.00	−0.80**	0.36
Sensory tenderness score (STS)				0.50*	0.01	−0.93**	0.27
Connective tissue score (CTS)					0.06	−0.47*	0.26
Juiciness (J)						−0.04	−0.22
Warner-Bratzler shear (W-b)							−0.17

*P < 0.05
**P < 0.01

Table 3—Quantity of 30,000-dalton component of tender and tough bovine longissimus muscle from A- and E-maturity carcasses

A-Maturity		E-Maturity	
Tender	Tough	Tender	Tough
102[a]	0	55	48
96	0	75	96
61	0	29	38
84	0	86	20

[a] Ratio of 30,000 to actin x 10^3

support of this hypothesis, however, is that the longissimus muscle samples used in this study were postmortem aged from 10-14 days. It may well be that differences in CAF activity occur early in the postmortem aging cycle and that, as these results indicate, CAF activity from postmortem aged muscle of about 2 wk between two tenderness groups is not of significance.

CAF activity was slightly ($P < 0.05$) related to free calcium levels (Table 2). Cheng and Parrish (1977) have demonstrated previously that addition of calcium to minced muscle hastened the appearance of the 30,000-dalton component, which is associated with tender bovine longissimus muscle (MacBride and Parrish, 1977). Consequently it was of interest to determine the relationship between CAF and free calcium. These results suggest that free calcium is only partly related to activation of CAF (r=0.49). These results further suggest that calcium in bound form could be involved in CAF activity, although the amount of free calcium approached the quantity needed for optimum activity (Dayton et al., 1976). Other relationships between the other variables and either CAF or free calcium were not significant. On the other hand, MFI, sensory tenderness score (STS) and W-B shear values were highly correlated with each other (Table 2). These results further substantiate previous reports (Moller et al., 1973; Olson and Parrish, 1977; MacBride and Parrish, 1977; Culler et al., 1978).

MacBride and Parrish (1977) reported that tender bovine longissimus muscle from A-maturity carcasses had a 30,000-dalton component at 1-day postmortem, but it was absent from the tough group. These results suggest that tenderization, and the reactions associated with tenderization, occur early postmortem. Moreover, these observations raised the question as to why a 30,000-dalton componnet was present in tender longissimus, but not tough. Quantitation of the 30,000-dalton component as a ratio of 30,000-dalton component to actin in this study showed the presence of the 30,000-dalton component in tender, but not in tough, samples from A-maturity carcasses and postmortem aged at 2°C for 10-14 days (Table 3). This result extends and substantiates our previous report on tender and tough longissimus muscle postmortem aged for 1 day at 2°C (MacBride and Parrish, 1977). Hence, those muscles classified as tough lacked a 30,000-dalton component even after 10-14 days of postmortem aging. Obviously, the question as to why the tender group has a 30,000-dalton component and the tough group does not, is unresolved. Further studies, starting at 1-day postmortem and proceeding into the postmortem aging period, need to be conducted on tender and tough muscle to determine if there are differences in CAF and free calcium. On the other hand, an explanation for differences in the appearance of the 30,000-dalton component may be more subtle and complex than the determination of differences in CAF activity and (or) free calcium. It may involve a CAF inhibitor (Okitani et al., 1976). The 30,000-dalton component, however, was present in both tender and tough bovine longissimus muscle from E-maturity carcasses (Table 3). Although there was a significant difference in STS, W-B shear and MFI tender and tough E-maturity bovine longissimus muscle, background toughness (Marsh and Leet, 1966) could confound tenderness differences, and this could partly explain the appearance of the 30,000-dalton component in both tender and tough E-maturity bovine longissimus. These results do, however, substantiate the use of the term "myofibril

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A Research Note

Observations on the Weeping of Double Boiler Scrambled Egg Products and Its Prevention

T. C. CHEN

ABSTRACT

Experimental scrambled egg products were prepared with liquid whole eggs or liquid egg whites as the major ingredient. The effects of ingredients, length of cooking time and holding time on the weeping of double boiler cooker products were observed. For both types of products, longer cooking time resulted in products with lower weeping volumes. The presence of yolk greatly reduced the weeping. Most of the weeping occurred within the first 5 min after cooking and reached a maximum between 20 and 30 min of holding. A slight reabsorption of the weeping fluid was observed for some of the products after 40 min of holding. The addition of carboxymethylcellulose either reduced or prevented weeping.

INTRODUCTION

IN 1951, each person in the U.S. consumed an annual average of 389 eggs. This number dropped to 272 in 1977 (USDA, 1978). One of the obvious reasons for this decline was the inconvenience of eggs as breakfast food, since eggs are generally considered by consumers as a basic breakfast food item. There is a need for quality convenient egg products to put eggs back on the breakfast table.

In recent years, several scrambled egg mixes have been developed and marketed using liquid whole eggs or liquid egg whites as the major ingredient (Stadelman and Cotterill, 1977). One of the major problems involved in the acceptability of such items for household consumption is the weeping of cooked products, especially for yolk-free products.

According to our preliminary experiments, when a whole-egg product was scrambled to complete doneness, the percentage of weeping volume from double boiler scrambled product was approximately triple that of the pan scrambled in a Teflon skillet. Double boiler cooking has long been used as a common method for scrambled egg preparation in the household. This study was designed to observe weeping of double boiler cooked products and to search for a suitable food additive capable of reducing or preventing this weeping problem.

EXPERIMENTAL

EXPERIMENTAL liquid egg products for scrambling were prepared in our laboratory. Ingredients for each recipe, as listed in Table 1, were measured into a Waring Blendor and blended at "low" speed setting for one min. Week old "A" quality large eggs were used in this study. The lactic acid used had an acidity equivalent to 9.47 ml of 1.0N NaOH per ml sample. Two hundred gram portions of the prepared products were packaged in polyethylene pouches, heat sealed and stored at −18°C. For the evaluation of weeping preventive additives, carboxymethylcellulose (CMC-7H3XF, Hercules, Inc., Ill.) was added at 0.10, 0.25, and 0.40% levels.

The prepared frozen premixes were thawed overnight in a refrigerator before scrambling in a preheated 16-cm diameter double boiler. No cooking oil was used for scrambling. After cooking, products were transferred immediately into 500 ml beakers and held at room temperature (24°C). The amount of weeping fluid present in products after 5 and 10 min of hold time was measured and returned to the sample and then measured again every 10 min for a total of 60 min. The percentage of weeping fluid was calculated against the weight of the cooked product. To determine the effectiveness of food additives on weeping prevention, yolk-free and whole-egg products were cooked for 5 and 4 min, respectively.

Author Chen is with the MAFES, Poultry Science Dept., Mississippi State Univ., Mississippi State, MS 39762.

RESULTS & DISCUSSION

THE FINAL pH VALUES of the experimental scrambled egg products were 6.63 and 8.07 for the whole-egg and the yolk-free products, accordingly. Monosodium glutamate and salt were added to the yolk-free product to aim for a better flavored product. These two ingredients can also be incorporated into the whole-egg product.

The length of cooking time affected the weeping volume of the yolk-free product greater than did that of the whole-egg product (Fig. 1). For both types of products, longer cooking time resulted in products with lower weeping volumes. Weeping was reduced from 15.5 to 4.8% in yolk-free product by increasing the cooking time from 3 to 7 min. The presence of yolk greatly reduced the weeping of the products. Weeping was reduced from 7.0 to 4.0% in whole-egg product by increasing the cooking time from 3 to 6 min. In most cases, a 5 min cooking time resulted in products that appeared done. Cooking for 6 min resulted in overcooked products, especially for the whole-egg product.

The length of holding time also affected the amount of weeping in cooked scrambled egg products. In general, most of the weeping occurred within the first 5 min after cooking and reached a maximum between 20 and 30 min. A slight reabsorption of the weeping fluid was observed for some products after 40 min of holding. It was expected that, due to the evaporation effect, the volume of weeping fluid would be reduced if the cooked products were kept on a steam table.

The addition of carboxymethylcellulose (CMC) reduced or prevented the weeping of the cooked products although higher quantities were required for the yolk-free product than for the whole-egg product (Fig. 2). For the whole-egg product, the presence of 0.1% CMC reduced the weeping from 7.0% to 0.4%. Under the same condition, a reduc-

Table 1—Composition of experimental scrambled egg products

Ingredients	Percentage Composition: Whole-egg product	Percentage Composition: Yolk-free product
Liquid whole eggs	80.58	–
Liquid egg whites	–	87.04
Nonfat milk solids	3.20	6.00
Water	12.11	–
Corn oil	4.00	6.50
Monosodium glutamate	–	0.20
Salt	–	0.25
Food color (Marigoldine Yellow, H. Kohnstamm & Co., New York)	–	0.007
Lactic acid	0.10	–

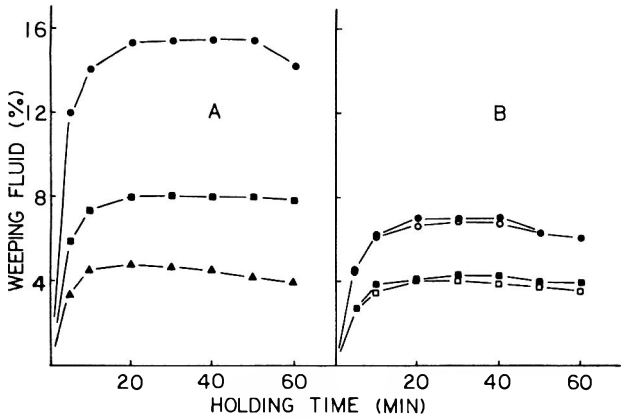


Fig. 1—Effects of length of cooking and holding time on weeping of cooked scrambled egg products. Each point represents the average of 4 observations. A, yolk-free product; B, whole-egg product; ●, 3 min cooking; ○, 4 min cooking; ■, 5 min cooking; □, 6 min cooking; and ▲, 7 min cooking.

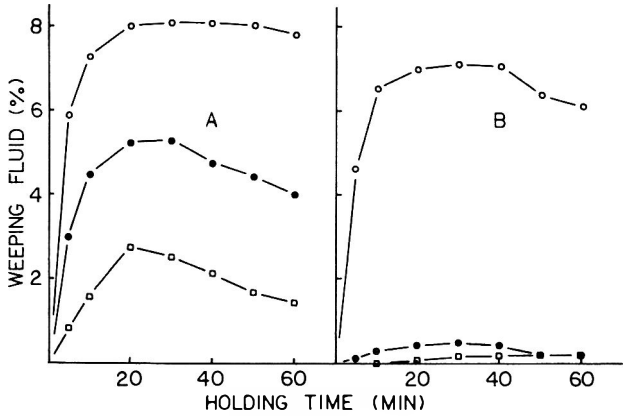


Fig. 2—CMC and weeping of cooked scrambled egg products. Each point represents the average of 4 observations. A, yolk-free product; B, whole egg product; ○, control; ●, 0.1% CMC added; and □, 0.25% CMC added.

tion from 8.0% to 5.2% was observed for the yolk-free product. The addition of 0.4% CMC prevented the weeping for both types of products.

In a separate study, the addition of corn starch to products also reduced weeping. However, its effectiveness was approximately one-tenth of that of the CMC-treated sample. The presence of 1.0% corn starch reduced the weeping percentage of cooked yolk-free product by half. However, high corn starch content (greater than 1.0%) occasionally produced a detectable corn starch flavor in the products.

Results clearly indicated that the weeping of the cooked scrambled egg products depended on the product formulation, length of cooking and holding time. The addition of CMC either reduced or prevented this weeping problem.

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fragmentation tenderness" to describe a state of tenderness in bovine longissimus muscle of conventionally handled A-maturity carcasses (MacBride and Parrish, 1977).

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A Research Note

Quantitative Determination of Ascorbic Acid in Potatoes and Potato Products by High Performance Liquid Chromatography

JORG AUGUSTIN, CLAUDIA BECK and GAIL I. MAROUSEK

ABSTRACT

A simple, rapid and accurate method for the quantitative determination of ascorbic acid in potatoes and potato products has been developed. The system is based on the reverse-phase, ion-pairing concept of high-performance liquid chromatography. Sample preparation is minimal and involves a one-step extraction followed by filtration and/or centrifugation. Recoveries were near the 100% level. As low as 0.02 mcg ascorbic acid could be determined.

INTRODUCTION

ASCORBIC ACID is commonly determined by the 2,6-dichlorindophenol visual titration method (AOAC, 1975). Working with deep-fat fried products such as potato chips and French fries, investigations performed in this laboratory, aside from obtaining unrealistically high values, found it to be difficult, if not impossible to visually determine the titration endpoint with this method. Similar problems were encountered with potato granules and flakes which had turned brown when stored at elevated temperatures for prolonged periods of time. Among other methods of ascorbic acid analysis such as the fluorometric method (AOAC, 1975), turned out unrealistically high values, and so did the polarographic method (Jadhav et al., 1975), whereas gas liquid chromatography (Schlack, 1974) is rather time consuming.

Sood et al. (1976) reported on a method of ascorbic acid analysis using high-performance liquid chromatography (HPLC) in a reverse phase ion pairing system. We have further developed, modified, and evaluated their method for potatoes and potato products.

MATERIALS & METHODS

Chromatographic equipment

A Model ALC/GPC 204 Liquid Chromatograph equipped with a Model 6000 pump, a U6K injector and a 254 nm detector (Waters Associates, Milford, MA) was used preceded by a precolumn containing Bondapak C18/Corasil (Waters Associates).

Column

A 30 cm x 3.9 mm u-Bondapak C_{18} column was used (Waters Associates).

Mobile phase

5.0×10^{-4} tridecylammonium-formate in 40/60 v/v water/methanol, pH 4.5., was prepared as follows: 220 mg tridecylamine (Eastman) was dissolved in 600 ml methanol followed by the addition of an excess of formic acid (0.05 ml); finally under constant stirring 400 ml water was added slowly to the system.

Calibration curves

Ascorbic acid standard samples were prepared and diluted to 20 mg per 100 ml in 6% HPO_3. The final solution containing 0.5 mg/100 ml was prepared by diluting 5 ml stock solution to 100 ml with water. Standard curves were prepared by plotting ascorbic acid concentration against peak height.

Sample preparation

Raw potatoes, French fries and chips. A fresh amount equivalent to approximately 20g dry weight basis macerated in 200 ml 6% HPO_3 using first a Waring Blendor and then a Polytron homogenizer (Brinkmann Instruments). The resulting slurry was filtered first through Miracloth (Chicopee Mills, New York, N.Y.) followed by filtration through Whatman No. 40 filter paper in the case of raw potatoes. French fries and potato chips were each mixed 1:1 with ethanol and centrifuged.

Dehydrated flakes and granules Approximately 1g was macerated in a 20 ml of 6% HPO_3 using a Polytron homogenizer. The slurries containing fortified and nonfortified granules were quantitatively transferred to 200 and 100 flasks respectively, diluted to volume with water and filtered through a Whatman No. 40 filter paper. In the case of flakes, after transfer to a 100 ml volumetric flask, 20 ml of 95% ethanol were added before filling the volumetric flask to volume with water. In this case the slurry was centrifuged and the supernatant decanted. The filtrates or supernatants were then filtered through a Millipore (average pore size 0.45 millimicrons) filter prior to injection into the HPLC.

RESULTS & DUSCUSSION

THE PROCEDURES described by Sood et al. (1976) using 0.001M tridecylammonium formate failed to produce complete separations of ascorbic acid from interfering compounds in potato extracts. Increasing the salt concentration from 0.001M to 0.002M also failed to improve separations. The same held true when using other ion-pairing compounds, notably the quaternary ammonium compounds, e.g. tetrabutyl ammonium phosphate (PIC A, Waters Associates).

Using the system outlined earlier resulted in a complete separation of ascorbic acid from interfering substances (Fig 1). Detector response was linear in the range 0.02-1.125 mcg ascorbic acid. Increasing the flow rate from 1.0 ml/min to 3.0 ml/min reduced retention values from 9 min to 3.5 min. Peak identification for ascorbic acid was verified by spiking samples with ascorbic acid. Furthermore, eluent samples coinciding with ascorbic acid peaks were collected from replicated runs, pooled and tested qualitatively for their ability to discolor dichloroindophenol solutions.

The separation of ascorbic acid from extracts of various potato products are shown in Figure 1, and the results of the determinations of ascorbic acid using HPLC and the 2.6 dichloroindophenol titration method are summarized in Table 1. These data show the HPLC method in general to compare favorably with the titration method. Only in the cae of potato chips did the titration method turn out unrealistically high values. This is believed to be due to the compounds which not only affect the reduction of dichloroindophenol but also obscure the titration endpoint. The reason for this conclusion is based on earlier observations made in the laboratory: A comparative analysis of ascorbic acid in commercially parfried French fries and corresponding finish fried product showed in spite of a clear titration endpoint that the final product contained significantly more ascorbic acid than its parfried counterpart (Augustin, 1979).

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The authors are with the Food Research Center, Univ. of Idaho, Moscow, ID 83843

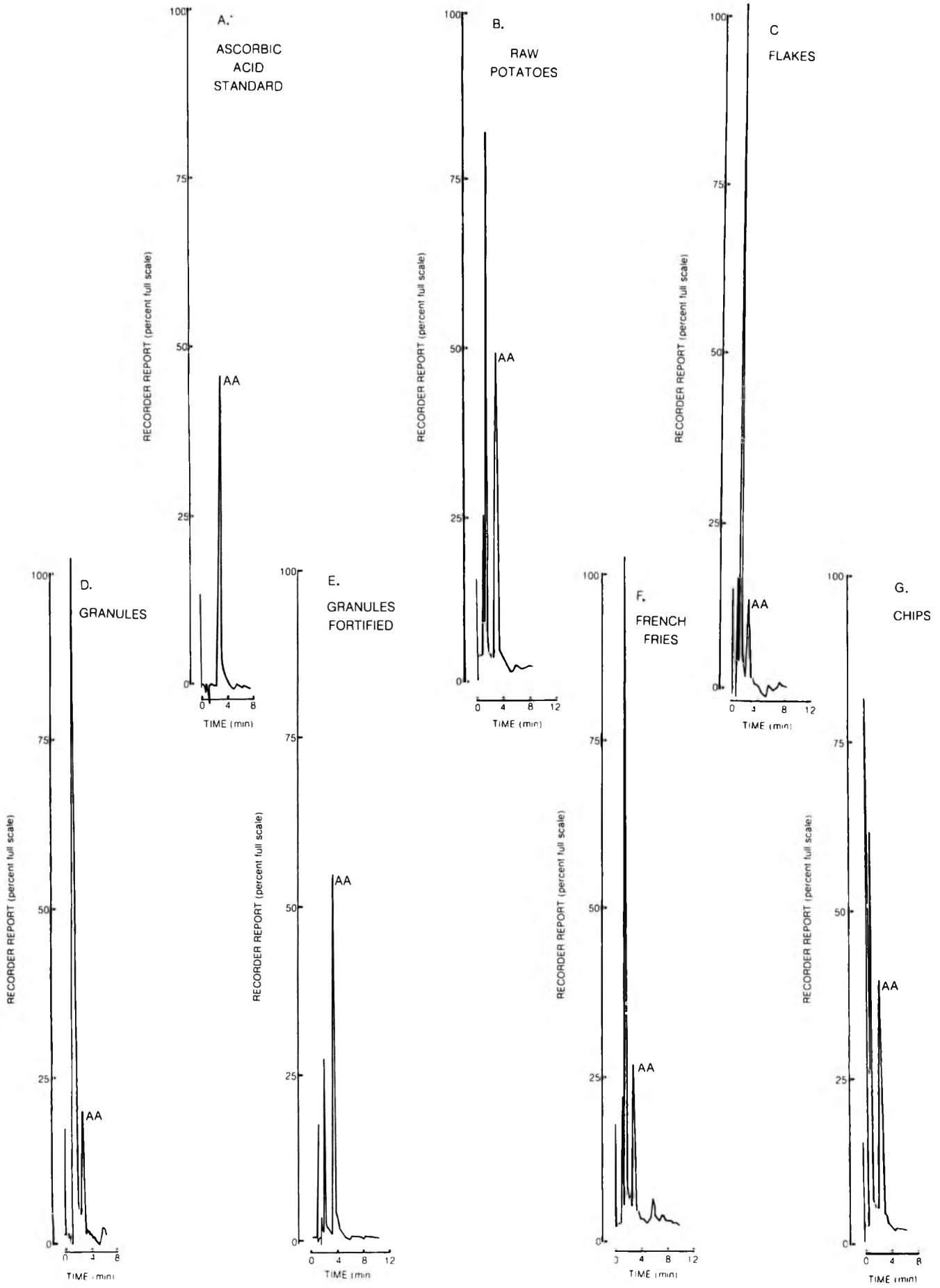


Fig. 1—Chromatograms of ascorbic acid in potatoes and potato products: (A) ascorbic acid standard; (B) raw potatoes; (C) flakes; (D) granules; (E) granules fortified with ascorbic acid; (F) French fries; (G) chips. AA: ascorbic acid.

A Research Note

Antimicrobial Activity of Butylated Hydroxyanisole, Tertiary Butylhydroquinone, and Potassium Sorbate in Combination

P. M. DAVIDSON, C. J. BREKKE, and A. L. BRANEN

ABSTRACT

Butylated hydroxyanisole (BHA), tertiary butylhydroquinone (TBHQ), and potassium sorbate, alone and in combination, were tested for antimicrobial activity against *Staphylococcus aureus* and *Salmonella typhimurium* in trypticase soy broth. Concentrations of the antimicrobials which resulted in 0-50% growth inhibition of the test organisms after 72 hr at 32°C were used in combination. Sorbate-BHA and sorbate-TBHQ combinations caused a 12 hr delay in growth initiation of *S. aureus*. No delay in growth initiation was found with BHA-sorbate combinations against *S. typhimurium*. but all combinations had synergistic antimicrobial activity against the microorganism. TBHQ showed little or no effect on growth of *S. typhimurium*.

INTRODUCTION

SORBIC ACID and its potassium salt have recently been studied for their antimicrobial activity against various food poisoning bacteria. Tompkin et al. (1974) found that 0.1% (w/w) potassium sorbate delayed the growth of *Staphylococcus aureus* and *Salmonella* sp. as well as growth and toxin production of *Clostridium botulinum* in uncured pork sausage held at 27°C. In cured meat products, combinations of nitrite and potassium sorbate have been found to delay or prevent the growth and toxin production of *C. botulinum* at 27°C (Price and Stevenson, 1979; Sofos et al., 1979a, b). Robach and Hickey (1978) showed that 0.05-0.1% sorbic acid delayed or prevented the growth of *Vibrio parahaemolyticus* at 35°C in crab and flounder homogenates adjusted to pH 6.2.

Microorganisms important in food spoilage have also been found susceptible to sorbate. Moustafa and Collins (1969) showed that *Pseudomonas fragi* growth was delayed by 0.1% sorbate in skim milk adjusted to pH 5.2. In trypticase soy broth at pH 6.0, 0.2% sorbate was found to be effective in inhibiting or delaying the growth of *Pseudomonas fluorescens* and *Pseudomonas putrefaciens* (Robach, 1978; 1979). In a related study, Robach and Ivey (1978) found that a 2.5% potassium sorbate dip significantly reduced the number of total viable microorganisms on poultry parts stored at 6°C and 10°C.

Butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) are commonly used as antioxidants to prevent the autoxidation of lipids. These phenolic antioxidants have been found to possess dual functions as antioxidants and antimicrobials. The antimicorbial activity of BHA extends to a variety of bacteria, fungi, and viruses (Branen et al., 1980). Klindworth et al. (1979) studied the effect of combinations of BHA and sorbic acid on *Clostridium perfringens*. They found a possible synergistic antimicrobial effect of the compounds against the microorganism. Eriksen and Tompkin (1977) also found TBHQ to be an effective antimicrobial towards several bacteria. They were able to delay spoilage of a 30% gelatin solution using 0.01% TBHQ and prevent the growth of normal spoilage bacteria in milk at 2°C with 0.005% TBHQ.

It is important to evaluate antimicrobial compounds in a defined system before attempting to apply the compounds to a highly variable food system. This study was designed to determine the limits of antimicrobial effectiveness of several compounds against two bacterial species in a defined system.

Authors Brekke and Branen are with the Dept. of Food Science & Technology, Washington State University, Pullman, WA 99164. Author Davidson, formerly with Washington State Univ., is now affiliated with the Dept. of Food Technology & Science, Univ. of Tennessee, P.O. Box 1071, Knoxville, TN 37901.

EXPERIMENTAL

STOCK CULTURES of *Staphylococcus aureus* and *Salmonella typhimurium* were obtained from the Washington State Univ., Dept. of Food Science & Technology Culture Collection. The microorganisms were grown on Trypticase Soy Agar (TSA) Slants (BBL, Cockeysville, Md.) at 32°C for 24 hr. The slants were stored at 4°C and transferred every 2 wk to maintain viability. All cultures used were grown in Typticase Soy Broth (TSB) (BBL) for 24 hr before being inoculaed into the test media.

Butylated hydroxyanisole (BHA) and tertiary butylhydroquinone (TBHQ) were obtained from Eastman Chemical Products (Kingsport, Tenn.). A powder of BHA was made by grinding the original tablets in a Sorvall OmniMixer (Newton, Conn.). The BHA powder and TBHQ were then dissolved in 95% ethanol to a concentration of 4% (w/v) to prepare the stock solutions. The stock solutions were stored at 4°C when not in use. Potassium sorbate (Monsanto Co., St. Louis, Mo.) was added directly to the culture medium.

Growth inhibition tests were run by preparing 100 ml of TSB and adjusting the pH to 6.0 with concentrated HCl. Appropriate aliquots of BHA or TBHQ stock solution and/or solid potassium sorbate were added to the TSB and 5 ml of the medium was dispensed into 13 x 100 mm screw-capped culture tubes. Previous studies have indicated that autoclaving the inhibitors in broth does not influence the antimicrobial activity (Klindworth et al., 1979). The medium was autoclaved at 121°C for 15 min. Triplicate tubes of sterilized medium were inoculated with 0.1 ml of a 1:100 dilution of a 24 hr TSB culture (pH 6.0) of the appropriate microorganism. This corresponded to approximately 2.4×10^5 *S. aureus*/ml and 1.6×10^5 *S. typhimurium*/ml. The tubes were incubated at 32°C for 72 hr and growth was monitored by measuring the absorbance (OD) increase at 600 nm on a Bausch and Lomb Spectronic 20 Spectrophotometer (Bausch and Lomb, Inc., Rochester, N.Y.).

Concentrations of the inhibitors to be used in combination were determined by first adding the inhibitors alone to the TSB. The concentrations of each antimicrobial used were: BHA or TBHQ (ppm) 10, 25, 50, 100, 150, 200, and 400; potassium sorbate (ppm) 500, 1000, 2000, and 3000. Concentrations which resulted in approximately 0-50% inhibition after 72 hr incubation were used in combination testing. For combination tests, the inhibitors were simply added together in TSB and growth inhibition tests repeated. Percentage inhibition (PI) was measured using the following formula:

$$PI = \frac{(OD\ Control - OD\ Blank) - (OD\ Inhibitor - OD\ Blank)}{(OD\ Control - OD\ Blank)} \times 100$$

Blanks were either the OD at the time 0 of incubation or the OD of an uninoculated tube. All treatments were run in duplicate.

The total inhibition theoretically possible from a combination of BHA and sorbate (theoretical PI) was estimated as follows:

$$\text{Theoretical PI} = \text{PI of sorbate} + [100 - \text{PI of sorbate}] \times [\text{PI of BHA}/100]$$

The actual mean PI of the combinations (actual PI) was then compared to the theoretical PI and differences were statistically anal-

yzed using Student's t test (Brownlee, 1965). If the actual PI was statistically greater than the theoretical PI the combination was considered synergistic; if the actual PI was statistically less than the theoretical PI the combination was considered antagonistic; and if the actual PI was statistically equal to the theoretical PI the combination was considered additive.

RESULTS & DISCUSSION

THE CONCENTRATIONS of potassium sorbate tested which resulted in less than 50% inhibition after 72 hr at 32°C were 500 and 1000 ppm for both microorganisms. None of the concentrations of sorbate used at pH 6.0 resulted in total inhibition. This could be expected since pH 6.0 is near the upper limit of effectiveness for potassium sorbate (Chichester and Tanner, 1968). At lower pH levels the activity of sorbate would probably be increased. These results are in agreement with Tompkin et al. (1974) who found that potassium sorbate at 1000 ppm did not totally inhibit the growth of *S. aureus* and *S. typhimurium* at pH 6.2.

Concentrations of BHA which resulted in less than 50% inhibition of *S. typhimurium* were 50 and 100 ppm. The microorganism was totally inhibited by 150-200 ppm. These results are in contrast to Chang and Branen (1975) who found 400 ppm BHA necessary for total inhibition of *S. typhimurium*. The differences could be due to the strain used, media, or cultural conditions since these factors have been shown to influence BHA activity in other studies (Branen et al., 1980). *Staphylococcus aureus* was inhibited less than 50% by 50 ppm BHA (Fig. 1). Greater than 95% inhibition was achieved at concentrations of 150 ppm BHA and above. These findings agree with previous reports which generally showed *S. aureus* to be totally inhibited by concentrations of 150 ppm and above (Chang and Branen, 1975). Tertiary butylhydroquinone had dramatically different effects on the two bacteria. *Salmonella typhimurium* was able to grow even at 400 ppm TBHQ, whereas *S. aureus* was totally inhibited by as little as 25 ppm TBHQ. The results on *S. aureus* are in general agreement with Erickson and Tompkin (1977) who found that 50 ppm TBHQ inhibited spoilage bacteria in milk. It is likely that the difference between the organisms is due to the ability of gram negative organisms such as *S. typhimurim* to screen out TBHQ as theorized by Branen et al. (1980).

The concentrations to be used in the combination tests were determined from the above results and were as follows: *S. aureus*–500 ppm sorbate-25 ppm BHA, 500 ppm sorbate-50 ppm BHA, 1000 ppm sorbate-25 ppm BHA, 1000 ppm sorbate-50 ppm BHA, 500 ppm sorbate-10 ppm TBHQ and 1000 ppm sorbate-50 ppm TBHQ; *S. typhimurium*–500 ppm sorbate-50 ppm BHA, 500 ppm sorbate-100 ppm BHA, 1000 ppm sorbate-50 ppm BHA and 1000 ppm sorbate-100 ppm BHA. TBHQ was not used with *S. typhimurium* because of the high concentrations needed for inhibition.

Potassium sorbate or BHA alone demonstrated little or no delay in the initiation of growth of either *S. aureus* or *S. typhimurium*. However, all of the combinations of BHA and sorbate, except the lowest concentration (500 ppm sorbate-25 ppm BHA), delayed initial growth of *S. aureus* by 12 hr. With TBHQ as an antimicrobial, both the sorbate-TBHQ combinations and TBHQ alone caused a 12 hr delay in the growth initiation of *S. aureus*. In contrast to *S. aureus*, none of the combinations of BHA and sorbate affected growth initiation of *S. typhimurium*. The growth rate of *S. typhimurium*, however, was slower in the presence of the three highest concentrations of BHA and sorbate.

The percentage inhibition of the antimicrobials in combination (actual PI) at 72 hr of incubation was then compared with the sum of the percentage inhibitions calculated for the antimicrobials when used alone (theoretical PI).

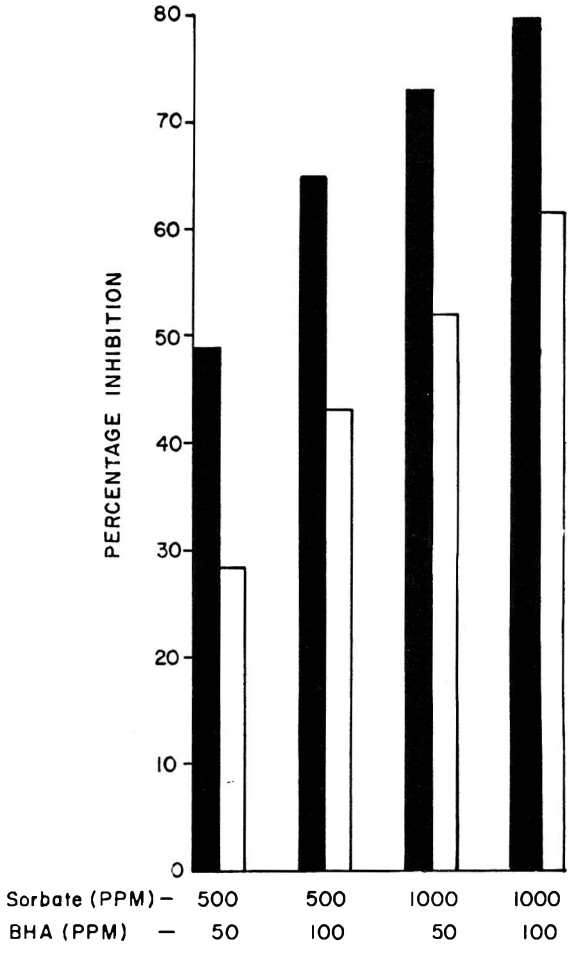


Fig. 1–Percentage inhibition of Salmonella typhimurium *by potassium sorbate and butylated hydroxyanisole after 72 hr incubation at 32° C in Trypticase Soy Broth, pH 6.0 (■–Actual inhibition of sorbate and BHA in combination; □–Theoretical inhibition of sorbate and BHA when added alone.)*

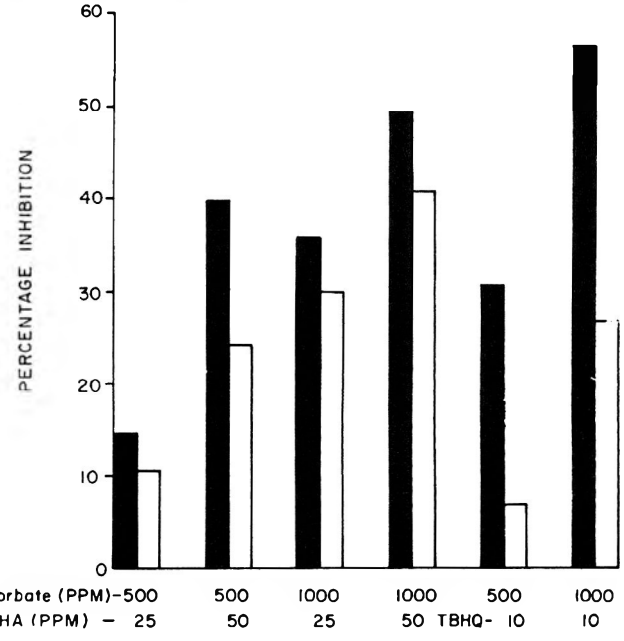


Fig. 2–Percentage inhibition of Staphylococcus aureus *by potassium sorbate and butylated hydroxyanisole after 72 hr incubation at 32° C in trypticase soy broth, pH 6.0. (■–Actual inhibition of sorbate and BHA (or TBHQ) in combination; □–Theoretical inhibition of sorbate and BHA (or TBHQ) when added alone.)*

The actual PI's for 500 ppm sorbate-50 ppm BHA and 1000 ppm sorbate-25 ppm BHA were both significantly higher ($P < 0.01$) than the theoretical PI's against *S. aureus* (Fig. 2). At 500 ppm sorbate-25 ppm BHA, the actual PI was also significantly higher than the theoretical PI but only at the 95% level ($P < 0.05$). There was no significant difference between the actual PI and the theoretical PI of 1000 ppm sorbate-50 ppm BHA. The reason for the highest concentrations of BHA and sorbate showing an additive inhibition is unknown. Both levels of TBHQ and sorbate had significantly greater ($P < 0.01$) actual PI's than their corresponding theoretical PI's.

The combinations of BHA and sorbate were even more effective against *S. typhimurium* (Fig. 1). All the combinations tested had significantly higher ($P < 0.01$) actual PI's than their corresponding theoretical PI's.

The results demonstrate that combinations of potassium sorbate, BHA and TBHQ were, in general, effective in synergistically delaying growth initiation and total growth of *S. aureus*. Combinations of potassium sorbate and BHA were also synergistic in suppressing final growth of *S. typhimurium*. The importance of these findings is twofold. First, this could allow for a reduction of potassium sorbate when used as a preservative if BHA and/or TBHQ are present. This is, of course, dependent upon the type of food since it has been sh wn that the presence of lipid decreases the antimicrobial activity of BHA (Klindworth et al., 1979). It is important not to attempt to extrapolate these results directly to a complex food system. The effect of many food components, especially lipids, on the antimicrobial effectiveness of potassium sorbate, BHA or TBHQ cannot be predicted. Second, if BHA and/or TBHQ are present they may perform a dual function as antimicrobials and antioxidants. The antimicrobial activity would, most likely, be less than potassium sorbate in a food and would merely contribute to preservation. The antimicrobial effectiveness of the antioxidants is presently being evaluated in food systems.

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ASCORBIC ACID IN POTATOES BY HPLC . . . From page 313

Table 1—Comparative analyses of ascorbic acid (AA) in potato products

	HPLC method		Titrimetric method	
	mg/100g	% Recovery of added AA	mg/100g	% Recovery of added AA
Raw potatoes	12.5 (2.6)[a]	100.0	11.9 (2.1)	106.9
Flakes	26.6 (5.6)	99.5	24.4 (2.4)	101.4
Granules				
Nonfortified	5.6 (5.7)	99.3	5.8 (1.4)	100.0
Fortified	201.6 (1.6)	–	226.1 (2.6)	–
French fries	3.9 (2.5)	102.3	4.0 (6.3)	98.2
Chips	20.2 (5.1)	97.7	45.5 (6.7)	109.5

[a] Percent standard deviation

In conclusion, the HPLC procedure described above using a reverse phase, ion pairing system is a simple, rapid and accurate method to determine ascorbic acid in potatoes and potato products.

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