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Volume 53: Number 4

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Letters

Effect of AA and O_2 on Nonenzymatic Browning of Orange Jucie

Re the paper Nonenzymatic browning in aseptically packaged orange juice and orange drinks: Effect of amino acids, deaeration, and anaerobic storage by Kacem et al. (J. Food Sci. 52:1665, 1987), the authors studied the effect of amino acids on browning and loss of ascorbic acid in aseptic orange juice. What is in error in the article is something very basic. The authors plot loss of ascorbic acid under different conditions with time on linear coordinate graph paper. This in itself is correct. In most cases, they have less than a 20% loss or change occurring. They then note that a number of authors, showed that ascorbic acid loss was first order. They then said, about their own results, that "The retention of ascorbic acid followed a first order kinetic model since the data fit a straight line." This is correct but since they show a plot (linear coordinates) of % concentration vs time their proof is incorrect. A first order degradation mechanism will give a straight line on a log (% retention) plot, not on a linear plot. Thus, their basic premise is false since they did not apply kinetics properly with respect to the difference between zero and first order. Secondly, they should have reported the results as a pseudo first order since the molecularity of the reaction is more complex and should include the concentration of oxygen in the equation [e.g., See J. Food Processing Presv. **11:** 197]. Calling it a pseudo (zero or first) order gets around the need to do complete reaction sequencing. Interestingly, since they did not carry out the experiment beyond 20%, they could use either pseudo-zero or -first order. A linear plot as they presented, in fact, is pseudo-zero order and should be treated as such. It should be noted that, in order to prove that a reaction is truly first order, one must go beyond two half lives reduction (i.e., >75% loss); otherwise, the error in the rate constant is large. Based on their going to only 20% loss, the theoretical maximum error in the rate constant is greater than ± 70%, assuming that the analytical precision for ascorbic acid is ± 5% [J. Chem. Education **61:** 348 (1984)].

They further carry out their incorrect reasoning when they calculated the rate constant. For zero order the equation is:

$$\frac{dC}{dt} = -k_o \qquad (1)$$

integrating over time we get $[C_o - C] = k_o t$ (2)

thus: $k_o = [C_o - C]/t$ = amt/time (e.g., mg/day) (3)

where: t = time; C_o = initial concentration; C = concentration at time t.

To get k_o one does a linear regression of concentration with time which gives units of concentration/time, e.g., mg/day. One should not regress % loss or % remaining with time if the data are to be compared between systems. To prove this, if we divide both sides of Eq. (2) by C_o and multiply by 100 then we have:

$$100\,[1 - \text{fraction remaining}] = 100\,[k_o/C_o]\,t \qquad (4)$$

where: $\text{fraction remaining} = C/C_o$ (5)

also: $100 - \%\ \text{remaining} = 100\ k'\ t$ (6)

where: $k' = k_o/C_o$ (7)

thus: $\%\ \text{remaining} = 100\ C/C_o = 100\,[1 - k'\ t]$ (8)

This is still a straight line, the slope of which is 100 k' where k' as noted is k_o/C_o. Thus, it can be seen that the value of the slope of % remaining vs time depends on the initial concentration, and is not comparable between systems which are zero order whereas k by itself is independent of initial concentration.

For a pseudo-first order equation we have:

$$\frac{dC}{dt} = k_1 C \qquad (9)$$

Integrating we have: $\ln [C/C_o] = k_1 t$ (10)

Thus, a plot of log concentration or log % remaining gives a straight line, the negative slope of which is k_1, the rate constant. The units of k_1 are reciprocal time. Thus, the pseudo-first order rate constant also does not depend on the initial concentration.

In their article, they report, in Table 2, the rate constants for ascorbic acid loss which they call first order rate constants with units of reciprocal weeks. In evaluating the data, it can be seen that what they did to get the numbers was run a linear regression on % remaining with time, which should give pseudo-zero order rate constants, not first order as they state. However, at this low extent of degradation, it is very hard to truly distinguish between either order, unless one has very good precision and had at least eight concentration vs time points after zero time. They have only four. The fact that they get a straight line thus does not prove which order is correct, and in fact, they report no r^2 values. They make a point that their slopes are significantly different than zero (i.e., no loss) but that is sort of a trivial statistical argument. Either one gets no loss or there is a loss with time.

An attempt was made to calculate the actual pseudo-zero order rate constants using the value of C_o from their Fig. 2 for zero time (34 mg/100 mL for the deaerated and anaerobically stored products). The results are presented in Table 1. The values for zero order are very different from those in the Kacem table because they were calculated as mg/week rather than as %/week (note again their table lists the units as weeks^{-1} which is wrong). One can convert the new values to theirs by dividing k_o by 34 and multiplying by 100. Table 1 also contains the calculated k_1 by linear regression of ln C/C_o vs time. Obviously, the actual pseudo first order rate constants are very different than what they report because of the error in interpretation. The r^2 values for both pseudo orders also are reported in Table 1. As can be seen, it is really impossible at this level of degradation to distinguish between zero and first order.

As a last note, another common error made in calculations for pseudo-first order is to use semi-log graph paper, get the slope for the value of k, but forget to multiply by 2.303, which is the conversion from log base 10 to natural log [J. Chem. Education. **61:**348 (1984)]. Another common error is to conclude that if the rate constants plotted on semilog paper vs. inverse absolute temperature give a straight line (the Arrhenius plot), the data, therefore, are first order. In fact, all orders should fit this type of plot unless there is a change in mecha-

Table 1—Ascorbic acid in aseptic orange juice

Time Weeks	Sample designation ODOAE	ODOAN	OD4AE	OD4AN	OD8AE	OD8AN
			(mg/100 mL)			
1: 0	34	34	34	34	34	34
2: 4	32.88	34	32.54	34	32.03	33.01
3: 8	31.45	33.76	31.21	33.80	30.97	31.82
4: 12	30.53	32.1	30.26	32.78	29.27	31.59
5: 16	29.31	32.1	28.59	32.27	27.44	30.36
k_{zero}	0.2932	0.129	0.328	0.117	0.397	0.218 (mg/wk)
linear C_o	33.98	34.32	33.94	34.31	33.91	33.90
r_2	0.9967	0.8681	0.9943	0.863	0.993	0.9718
k_{1st}	0.009275	0.003896	0.0105	0.00335	0.01297	0.00676 $(wk)^{-1}$
ln C_o	3.527	3.3536	3.5263	3.5355	3.5267	3.5239
r^2	0.9972	0.869	0.9929	0.8688	0.9909	0.972

nism, etc., [see Labuza and Schmidl, Food Technol. **39:** 57 (1985)].

The writer hopes that this exercise will be instructive to those who include kinetic calculations in their papers in the future.

—*Theodore Labuza,* Professor, Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108.

And the response. . .

We appreciate Dr. Labuza's pointing out errors in the application of kinetics in this paper and the opportunity to correct them. The main objectives of this paper were to measure the effects of amino acids and oxygen on the nonenzymatic browning of orange juice. We were most interested in those changes which would have practical importance in terms of time and temperature. Our experimental design called for measurements to be taken on the degradative changes in each of 6 treatments, each with 3 replicates for a period of 16 weeks. This time frame for storage at room temperature is reasonable for a commercial product.

In order to make comments on the kinetics of ascorbic acid (AA) degradation, the experiment should have been run to the point where more than 75% of the initial levels of AA had disappeared. Dr. Labuza's point is valid, it is incorrect to determine whether the reaction kinetics are first order, second order or pseudo-first order, without these data.

Nagy [J. Ag. Food Chem. 28(1): 8–18 (1980)] presents a review on vitamin C and shows a plot of ln vitamin C retention and storage time as being linear up to 30°C. Using this information presented here and assuming other things as equal it would require more than 104 weeks at 24°C, more than 80 weeks at 32°C, and more than 24 weeks at 37°C in order to reduce the initial vitamin C levels by more than 75%. These times and temperatures were impractical in terms of the design standpoint of this experiment.

For data showing limited degradation of ascorbic acid, regression analysis of linear and semilogarithmic plots gives nearly equivalent correlation coefficients. The data of Fig. 1 were graphed on axes with linear coordinates (Y axis = % AA retention, X axis = storage time in weeks). As stated in the caption, the regression equations were of the form: % AA retention = Y intercept − slope * time. In view of limited degradation of AA that occurred during the experiment, other graphical presentations (e.g. semilogarithmic), which imply a knowledge of a reaction order, are kinetically and statistically uncertain. The slopes of such linear plots of % retention vs time are shown in Table 2 of the paper. The units of these slopes (i.e., zero-order rate constants), should be % loss/week, not $weeks^{-1}$ as shown. Dr. Labuza's calculated zero-order rate constants are equivalent to the values of Table 2 if expressed in terms of % loss/week.

It is important not to confuse the use of R^2, the square of the correlation coefficient, which is a measure of the closeness of fit, when comparing two equations which have different units of measurement, mg/100 mL vs ln (mg/100 mL). R^2 is dependent on the units in which the variables are measured. Additionally, the R^2 values presented in Dr. Labuza's Table 1 are inflated because they are based on the averages of 3 packs, not on the individual observations.

In our discussion of Fig. 3 we state there is a significantly greater slope at the 0.8% level of amino acid addition than the 0.0 or 0.4% levels.

We hope this explains our experimental design and data. We believe all of the conclusions in the paper remain valid. The application of kinetics should not have been included.

—*B. Kacem, R. F. Matthews, P. G. Crandall, and J. A. Cornell,* Citrus Research & Education Center, University of Florida, Lake Alfred, FL 33850.

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Evaluation of the Moisture Permeability Characteristics of Chocolate Films as an Edible Moisture Barrier

B. BIQUET and T.P. LABUZA

ABSTRACT

The moisture barrier properties of a semisweet dark chocolate film, including effective diffusion coefficient and effective permeability constants, were determined at variable film thickness, temperature and relative humidity in the intermediate moisture range. In addition, both adsorption and desorption isotherms were determined. It was found that this chocolate film 0.6 mm (24 mil) thick was a better moisture barrier than a 1 mil thick low density polyethylene film. The mathematical packaging model for nonedible packaging materials worked well for a typical outer barrier coated model system while a new mathematical model predicted well the moisture transfer within a bicomponent system of different initial a_w with the barrier between them.

INTRODUCTION

THE SHELF LIFE of a food product is determined by numerous and complex interactions between parameters related to the product itself and/or associated with the external environment. Among those factors, the interaction between the moisture and the food is critical and moisture transfer in finished food products frequently leads to deleterious changes in quality. Moisture transfer can occur between a food and its environment (Duckworth, 1975; Labuza et al., 1972; Labuza and Contreras-Medellin, 1981; Matz, 1965) and/or within a nonhomogeneous structured food system (Genctruk et al., 1986; Hong et al., 1986; Iglesias et al., 1979; Salwin and Slawson, 1959). In both cases it results in a variation of the water activity (a_w) and the moisture content of the product as a function of time.

The kinetics of chemical and enzymatic reactions in foods as well as the microbial stability of food products are strongly influenced by the water activity and are well documented. Textural properties and physical stability of foods are also related to the bound state of the water and can change drastically as a result of loss or gain of moisture during processing and/or storage (Kapsalis, 1975; Labuza, 1985b; Kapsalis et al., 1970; Saltmarch and Labuza, 1980). These changes can result in reduction of shelf life. For example, products such as raisins (Bolin, 1976; Kochhar and Rossell, 1982; Lowe et al., 1963; Marston, 1983; Watters and Brekke, 1961), candies and chocolate-coated products (Andres, 1984; Barron, 1977; Cosler, 1957; Feuge, 1970; Jokay et al., 1967) and dry crackers, biscuits, pizza crust, and filled bakery crust (Heiss, 1968; Katz and Labuza, 1981; Labuza, 1985a;) may become unsatisfactory as a result of moisture gain or loss.

The properties and mechanisms governing moisture transfer in finished food systems have been reviewed elsewhere (Hallstrom and Skjoldebrand, 1983; Karel, 1972; Labuza and Contreras-Medellin, 1981). It can be demonstrated that moisture transfer between a food and its environment and/or within its different components is caused by water chemical potential gradients, i.e., water activity gradients. The transfer occurs from the phase with a high a_w to the phase with a low a_w (Hong et al., 1986; Matz, 1965; Oswin, 1976; Salwin and Slawson, 1959; Stewart, 1975; Szulmayer, 1973). At equilibrium all components of the system will have the same a_w, i.e., the vapor pressure gradient will be zero at constant temperature and pressure.

Moisture exchange between a food product and its surrounding atmosphere can be controlled provided adequate packaging material is used. A more challenging problem is to prevent or slow down moisture exchange within multicomponent food products such as mixtures of dehydrated foods, jelly-filled cookies, pies, pizza. By appropriate manipulation of certain parameters, the rate of moisture exchange and/or the overall moisture gain or loss may be reduced. This can be achieved by: (1) eliminating or reducing the a_w gradient by changing the product formulation and, 2) separating the different product components with a nonedible or an edible moisture barrier. The use of humectants to reduce the a_w gradient, however, is not always possible and may result in drastic modifications of the sensory and physiochemical characteristics of the product (Guilbert, 1985; Karel, 1976). Other parameters such as process and storage parameters may be difficult to control or have little effect. The use of a nonedible barrier is not possible in most cases. Thus, the most logical alternative solution is the use of an edible moisture barrier placed at the interface between the different components of the system.

Edible films and coatings and edible moisture barriers have been comprehensively reviewed elsewhere (Biquet, 1987; Daniels, 1973; Guilbert, 1986; Kester and Fennema, 1986b; Kroger and Igoe, 1971; Morgan, 1971). The basic properties needed for a good moisture barrier include: (1) a physical structure which interferes with the ease of the diffusion process, e.g., very small pores, (2) the absence of structures or functional groups similar to the permeant molecule, and (3) the wettability of the film surface with respect to adhesion to the food itself. Studies done on the waxes found in plant cuticles have shown that the surface structure plays a critical role (Bain and McBean, 1967; Martin and Juniper, 1970; Possingham, 1972). It has been shown that the most efficient cast film edible moisture barriers are lipid- and wax-based film coatings (Archer and la Mer, 1955; Kamper and Fennema, 1984a; Kester and Fennema, 1986b, b; Landman et al., 1960; La Mer et al., 1964; Lovegren and Feuge, 1954).

Other potential edible moisture barrier materials include: (1) surface active agent films (Roth, 1982; Roth and Loncin, 1985), (2) composite films and coatings using oil emulsions (Daniels, 1973; Guilbert, 1986; Hocquard, 1986; Luft and Murray, 1974; Silva et al., 1981), and (3) multicomponent systems combining proteins and/or various polysaccharides with fatty emulsions or fatty layers (Cole 1969; Cosler, 1957; Daniels, 1973; Guilbert, 1986; Hamdy and White, 1969; Harris and Lee, 1974; Hocquard, 1986; Kamper and Fennema, 1984b, 1985; Kester and Fennema, 1986a; Lowe et al., 1963; Schultz et al., 1949; Silva et al., 1981; Ukai et al., 1976). Kamper and Fennema (1984b, 1985) studied a blend of palmitic and stearic acids with a hydroxypropylmethylcellulose (HPMC) support which was cast into a film. The film-forming system involved polyethylene glycol and a water-ethanol solvent. Their film (0.04 mm thick) had very good moisture barrier properties, especially when tested in a pizza model system. Kester and Fennema (1986a) used a similar technique but a thin layer of bees

Authors Biquet and Labuza are with the Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108.

wax was laminated onto the surface of the HPMC/fatty acid matrix. The permeability constant was in the same range as that of the Kamper and Fennema (1986a) film even at high relative humidities. Cast films have some disadvantages, however, their application is limited to layered foods and the technique of preparation and application may be difficult to develop on an industrial scale. Good sealing and adhesion on a commercial product may be impossible to achieve. Unfortunately, the crystalline structure and molecular packing and the chemical composition of many fatty materials often result in a waxy, mouth-feel. One of the solutions to modify these undesirable properties is to incorporate desirable solids in the fat phase; such as the basis for chocolate manufacturing.

Solid dark chocolate may be defined as a continous dispersion of cocoa solids and sucrose suspended in cocoa butter with some stability provided by the addition of lecithin, a surfactant. The water activity of plain chocolate is generally in the range 0.2 to 0.4 with a moisture content between 0.4 to 1g H_20/100g dry solids. Moisture sorption isotherms for cocoa products (Fincke, 1965; Gane, 1950; Gough and Pilliatt, 1979; Heiss, 1968; Lomauro et al., 1985b; Reade, 1969; Van den Berg, 1983), sucrose (Iglesias and Chirife, 1982; Labuza, 1984) and lecithin (Elworthy, 1961, 1962; Wilkinson et al., 1977) have been published. No moisture sorption isotherm has yet been published for cocoa butter, however, Niediek (1981) reported approximately 0.8% moisture at 60°C and 46% relative humidity. Heiss (1968) determined the adsorption isotherm for a dark chocolate and Ogunmoyela and Birch (1984) studied the moisture sorption properties of chocolate systems with various sweeteners.

The unique properties of chocolate indicate that good moisture barrier properties may be expected. Practical evidence somewhat supports this hypothesis; many dry and semimoist products are coated with chocolate (Minifie, 1980) and can have considerable shelflife unpackaged. In addition, several studies have confirmed the efficiency of chocolate and cocoa butter coatings as moisture barriers (Kempf, 1967; Landman et al. 1960; Neidiek, 1981; Soboleva and Chizhikova, 1978; Tiemstra and Tiemstra, 1974). The best study to date is the work of Landman et al. (1960). Using the cup method and saturated salt solutions, they determined the water vapor transmission rate (WVTR) and permeability constants of cocoa butter, chocolate liquor and chocolate milk films. Evidence was shown that chocolate was a good moisture barrier and that the cocoa butter continuous phase was the main reason for it. The importance of a good tempering process was also pointed out.

The objectives of this study were: (1) to assess the moisture barrier properties of a chocolate film under various environmental conditions and (2) to predict and test the rate of moisture transfer through a chocolate layer in two intermediate moisture food model systems.

MATERIALS & METHODS

Preparation of the chocolate films

Samples of semisweet paste (dark chocolate) were obtained from Dr. Darrell Medcalf of the Hershey Foods Corporation (Hershey, PA) and stored in closed containers at 6°C. To reduce the viscosity of the melted chocolate paste, it was necessary to increase its fat content from 25.4% to 29.6% by addition of cocoa butter. The final composition was 29.6% cocoa butter, 51.4% sucrose, 18.5% cocoa solids and 0.4% moisture. The mixture was homogenized at 55°C in a water bath with a hand mixer (Moulinex Regal, Model No. V748 with a modified dough hook) for 90 min. The mixer was set on its low speed, and a rheostat (Powerstat, Type 2PF10) was used for further adjustment of the agitation. From this, 370g melted chocolate was then poured into a metallic mold (18 × 29 × 1.5 cm) and vigorously tapped. The resulting thin layer (0.4 cm) of chocolate was next dried at 55°C and 29" vacuum for 16 hrs (National Appliance Company, Portland, OR) and stored over desiccant (Drierite).

Then, exactly 350g of the above prepared chocolate was broken up and placed into a 600 mL plastic beaker and heated to 55°C. The next step was to temper the paste to produce the stable polymorphic forms of the cocoa butter. The melted chocolate paste (55°C) in the 600 mL beaker was cooled down to 34°C with continuous hand-stirring in a 6°C cold room. The beaker was then placed in a water bath at 27.3°C (± 0.1°C). Continuous agitation was applied using the system described previously. The temperature in the center of the paste was continuously recorded and monitored to the nearest 0.1°C. The chocolate was cooled down to and stabilized at 28°C for 10 min, then heated to and stabilized at 31°C for 20 min.

A specially built glass-mold was used in which to prepare the chocolate film (Contact T.F. Labuza for design). The mold was covered with thin commercial aluminum foil (1851 Anaconda; Arco Packaging Products, Louisville, KY) which was changed after each use. The depth of the mold (and, therefore, the thickness of the chocolate layer) could be adjusted with rigid plastic sheets and tape. The tempered chocolate at 31°C was quickly placed into the mold and spread with a spatula. This was done in a room at 20–23°C (temperature of the mold). The mold was tapped sharply to rid the chocolate of air bubbles. A thin (2 mm thick) and rigid plastic sheet was placed at a right angle at one end of the mold and passed over the whole length of the mold to uniformly smooth out the surface and remove the excess chocolate. The mold was then vigorously tapped again very carefully to further remove air bubbles and homogenize the layer. The overall operation was done as quickly as possible. The mold was placed into a plastic styrofoam cooler (internal dimension: 45 × 27 × 28 cm) with desiccant on the bottom and the whole system was put into a cold air room at 6°C. When the temperature of the air inside the cooler had reached 15°C, it was removed from the cold room and placed at room temperature (20–23°C). The cooler was again moved into the cold room, and the layer was then held for at least 12 hrs after it reached 6°C. After this operation, the layer was then held for at least 12 hrs after it reached 6°C. After this operation, the layer was cut on the edges of the mold. The bottom of the mold with the layer was separated from the outside perimeter frame. A sharp, metallic, hollow cylinder, slightly heated, was used to cut up the layer into disks of the desired diameter (5.3 or 5.9 cm). The latter were carefully separated and placed over desiccant at 6°C. This system allowed one to prepare layers as then as 0.6 mm in a reproducible way. The thickness of all layers was carefully measured to the nearest 0.02 mm with a caliper guage. All layers were used within 5 days after their preparation to limit changes in the crystallization state of the fat phase.

Model system preparation

Freeze-dried agar/microcrystalline cellulose (MCC) gels with a constant MCC to agar ratio of 5/1.5 (w/w) were prepared according to the procedure described by Biquet and Labuza (1988). Purified agar (Difco Laboratories, Detroit, MI) and microcrystalline cellulose Avicel PH 105 with an average particle size of 20 microns (F.M.C. Corporation, Philadelphia, PA) were used.

Moisture content determination

Moisture was determined by the Karl Fischer method (Mitchell and Smith, 1980; Scholz, 1984), using the Aquatest IV titrator (Photovolt Corporation, New York, NY) with Hydranal-Coulomat reagent (Fisher Scientific Company, Fair lawn, NJ). Extraction of the moisture was achieved by letting the samples stand in anhydrous methanol (ACS grade, Columbus Chemical Industries, Inc., Columbus, WI) for 12 hr at 6°C (model gels) or for 12 hr at 50°C (chocolate).

Determination of moisture sorption isotherms

Saturated salt solutions were prepared according to the procedure described by Multon (1984) and their water activity at 20°C was taken from the literature (Greenspan, 1977; Multon, 1984; Stamp et al., 1984). Isotherms were determined using the method described by Labuza (1984), at a temperature controlled to 20 ± 1°C using triplicate samples (1.8g chocolate and 1.1g gel). Equilibrium was assumed to be reached when the change in weight expressed on a dry basis did not exceed 0.1%, i.e., was less than 0.001 g/g dry solids for three consecutive weighings at no less than 5 day intervals. After equilibrium, each sample was immediately dropped into methanol for Karl Fischer titration.

Determination of the diffusion coefficients

Since the kinetics of sorption were expected to be slow for the chocolate films, discontinuous weighing could be done. Mason jars

(1 quart) were used to hold the samples at a give relative humidity. The films used were 0.6 mm thick and 5.3 cm in diameter. They were placed on preweighed glass dishes into the jars (1 sample per jar) and held at 20 ± 1°C. Samples were weighed to 0.00001 g (Mettler HSI, Mettler Instrument Corporation, Princeton, NJ) at time intervals never smaller than 2 hr apart. Initial moisture was determined by the Karl Fischer method on five samples prepared under the same conditions. Due to the small thickness and large diameter of the chocolate disks, a monodirectional transfer could be assumed. In this system, the chocolate layers can be described geometrically as infinite slabs with moisture sorption taking place on one side only. For such a system and under certain assumptions, the solutions of Fick's second law (Crank, 1975) converges rapidly for large values of time. The first term of the series solution can be used with very little error (Schwartzberg, 1975). Thus, from the slope of a plot of lnΓ vs time, D_{eff} can be found as shown in Eq (1):

$$\ln \frac{m - m_e}{m_i - m_e} = \ln \Gamma = \frac{-\pi^2 D_{eff}}{L^2} \cdot t + \ln \frac{8}{\pi^2} \quad (1)$$

Determination of water vapor permeability constants

The procedure used was adapted from the ASTM method (E96-80). However, due to the low melting point of the chocolate, a different sealing system had to be developed. The chocolate films were prepared as indicated before. A 5% variation from the desired average thickness was tolerated and the film diameter was about 5.9 cm. Twing Albert cups were filled with desiccant (Drierite) or a saturated salt solution (81% RH) and stabilized at the temperature of the experiment for 12 hr. The films were also stabilized at the temperature of the experiment for 6 hr over desiccant. The cup lips were filled in slight excess with chocolate coating at 52–54°C with a curved spatula. The selected layer was then added and gently pressed so that the melted chocolate overlapped on the edges. The system was allowed to stand at room temperature for 2 min, after which it was placed at 6°C over desiccant or a saturated salt solution (such that there was no a_w gradient) for 5 min. Melted chocolate coating (52–54°C) was next spread with the curved spatula on the edges of the film and let set for 2 min at room temperature. The cup was then placed over desiccant or a saturated salt solution at 6°C for another 10 min. Final stabilization was done at the temperature of the experiment for 1 hr. Moisture adsorption by the chocolate seal was prevented by coating with high vacuum grease (Silicone Dow Corning Corp., Midland, MI). The effective surface areas was measured at the end of each experiment (5 measurements per cell, ± 0.02 mm). The sealed cups were placed individually in small desiccators at the desired temperature (± 1°C). The relative humidity within the desiccator was determined by the saturated salt solution used. Steady state conditions were assumed to be reached when the change in weight of the cup over time became constant as determined by visual plotting of the data. Under steady state conditions, the permeability constant k is calculated from a plot of moisture (actually total weight of cup) gain or loss (w) versus time using the following equation (Karel et al., 1959; Lebovitz, 1966) which is a derivation from a combination of Fick's and Henry's laws for diffusion of a gas in a semi-permeable isotropic medium.

$$\frac{dw}{dt} = (k/x)\, A\, (p_{out} - p_{in}) \quad (2)$$

Therefore, the water vapor transmission rate is:

$$WVTR = \frac{slope}{A} = \frac{g\ H_2O}{day\ m^2} \quad (3)$$

Where slope is the slope of the weight gain (or loss) curve. The film permeability is:

$$k = \frac{slope}{A\,(p_{out} - p_{in})} = \frac{g\ H_2O\ mil}{day\ m^2\ mm\ Hg} \quad (4)$$

Conversely, the overall film permeance k/x is given by:

$$k/x = \frac{slope}{A\ x} = \frac{g\ H_2O}{day\ m^2\ mm\ Hg} \quad (5)$$

Model system moisture transfer experiments

The freeze-dried agar/MCC gels were used to simulate two different low or intermediate moisture food model systems to evaluate the moisture barrier properties of the chocolate coating in more realistic conditions. The chocolate films (5.2 cm diameter and 0.6 mm thick) were prepared as indicated before and preequilibrated at 20 ± 1°C for 6 hr over desiccant (Drierite). The freeze-dried agar/MCC gels were equilibrated to the desired water activity level (0.33 for low a_w and 0.85 for high a_w) at 20°C for at least 15 days. A method similar to that used for the permeability test was used to seal the cells. In the *monocomponent* test configuration the initial water activity of the gel was set to 0.33. This was placed in a special glass test cell made at the University of Minnesota (similar to a Twing Albert Cup but with a plane rather than a curved edge), covered with the chocolate film and was held at 80.8% RH (ammonium sulfate) and 20°C. Duplicate samples were removed at appropriate intervals and the moisture of the chocolate film and the gel were measured by Karl Fischer. For the *bicomponent* test system, the low water activity gel was placed into the bottom cell of a special glass cell and the chocolate film was immediately sealed on it with the "tempered" surface on top. The high a_w gel was then quickly placed into the top cell sealed with chocolate onto the bottom cell. Silicone grease was spread on the edges of the system to insure a moisture proof seal prior to being put at 20°C. Duplicate samples were removed for moisture measurement at suitable times.

RESULTS & DISCUSSION

Moisture sorption isotherm

Equilibrium times for moisture adsorption and desorption of the chocolate ranged between 40 and 60 days, depending on the relative humidity (Fig. 1). This is in contradiction with the results published by Ogunmoyela and Birch (1984) who reported that constant weights were usually observed after 14 days. Because of the complexity of chocolate, due to the presence of sucrose and the presence of a continuous fat phase slowing sorption and the small moisture encountered, small weight changes were not negligible. As noted in Fig. 1, some amorphous to crystalline transfer is occurring, probably the sucrose (Labuza, 1984). The moisture adsorption and desorption isotherms of a dark chocolate film at 20°C are shown in Fig. 2. The values found on adsorption compare well (on a non-fat dry basis) with those found by Heiss (1968) and are in the same range as those reported by Ogunmoyela and Birch (1984) for a dark commercial chocolate. As noted, the adsorption isotherm was determined by measuring the moisture of the equilibrated samples by both weight change and the Karl Fischer method. The initial a_w for the desorption isotherm was 0.808. As can be seen from Fig. 2, chocolate exhibits an important hysteresis. Crystalline sucrose shows only a limited hysteresis (Labuza, 1984) but some components of the cocoa solids such as starch exhibit a large one (Van den Berg and Leniger, 1978). The Guggenheim-Anderson-De Boer (GAB) equation (Aguerre et al., 1986; Bizot, 1983; Lomauro et al., 1985; Schar and Ruegg, 1985; Van den Berg, 1981, 1983, 1985) was evaluated for goodness of fit of moisture content versus water activity for the adsorption and desorption iso-

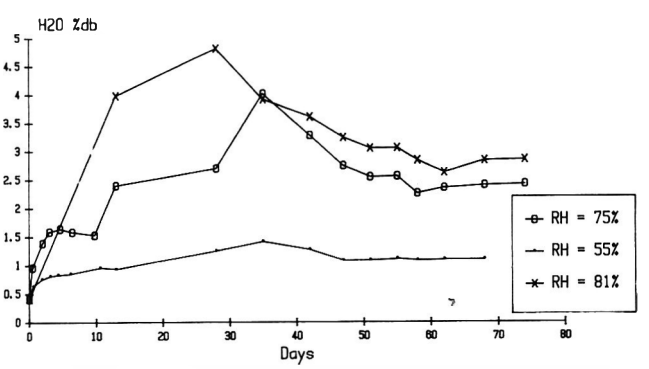

Fig. 1—Kinetics of moisture adsorption for a dark chocolate film at different relative humidities (20°C, thickness = 0.6 mm).

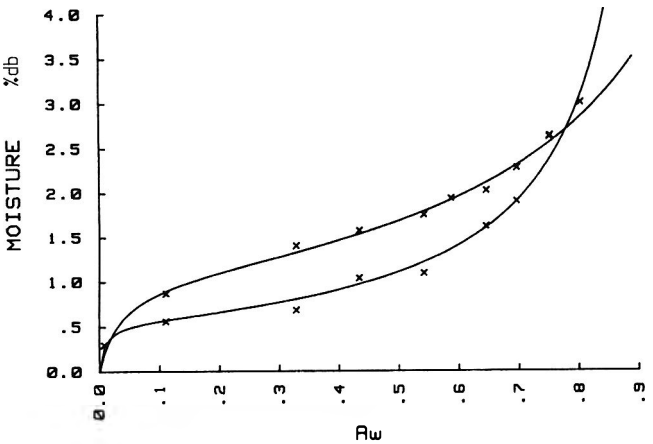

Fig. 2—Moisture adsorption (lower curve) and desorption (upper curve) isotherms of dark chocolate (20°C, Karl Fischer method) fitted with the GAB equation.

Table 1—GAB constants calculated by non-linear regression analysis of the average moisture contents at each a_w for dark chocolate

Mode of sorption	Initial a_w	a_w Range of test	m_o $gH_2O/100g$ solids	K	C
Adsorption	0.01	0.01 –0.808	0.545	1.024	103.857
Desorption	0.81	0.754–0.112	1.067	0.785	34.262

Table 2—Effective diffusion coefficient of a dark chocolate film at 20°C (Average of four determinations; values in brackets are the standard deviations)

Sorption mode	Initial a_w	RH (%)	Thickness (mm)	$D_{eff} \cdot 10^{13}$ ($m^2 \cdot sec^{-1}$)
Adsorption	0.01	75.4	0.607 (0.05)	1.08 (0.22)
		64.8	0.594 (0.04)	0.82 (0.31)
		54.4	0.607 (0.04)	0.87 (0.09)
Desorption	0.81	33.0	0.599 (0.04)	1.33 (0.13)

therms of dark chocolate. The GAB equation as applied to water vapor sorption can be written as:

$$m = \frac{m_o C K a_w}{(1 - K a_w)(1 - K a_w + C K a_w)} \quad (6)$$

A direct nonlinear regression procedure (using the statistical library for Hewlett Packard 200 Series on a Hewlett Packard 9816 Computer) on the average Karl Fischer moisture for each a_w was used. The solid lines in Fig. 2 are the predicted equation and the GAB constants are given in Table 1.

Effective moisture diffusion coefficients

The effective diffusion coefficients (D_{eff}) for both adsorption and desorption at 20°C for a dark chocolate film are given in Table 2. Comparison with values of D_{eff} for common foods (Hong et al., 1986) indicates that the internal resistance to moisture transport in chocolate is high, similar to that of raisins. D_{eff} is basically independent of the surrounding relative humidity which is in agreement with the theory of diffusion of gases in a porous medium. D_{eff} for desorption is slightly larger than that for adsorption which may be due to a less dense packing of the fat crystals at the higher moisture, however, the values are not significantly different.

Effective water vapor permeability of chocolate

In the permeability tests the times at which an apparent steady state was established differed depending on the thickness of the chocolate film; (ca. 15 hr for a thickness of 0.6 mm and 45 hr for ..2 mm). In the case where the desiccant was placed in the desiccator and the saturated salt solution was placed in the cup, the establishment of an apparent steady state required about 75 hr for a thickness of 0.6 mm at 81% RH and 20°C. Landman et al. (1960) reported equilibrium times of one to two days under similar conditions with a larger thickness.

Because Landman et al. (1960), in their early work, used a cup configuration where the saturated salt solution was placed inside the cup instead of desiccant as in the usual method, it seemed important to investigate whether or not the placement of the highest a_w could influence the results. Another reason for looking at this is that a chocolate film may not behave like an inert hydrophobic material, thus not following Eq. (1). The water vapor transmission rate (WVTR) and effective permeability constants (k_{eff}) are presented in Table 3. A five- to seven fold difference between the two methods is shown, the lowest value being obtained with the salt solution inside the cup. There should be no difference between the two methods since the same chemical potential exists across the barrier for either configuration. A possible explanation is that, as will be shown later on, the chocolate film adsorbs moisture during the experiment, thus, not behaving ideally. This difference could also be due to a difference in the surface structure of the two sides of the chocolate film. The side in contact with the bottom of the mold when it was made appeared to be smooth and glossy while the other side looked rough. This can be related to the difference reported by Minifie (1980) in the surface structure of chocolate coatings prepared by enrobing and molding when observed under the light microscope. However, we could not find a significant effect of the film configuration in the cup method on its moisture barrier properties as also seen in Table 3. Similar results were obtained for a similar experiment with the salt solution in the cup, i.e., no difference with the placement of the surface sheen. However, further investigation is needed on this point, thus, the term "effective" permeability constant (k_{eff}) is used.

The reproducibility of the method (Drierite in the cup) was tested at 20°C and was found to be satisfactory as seen in Table 4. The average values for two independent experiments obtained under similar conditions (temperature, vapor pressure gradient and film thickness) were not significantly different, however, for a smaller Δp but at higher absolute vapor pressure, the k_{eff} was about two times higher. Increasing the water vapor pressure differential (RH gradients) across the chocolate film at constant temperature (20°C) in the range of 0-14.1 mm Hg (0-81% RH) increases the WVTR (Table 5). The WVTR increased from 1.20 to 3.21 g/day·m² when the water vapor pressure gradient was increased from 0–5.8 to 0–14.1 mm Hg at 20°C, however, the effective permeability constant is not significantly different (4.9 vs 5.6 g·mil/day·m²·mmHg) in agreement with Fick's Law that there should be no change (Lebovitz, 1966). Measurement at higher vapor pressures has a greater effect on the permeability constant. With about the same water vapor pressure gradient of 0–5.9 vs 9.5–14.1 mm Hg, but the latter at higher absolute pressure, the WVTR increases from 1.2 to 2.1 g/day·m² while k_{eff} increases more drastically from 4.9 to 11.0 g·mil/day·m²·mm Hg (Table 4) The same effects can also be observed by looking at the k/x values. A similar effect is seen for a Δp = 9 mm Hg (second vs fifth data set).

Kamper and Fennema (1984b) reported similar results for their fatty acid film; the permeability value increased from 0.69 g·mil/day·m²·mm Hg for the 65–33% RH range to 10.1 g·mil/m²·day·mm Hg for the 97–65% RH range at 25°C. The increased value at higher relative humidity may have been due to structural changes resulting from the sorption of moisture by the hydrophilic support of their film. The same explanation probably holds for the phenomenon observed with chocolate. Landmen et al. (1960) observed a tremendous increase of the permeability constant at high relative humidities together with

Table 3—Effective water vapor permeability constants (k_{eff}) and water vapor transmission rate (WVTR) for a dark chocolate film at 20°C as determined by the cup method in two different configurations: (1) Drierite in the cup and saturated salt solution outside, (2) saturated salt solution in the cup and Drierite outside

Configuration	Glossy side	RH (%)	ΔP (mmHg)	Number of determinations	Thickness (mm)	WVTR (g/day · m²)	$\frac{k}{x}$ (g/day · m² · mm Hg)	k_{eff} (g · mil/day · m² · mm Hg)
(1)	random	0-80.8	0-14.13	7[a]	0.612 (0.017)[b]	3.21 (0.30)	0.227 (0.30)	5.56 (0.58)
				5	1.192 (0.017)	2.30 (0.18)	0.162 (0.01)	7.74 (0.64)
(2)	random	80.8-0	14.13-0	3	0.610 (0.007)	0.67 (0.23)	0.047 (0.02)	1.15 (0.38)
				4	1.228 (0.009)	0.32 (0.03)	0.022 (<0.01)	1.09 (0.08)
(1)	inside	0-80.8	0-14.13	4	1.114 (0.023)	3.41 (0.35)	0.241 (0.03)	10.73 (1.09)
(1)	outside	0-80.8	0-14.13	4	1.113 (0.022)	3.25 (0.35)	0.230 (0.01)	10.22 (0.17)
(2)	inside	80.8-0	14.13.0	4	1.020 (0.008)	0.30 (0.03)	0.021 (<0.01)	0.86 (0.09)
(2)	outside	80.8-0	14.13.0	4	1.228 (0.009)	0.38 (0.03)	0.023 (<0.01)	1.09 (0.08)

[a] From two independent experiments
[b] Values in brackets are the standard deviations

Table 4—Reproducibility of the permeability experiments for a dark chocolate film at 20°C

RH (%)	ΔP (mm Hg)	Run	Number of determinations	Thickness (mm)	WVTR (g/day · m²)	$\frac{k}{x}$ (g/day · m² · mm Hg)	k_{eff} (g · mil/day · m² · mm Hg)
0-64.8	0-11.4	1	4	0.611 (0.010)[a]	2.84 (0.18)	0.250 (0.01)	6.12 (0.24)
		2	4	0.604 (0.034)	2.63 (0.47)	0.231 (0.04)	5.58 (0.94)
0-80.8	0-14.13	1	3	0.611 (0.020)	3.06 (0.24)	0.217 (0.02)	5.30 (0.55)
		2	4	0.613 (0.012)	3.31 (0.33)	0.235 (0.02)	5.76 (0.60)
54.4-80.8	9.54-14.13	1	4	0.596 (0.016)	2.09 (0.09)	0.455 (0.03)	10.84 (0.64)
		2	4	0.599 (0.020)	2.15 (0.40)	0.468 (0.08)	11.20 (1.92)

[a] (standard deviation)

Table 5—Effective water vapor permeability constant (k_{eff}) and water vapor transmission rate (WVTR) as a function of the water vapor pressure gradient ('Δp) for a dark chocolate film at 20°C

RH (%)	ΔP (mm Hg)	Thickness (mm)	Number of determinations	WVTR (g/day · m²)	$\frac{k}{x}$ (g/day · m² · mm Hg)	k_{eff} (g · mil/day · m² · mm Hg)
0-33.0	0- 5.79	0.594 (0.011)	3	1.20 (0.12)[a]	0.208 (0.02)	4.94 (0.51)
0-54.4	0- 9.54	0.598 (0.033)	4	1.34 (0.05)	0.141 (0.01)	3.38 (0.13)
0-64.8	0-11.40	0.608 (0.024)	8[b]	2.74 (0.43)	0.241 (0.03)	5.85 (0.70)
0-80.8	0-14.13	0.612 (0.017)	7[b]	3.21 (0.30)	0.277 (0.02)	5.56 (0.58)
33.0-80.8	5.79-14.13	0.590 (0.016)	4	2.35 (0.43)	0.282 (0.06)	6.65 (1.32)
54.4-80.8	9.54-14.13	0.597 (0.017)	8[b]	2.12 (0.27)	0.462 (0.06)	11.02 (1.34)

[a] From two independent experiments
[b] (standard deviations)

a destruction of the film integrity. Their values for k_{eff} ranged from 5.1 g·mil/m²·day·mm Hg for a vapor pressure gradient of 26.3–0 mm Hg at 26.7°C using a milk chocolate-type coating. Film thicknesses were between 1.7 and 1.9 mm.

As shown in Table 6, doubling the film thickness from 0.6 to 1.2 mm at 81% RH and 20°C results in a reduction of the WVTR from 3.2 to 2.3 g/day·m² while the permeability constant increases from 5.6 to 7.7 g·mil/day·m²·mm Hg. However, an increase of the thickness from 0.6 to 0.9 mm reduced the WVTR from 3.2 to 2.0 g/day·m² but did not significantly change the k_{eff} value as it should not. Landman et al. (1960) also observed the same phenomenon with cocoa butter for thicknesses ranging between 1.6 and 2.9 mm at 100% RH and 26.7°C but in their case the k_{eff} values increased. They were 11.5 for $\times$ = 1.6 mm, 15.4 for $\times$ = 2.1 mm and 27.7 for $\times$ = 2.9 mm. An increase of the WVTR was also observed by Landman et al. (1960) when the thickness of the cocoa butter film was increased. This may indicate a change in the structure of the cocoa butter film under these conditions as its thickness increases. As seen in Table 6, doubling the chocolate thickness caused a 50% increase in k_{eff}. According to Fick's law of diffusion in a film, the permeability constant k should be independent of the film thickness. A positive deviation from ideality with increasing thickness (k_{eff} increases as shown) indicates that the film material has an affinity for moisture (accelerated permeation) that is not taken into account in Fick's and Henry's laws (Swarbrick and Amann, 1968). This can be attributed to the hygroscopic solids (sugar) of the chocolate but remains apparently limited, at the relative humidity level used (81% RH) which seems to indicate that the transport of the moisture takes place mostly in the hydrophobic fat phase.

A common problem encountered in studying the temperature effect on permeability is that it is not possible practically to maintain a constant vapor pressure differential at different temperatures when using saturated salt solutions. Glycerol or sulfuric acid solutions of variable concentrations would be more

Table 6—Effective water vapor permeability constant (k_{eff}) and water vapor transmission rate (WVTR) of a dark chocolate film at 20°C for three different thicknesses

RH (%)	ΔP (mm Hg)	Number of determinations	Thickness (mm)	WVTR (g/day · m²)	$\frac{k}{x}$ (g/day · m² · mm Hg)	k_{eff} (g · mil/day · m² · mm Hg)
0-80.8	0-14.13	7[a]	0.612 (0.017)[b]	3.21 (0.30)	0.277 (0.03)	5.56 (0.58)
		3	0.926 (0.015)	2.01 (0.26)	0.143 (0.02)	5.28 (0.74)
		5	1.192 (0.017)	2.30 (0.18)	0.162 (0.01)	7.74 (0.64)

[a] From two independent experiments
[b] (standard deviations)

Table 7—Effective water vapor permeability constant (k_{eff}) and water vapor transmission rate (WVTR) for a dark chocolate film at three temperatures

RH (%)	ΔP (mm Hg)	Number of determinations	Thickness (mm)	Temperature (°C)	WVTR (g/day · m²)	$\frac{k}{x}$ (g/day · m² · mm Hg)	k_{eff} (g/ · mil/day · m² · mm Hg)
0-82.1	0- 7.56	3	0.584 (0.008)[a]	10	3.34 (0.58)	0.441 (0.07)	10.30 (1.74)
0-80.6	0-14.13	7[b]	0.612 (0.017)	20	3.21 (0.30)	0.227 (0.024)	5.56 (0.58)
0-80.4	0-20.27	3	0.593 (0.019)	26	10.38 (1.77)	0.512 (0.09)	12.14 (2.12)

[a] (standard deviations)
[b] From two independent experiments

appropriate but their vapors would be adsorbed by the chocolate. The films of three thicknesses were tested at 10°, 20°, and 26°C and about 80% RH differential with multiple replicates. The results are summarized in Table 7. The WVTR and k_{eff} values increased markedly with increased temperature from 20°C to 26°C. However, there is not much difference between 10°C and 20°C. In fact the permeability increased slightly at 10°C compared to 20°C. Labuza and Contreras-Medellin (1981) found a similar increase of the permeability of plastic films below −5°C, attributing it to a glass transition. Clearly, the temperature effect in the range of temperature studied did not obey an Arrhenius type law; the WVTR remained constant when the temperature was reduced from 20°C to 10°C while the permeability constant increased above 20°C. The solid fat index (SFI) of cocoa butter decreases from about 90% to about 80% when increasing temperature from 20°C to 26°C but changes only slightly between 10°C and 20°C (Biquet, 1987). Other changes in the crystalline structure of the cocoa butter phase might also occur. At low temperatures such as 10°C and under the conditions of the cup method, microscopic cracks in a thin film can appear to increase the permeability constant (Kamper and Fennema, 1984b). This may not have been completely prevented by the pre-equilibration of the film at the temperature of the experiment since the sealing process requires a partial melting of the film on the edges. Determination at higher temperatures was not possible for such a small thickness. An increase of the water vapor permeability constants of fatty film materials at low temperatures was also reported by Kamper and Fennema (1984b) and Landman et al. (1960). Landman et al. (1960) reported an increase of the k values for cocoa butter from 5.1 g·mil/day·m²·mm Hg at 26.7°C to 12.2 g·mil/day·m²·mm Hg at 3°C at about 65% RH and for a film thickness of 1.6 mm (k/x increases from 0.08 to 0.19). From those results, it can be concluded that reducing the temperature may not have a favorable effect with respect to the moisture barrier properties of a chocolate coating.

Table 8 compares the moisture barrier properties of chocolate to other literature data. A chocolate film 0.6 mm thick is a much more efficient barrier in terms of WVTR than a typical low density polyethylene film (0.025 mm thick = 1 mil). On a k/x basis, the 0.6 mm (24 mil) chocolate film is about 2 to 8.5 times less permeable than the 1 mil polyethylene film depending on the configuration used with the cup method. It is better or at least as good as the film of Kamper and Fennema (1984b).

Moisture transfer experiments

The monocomponent system studied simulated a chocolate-coated wafer or a dried food enrobed in an edible packaging film. No previous study has been published attempting to predict the moisture gain in a system using an edible film. However, most of the theoretical requirements of the basic mathematical model for the prediction of moisture gain and loss in packaged food products, using nonedible packaging materials can still be considered true for this model system (Karel, 1967; Labuza et al., 1972; Mizrahi et al., 1970a,; Mizrahi and Karel, 1977a, 1978). More specifically, the respective diffusion coefficients of the model gels ($1.0 - 2.1 \times 10^{-9}$/m² sec; Biquet and Labuza 1987) and of chocolate ($0.8 - 1.3 \times 10^{-13}$ m²/sec) as well as the permeability constant of a chocolate film compared to those of some packaging materials (Table 8) support the hypothesis that the chocolate film can be considered as the controlling resistance to moisture transfer while the model gel alone would equilibrate very quickly with the internal atmosphere. Figure 3 shows that a gel at an initial a_w of 0.3 (7.5% moisture) will equilibrate to 81% RH in less than 12 hrs with no chocolate film. Thus, its use should uphold the assumption that the film is the major resistance. Assuming A, k, x, p_{out} and the temperature to be constant and assuming a "pseudo steady state" (i.e., a succession of small time intervals where steady state is reached), and using a linear equation for modeling the model gel isotherm (Labuza, 1968), one can write for moisture gain (Labuza, 1972):

$$\ln \frac{m_e - m_i}{m_e - m} = \frac{k}{x}\frac{A}{W}\frac{p_o}{b}t \qquad (7)$$

which can be solved for the moisture content of the food (i.e., the model gel) at any time.

Figure 4 shows the moisture gain of the model gel and of the chocolate film as a function of time for the monocomponent system while Fig. 5 represents the same data expressed in terms of water activity using the respective adsorption isotherms of chocolate and of the model gels. Comparison of the control and "coated" systems confirms the good moisture barrier properties of chocolate since it took more than 35-40 days of storage at 20°C and 81% RH for the "coated" gel to reach an $a_w = 0.45$ and 70 days to reach an $a_w = 0.55$, while it took only 1 and 2 hrs, respectively, if the chocolate film was removed. Figure 4 also indicates that the chocolate film adsorbs moisture up to about 1.55 g H_20/100g dry solids at which point a pseudo-equilibrium seems established for it.

Prediction of the moisture gain in the above systems was done by programming the solution of equation (8) on a programmable pocket calculator (Hewlett-Packard 15C). One problem is that there is no objective criteria for linearizing the model gel isotherm as is required in the mathematical model. The theoretical requirements are that m_e and m_i are calculated from the linear isotherm. It is obvious that the linear isotherm should at least pass through the actual initial point. However, it is also common sense to discard the actual equilibrium point as the second point for linearization since at that point, the critical quality loss would have occurred previously. A more meaningful choice is to use the critical point for quality loss. The loss of texture of the internal component at an a_w of 0.6 was chosen as the critical quality parameter. The linearization was done by hand on the model gel adsorption isotherm (Biquet, 1987), between the two points, respectively, at $a_w = 0.33$ and $a_w = 0.60$. The slope and intercept are in Table 9.

Calculations were carried out with each permeability constant as determined by the cup method in the two configurations (from Table 3). The other characteristics of the model system necessary for the modeling, such as dry weight of the gels, surface, area of transfer and film thickness, are given in Table 9.

The predicted moisture gain for the model gel is shown as the solid line in Fig. 4. When the permeability constant, as determined with the salt solution outside the cup (k_o), was used, the moisture gain was over predicted. These results tend

Table 8—Relative permeability constants and WVTRs for various edible and nonedible film.

Film		Temperature (°C)	ΔP (mm Hg)	Thickness (mm)	WVTR (g/day · m²)	$\frac{k}{x}$ (g/day · m² · mm Hg)	k_{eff} (g/ · mil/day · m² · mm Hg)
Chocolate							
This study	(0)[f]	20	0-14.1	0.610	0.67	0.048	1.2
	(1)	20	14.1-0	0.612	3.21	0.228	5.6
Landman et al.[a]	(2)	27	11.5-0	1.58	0.92	0.808	5.1
	(3)	27	19.8- 5.9	1.77	1.00	0.072	5.1
Fatty acid C_{16} - C_{18}[b]		25	20.2-0	0.04	3.05	0.151	0.2
Casein-Gelatin[c]		30	28.9-18.5	0.25	295.4	28.4	284
Cellulose acetate[d]		37.7	44.3-0	0.025	200	4.5	4.5
Polyethylene[e] (Low density)		37.7	44.3-0	0.025 (1 mil)	18.0	0.406	0.4
Foil laminate[d]		37.7	44.3-0	—	0.1	0.002	—

[a] Landman et al. (1960)
[b] Kamper and Fennema (1984b)
[c] Hocquard (1986)
[d] Labuza (1987)
[e] Myers et al. (1961)
[f] (0) Tempered dark chocolate (salt solution in the cup);
(1) Tempered dark chocolate (desiccant in the cup);
(2) Tempered cocoa butter;
(3) Tempered sweet milk chocolate coating.

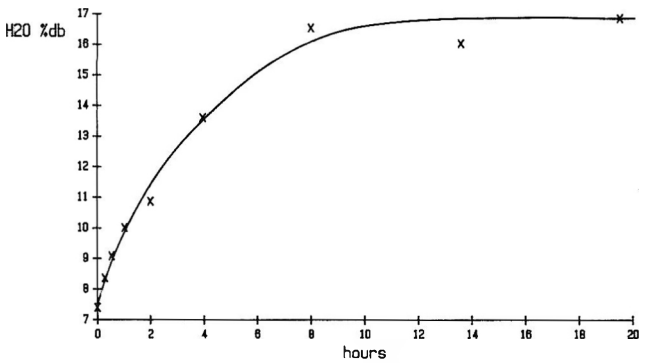

Fig. 3—Moisture content of the model gel in the monocomponent system test as a function of time for the control experiment (no chocolate film) (20°C, 81% RH).

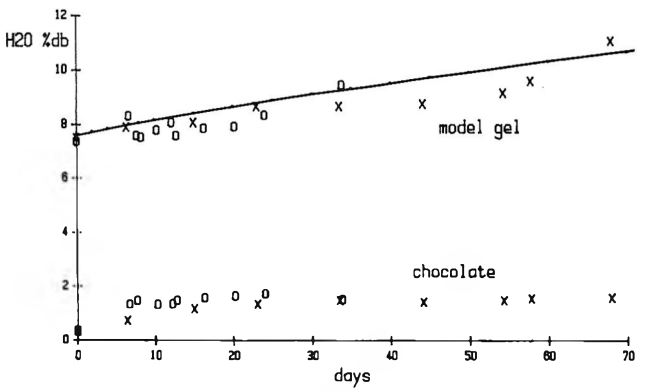

Fig. 4—Predicted (continuous line) versus actual moisture content for the model gel in the monocomponent system using the chocolate permeability constant as determined with the salt solution in the cup (20°C, 81% RH, film thickness = 0.604 mm). The Xs and Os refer to two independent tests.

to support the hypothesis that, at least in the specific case of chocolate, permeability constants as determined by the cup method with the salt inside the cup (k_i) are more representative of the actual phenomenon and are thus of greater practical value.

The bicomponent system was composed of a moist (high a_w) and a dry (low a_w) component (model gel) separated by the chocolate film. The system was assumed to be isolated (no exchange with the surroundings) and the film between the two

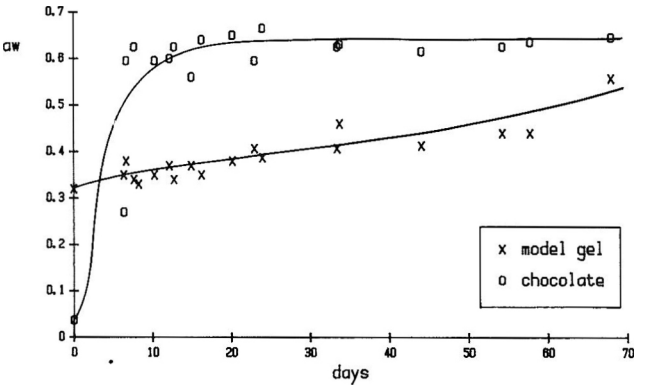

Fig. 5—Apparent water activity of the chocolate film and the model gel as a function of time for the monocomponent system (20°C, 81% RH, film thickness = 0.604 mm).

components was considered to be the controlling resistance to moisture transfer. The two components were assumed to equilibrate instantaneously with any transferred moisture, thereby, determining the instantaneous vapor pressure on each side of the film. The other assumptions were similar to those presented earlier for the monocomponent model, i.e., constant permeability of the chocolate film and constant temperature.

The weight gain for the dry component in a system such as this is discussed elsewhere (Taoukis et al., 1987). The equation is:

$$\frac{dM_l}{dt} = \frac{k}{x} A (p_h - p_l) \quad (9)$$

where the subscript (h) refers to the higher a_w system and subscript (l) refers to the low a_w system and subscript (e) refers to the value at equilibrium for all terms in Eq. (9) through (15). A mass balance can also be done on the moisture and the dry solids components in the system.

$$M_h + M_l = M_T = \text{total moisture} \quad (10)$$

$$W_h + W_l = W_T = \text{total weight of solids} \quad (11)$$

Eq. (10) can be rearranged so that:

$$m_T = m_h \cdot W_h + m_l \cdot W_l = m_{ih} \cdot W_h + m_{il} \cdot W_l \quad (12)$$

where m_{il} is the initial moisture of the low moisture component and m_{ih} is the initial moisture of the high moisture component.

Table 9—Characteristics of the model systems used for the moisture transfer experiments

		Average dry weight of gel (g)		Average effective surface area (cm^2)	Average thickness of the chocolate film (mm)	Linear isotherm	
		High a_w gel	Low a_w gel			Slope	Intercept
Monocomponent system	Run 1	—	1.11	15.11 (0.57)[a]	0.609 (0.014)	16.0	2.3
	Run 2	—	1.10	15.45 (0.91)	0.599 (0.018)	16.0	2.3
Bicomponent system	Run 1	1.15	1.15	14.95 (0.50)	0.602 (0.019)	23.8[b]	4.0[b]
	Run 2	1.08	1.06	14.96 (0.39)	0.600 (0.014)	23.8[b]	4.0[b]

[a] (standard deviation)

[b] Values are for high a_w component. The values for the low a_w system are the same as for the monocomponent study. Units are g N_2O/100g solids.

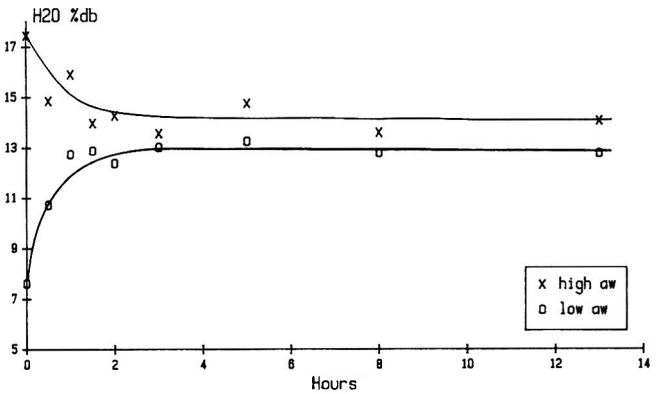

Fig. 6—Moisture content of the model gels as a function of time for the bicomponent system for the control experiment (20°C, no chocolate film).

Assuming linear isotherms for the two components (model gels) in the system and integrating between the limits, the solution for the moisture content of the dry component any time t is given by:

$$m_l = \left(\left[m_{il}\ \text{B-D}\right]/\left[\text{B}\exp\left[\frac{k}{x}\frac{A}{W_e}p_o t\right]\right]\right) + \frac{D}{B} \quad (13)$$

with:

$$B = \left(\frac{W_e}{W_h\, b_h} + \frac{1}{b_l}\right) \quad (14)$$

$$D = \left(\frac{M_T/W_h - c_h}{b_h} + \frac{c_l}{b_l}\right) \quad (15)$$

and m_h is calculated through Eq. (12).

When no chocolate is present, as shown in Fig. 6, the two gels quickly equilibrate within 2 hr. Figure 7 shows the moisture content of the dry and moist component and of the chocolate film as a function of time for duplicate tests. Figure 8 illustrates the transfer in terms of water activity. The adsorption isotherm was used for the dry component and the desorption isotherm (preequilibrium at $a_w = 0.85$) was used for the moist component since they showed a marked hysteresis (Biquet and Labuza, 1987). As with the monocomponent experiment, comparison of the control and of the "coated" experiment points out the good barrier properties of chocolate. It took about 90 days for the dry component to reach an $a_w = 0.45$ versus less than 2 hr without the chocolate film. As seen, the chocolate film adsorbs moisture up to about 1.4 g/100g solids. One interesting point is that the "water activities" of the moist gel and of the chocolate film rapidly reach values of the same order of magnitude.

Calculations of moisture gain were done using the derived equations, and linearization of the gel adsorption isotherm as before. Linearization of the desorption isotherm (preequilibrium at $a_w = 0.85$) of the model gels was done in the range $a_w = 0.83 - 0.63$ as used before. The values are in Table 9.

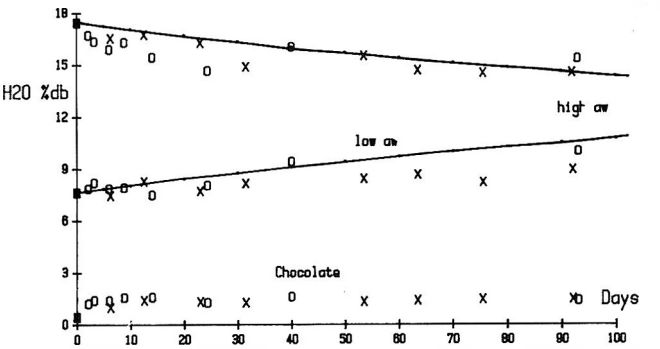

Fig. 7—Predicted (continuous line) versus actual moisture contents for the model gels in the bicomponent system using the chocolate permeability constant as determined with the salt solution outside the cup (20°C, film thickness = 0.601 mm). The upper line is for the high a_w component, the middle line is the low a_w component. The Xs and Os refer to two separate experiments.

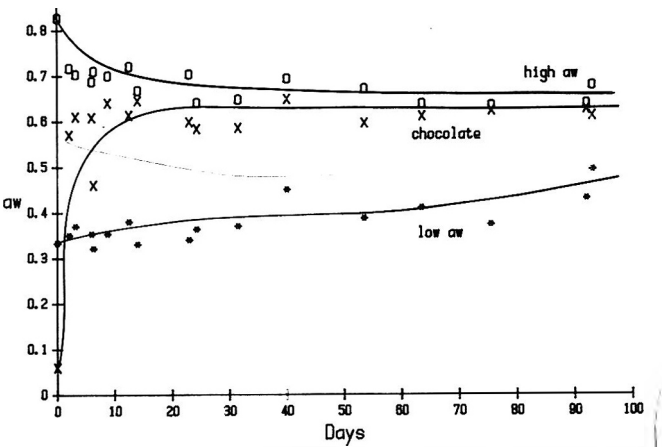

Fig. 8—Apparent water activity of the model gels and the chocolate film as a function of time for the bicomponent system (20°C, film thickness = 0.601 mm). The upper line is the predicted apparent a_w for the high a_w component, the lower line is for the low a_w component and the middle line is the a_w for the chocolate.

Other parameters of the system such as dry weights are also given Table 9. As with the monocomponent system, the modeling was done with both k_o (salt solution outside the cup) and k_i (salt solution inside the cup).

The predicted moisture change for the dry and moist gels is shown in Fig. 7 and 9. Interestingly, both permeability values (k_o and k_i) give a relatively good prediction for the moisture gain for the dry component which is the critical one from a quality standpoint, where k_i (salt solution in the cup) underpredicts the moisture gain while k_o (salt solution outside the cup) slightly overpredicts it. These results obtained in "ideal" conditions substantiate the potential of chocolate coatings as a

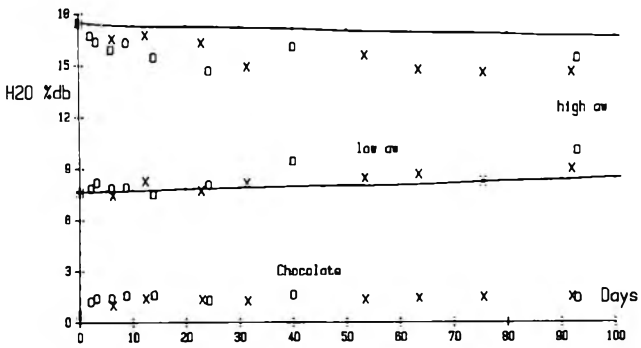

Fig. 9—Predicted (continuous line) versus actual moisture contents for the model gels in the bicomponent system using the chocolate permeability constant as determined with the salt solution in the cup (20°C, film thickness = 0.601 mm). The upper line is for the high a_w component, the middle line is the low a_w component. The Xs and Os refer to two separate experiments.

moisture barrier in heterogeneous mixed moisture foods. It also points out the complexity of the mechanisms involved in moisture transport through chocolate. Since chocolate interacts with moisture and the mechanisms of transfer through it appear to be complex, the results presented in this study cannot be considered a sufficient proof of the theoretical basis for the bicomponent model but the model seems to be useful.

NOMENCLATURE

A	= surface area of the film
a_w	= water activity
b	= slope of the linear isotherm
B,D	= constants for moisture transfer equation
c	= intercept of the linear isotherm at $a_w = 0$
C,K	= adsorption constants for GAB equation
k	= water vapor permeability of the film
k_{eff}	= effective water vapor permeability of the film
L	= chocolate slab thickness
m	= moisture content (g/g solids)
M	= water weight (g)
m_e	= equilibrium moisture content (g/g solids)
m_i	= initial moisture content (g/g solids)
m_o	= monolayer moisture content
p	= water vapor pressure
p_{in}	= vapor pressure inside
P_{out}	= vapor pressure outside
p_o	= water vapor pressure of pure liquid water
slope	= slope of weight gain (or loss) vs time curve
t	= time
W	= dry weight of solids (g)
WVTR	= water vapor transmission rate (gH_20/day m^2)
w	= weight of system
x	= thickness of the film

Subscripts

e	= equilibrium with external atmosphere
h	= high water activity component
i	= initial time (zero)
l	= low water activity component
T	= total (overall system)

Greek symbols

Δ	= difference
Γ	= unaccomplished moisture content

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Effect of Cryoprotectants on Frozen Whitefish Fillets

MARK KRIVCHENIA and OWEN FENNEMA

ABSTRACT

Whitefish fillets (*Coregonus cupleaformis*) were treated with sodium tripolyphosphate (STP), monosodium glutamate (MSG) or a high-pH buffer by high pressure injection, then frozen and stored at −12°C. Control samples included untreated fillets stored at −12°C and −60°C, and STP-dipped fillets (−12°C). Samples treated with STP or high-pH buffer showed better textural properties (reduced centrifugal drip, cooked drip and firmness) than untreated control samples. High pressure injection of STP was not significantly better and sometimes less effective than surface application. Sensory analysis at 18 wk revealed that STP-treated samples (−12°C) were significantly preferred over untreated control samples (−12°C) but not over −60°C control samples.

INTRODUCTION

FREEZING is generally the best method for achieving long-term preservation of fish. However, after prolonged storage of fishery products at −20°C or above, significant undesirable sensory changes take place. In high fat species, shelf life is limited by oxidative changes in the lipids and pigments, which cause off-flavors and discoloration of the flesh. In lean fish species, quality loss often involves alteration of proteins accompanied by loss of water-holding capacity and toughening of the muscle (Sikorski et al., 1976).

On a practical level, it has been found that cryoprotectants reduce the rate of deteriorative changes in fish and fishery products during frozen storage. Fish fillets are commonly dipped in sodium tripolyphosphate (STP) or sodium hexametaphosphate to reduce drip and increase tenderness (Ellinger, 1972; Mahon et al., 1971). The addition of sucrose, sorbitol, polyphosphates and/or sodium glutamate to surimi has been shown to maintain protein functionality during long periods of frozen storage (Suzuki, 1981). A problem encountered when applying chemicals to intact fish is that only the surface of the tissue can be treated.

Although some studies have been conducted on changes which occur in freshwater fish during frozen storage, these have generally focused on off-flavor development resulting from lipid oxidation, and relatively little attention has been given to textural changes in the presence or absence of cryoprotectants.

In this study, whitefish (*Coregonus cupleaformis*), a medium fat (3–6%) freshwater fish of significant commercial importance was chosen for study, and major attention was given to changes in textural properties during frozen storage. Awad et al. (1969) studied the deterioration of untreated whitefish muscle stored for 16 wk at −10°C. They found that protein extractability and water-holding capacity of the muscle decreased with time and that these changes were accompanied by increases in free fatty acid concentration, sensory toughness and oxidative rancidity. Manohar et al. (1973) dipped whitefish in a solution containing STP and NaCl (8% and 2% w/v, respectively), stored these samples for 2 wk at −20°C, and found that this treatment significantly reduced free drip. Josephson et al. (1985) studied the effect of handling and packaging on the quality of frozen whitefish. They recommended vacuum packaging and glazing as a means to protect against oxidation and they also noted that this species became firmer and less flakey during frozen storage.

The purposes of this research were to (1) determine if the effectiveness of cryoprotectants used to inhibit freeze toughening of intact whitefish fillets was dependent on the method of application (injection versus dipping) and (2) determine which of several chemical treatments were most effective in stabilizing textural and other properties of whitefish during frozen storage.

Author Fennema is with the Dept. of Food Science, Univ. of Wisconsin, Madison, WI 53706. Author Krivchenia's current address is: General Mills, Inc.; 9000 Plymouth Ave. North, Minneapolis, MN 55427.

MATERIALS & METHODS

Fish

Fresh whitefish (*Coregonus cupleaformis*), ranging in length from 43–61 cm, were obtained from Lake Michigan in December, 1985. The fish were caught in live entrapment nets and iced immediately upon capture. They were gutted, washed, iced and shipped to Madison on the same day of capture. The next day, the round fish were washed, filleted and skinned. The belly flap and all visible fat were removed from the fillet. Two-pound lots of washed fillets, selected at random, were put in Ziplock polyethylene bags (Dow Chemical, 16.5 × 15 cm × 1.1 mils) and stored on ice until treated.

Preparation of chemical solutions

Sodium glutamate (MSG; Sigma grade, G 1376) was dissolved in distilled water to obtain a 25% w/v solution, pH 8.0. Sodium tripolyphosphate (STP; Sigma, 90–95%) was dissolved in distilled water to obtain an 11.8% w/v solution and the pH was adjusted to 8.0 with concentrated phosphoric acid. K_2HPO_4 or $K_2HPO_4 - 3H_2O$, potassium phosphate dibasic (high-pH buffer; Mallinckrodt) was dissolved in distilled water to obtain a 1.8M solution and the pH was adjusted to 8.0 with concentrated phosphoric acid.

Treatment of samples

The treatments applied are summarized in Table 1. Fish samples to be injected with chemicals were promptly shipped on ice to the Utilization Research Division, Northwest and Alaska Fisheries Center, U.S. Department of Commerce in Seattle, WA. A pilot-plant-sized, high-pressure injection machine (Intermediate Technology Manufacturing, Inc.) was used to inject chemicals into the samples (Fig. 1). Approximately 900g (2 lb) whitefish fillets were placed in a 21 × 10 × 5 cm box with an aluminum foil interior (Dixie/Marathon Products,

Table 1—List of treatments applied to whitefish

Treatment designation[a]	Description (Abbreviation)
TR-1	−60°C control (C-60)
TR-2	−12°C control, vacuum packaged[b] (C-12V)
TR-3	−12°C control (C-12)
TR-4	−12°C, dipped sodium tripolyphosphate[c] (D-STP)
TR-5	−12°C, injected sodium tripolyphosphate[d] (I-STP)
TR-6	−12°C, injected monosodium glutamate[e] (I-MSG)
TR-9	−12°C, injected high-pH buffer[f] (I-High pH)

[a] Treatments 7 and 8 involved antioxidants, but the concentrations utilized were too low (inadvertently) to achieve meaningful results.
[b] FreshTuff bags (American Can Co.; coextruded, 1.2 mil type 6 Nylon/2.8 mil Surlyn 1625).
[c] STP (11.8% w/v), 20°C, 2-3 min
[d] STP (11.8% w/v)
[e] MSG (25% w/v)
[f] Potassium phosphate buffer solution (1.8M), pH 8.0

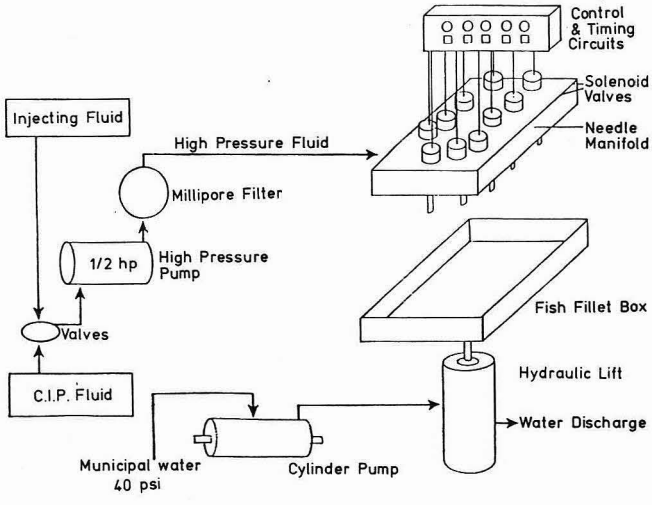

Fig. 1—High-pressure injection machine used to apply chemicals to fish fillets.

Dine/out #906) and injected (lid open) with STP, MSG or the high-pH buffer solution. Each lot (900g) was penetrated by ten equally spaced needles, each having an inner diameter of 0.1 mm and constructed to have four exit ports arranged at 90° intervals around the tip of the needle and located 1.3 cm from the tip. The amount of chemical injected into each sample was regulated by varying the number of pulses of injected fluid, the pressure of each pulse (4,000 psi), and the speed of the hydraulic lift. The concentration of chemicals added to the fish tissue was usually calculated from the weight gain of the fish and the concentration of chemicals in the solutions used.

Injected samples were frozen in a −20°C circulating air freezer and were then placed at −12°C. Samples that were not injected were similarly packaged and frozen and then stored at either −60°C or −12°C. Control STP samples (TR-4; Table 1) were dipped in an 11.8% w/v STP solution for 2-3 min to obtain a mean tissue concentration of STP that was approximately equal to that of the injected samples. Some samples were packaged in FreshTuff bags (American Can Co.; coextruded, 1.2 mil, type 6 nylon, 2.8 mil Surlyn 1625) and vacuumized using a multivac vacuum packager (Sepp Haggenmuller, KG).

Approximately 225g fish muscle for each replicate of each treatment were tested at 4, 10, 16, and 28 wk of frozen storage.

Preparation of samples for testing

Each 225g sample was thawed in air at 4°C, allowed to stand at room temperature for 1 1/2 h and then cut into approximately 1 cm cubes. A 40–50g quantity was passed through an Oster grinder (Oster Kitchen Center) equipped with a perforated plate (4 mm diam holes). Minced samples were placed in glass sample jars and stored on ice until utilized (usually within 48 hr). The remainder of the cubed samples were stored on ice and were used later for cooked drip and texture measurements.

Methods of analysis

Sample pH was determined by the method of Connell and Howgate (1968). The method of Jauregui et al. (1981) was employed to measure centrifugal drip (CD). Samples were centrifuged for 10 min at 2,000 rpm (495 × *g*) in a Sorvall RC-5 centrifuge (DuPont). CD was calculated as % moisture lost from the original sample.

Cooked drip values were obtained by placing an accurately weighed amount (100–200g) of 1 cm cubes of raw fish muscle in double Ziplock bags and immersing them in a 70–80°C circulating water bath until the internal temperature of the fish reached 65°C (20–30 min). The fish and juice were poured into a funnel and the juice was allowed to drain into a beaker for 5 min. "Percent cooked drip" (CKD) was the weight of drained fluid divided by the original sample weight, with the resulting weight multiplied by 100.

Protein extractability was determined according to a modified method of Dyer (1951). Fish muscle was extracted with an aqueous solution containing 0.6M KCl (Columbus Chemical Industries, Inc.) and 50 mM phosphate buffer adjusted to pH 7.0 with 0.1M NaOH (hereafter referred to as the "extracting solution").

Two grams minced fish muscle and 30 mL extracting solution were blended at high speed for 15 sec in a jacketed Waring blender (jacket fluid −5°C). The blended fish muscle was transferred to a 50 mL polyurethane centrifuge tube along with 10 mL extracting solution that was used to rinse the blending jar. This mixture was centrifuged for 30 min at 27,750 × *g* in a Sorvall RC-5 centrifuge operated at 0–5°C. Two 1-mL aliquots of the supernatant fluid were removed from each centrifuge tube and each was mixed with 9 mL extracting solution (1:10 dilution). An 0.1-mL aliquot of diluted solution was mixed with 5 mL Bio-Rad reagent (Bio-Rad Laboratories) and absorbance at 595 nm was determined within 30 min. Protein concentration was determined using crystallized, lyophilized bovine serum albumin (Sigma, 99% albumin) as a protein standard. Results are reported as μg protein per 0.1 mL extracting solution (undiluted).

Objective texture measurements were accomplished with an Instron texture testing instrument, Model 1132, equipped with a Kramer shear-compression cell (CS-1). Chart and crosshead speed were 200 mm/min. For each texture test, 50g diced, drained and cooked fish (cooking method described above) were placed in the Kramer cell in a random fashion and sheared once. Samples were tested at room temperature within 2-4 hr after cooking and were covered during storage to avoid moisture loss. Results were reported as pounds-force at peak height/50g sample. These values are referred to as "texture" or "firmness" in the subsequent discussions.

Sensory properties of fish samples were determined in the University of Wisconsin-Madison Sensory Analysis Laboratory. Fish samples were cooked as described above (for sensory tests the intact fillet was cooked) and were divided into approximately 20-30 g portions and placed on paper plates coded with three-digit random numbers. All samples were served warm, within 5 min of cooking. Fish samples were evaluated using semi-structured, quantitative descriptive analysis (QDA) (Stone et al., 1975) including scales for selected descriptive attributes and overall preference. The overall preference scale was included to provide an indication of the acceptability of the product to the panel. Panel members (29) were semi-experienced in the sensory evaluation of food products, so their judgments with regard to overall acceptability of the products should not be construed to accurately reflect acceptability by the general public. Evaluations were conducted in a room equipped with individual booths and indoor fluorescent lighting (about 78 ft-c).

QDA and hedonic scales were coded on a seven-point basis for each attribute, with not flaky to very flaky, not firm to very firm, very dry to very moist, not chewy to very chewy, not fibrous to very fibrous, off-flavor absent to off-flavor pronounced and dislike extremely to like extremely, being 1 and 7, respectively. Coded values were analyzed by one-way analysis of variance suitable for a completely random design. An individual analysis was performed for each quality attribute, and least significant differences (LSD) were determined for all pairs of samples (Steel and Torrie, 1960).

Experimental design

This experiment was designed as a split-plot analysis of variance with treatments as the main effect and time as the secondary effect. Each of the nine treatments was tested in triplicate. An experimental unit was defined as a 900g (2 lb) block of fish either injected or not injected with a specific chemical treatment. The four subunits of the experimental unit were sampled over time. The model for this design is:

$$X(ijk) = \mu + TRT(i) + REP(j) + TRT*REP(ij) + TIME(k) + TRT*TIME(ik) + E(ijk)$$

where: x is response variable for the ith treatment and the jth replicate at the kth time; μ is overall mean; TRT is treatment effect; REP is replicates; Time is time effects; E is subplot error.

A software program from Statistical Institute, Inc. (SAS) was used to determine the main and sub-effects of the data (Steele and Torrie, 1960). LSD pair-wise comparison tests were used on planned treatment comparisons. Scheffe's multiple comparison test was used to compare all the treatment and time effects (Neter and Wasserman, 1974).

RESULTS & DISCUSSION

SIGNIFICANT treatment-time interactions occurred with all response variables except cooked drip. Therefore, it was nec-

Table 2—Pearson correlation coefficients for whitefish

Property	Cooked drip	Texture	pH	Protein extractability	TBARS[a]
Centrifugal drip	0.60 S[b]	0.65 S	−0.13 NS	0.13 NS	0.02 NS
Cooked drip		0.66 S	−0.24 NS	−0.06 NS	0.10 NS
Texture			−0.21 NS	−0.31 S	0.39 S
pH				0.05 NS	−0.01 NS
Protein extractability					−0.55 S

[a] 2-thiobarbituric acid reactive substances
[b] Coefficients are significant (S) at the $P \leq 0.05$ level.

essary to make a series of pair-wise comparisons of treatment means at a given time or time means for a given treatment.

Centrifugal drip (CD), cooked drip (CKD), texture (TE)

Treatment effects: Strong correlations existed among CD, CKD, and TE (Pearson's correlation coefficient > 0.60; Table 2), so these attributes are grouped together for discussion. As is evident from Fig. 2a, b, and c, samples treated with the high-pH buffer (TR-9) and those treated with STP (TR-4,5) usually exhibited significantly ($P \leq 0.05$) less drip and were less firm than the untreated control samples (TR-3; Fig. 2a,b,c). However, it is not clear from this study whether the desirable effects were caused by elevation of pH or some specific attribute of phosphates. This finding is in general agreement with the results of several other studies (Boyd and Southcott, 1965; MacCallum et al., 1964a,b; Mahon and Schneider, 1964; Manohar et al., 1973).

Samples dipped in STP (TR-4) exhibited significantly less CD, averaged over all storage periods, than samples injected with STP (TR-5).These two treatments did not result in significantly different values for CKD and firmness. Samples dipped in STP contained a mean concentration of 0.4% STP and a pH of 6.7 after 4 wk of storage, whereas injected samples contained a mean STP concentration of 0.31% and a pH of 6.5 (Table 3). These differences in mean concentration of STP and pH can reasonably account for the ability of the dipping procedure to lessen CD to a greater extent than the injection procedure. Furthermore, the fact that the pH of samples injected with STP was the same as that of the control samples suggests that the injection process, as administered, was not a very effective method for incorporating STP. However, at all time periods after 4 wk, injection of STP (TR-5) significantly reduced CD and firmness as compared to corresponding values of the untreated control samples (TR-3; Fig 2a,c). It is likely that the mean concentration of STP in the tissue as achieved by injection could be greatly increased by altering the injection conditions.

In general, the −60°C control sample (TR-1) exhibited less CD (but not less CKD) and was less firm than the −12°C control sample (TR-3).

Samples injected with MSG (TR-6; data not shown) had values for CD, CKD and firmness that were comparable to those of the untreated control samples (TR-3). This result appears to contradict the findings of Noguchi and Matsumoto (1970). However, in their work, MSG at a concentration of 3.74% was used in minced fish tissue, whereas in this research MSG was used at a concentration of 0.41% in intact fillets. This lower concentration was used because it is more typical of acceptable use levels in food products (Motono, 1982).

Time effects: CD values at 28 wk storage were significantly larger than the 4-wk values only for the untreated, vacuum-packaged control samples stored at −12°C (TR-2; results not shown) and the untreated control samples (TR-3). For these

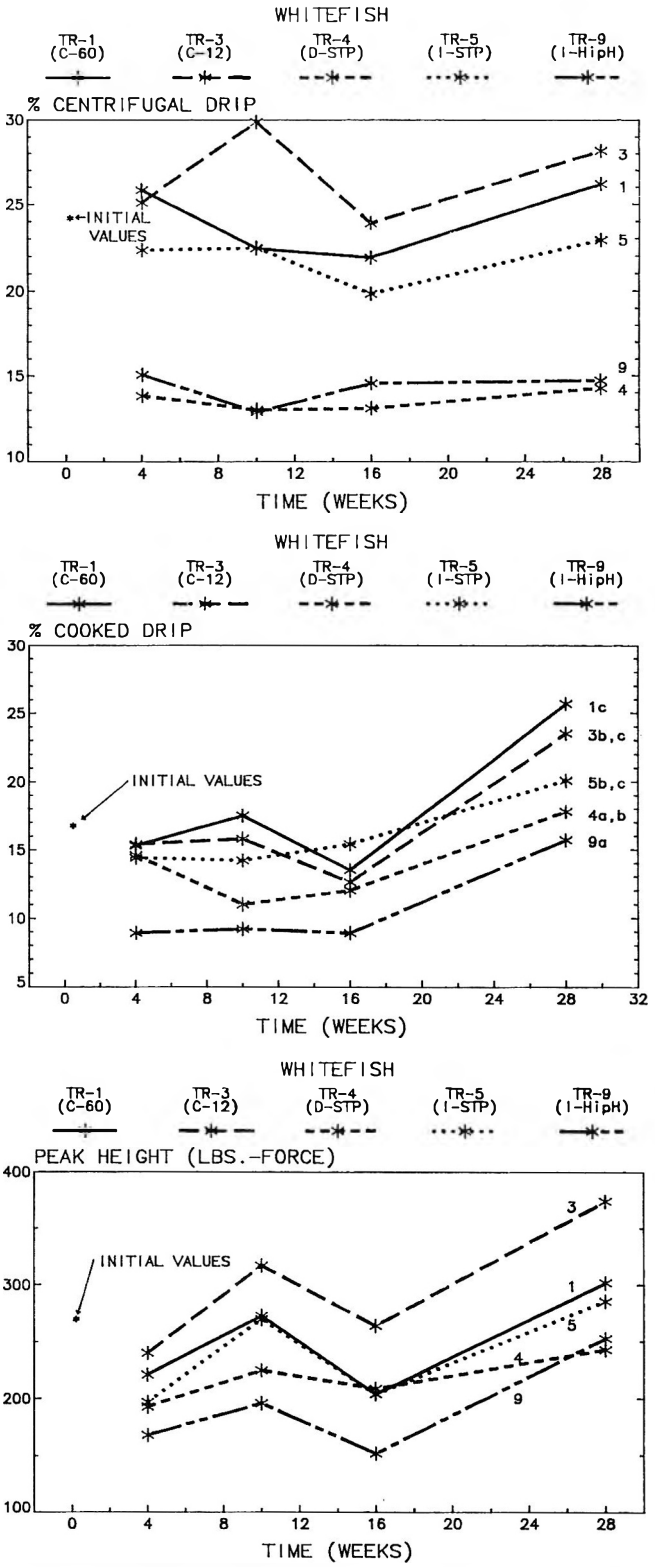

Fig. 2—Effect of various treatments on three attributes of frozen-thawed whitefish fillets: (A) % centrifugal drip (CD), (B) % cooked drip (CKD); Lines followed by different letters indicate that the treatments are significantly different at the $P \leq 0.05$ level. (C) Texture (TE; firmness). Curve 1 represents treatment 1 (−60°C control samples), Curve 3 represents treatment 3 (−12°C control samples), Curve 4 represents treatment 4 (samples dipped in sodium tripolyphosphate and stored at −12°C), and Curve 9 represents treatment 9 (samples injected with a high-pH buffer and stored at −12°C).

samples, CD increased to 29% from 25% during the entire storage period, which was statistically significant, but probably

of no practical importance.

Awad et al. (1969) reported that CD measurements for raw, untreated whitefish, stored at −10°C, increased to 32.5% from 19.0% during the first six wk of frozen storage and leveled off at 34.5% by 16 wk. Thus, in comparable samples from the two studies, a similar pattern of increasing CD was observed. Quantitative differences between the two studies can be accounted for by differences in the CD procedure and differences in the fish.

Averaged over all treatments, CKD values at 28 wk were significantly ($P \leq 0.05$) larger than at any other time periods. However, at 16 wk, CKD values were significantly ($P \leq 0.05$) smaller than those at 4 and 10 wk. This pattern was also observed with the CD data.

Awad et al. (1969) reported that the CKD of untreated, whitefish stored at −10°C increased from 24% to 48% over a 16 wk period. Here, the CKD of untreated whitefish (TR-2 and 3) increased to 23.4% from 16.5% during 28 wk of frozen storage at −12°C. The large difference in CKD values is attributable to the different methods used to measure cooked drip. Awad et al. ground samples, cooked them for 60 min in a 70°C water bath, centrifuged them and then measured drip. In contrast, we measured free drip from 1 cm cubes of fish muscle that had been cooked to an internal temperature of 65°C.

In general, all samples were significantly firmer at 28 wk than at 4 wk. However, a discontinuity occurred during the 10–16 wk interval and an explanation for this occurrence is not obvious. Since this behavior is consistent with several other response variables the changes would appear to be attributable to actual behavioral characteristics of the fish.

If one considers the untreated control samples (TR-1 and 3) during the entire storage period, the mean of these samples increased from about 225 lb-force at 4 wk to about 340 lb-force at 28 wk. Samples treated with STP (TR-4 and 5) exhibited mean force values of about 200 at 4 wk and about 275 at 28 wk. Thus, samples treated with STP were, on the average, about one-third less firm than their untreated counterparts.

pH

Tissue pH declined 0.1-0.2 pH units for all samples between 4 and 28 wk of frozen storage (Table 3). A similar result was obtained by Botta and Richards (1973) with Pacific halibut and chinook salmon. Changes in pH were not significantly correlated with CD, CKD or firmness (Table 2).

Protein extractability

Protein extractability often but not always correlates well with textural changes in frozen fish (Connell and Shewan, 1980). In our study we found that PE decreased significantly ($P \leq 0.05$) during the early stages of frozen storage, exhibiting values of about 500–700g protein/0.1 mL at 4 wk, about 300-400 μg protein/0.1 mL at 10 wk, and remaining relatively constant thereafter (Fig. 3). The −60°C control sample (TR-1) exhibited significantly greater PE values than those of other samples at each time period. However, it is interesting that PE from the −60°C control sample did not remain constant throughout the storage period, but rather followed the same pattern exhibited by other samples. Samson et al. (1985) also observed changes in minced cod during storage for 35 days at −40°C.

Awad et al. (1969) measured the PE of whitefish and observed a steady decrease to 43% from 93% of total fish protein during 16 wk of storage at −10°C. We found a similar but more rapid decline in PE.

In addition, Awad et al. (1969) found a linear relationship between PE and the sensory texture of baked whitefish, with the fish becoming tougher as PE decreased. However, this relationship was not evaluated statistically. In this study, we found a weak negative correlation (−0.31) between PE and TE.

Table 3—pH values of whitefish fillets at 4 and 28 weeks of frozen storage

Treatment[a]	pH (±0.05)[b] 4 Weeks	28 Weeks
TR-1, −60°C control	6.7	6.5
TR-3, −12°C control	6.7	6.5
TR-4, dipped STP	6.9	6.7
TR-5, injected STP	6.7	6.6
TR-9, injected high-pH buffer	7.0	6.9

[a] Treatments are described more fully in Table 1.
[b] Means of three determinations from three samples

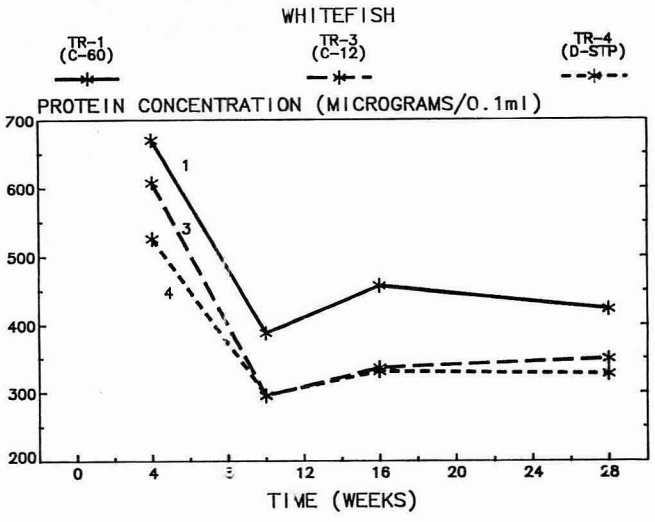

Fig. 3—Protein extractability from frozen-thawed whitefish fillets as influenced by storage temperature and treatment with sodium tripolyphosphate. Curve 1 is treatment 1 (−60°C control samples), Curve 3 is treatment 3 (−12°C control samples) and Curve 4 is treatment 4 (samples dipped in sodium tripolyphosphate and stored at −12°C).

Table 4—Mean sensory scores for poached whitefish fillets

Trial	Sample	Sample attribute: Firmness[a]	Moistness[b]	Off-flavor intensity[c]	Overall preference[d]
1	TR-1, −60°C	4.10a[e]	3.83a	2.79a	4.30a
	TR-3, −12°C	4.68b	3.81a	3.47b	3.56b
	TR-4, STP dipped	3.56c	4.90b	2.94a,b	4.55a
2	TR-1, −60°C	4.02a	3.82a	3.03a	3.87a
	TR-5, STP injected	3.43b	4.77b	2.99a	4.08a

[a] Scale: 1 = not firm; 7 = very firm
[b] Scale: 1 = very dry; 7 = very moist
[c] Scale: 1 = absent; 7 = very pronounced
[d] Scale: 1 = dislike extremely; 7 = like extremely
[e] n = 29. Within a trial, in a given column, scores followed by the same letter do not differ significantly at the 5% level of probability.

Sensory results

Sensory attributes of poached whitefish were evaluated after 18 wk of frozen storage (Table 4). The −60°C control samples were used as a common reference material in the the two trials.

Both panels found the STP-treated samples to be significantly ($P \leq 0.05$) moister and less firm than the −60°C control samples. However, both panels found no significant difference in overall preference between the STP-treated samples and the −60°C control samples. The differences between the dipped STP samples and the −12°C control samples were significant

($P \leq 0.05$) in terms of firmness, moistness and overall preference, indicating a marked superiority of the STP-treated fish over the untreated fish stored at −12°C. Although sensory properties of samples dipped and injected with STP were not directly compared, one can infer, using the −60°C control samples as a reference, that they would not have differed significantly.

These results are in accord with some of the results obtained from the physical and chemical tests and in disagreement with others. TE, CD and CKD tests showed significant ($p \leq 0.05$) differences between −60°C control samples (TR-1) and samples dipped in STP (TR-4; Fig. 2a,b,c,) and panelists indicated a significant ($P \leq 0.05$) preference for the moistness and firmness of the STP-dipped samples as compared to these attributes of the −60°C control samples. However, TE, CD and CKD tests failed to show a significant difference between the −60°C control samples and the samples injected with STP (TR-5), yet in the sensory analysis, panelists found the STP injected samples to be significantly ($P \leq 0.05$) moister and less firm than the −60°C control samples.

Comparisons between samples dipped in STP (TR-4) and the −12°C control samples (TR-3) indicate a strong correlation between sensory and objective tests. Objective tests indicated that the STP-dipped samples were moister (lower CK, CKD and less firm) than the −12°C control samples, and the panelists made similar judgements.

It is interesting to note that the only overall preference expressed by the sensory panelists (Table 4) was for the dipped STP and −60°C control samples over the −12°C control samples. It appears that the panelists were able to distinguish the textural qualities of the STP-treated samples (especially moistness and firmness) from the textural qualities of all other samples, but that this did not always lead to an overall preference for these samples (e.g. TR-5 was not preferred to TR-1). This suggests that while textural differences in whitefish, over the range encountered in this study, can be detected, they are not of paramount importance in determining an overall preference for the fish.

Values of PE at 16 wk did not correspond in any meaningful way to any of the sensory attributes at 18 wk. However, the large changes in PE occurred during the first 4 wk of storage and sensory data collected during this period would have been necessary to establish whether changes in sensory properties were associated with changes in PE.

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Effect of Cryoprotectants on Frozen Burbot Fillets and a Comparison with Whitefish Fillets

MARK KRIVCHENIA and OWEN FENNEMA

ABSTRACT

Burbot fillets (*Lota lota*) were injected with sodium tripolyphosphate (STP), sodium glutamate (MSG), a high pH buffer or an antioxidant mixture (BHA, propyl gallate and citric acid) under high pressure, then frozen and stored at −12°C. Control samples included untreated fillets stored at −12°C and −60°C, and STP dipped fillets. Samples treated with STP or the high-pH buffer had better textural properties than untreated control samples. Samples injected with STP exhibited no improvement as compared to the dipped STP samples and in some respects were worse. Burbot and whitefish have very different textural properties; however, the patterns of change during frozen storage are quite similar.

INTRODUCTION

IN PREVIOUS WORK (Krivchenia, 1987; Krivchenia and Fennema, 1988) we examined the textural changes of whitefish (*Coregonus cupleaformis*) during frozen storage. This is a medium-fat (3–6%) freshwater fish that has tender muscle when fresh but becomes "dry" after prolonged periods of frozen storage. In this study we examine the textural changes in burbot (*Lota lota*), a lean freshwater fish (muscle lipids <1%) which is known for its firm texture and for its pronounced tendency to toughen during frozen storage. It is also the only freshwater member of the gadoid species (e.g., cod, hake, pollock, haddock).

Samson (1983) compared the textural changes in burbot minces with cod minces and found that burbot underwent textural changes similar to those in traditional gadoid species and that burbot also produces dimethylamine (DMA), an enzymatic breakdown product of trimethylamine oxide (TMAO), which is thought to be responsible for textural changes in some gadoid species (Gill et al.,1979). Lindsay et al. (1981) evaluated several methods of processing burbot for the purpose of increasing its acceptability. They found that sodium tripolyphosphate (STP) dips significantly decreased free drip and cooking loss from burbot fillets. Sensory analysis showed that interior portions of burbot fillets were of comparable eating quality to commercially obtained cod, haddock and pollock.

The purpose of this research was: (1) to determine whether high-pressure injection of cryoprotectants into intact fillets of burbot will decrease the rate of textural deterioration during frozen storage compared to that which normally occurs in untreated samples, and (2) to compare the properties, especially texture, of whitefish and burbot during frozen storage.

MATERIALS & METHODS

Materials

Burbot (*Lota lota*), ranging in length from 43–69 cm, was obtained from the Green Bay area of Lake Michigan during February, 1986. The fish were caught in gill nets, then gutted, washed, iced and shipped to Madison. The following day, the fish were washed, skinned, and filleted. The belly flap was removed from the fillet and all other portions of the fillet were used. Two-pound lots of washed fillets, selected at random, were placed in polyethylene ziplock bags (Dow Chemical, 16.5 × 15 cm × 2.1 mil) and stored on ice until treated and frozen.

Author Fennema is with the Dept. of Food Science, Univ. of Wisconsin, Madison, WI 53706. Author Krivchenia's current address is: General Mills Technical Center, 9000 Plymouth Ave. North, Minneapolis, MN 55427.

Preparation of chemical solutions

Solutions of sodium tripolyphosphate, sodium glutamate and a high pH phosphate buffer were prepared as previously described (Krivchenia and Fennema, 1988).

An antioxidant mixture (Krivchenia, 1987) was formulated by mixing 1.50g butylated hydroxyanisiole (BHA; Sigma), 4.50 g n-propyl gallate (Sigma), 4.50 g citric acid (Sigma), 27.0 g propylene glycol and 17.0 g Tween-80 [polyoxyethylene (20) sorbitan monoleate; EM Science]. A 1.8-g portion of the antioxidant mixture was dissolved in 5 L distilled water.

Injection and post-treatment handling of samples

Burbot fillets were handled in a manner similar to that of whitefish fillets (Krivchenia and Fennema, 1988); however, the injected solution was less well retained by burbot tissue than it was by whitefish. Therefore, burbot fillets were injected twice. After the first injection, the box of fish fillets was adjusted laterally so that the second injection occurred at different points in the fish tissue than the first.

All subsequent handling of samples was done as described previously (Krivchenia and Fennema, 1988). Burbot samples were tested at 4, 10, 17, and 23 wk of frozen storage.

Methods of analysis

Methods for centrifugal drip (CD), cooked drip (CKD), protein extractability (PE), texture (TE), pH and sensory analysis of burbot samples were described previously (Krivchenia and Fennema, 1988).

The method of Robles-Martinez et al. (1982) was used to determine 2-thiobarbituric acid reactive substances (TBARS).

To determine the concentration of TMAO in fish tissue, TMAO was first reduced to trimethylamine (TMA) using a 1% solution of titanium (III) chloride (Yamagata et al., 1969). A 0.5-mL volume of TCA extract was added to 0.5 mL of a 1% $TiCl_3$ solution and heated for 10 min. at 60°C. Then 0.5 mL of this mixture was made up to 4.0 mL with distilled water. The remaining procedure was exactly the same as that for TMA (Murray and Gibson, 1972). To determine the amount of TMAO in fish tissue, the amount of reduced TMA-N was subtracted from the amount of unreduced TMA-N.

Dimethylamine (DMA) concentrations were determined according to the method of Samson (1981).

Experimental Design

The experimental design was the same as that previously described for whitefish (Krivchenia and Fennema, 1988).

RESULTS & DISCUSSION

THE TREATMENTS applied to burbot fillets are described in Table 1.

Significant treatment-time interactions occurred only with cooked drip. For all other response variables, treatment and time effects were calculated according to the equation presented in the previous paper (Krivchenia and Fennema, 1988).

Table 1—List of treatments applied to burbot

Treatment designation	Description (Abbreviation)
TR-1	−60°C control (C-60)
TR-2	−12°C control, vacuum packaged (C-12V)[a]
TR-3	−12°C control (C-12)
TR-4	−12°C, dipped sodium tripolyphosphate (D-STP)[b]
TR-5	−12°C, injected with sodium tripolyphosphate (I-STP)[c]
TR-6	−12°C, injected with monosodium glutamate (I-MSG)[d]
TR-7	−12°C, injected with antioxidants, vacuum packaged (I-AOV)[e]
TR-8	−12°C, injected with antioxidants (I-AO)[f]
TR-9	−12°C, injected with high-pH buffer (I-High pH)[g]

[a] FreshTuff bags (American Can Co.; coextruded, 1.2 mil type 6 Nylon/2.8 mil Surlyn 1625).
[b] STP (11.8% w/v), 20°C, 2-3 min.
[c] STP (11.8% w/v)
[d] MSG (25% w/v)
[e] 36 ppm BHA, 109 ppm each for propyl gallate and citric acid based on fat in tissue; same film as TR-2.
[f] Same antioxidant conc. as TR-7
[g] Potassium phosphate buffer solution (1.8M)

Table 2—Pearson correlation coefficients for burbot

Property	Cooked drip	Texture	pH	Protein extractability	TBARS[a]	Dimethyl amine
Centrifugal drip	0.73 S[b]	0.67 S	−0.68 S	0.08 NS	0.20 S	0.27 S
Cooked drip		0.72 S	−0.59 S	−0.05 NS	0.24 S	0.28 S
Texture			−0.71 S	−0.04 NS	0.33 S	0.47 S
pH				0.19 NS	−0.41 S	0.13 NS
Protein Extractability					−0.07 NS	−0.07 NS
TBARS[a]						0.20 S

[a] 2-thiobarbituric acid reactive substances.
[b] Coefficients are significant (S) at the P≤0.05 level.

Centrifugal drip (CD), Cooked drip (CKD), Texture (TE), and pH

Based on Pearson's correlation coefficients (Table 2), TE, CKD, pH and CD are all strongly correlated. This suggests that firmness of the muscle is highly correlated with its water-holding capacity (WHC) as determined by CD and CKD. Also, muscle pH appears to have an important influence on the texture and WHC of burbot.

Treatment effects. Samples that were adjusted to a high pH by the addition of phosphate buffer (TR-9) exhibited significantly less CD, CKD, and firmness then most of the samples treated with STP (TR-4 and 5) and all of the untreated control samples (TR-1 and 3; Fig. 1a, b, c).

Samples dipped in STP (TR-4) exhibited significantly ($P \leq 0.05$) less CD and were less firm than samples that were injected with STP (TR-5; Fig 1a,c). Dipped and injected samples did not differ significantly in CKD (Fig. 1b). The calculated concentration of STP in the burbot tissue was 0.37% and 0.36% for the dipped and the injected samples, respectively. Thus, it does not appear that mean STP concentration can explain the difference between treatments. However, in the dipped samples, much of the STP would have been on the surface, whereas in the injected samples the STP presumably would have been distributed more uniformly throughout the tissue (Scheuer,

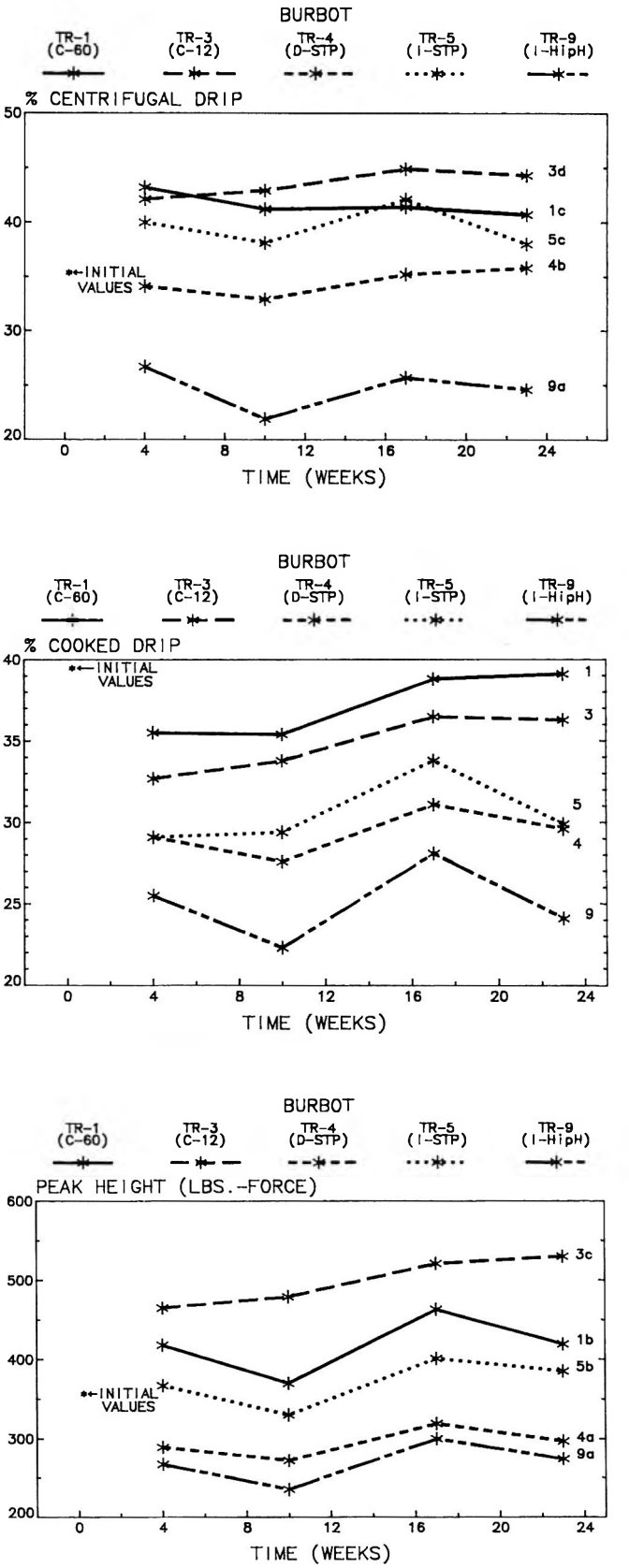

Fig. 1—(A) % centrifuged drip (CD), (B) % cooked drip (CKD), and (C) texture (TE; firmness) of frozen burbot fillets given various treatments. Curve 1 is treatment 1, −60°C control samples; Curve 3 is treatment 3, −12°C control samples; Curve 4 is treatment 4, samples dipped in sodium tripolyphosphate and stored at −12°C; Curve 5 is treatment 5, samples injected with sodium tripolyphosphate and stored at −12°C; Curve 9 is treatment 9, samples injected with high-pH buffer and stored at −12°C. Lines followed by different letters indicate that the treatments are significantly different at the $p < 0.05$ level.

Table 3—pH values of burbot fillets at 4 an 23 weeks of frozen storage

Treatment[a]	pH ± 0.05[b] 4 weeks	23 weeks
TR-1, −60°C control	6.5	6.2
TR-3, −12°C control	6.5	6.1
TR-4, dipped STP	6.8	6.5
TR-5, injected STP	6.7	6.3
TR-9, injected high pH	7.1	6.9

[a] Treatments are described more fully in Table 1.
[b] Means of three determinations on three different samples.

Table 4—Mean sensory scores for poached burbot fillets

Trial	Sample	Sample Attribute: Firmness[a]	Moistness[b]	Off-flavor intensity[c]	Overall preference[d]
	TR-1, −60°C	4.41a[e]	4.04a	3.33a	3.35a
1	TR-3, −12°C	4.47a	3.71a	3.23a	3.77a
	TR-4, STP, dipped	3.94a	5.07b	2.59b	4.42b
	TR-1, −60°C	4.13a,b	4.00a	2.67a	4.00a
2	TR-5, -STP, injected	3.86a	5.05b	2.89a,b	4.21a
	TR-8, antioxidant injected	4.54b	3.27c	3.19b	3.36b

[a] Scale: 1 = not firm; 7 = very firm
[b] Scale: 1 = very dry; 7 = very moist
[c] Scale: 1 = absent; 7 = very pronounced
[d] Scale: 1 = dislike extremely; 7 = like extremely
[e] n = 29; within a trial, mean scores in the same column with the same letter are not significantly different at 5% level.

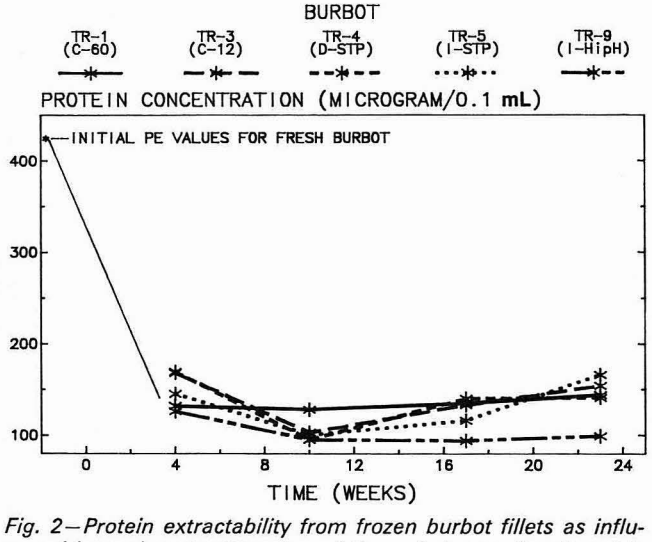

Fig. 2—Protein extractability from frozen burbot fillets as influenced by various treatments and time of storage (treatment 1, −60°C control samples; treatment 3, −12°C control samples; treatment 4, samples dipped in trisodium polyphosphate and stored at −12°C; treatment 5, samples injected with trisodium polyphosphate and stored at −12°C; treatment 9, samples injected with a high-pH buffer and stored at −12°C).

1968). However, the small size of the fillets (3/4 lb.) would tend to lessen the presumed advantage of injection since Mahon et al. (1971) reported that STP applied by dipping penetrated effectively in small fillets. Love and Abel (1966) suggested that STP surface treatments seal fluids in the fillet, thus causing the lower drip values as was seen in this study.

Phosphate treatments elevated muscle pH in all instances, and this is evident at the 4 wk storage period (TR-4, 5 and 9 vs. TR-1 and 3; Table 3). Samples dipped in STP (TR-4) had higher pH values than those injected with STP (TR-5), and in general, the former were found to be less firm and to exhibit smaller drip values than the latter (Fig. 1a, b, c).

Samples treated with sodium glutamate (TR-6), antioxidants (TR-7 and 8) and/or vacuum packaged (TR-2 and 7) did not cause a reduction in CD, CKD or firmness when compared to corresponding values for the −12°C untreated control samples (TR-3; data for TR-2, 6, 7, and 8 are not shown). This would suggest that lipid oxidation, to the extent that it occurred, had no significant effect on the water-holding capacity (WHC) and texture of burbot muscle. This also demonstrates that MSG at the concentration used in this study (0.4% w/w in fish muscle) is an ineffective cryoprotectant—a result differing from that of Noguchi and Matsumoto (1970). However, it should be noted that Noguchi and Matsumoto studied minced fish and used a much higher concentration of MSG (3.74% in the tissue) than was used here.

Time effects. During the first 4 wk of storage, CD values of untreated samples increased from 35% to about 40-45% (Fig 1a; TR-1 and 3) and then remained essentially unchanged for the remainder of the storage period. CD values of samples injected with STP (TR-5) increased to about 40% after 4 wk of storage then remained almost constant through 23 wk of storage. CD values of samples dipped in STP (TR-4) remained unchanged throughout the entire storage period, whereas CD values for samples given the high-pH treatment (TR-9) decreased to about 25% after 4 wk storage and remained virtually unchanged thereafter.

Samson (1983) studied CD of burbot and cod using a method similar to that used in this study. It should be noted, however, that the burbot used in Samson's study had been frozen for 6 mo at −40°C prior to the first analyses. He found that burbot stored at −7°C or −40°C maintained a relatively constant CD of 57–58% during 45 days of storage. Comparable values in the present study were 40-45%. The larger CD values obtained by Samson probably occurred because he centrifuged samples at a higher G force and for a longer period of time than was done in this study.

All CKD values decreased during the first 4 wk of storage, with only minor changes occurring during the 4–23 wk interval (Fig 1b). One would not expect CKD values of untreated samples to decrease during the first 4 wk of storage and this brings into question the validity of the initial CKD value.

The pH values (Table 3) of all samples followed a similar trend during frozen storage, each sample exhibiting a decline in pH of 0.2 to 0.4 units during the 4- through 23-wk period.

For untreated samples (TR-1 and 3) firmness increased greatly during the first 4 wk of storage and more gradually during the remainder of storage (Fig 1c). Samples injected with STP (TR-5) became slightly firmer during storage, whereas samples dipped in STP or injected with the high pH buffer decreased in firmness during the first 4 wk of storage then became slightly firmer during the remainder of the storage period.

Averaged over all treatments, fillet firmness was significantly greater at 17 and 23 wk than at 4 and 10 wk. Burbot fillets attained maximum firmness by 17 wk of frozen storage and changed insignificantly from 17 through 23 wk of storage.

If one compares the firmness of fresh, cooked burbot—about 350 lbs-force—to that of the untreated control samples (TR-1, 2 and 3) at 4 wk—about 450 lbs-force—it is evident that significant textural changes occurred during the first month of frozen storage regardless of storage temperature. This parallels a sharp increase in CD and an abrupt decline of PE during the first month of frozen storage.

Protein extractability (PE)

The initial value for PE from fresh, untreated burbot muscle was 435 μg protein/0.1 mL extraction solution (Fig. 2). There was a significant decline in PE during the first 4 wk of frozen storage with all samples, regardless of treatment, exhibiting PE values of less than 170 μg protein/0.1 mL at the 4 wk

Table 5—A comparison of selected properties of whitefish and burbot muscle during frozen storage

Objective test[a]		Whitefish			Burbot		
		Initial	4 Wk	28 Wk	Initial	4 Wk	23 Wk
Centrifugal drip (%) (CD)	UNTRT	24	25.6	29	35	42.2	42.3
	PHOS. TRT	NM	18.1	18.6	NM	37.1	37
Cooked drip (%) (CKD)	UNTRT	16.5	14.5	23.4	40	32	34
	PHOS. TRT	NM	14.5	19.0	NM	29.1	29.8
Texture (lb-force) (TE)	UNTRT	265	238	377	350	460	546
	PHOS. TRT	NM	195	265	NM	328	341

[a] For CD, CKD, TE: UNTRT = Mean of untreated vacuum packaged samples (Treatment 2, stored at −12°C) and untreated control samples (Treatment 3, stored at −12°C). PHOS. TRT = Mean of samples dipped in or injected with STP and stored at −12°C (Treatments 4 and 5). See Table 1 for further details. NM = not measured.

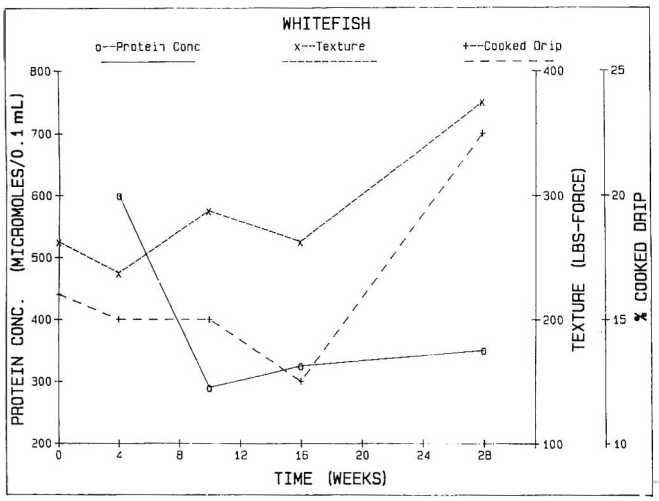

Fig. 3—Protein concentration (μ moles/0.1mL), texture (lb-force) and cooked drip of untreated whitefish fillets stored at −12°C.

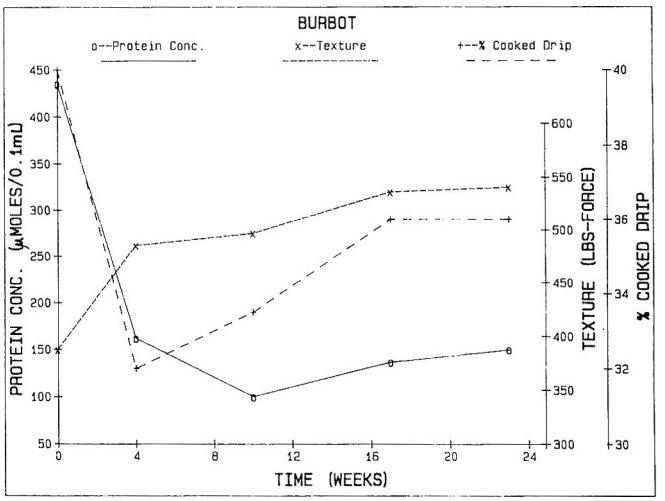

Fig. 4—Protein concentration (μ moles/0.1 mL), texture (lb-force) and cooked drip of untreated burbot fillets stored at −12°C.

examination time. Thereafter, PE values remained virtually unchanged with all samples having values in the range of 100–150 μg protein/0.1 mL.

The sharp decline in PE during the first 4 wk of storage is accompanied by abrupt increases in CD and firmness (Fig. 1a, c), but these correlations are not significant (Table 2). This lack of correlation is most likely due to the fact that the major changes in PE, CD and TE occurred during the first 4 wk of storage and data for these variables were not collected during this period.

red hake, a marine gadoid species known for its firm texture and rapid deterioration during frozen storage, also exhibits a rapid decline in PE during frozen storage (Castell et al., 1971).

Thiobarbituric acid reducing substances (TBARS)

Averaged over all storage periods, samples treated with antioxidants (TR-7 and 8) or STP contained significantly ($P \leq 0.05$) smaller amounts of TBARS than untreated control samples or vacuum-packaged control samples (TR-2) (data not shown). Samples treated with antioxidants or dipped in STP were most resistant to the formation of TBARS and samples injected with STP were somewhat less resistant than the most resistant samples. The antioxidant effect of STP, as observed here with burbot, has been reported by several researchers (Ellinger, 1972). However, this effect was not observed in whitefish treated with STP (Krivchenia, 1987).

In general, the amounts of TBARS did not correspond closely to the sensory quality of the fish (Table 4). For example, at 23 wk of frozen storage, many of the samples smelled highly oxidized, yet qualified as "good" according to the general guidelines for the TBARS test (Robles-Martinez et al., 1982). However, these authors noted that TBARS must be used in conjunction with other evaluation methods to provide a valid measure of fish quality.

Trimethylamine oxide (TMAO) and dimethylamine (DMA)

TMAO and DMA were measured to determine if the formation of DMA from TMAO might play a role in the freeze-toughening of burbot muscle. The concentration of TMAO in untreated burbot muscle was about 1.0 mM/100g muscle after 4 wk of frozen storage and this concentration is low compared to that present in marine species of fish (Regenstein et al., 1982). TMAO concentration also did not change significantly during the 4–23 wk of frozen storage. Hebard et al. (1982) reported a value of 1.5 mM TMAO/100g burbot tissue which agrees reasonably well with the value found in this study. Generally TMAO declines over time, as endogenous TMAOases degrade TMAO to DMA and FA. This was not observed in the present study during the period after 4 wk of frozen storage (we did not collect TMAO data on fresh burbot).

The concentration of DMA in untreated burbot muscle was about 6 μM DMA/g tissue after 4 wk of frozen storage at −12°C and did not change significantly during 4–23 wk of storage. Samson (1983) reported DMA concentrations of 3-7 μM DMA/g of burbot mince which corresponds quite well with the DMA concentrations of 2–7 μM DMA/g found in this study. Samson reported a large variability in DMA values with no net change during 45 days of frozen storage at −7°C or −40°C. We also found a very large coefficient of variation (28.2%) in DMA measurements. Samson suggested that the large variability in the data is probably due to the varied distribution of TMAO in fish tissue, volatility of DMA during storage and sampling, and shortcomings in the extraction procedure.

It is important to point out that while the TMAO concentration in burbot is very low compared to that of other gadoid species, the DMA concentrations reported in this study and in Samson's work are similar to those found in marine gadoid fish such as cusk and pollack (Castell et al., 1971). Thus, one cannot dismiss the possibility that FA may cause textural changes in burbot. The failure to obtain significant correlation coefficients between DMA versus PE, firmness and WHC measurements may have resulted because DMA concentrations were not measured during the first 4 wk of the study when the greatest changes in PE, firmness and CD occurred.

Sensory analysis

Two descriptive sensory analyses of poached burbot were conducted at 12 wk of frozen storage (Table 4). The −60°C control samples were used as a common reference material for the two studies.

Panelists found that the samples dipped in STP (TR-4) were significantly moister, contained fewer off-flavors and had better overall quality (overall preference) than the −60°C and −12°C control samples. Surprisingly, they were unable to distinguish between the −60°C and −12°C control samples in any of the test categories. Panelists also found that samples injected with STP (TR-5) were moister than the −60°C control samples, however, panelists did not express an overall preference for either of these two samples. The samples injected with antioxidants (TR-8) were, for most attributes, found to be the least preferred among all of those tested. A reason for this cannot be offered.

While panelists were unable to distinguish differences in firmness between −60°C control samples and samples given either of the STP treatments, they did judge the STP-treated samples to be moister. Based on "overall preference," panelists preferred samples dipped in STP to the −60°C and −12°C control samples, while they showed no preference for samples injected with STP as compared to the −60°C control samples. This suggests that samples dipped in STP were superior in quality to those that were injected with STP.

Some of these results are in accord with the physical and chemical tests performed, and some are not. The moderately poor "flavor" scores assigned to samples injected with antioxidants suggest that the test for TBARS may not be a good indicator of burbot quality. The amounts of TBARS in the antioxidant-treated samples qualified them for an "excellent" rating and indicated that they were superior to the −60°C control samples, yet the panelist's judgement did not confirm this.

Based on CD, CKD, and firmness values, samples dipped in STP were superior to the −60°C and −12°C control samples. Sensory results were in fairly good accord with these objective results since panelists found the STP-dipped fish to be moister and preferred overall to both of the control samples. It is interesting to note, however, that panelists failed to detect a difference between the −60°C control sample and the −12°C control samples, whereas, these samples differed significantly in CD and firmness measurements.

Panelists found samples injected with STP to be significantly moister than the −60°C control samples, but these results are not fully in accord with the fact that CD and firmness values for these two samples did not differ significantly (CKD was significantly different; Fig 1a, b, c).

It does not appear that any one of the objective tests was able to consistently predict the sensory results for burbot.

Comparison between whitefish and burbot

Burbot and whitefish (Krivchenia and Fennema, 1988) were chosen for this study because they were believed to have markedly different textural properties. Whitefish is a medium-fat, high quality fish. Its muscle is tender when fresh but reportedly becomes "dry" during frozen storage. Burbot, on the other hand, is a lean fish. Its muscle is firm and "lobster-like" when fresh, but reportedly rapidly becomes tough and "spongy" during frozen storage. It is also the only known freshwater species of the gadoid family.

The two species differed greatly with respect to CD, CKD, and TE (Table 5). A comparison of the properties of untreated burbot and whitefish averaged over the initial, 4- and 23/28-wk storage periods reveals that CD from burbot was 52% larger than that of whitefish, CKD from burbot was 95% larger than that of whitefish and burbot was 54% firmer than whitefish. Thus, it can be seen that burbot muscle is significantly firmer and has a significantly lower WHC than whitefish muscle. Some indication of the comparative effect of phosphates on burbot versus whitefish can also be gained from Table 5. Treatment with phosphate reduced the CD values of whitefish more than those of burbot, whereas reduction in CKD values of the two species were fairly similar except for the failure of phosphates to influence CKD from whitefish at the 4-wk storage period. Phosphates decreased the firmness of burbot to a substantially greater degree than it did for whitefish, perhaps because whitefish had an inherently more tender flesh initially.

Although the initial properties of the two species differed greatly, both species were affected similarly by the various treatments and by storage. In both species, injection with a high-pH buffer (TR-9) resulted in the lowest CD, CKD, and TE values of any of the samples tested, samples dipped in STP (TR-4) had lower CD, CKD and TE values than samples injected with STP (TR-5), and the −60°C control samples (TR-1) had lower CD, CKD and TE values than the −12°C control samples (TR-3) (These comparisons are not shown in Table 5).

The fact that the tests for CD, CKD, and TE are significantly correlated for each species suggests (but does not prove) that they are measuring similar properties of the fish muscle. It also suggests that the WHC of these fish is strongly correlated to their textural properties as measured in a Kramer shear-compression cell. However, this relationship is not a simple one. For example, values of CKD for burbot were highest initially when firmness measurements were lowest, yet CKD values changed in a nonlinear fashion with time (they declined and then began to increase), whereas firmness increased with time.

The pH of untreated whitefish muscle was about 6.6 at 4 wk of frozen storage and 6.5 at 28 wk, whereas untreated burbot muscle had a pH of about 6.5 at 4 wk and about 6.2 at 23 wk. Interestingly, correlation analysis showed pH of burbot to be highly correlated with TE, CD, and CKD, whereas in whitefish, these correlations were not significant. Thus, in burbot, pH changes may have a significant role in the phosphate-induced changes in texture and WHC; however, such an explanation cannot be offered for the effects of phosphates in whitefish.

Changes in PE were qualitatively similar in both species, however, the rate of change and the absolute values differed substantially (Figs. 3 and 4). PE declined much more rapidly in burbot than it did whitefish. In both species, PE changed very little after the original large decrease had occurred.

Although the general patterns of changes observed in PE, CD and TE are similar for both species, as seen in Fig. 3 and 4, PE was not significantly correlated with CD, CKD or TE in either species. In burbot the pattern of rapid PE decline in the first 4 wk appears to coincide with the rapid increase in TE and CD. However, there is no evidence to suggest that there is a causal link between these events.

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—Continued on page 1050

Canned Salmon Curd Reduced by Use of Polyphosphates

JOHN C. WEKELL and FUAD M. TEENY

ABSTRACT

Significant reduction of curd in canned salmon prepared from frozen fish was achieved. Prior to being placed in the can, salmon steaks or pieces were given dips of a few seconds to 2 min in sodium polyphosphate in concentrations from 5% to saturated. Curd was effectively reduced in canned pink and sockeye salmon prepared from fish in frozen storage for up to a year, to a level at least equivalent to canned salmon prepared from fresh fish. Improvements in flavor and texture were noted. A simple scoring system is presented for evaluating canned salmon based upon visual scoring of curd present on the surface of canned product.

INTRODUCTION

UNTIL RECENTLY, most of the salmon landed was processed into canned salmon with the remainder going into fresh or frozen products. However, due to marketing demands in the past several years, there has been a steady decline in canned salmon production with a concomitant shift to the fresh and frozen products. For example, in 1984 nearly 43% of the landing was used for canning (assuming a 67% yield when salmon is canned), but in 1985 slightly less than 33% of the catch was processed into a canned product (Thompson, 1986). Due to the increased demand for frozen salmon, more freezing capacity is being added to processing plants.

Alaska accounts for almost 90% of all Pacific salmon landed in the United States (Thompson, 1986). Although most of the salmon canning in Alaska is done during the salmon season that extends from June to late September, the salmon runs occur in relatively specific and remote locations, where the season may be only 6 to 8 wk long. In some years, enormous landings are made in this brief "local season" that tax the local processing plant's operational capabilities. When salmon landings exceed canning capacity, the fish must be held until processing lines can clear the storage inventory. If frozen salmon could be successfully processed into a canned product at a later time perhaps some canning facilities could then be utilized throughout the year in place of the current 6- to 8-wk season.

In the past, attempts were made to use frozen salmon for canning, but the products had poor texture and flavor characteristics (Stansby and Dassow, 1951). Perhaps, more importantly, the use of frozen fish in canning results in a serious marketing defect--curd formation. Curd results from the heat coagulation of soluble proteins in cook-out liquid. The causes and mechanism of curd formation are complex, involving heat penetration, exudation of cook liquid from muscle cells, released minerals, solubilization of protein, and heat coagulation of protein. While curd is a visual defect, its presence can lead to substantially lower prices, and its presence in canned salmon has discouraged the salmon industry from using frozen fish.

Some of the earliest observations on curd formation were made over 40 years ago by Tarr (1942) and then by Tanikawa et al. (1952). Since then, a variety of techniques were proposed for controlling curd formation in canned salmon: tartaric acid dips (Dassow and Craven, 1955), pH and salt content (Seagran, 1956) and more recently proteolytic enzymes (Yamamoto and Mackey, 1981). While these methods appeared to be effective, their widespread adoption has been limited due to a combination of factors related to cost, sensory quality, regulations and marketing.

This study describes a simple procedure for effective reduction of curd in canned salmon made from fresh or frozen fish using a polyphosphate/salt dip. It also describes a simple scoring system for evaluating curd in canned salmon based upon visual observation of the curd on the surface of the fish. Our curd reduction method and the scoring technique were both found applicable to either the traditional salmon pack or to a new boneless-skinless canned salmon product.

Authors Wekell and Teeny are with the U.S. Dept. of Commerce, National Marine Fisheries Service, NOAA, Utilization Research Division, NWAFC, 2725 Montlake Boulevard East, Seattle, WA 98112.

MATERIALS & METHODS

Solutions

All chemicals were of food grade quality. Salt (Cargill Salt, Leslie Salt Company, Newark, CA), sodium tripolyphosphate (FMC Corporation, Philadelphia, PA), and Brifisol-512, a commercial polyphosphate blend (BK-Ladenburg Corporation, Cresskill, NJ) were used throughout the study. Sodium tripolyphosphate and Brifisol solutions (w/w) were prepared in a blender (4-L capacity) by dissolving the polyphosphates in cold tap water (10–15°C) first, and then adding the salt with high-speed mixing. Unfortunately, the limiting concentration of sodium tripolyphosphate in water is approximately 15%. At this high concentration and in the presence of 2% salt, these solutions became cloudy with apparently insoluble material. However, Brifisol-512 was readily prepared at 15% levels in the presence of 2% salt and the resulting solutions remained clear. Saturated solutions of Brifisol 512, containing 2% salt, occurred between 15–20%. When preliminary experiments were done using either sodium tripolyphosphate or Brifisol 512 dips, identical results were obtained. For the purposes of this study, it was assumed that the active ingredient in both additives was sodium tripolyphosphate; hereafter, we will use the term "polyphosphate" to refer to both Brifisol 512 and sodium tripolyphosphate.

Processing

Fresh and frozen sockeye (*Oncorhynchus nerka*) and pink (*Oncorhynchus gorbuscha*) salmon were purchased from local distributors and fish processing companies in the Seattle area. Prior to processing, all frozen fish were thawed in cold (10–15°C) running tap water.

Two styles of canned salmon were prepared: traditional steak pack and boneless-skinless chunked and diced packs. In the steak pack, the salmon were cut into approximately 4-cm steaks; for the chunked and diced packs, fish fillets were cut into either approximately 4-cm wide vertical strips (chunked pack) or into approximately 2-cm cubes (diced pack).

Fish samples were placed in the polyphosphate/brine solutions for varying lengths of time. For dip times up to 5 min, the fish/solution was given an initial gentle stir with a paddle for a few sec to insure good contact with the solution and, thereafter, stirred every minute. For dip times greater than 5 min and less than 30 min, the mixture was stirred every 5 min. For longer soak times, the fish were weighted down to insure the pieces were kept below the surface of the solution and placed in a cold room at 0 to 5°C. Control samples were given similar dips in 2% salt solutions. At the end of the dip time, the samples were drained of excess solution and packed in 1/2-lb 307 × 200.25 cans, each containing approximately 200g sample. Salt was added at a level of 1% by weight to each can where appropriate.

The cans were vacuum-sealed and placed in the retort basket upright with the newly applied lid as the top of the can and then retorted at

240°F according to National Food Processors Association recommendations (NFPA, 1982) for air-cooled canned salmon.

Curd evaluation and chemical analysis

Cans were stored for at least 2 wk before evaluating the contents for curd and other sensory characteristics. For comparative purposes, commercially canned salmon in 1/2-lb cans were purchased and evaluated.

Curd was evaluated by two techniques: visual scoring and direct weighing of the scraped curd as suggested by Yamamoto and Mackey (1981).

Since maximum curd formation ordinarily occurs at the "top" of the can contents, this portion was consistently evaluated for curd. In the evaluation, the cans were cut open, allowed to drain for 2 min, and the meat portion was left undisturbed in the can. A panel of 6 to 10 judges was instructed to evaluate the samples by estimating the area of the surface covered with curd. The scale used in scoring ranged from 0 (little or no curd) to 7 (maximum curd coverage of the surface). Any discoloration of the curd was noted. To evaluate our visual scoring method, cans were judged for curd and then the curd was scraped from the contents and immediately weighed.

Canned samples were also analyzed for total phosphorus using Inductively Coupled Argon Plasma (ICAP) emission spectrophotometer (Jarrell Ash, Model 975) (Teeny et al., 1984) and extracted orthophosphate by the method of Molins et al. (1985). *Total added phosphorus* was determined by subtracting the total phosphorus found in control samples (i.e., no polyphosphate treatment) from the total phosphorus of the samples. *Bound* or *retained phosphorus* was calculated by subtracting total extractable orthophosphate from the total phosphorus of the sample.

Curd scores, weights and treatments were examined for suitable mathematical relationships using a modified SIMPLEX algorithm described by Caceci and Cacheris (1984).

RESULTS

FREEZING FISH increases curd formation during the canning process, but added salt will reduce the amount of curd (Table 1). The addition of sodium tripolyphosphate directly to the can was ineffective in reducing curd, either by itself or in combination with added salt (Table 1). Even fresh fish, depending on holding conditions, will produce curd when canned, but the added salt appears very effective in reducing this curd (Table 1). In this study, it was found that dips or "soaks" were the most effective means in reducing curd formation in canned pink and sockeye salmon.

Curd scoring

The evaluation of curd by scraping and weighing, as suggested by Yamamoto and Mackey (1981), is both time consuming and laborious. Furthermore, curd, as a defect, is normally judged in commercial channels by visual inspection. Because of these commercial practices and the large number of samples in our study, a simple visual scoring system was employed.

The presence of curd as a visual defect in canned sockeye and pink salmon was easily evaluated by visual inspection. For example, commercially canned salmon made from unfrozen fish typically had scores of 0 to 3. In our studies, fresh salmon, when canned less than 72 hrs after catch, had scores ranging from 0 to 1. Canned sockeye or pink salmon steaks or chunks that were processed from fresh fish held in frozen storage for 2 to 6 months, and not treated with polyphosphates, yielded scores between 3 and 7. Pink salmon, held in frozen storage for about 1 year gave curd scores of nearly 7. The addition of sodium chloride to the can at a level of 1% to 2% reduced the score by up to 2 units; however, these reductions occurred only in the samples with the most heavy curd, i.e., scores of 6 to 7.

Table 1—Curd formation (g/can) in fresh and frozen canned sockeye salmon steaks

Treatment	Fresh (N = 4)	Frozen (N = 4)
No additives	1.0 (0.5 - 1.3)	1.9 (1.2 - 3.2)
Salt added (1%)[a]	0.4 (0.3 - 0.6)	1.0 (0.7 - 1.6)
NaTPP (0.5%)[b]	0.8 (0.3 - 1.2)	3.3 (1.9 - 6.0)
NaTPP (0.5%) + salt (1%)[a,b]	0.7 (0.3 - 1.8)	1.7 (0.6 - 2.9)

[a] Salt sprinkled on top surface of meat in can before sealing.

[b] Sodium tripolyphosphate (NaTPP) sprinkled on top surface of meat in can before sealing.

While the intent of this study was not to compare the curd forming potential of sockeye and pink salmon, it was noted that pink salmon appeared to form more curd than sockeye salmon. Others have reported similar observations (Stansby and Dassow, 1951; Dassow and Craven, 1955). However, curd was more noticeable in the sockeye due to the contrast between the relatively darker red of the sockeye tissue compared to the very light meat of the pink salmon.

In several experiments, curd was evaluated by both visual scoring and by direct curd weight determination. There appeared to be a direct relationship between visual score and curd weight (Fig. 1). The relationship was not expected to be linear since the judges were not permitted to evaluate the depth of the curd covering the surface of the fish. Nevertheless, these data were fitted to a convenient nonlinear mathematical model $F(x) = ax/(b+x)$. This model allowed us to transform visual curd scores to "calculated curd weights," a ratio scale quantity, permitting us to readily evaluate statistical differences between treatments.

While the visual curd evaluation worked very well with the traditional steak pack of salmon and the boneless-skinless large chunked products, its application to the smaller boneless-skinless diced product was not attempted for several reasons. Since the small diced product was a new product, industry or consumer guidance for curd as a visual defect was lacking. Furthermore, in the diced product, curd appeared most commonly in the geometric center of the can, particularly in the phosphate-treated samples. To observe the curd, it would have been necessary for the judges to pick apart the sample. Such action would have required a separate sample for each judge and would have been quite time consuming. Therefore, the visual scoring system was not applied to the small diced boneless-skinless product. Nevertheless, it was possible to note visual difference between polyphosphate-treated samples and non-treated samples of the small diced product, indicating that some scoring system was feasible. However, if such a system were employed, a different mathematical relationship would be required.

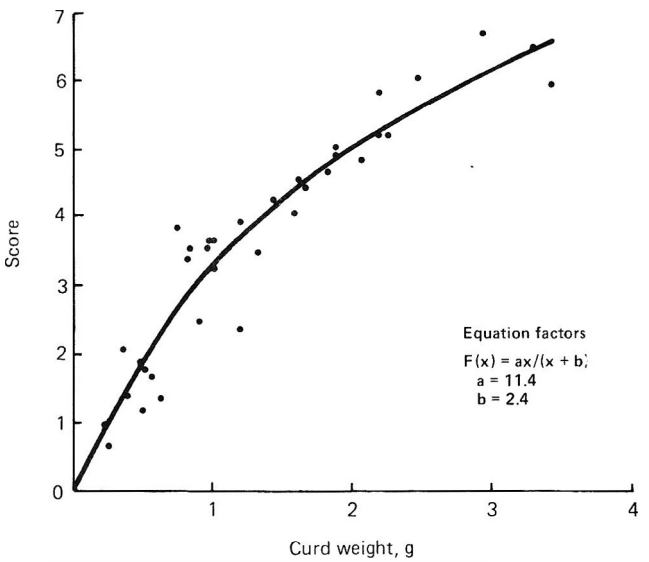

Fig. 1—Relationship between the sensory scores and weight of curd scraped from top surface of salmon steaks canned in 1/2-lb cans.

Steak pack

The steak pack is the traditional retail form for packing canned salmon, most commonly in "1/2-lb" and "1-lb" cans. The worst curd formation was observed in a pink salmon pack prepared from fish held in frozen storage for nearly a year. For the untreated samples, the curd was very thick and dark grey to brown in color. The judges scoring these samples gave unanimous scores of 7. However, by soaking the thawed steaks in a polyphosphate/brine (5%/3.5%) solution overnight at 5°C, the curd was completely eliminated and the meat was noticeably softer and moister compared to the control or untreated samples (Fig. 2). Some judges also noted a more intense salmon flavor in this product.

While the overnight soaks of salmon steaks could indeed eliminate curd formation, this procedure was considered somewhat limited based on current salmon processing practices. Therefore, methods that required shorter exposure times and higher concentrations of polyphosphate were examined.

When samples of sockeye salmon steaks were held for 2, 30, and 120 sec in Brifisol solutions ranging in concentration from 5% to saturated polyphosphate (i.e., "20%" Brifisol in Table 2), curd scores were reduced significantly (Table 2). A dip in a saturated polyphosphate solution (containing 2% salt), for between 2 and 30 sec reduced the calculated curd weight from 1.85 g/can to less than 0.8 g/can. Dips of 30 sec to 2 min required levels of 10-15% polyphosphate to reduce the curd below 1.0 g/can. Direct addition of polyphosphates to the canned salmon, either in solid or liquid form, resulted in no curd reduction over that observed in the controls (Tables 1 and 2).

Boneless-skinless product

Recently, a new boneless-skinless canned salmon product was introduced in the retail market. When such a product was prepared from frozen fish, large amounts of curd appeared on the surface and throughout the meat portion. In one sample, the *surface* curd alone represented about 4% of the total weight of the meat in the can. The curd produced in these untreated samples appeared to act as a binder that firmly bonded the pieces together. By contrast, only very small amounts of curd could be seen in polyphosphate-treated samples. When chunks or pieces were dipped for longer than 2 min, there was significant material loss to the hypertonic polyphosphate/salt solution.

Phosphorus concentrations

The more polyphosphate absorbed by the product, the less curd was observed (Fig. 3). Each data point represents one can in Fig. 3 to 5. It is assumed that phosphorus associated with the food matrix was responsible for curd reduction and other sensory improvements. Therefore, a partial index of this polyphosphate-food matrix association, after processing, should be nonextractable phosphorus, i.e., bound phosphate.

In the study on canned sockeye salmon, two untreated (i.e., no polyphosphate dip) controls were used: "salt added" and "no salt added." When no salt was added to the can, the control samples had maximum scores of 7, while the addition of salt to the cans resulted in scores between 3.6 and 5.4. In both kinds of control samples, background phosphorus in the meat portion was 2368 μg/g (range 2253-2562 μg/g) and the bound phosphorus was 869 μg/g (range 794–915 μg/g).

Total added phosphorus is defined as that portion of phosphorus added to the salmon above the background phosphorus (2368 μg/g) resulting from the application of the polyphosphate treatments. When total added phosphorus was plotted as a function of "calculated curd weight," a simple logarithmic function could be used to describe the curve (Fig. 4). This model predicted that a curd score of 0 would require 2557 μg/

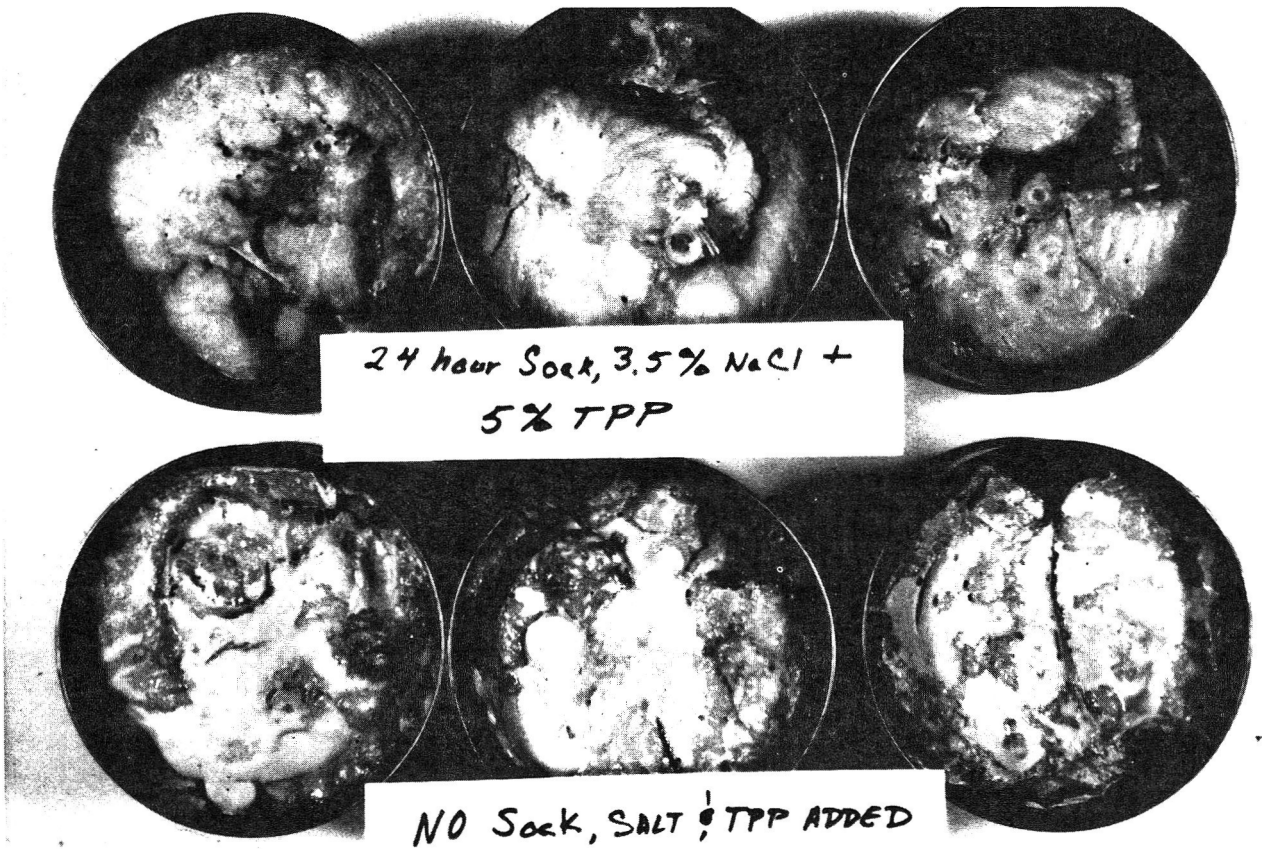

Fig. 2—Effect of polyphosphate dip upon elimination of curd (upper row) in canned pink salmon prepared from fish held in frozen storage for about 1 year.

Table 2—Calculated curd weight (g/can) in canned sockeye salmon treated with Brifisol

Treatment	Dip time (seconds) 0 (no dip)	2	30	120
	(grams per can)			
No treatment	3.91	*	*	*
1% NaCl - control	1.85	—	—	—
0.5% Brifisol®[a]	1.27	—	—	—
1% NaCl + 0.5% Brifisol®[a]	1.97	—	—	—
5% Brifisol® dip	—	1.35	0.75*	0.93*
10% Brifisol® dip	—	0.94	0.58*	0.53*
15% Brifisol® dip	—	0.63*	0.25**	0.53**
"20%" Brifisol® dip	—	0.79*	0.49**	0.32**

[a] Brifisol (dry, 0.5% by weight) added directly to the can by sprinkling on top surface.
* and ** $P \leq 0.05$ and $P \leq 0.01$ respectively, using student's t-test of data compared to "1% NaCl-control" treatment. In determining curd scores, 10 judges viewed 3 cans from each treatment.

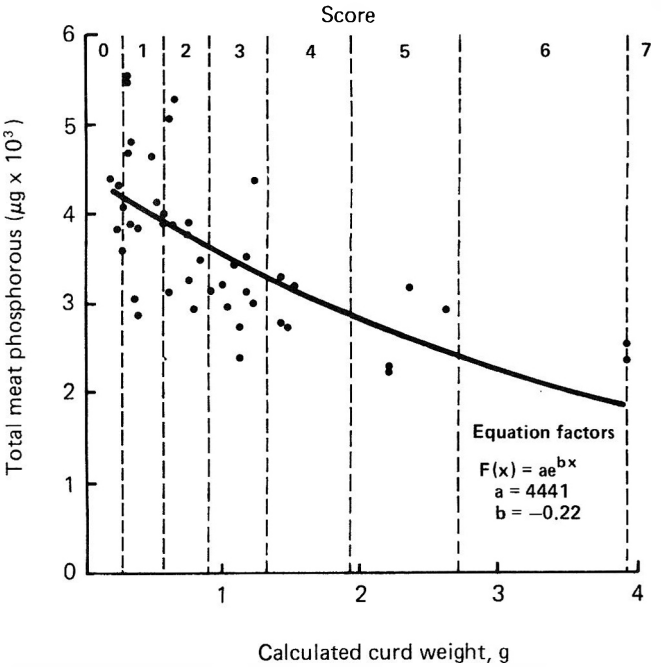

Fig. 3—Relationship between total meat phosphorus and calculated curd weight.

g of total added phosphorus (approximately 1% as sodium tripolyphosphate).

Curd reduction also appears to be related to bound phosphorus (Fig. 5). In this model, a level of 1470 μg/g bound phosphorus was required to reduce curd to 0. The four points in this figure that occurred at calculated curd weights above 2.0g were untreated control samples, with curd scores >5, and they contained 862 μg/g (range 794–915 μg/g) bound phosphorus. The two samples in the figure with calculated curd weights of 4.0 g received the maximum scores of 7.

DISCUSSION

MANY EFFORTS have been made to reduce or eliminate curd formation in canned salmon. Early work by Tarr (1942) and Tanikawa et al. (1952) demonstrated the close connection between drip and curd reduction. Dassow and Craven (1955) proposed the use of tartaric acid dips. Their findings were similar to ours in that dips were required for curd reduction, while spraying or application to the surface of the fish after it had been placed in the can was ineffective. These workers also found that their treatment preserved the apparent color of the salmon after retorting. Seagran (1956) demonstrated that controlling pH and salt could very effectively reduce curd. For example, by adjusting the pH of the salmon tissue below pH

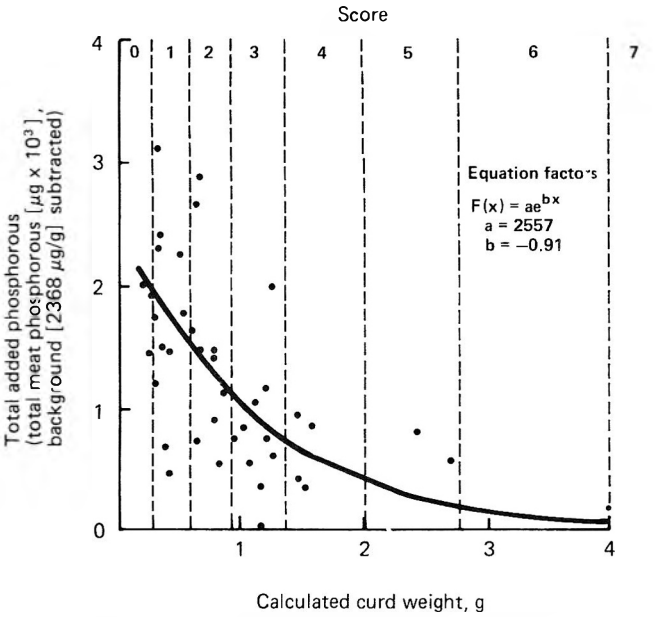

Fig. 4—Relationship between total added phosphorus (as polyphosphate) and calculated curd weight.

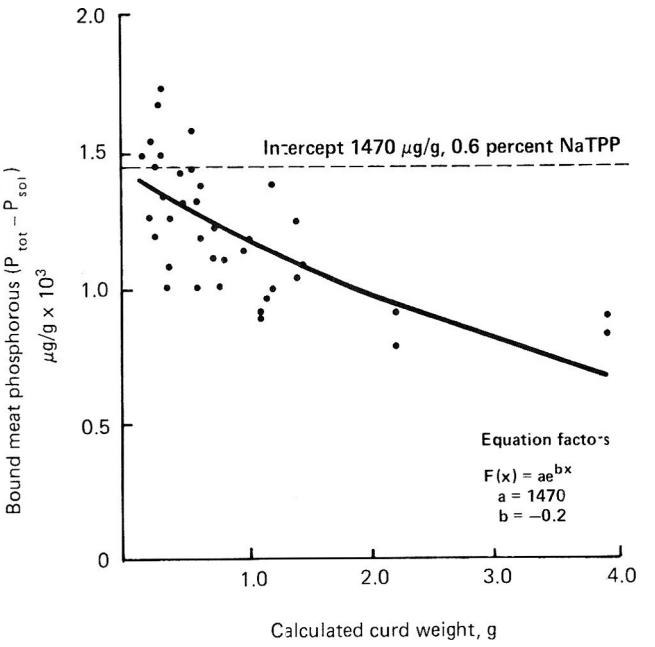

Fig. 5—Relationship between phosphorus bound to meat and reduction of curd.

5.5, no curd was observed after retorting the salmon. By adding salt in the form of brine dips and soaks, curd reduction could be attained in thinly sliced (1/8-in, 0.32 cm) salmon pieces that were subsequently canned.

Unfortunately, these earlier methods for the control of curd were dependent on the use of high salt brines and relatively long soaking times causing substantial uptakes of sodium. Recently, research using proteolytic enzymes added to the can just prior to sealing shows promising results (Yamamoto and Mackey, 1981) in the reduction of curd. In that report, papaya extract is added to the can and through enzymatic action, reduces the high molecular weight protein responsible for curd formation. The high retort temperature deactivates the enzymes.

While the addition of enzymes, salt or organic acids to canned salmon showed promise, it was thought that the expense, po-

tential regulatory difficulties, and concern for higher sodium levels made it useful to evaluate other food additives that the Food and Drug Administration considers as Generally Recognized a Safe (GRAS). Since the early work demonstrated an association between curd formation and expressed drip or moisture loss, it was felt that additives that were known to reduce these effects might be usefully applied to canned salmon. The most obvious additives in drip prevention in fishery products are the polyphosphates and salt. These studies encompassed the use of both sodium tripolyphosphate and a commercial polyphosphate mixture—Brifisol-512. Brifisol was used for two reasons: first, its rapid solubility in salt brines and secondly, as an arbitrary representative of commercial phosphate blends.

In the preliminary studies, the addition of sodium chloride to the polyphosphate solution increased the effectiveness of the polyphosphate dips for curd reduction. Since the salt enhancement of polyphosphate was known to the meat industry (Hamm, 1971), it was decided to include 2% salt in all of our polyphosphate solutions. However, no work was done to pursue the optimum level of salt for these solutions, but it appeared that 2% salt presented a reasonable compromise between polyphosphate activity and polyphosphate solubility problems at higher salt levels.

Soak or dip times of less than 1 min, and in some cases less than 30 sec, effectively reduced curd with only slight sodium uptake in the sample. The use of polyphosphates may reduce the requirement for the addition of salt to canned salmon for sensory or flavor purposes because of the association of the tripolyphosphate anion with the protein complex and subsequent sodium association with endogenous chloride ion.

The mechanical manipulation or "flexing" of seafood during polyphosphate treatment appears to increase the effectiveness of polyphosphates. Mahon et al. (1971) suggest that this "flexing" treatment of fish fillets or steaks, i.e., a gentle stretching and/or very gentle tumbling, allows the polyphosphate solution to penetrate more uniformly into the tissue by momentarily separating the myotomes or muscle layers in either fillets or steaks. In the current work, no effort was made to flex the fish steaks during dips or soaks other than by mixing with a paddle to insure good contact of fish and solution. However, some "flexing" of the fish probably occurred.

Past work (Tarr, 1942; Tanikawa et al., 1952; Dassow and Craven, 1955; Seagran, 1956) demonstrated a relation between the increased water-holding capacity of the tissue with curd reduction. As expected, the sensory observations that polyphosphate-treated samples were more moist than non-treated samples was consistent with these earlier findings. Also, like these past workers, it was observed that there were improvements in the color, i.e., a deeper pink, of the phosphate-treated salmon. In addition, it was found that the oil portion of the cook-out liquid in the cans given the polyphosphate treatment was redder in hue than that of the controls, which may indicate that the polyphosphates were conferring some protection from oxidative reactions (Farr and May, 1970), perhaps mediated by various metal ions such as iron.

The mechanisms by which polyphosphates reduce curd in canned salmon are not very clear. For example, when the polyphosphates are added to meats, they react with the surface protein immediately on contact with little penetration deep into the tissue mass (Hamm, 1971). In our study, we speculate that the ineffectiveness of the direct addition of polyphosphates to the can was probably due to the fact that the treated surfaces were nearest to the can sides where rapid heat penetration occurred. This rapid heat penetration would denature the surface protein almost immediately, hydrolyze much of the polyphosphate present and then bind the phosphorus within the denatured protein matrix, thereby reducing the effectiveness of the added polyphosphate. On the other hand, the effectiveness of the dips or soaks would seem to argue for a more complex mechanism that could involve deeply penetrated polyphosphates. This penetration, in which the polyphosphates may have entered the tissue between the abundant myotome structures present in salmon, could have been enhanced by a certain amount of "flexing" which occurred during stirring (Mahon et al., 1971).

The processing of the traditional canned salmon pack has evolved and been refined over the past 50 years and is somewhat entrenched. Due to the high degree of close competitiveness within the industry and the need to maintain low production costs, the inclusion of any new treatment involving a polyphosphate dip or soak will be difficult to implement for the traditional canned salmon product. However, with the current interest in the use of frozen fish and the advent of the new boneless-skinless canned salmon products, the inclusion of polyphosphates as sprays and dips is entirely possible and practical. With currently available heading, gutting, skinning, boning, filleting, and cutting machines, a highly automated process for the manufacture of a variety of boneless-skinless salmon canned products is possible.

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Protein Hydrolysis in Coho and Sockeye Salmon During Partially Frozen Storage

J.S. FRENCH, D.E. KRAMER, and J.M. KENNISH

ABSTRACT

Coho (*Oncorhynchus kisutch*) and sockeye salmon (*O. nerka*) were held at 0°, −1°, −2°, −3°, and −20°C for up to 25 days. Hydrolysis of individual soluble proteins was analyzed by SDS-PAGE. The highest rates of hydrolysis were in fish held at −2° and −3°C. Several proteins were present in nearly constant ratios. Significant changes were observed in nine other protein bands. A 35,000 dalton protein was present only in coho salmon. This protein appeared with a half-time of five days and then disappeared. Major changes were observed in 34,000 and 36,000 dalton proteins; half of which were hydrolysed in 7 to 20 days. A 32,000 dalton protein was half-hydrolyzed in 14–23 days in sockeye but very slowly in coho salmon.

INTRODUCTION

PARTIAL FROZEN STORAGE or superchilling is a method for holding food products at temperatures just below the freezing point of water so that some, but not all, of the tissue water is present as ice. This procedure has seen rapid growth especially for shipboard handling and transport of large numbers of a variety of marine fish. Partial freezing has been tested on many species of fish but has been used in the Pacific Northwest primarily for holding salmon (Roach et al., 1966; Roach and Tomlinson, 1969). Salmon have been transported using partial frozen storage from Bristol Bay, Alaska to Vancouver and Prince Rupert, British Columbia (Gibbard et al., 1981) and to Japan (Anonymous, 1982). The increased storage time obtained by lowering the temperature enough to partially freeze salmon, −1° to −3°C, results mainly from reduced bacterial action. The texture, moistness and other rheological properties of muscle tissue can be dramatically altered by the specific hydrolysis of a small amount of the total muscle protein (Goll et al., 1977). Several enzymatic reactions responsible for deterioration of fish muscle have been found to be more rapid at partially frozen temperatures (Toyomizu and Shono, 1972). In contrast, nucleotide degradation rates decrease with temperature (Roach et al., 1966; Kramer et al., 1988).

This study compares the rates of degradation of specific proteins at partially frozen temperatures in coho salmon and sockeye salmon muscle. This study in conjunction with those on nucleotide (Kramer et al., 1988) and lipid changes (Whitsett et al., 1987) determined at which temperatures biochemical reactions detrimental to salmon quality are the most rapid. The effects of 0° to −3°C storage on the rates of reactions catalyzed by proteinases were evaluated in an effort to maximize the overall stability of the salmon as a frozen product.

MATERIALS & METHODS

SOCKEYE AND COHO SALMON were caught by gill net off Coho Beach in Cook Inlet, Alaska. Each set of fish was caught within a few hours to maintain the same postmortem age. During the first phase of the study, 15 fish of each species were carefully selected for similar age and appearance. These fish were iced and taken to the plant where they were cut into 1 inch steaks, vacuum packaged, and held at 0°, −1°, −2°, or −3°C in constant temperature circulating water baths containing 6% sodium chloride. Control steaks were stored frozen at −20°C. Steaks, cut sequentially from head to tail from individual fish, were removed for biochemical evaluation after 0, 5, 10, 15, 20, and 25 days of constant temperature treatment. The lettering scheme of "a" for the shortest treatment to "f" for the longest was used in all figures representing electrophoretic results. During the second phase of the study, 90 sockeye salmon were placed into the constant temperature water baths, without packaging, and sampled as before except that a new fish was used at each storage time. Comparing the data from this phase to the previous samples provided more information on the individual variation and the effects of salt uptake on protein hydrolysis. A twenty gram sample of light muscle from the center of a dorsal quadrant of each steak was immediately homogenized with 80 mL of 50 mM sodium phosphate buffer pH 7.0 at 0°C, centrifuged 10 min at 10,000 x *g*, and the supernatant recentrifuged 90 min at 100,000 x *g*. The resultant supernatant was analyzed as the soluble protein fraction. These samples were stored frozen at −20°C and filtered through a 0.45 μm filter immediately before use. Total soluble protein was determined, prior to electrophoresis, by the dye binding method (Bradford, 1976) using bovine serum albumin as the standard.

Polyacrylamide gel electrophoresis was conducted in the presence of 0.2% sodium dodecyl sulfate (Laemmli, 1970) using 8% acrylamide, or 12% acrylamide when stated otherwise. Filtered soluble protein samples were denatured by heating 5 min at 95°C in the presence of sodium dodecyl sulfate and 2-mercaptoethanol. Ten microliter samples were analyzed when higher resolution was desired, whereas 0.030 mL samples were used to detect minor electrophoretic bands. Thus, samples represent an equal portion of the total soluble protein rather than equal amounts of protein. Proteins were either stained with Coomassie Blue R-250 or BioRad silver stain followed by Coomassie Blue, if so indicated. Individual polypeptide temperatures were determined from amount of dye bound for a series of samples where the sample volume was proportional to the amount of dye bound as determined using a BioRad model 620 densitometer and Hewlett-Packard model 3392A reporting integrator on gels soaked in 5% glycerol and dried between two pieces of cellulose acetate. Molecular weight standards were purchased from BioRad (Richmond, CA) and muscle protein standards from Sigma (St. Louis, MO). Proteins used as standards included: rabbit myosin (My), beta-galactosidase (104k), phosphorylase *b* (92k), bovine serum albumin (66k), rabbit actin (Ac), tropomyosin (Tm: alpha and beta chains), troponin (Tn), soybean trypsin inhibitor (21k) and lysozyme (14k), where the numerical abbreviations represent the apparent relative molecular weights in kilodaltons.

RESULTS

THE ELECTROPHORETIC PROTEIN profiles of coho salmon (Fig. 1 to 6) and sockeye salmon (Fig. 7) showed similar patterns in terms of temperature dependence with the exception of the hydrolysis of proteins of 34,000–37,000 dalton subunit molecular weights. In coho salmon there are three major proteins in this range as compared to two in sockeye salmon. The rate of change of these electrophoretic bands differed in coho and sockeye salmon especially at −1° and −2°C where the 35,000 dalton polypeptide was produced, with a half time of 5 days, and disappeared, with a half time of 5 to 12 days. The electrophoretic profile of soluble protein samples from consecutive steaks of three individual fish held in a 0°C constant

Author French is with the Fishery Industrial Technology Center, Univ. of Alaska, 202 Center St. Suite 201, Kodiak, AK 99615. Author Kramer is with the Marine Advisory Program, Univ. of Alaska, 2221 E. Northern Lights Blvd., Room 220, Anchorage, AK 99508. Author Kennish is with the Dept. of Chemistry, Univ. of Alaska, 3211 Providence Dr., Anchorage, AK 99508.

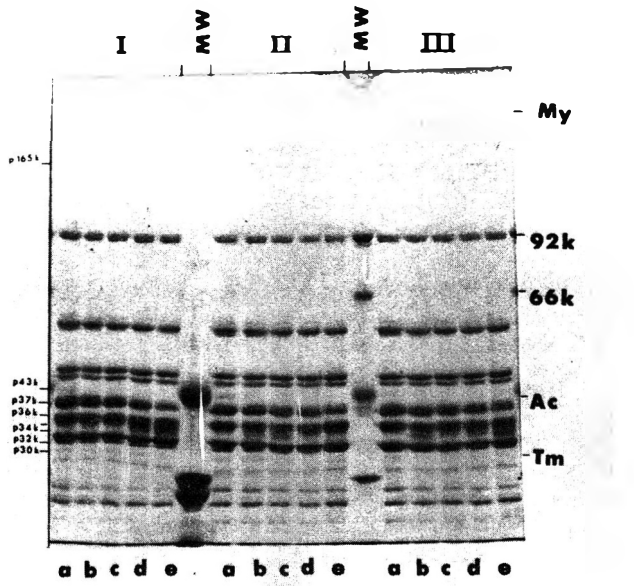

Fig. 1—SDS-PAGE profiles of coho salmon steaks held at 0°C. Each series, I, II, or III, presents samples from a single fish. The lanes marked MW, the left and right borders indicate the migration of standards.

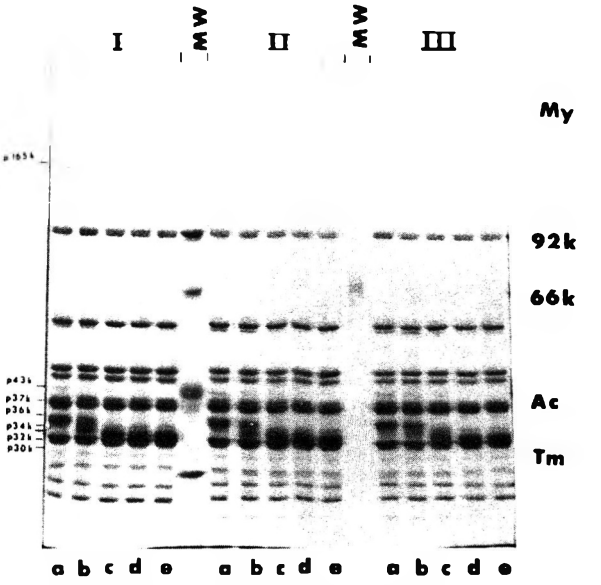

Fig. 3—SDS-PAGE profiles of coho salmon steaks held at −2°C. Each series, I, II, or III, presents samples from a single fish. The lanes marked MW, the left and right borders indicate the migration of standards.

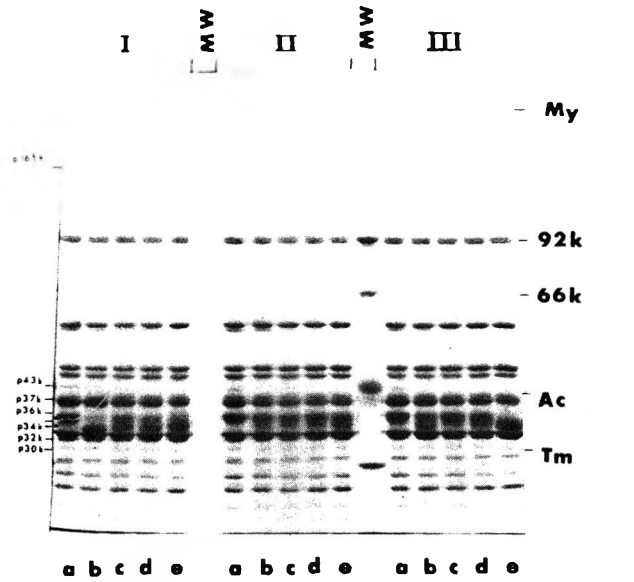

Fig. 2—SDS-PAGE profiles of coho salmon steaks held at −1°C. Each series, I, II, or III, presents samples from a single fish. The lanes marked MW, the left and right borders indicate the migration of standards.

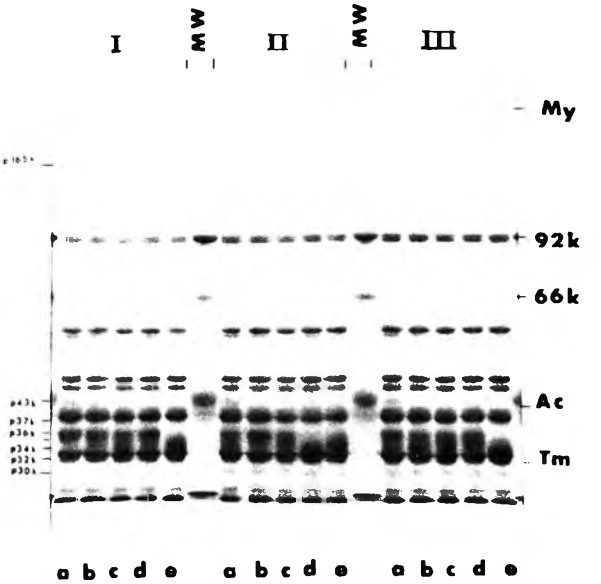

Fig. 4—SDS-PAGE profiles of coho salmon steaks held at −3°C. Each series, I, II, or III, presents samples from a single fish. The lanes marked MW, the left and right borders indicate the migration of standards.

temperature bath for 0 to 20 days are shown in Fig. 1. The most substantial changes in these and other coho salmon samples occurred in the proteins with apparent subunit molecular weights of 12,000 (p12k), 34,000 (p34k), 35,000 (p35k), 36,000 (p36k), 43,000 (p43k), and 165,000 (p165k) daltons, respectively, determined from the molecular weight standards on the gels. Figures 2 to 5 show parallel results for electrophoretic experiments with fish held at −1°, −2°, −3°, and −20°C, respectively. As can be seen from the rate of change in the degree of staining of the electrophoretic bands, hydrolysis of these specific proteins occurred most rapidly at −2° and −3°C. The electrophoretic patterns at −2°C with three times the amount of sample and a 12% acrylamide gel are shown in Fig. 6 which is representative of the changes in proteins p12k and p165k. The absence of a major new low molecular weight band demonstrated that a single large hydrolysis product (M_r > 5,000) could not account for the changes being seen in the major proteins. The variation between individual fish was small in both coho and sockeye salmon.

Sockeye salmon were subjected to partially frozen storage both as vacuum-packaged steaks and as whole fish, held in the round. Both sets of experiments gave practically identical results in terms of rates of protein hydrolysis. The electrophoretic profiles for the average of three sockeye salmon held 0 to 25 days at 0°, −1°, −2°, −3°, and −20°C are shown in Fig. 7. The temperature dependence of protein hydrolysis in sockeye salmon was similar to coho salmon in that the maximum rate of hydrolysis occurred at the lower end of the partially frozen temperature range. The most obvious changes were the disappearance of a protein with an apparent subunit molecular

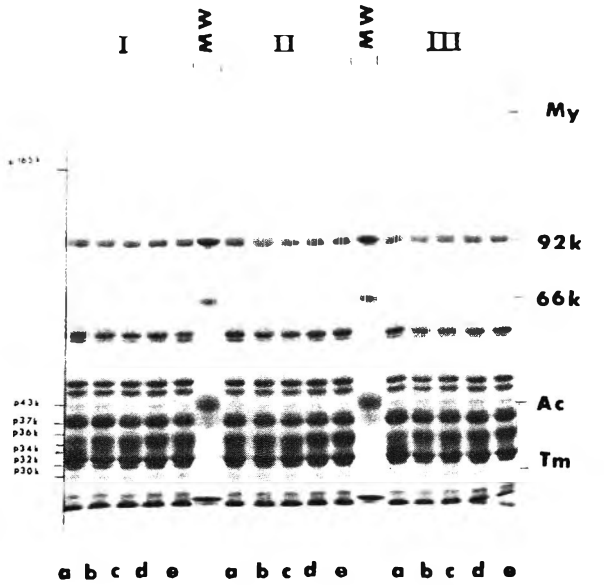

Fig. 5—SDS-PAGE profiles of coho salmon steaks held at −20°C. Each series, I, II, or III, presents samples from a single fish. The lanes marked MW, the left and right borders indicate the migration of standards.

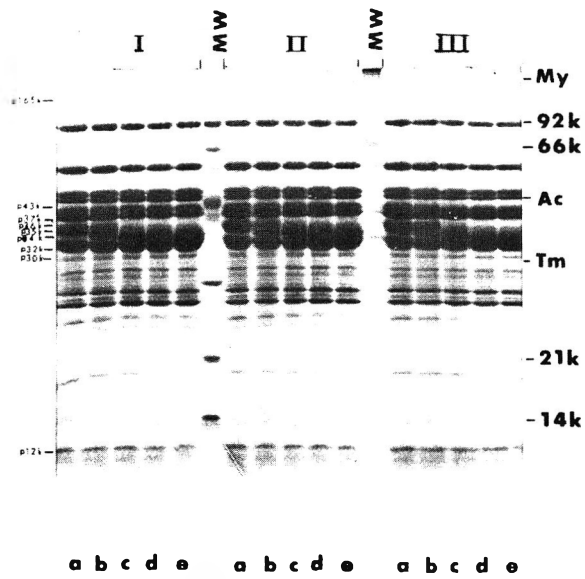

Fig. 6—SDS-PAGE profiles of coho salmon steaks held at −2°C. Each series, I, II, or III, presents samples from a single fish run by SDS-PAGE using an 12% acrylamide gel. The lanes marked MW, the left and right borders indicate the migration of standards.

weight of 36,000 daltons and the appearance of a protein of 34,000 dalton molecular weight. Unlike the coho salmon samples, no 35,000 dalton intermediate protein was seen. There were also significant changes seen in a pair of proteins with apparent molecular weights of 30,000 daltons and 32,000 daltons, respectively. Due to faulty thermostats, the −2° and −3°C experiments were terminated after 20 days. The remaining experiments were carried out to 25 days. On a basis of electrophoretic mobility and solubility after differential extraction, the protein with an apparent subunit molecular weight of 43,000 daltons has tentatively been identified as actin and the doublet at 30,000 and 32,000 daltons as the alpha and beta subunits of tropomyosin. The relative rates of hydrolysis of soluble proteins in coho and sockeye salmon muscle, as determined from densitometer scans of SDS-PAGE data, are summarized in Table 1. These values are given as the time, in days, which was necessary for half the observed change to take place. These changes do not fit simple zero or pseudo-first order kinetics. For times longer than 25 days, the values were estimates extrapolated from the initial rates. Proteins which had more variation between individual samples than was shown over holding-time were considered to have "half-lives" greater than the other proteins in the table. Although the temperatures which gave maximum rates of hydrolysis were not the same for all reactions, the most rapid rates of hydrolysis were seen in fish held at −2° and −3°C.

DISCUSSION

PROMPT, RAPID CHILLING has been shown to be important in the optimum handling of salmon. The questions at this time are primarily how to chill the salmon and what is the optimum holding temperature. This work confirmed that some enzymatic reactions were faster in salmon muscle held in partially frozen storage at −2° to −3°C, specifically, hydrolysis of some soluble muscle proteins might be significantly increased in salmon held in refrigerated seawater (RSW) at −2° to −3°C. The tentative identification of two of these proteins as water-soluble myofibrillar constituents (actin and tropomyosin) provides reason for concern about the effects of this treatment on the textural and moisture retaining properties of salmon after prolonged holding at these conditions (Goll et al., 1977; Asghar et al., 1985).

The observation that some enzymatic reactions, protein hydrolysis, alkylamine breakdown (Babbitt et al., 1986), and lipid hydrolysis (Whitsett et al., 1987) occur more rapidly at −2° to −3°C, whereas nucleotide degradation occurs more rapidly at 0°C may be explainable on the basis of kinetic differences in the mechanisms of the enzymes involved. With the exception of the oxidative breakdown of alkylamines, all the reactions studied involved the hydrolysis of a variety of ester, amide, and N-glycosidic bonds. This provides some gross similarities in the reaction mechanisms: (a) There is one substrate plus water; (b) The substrate binds to the enzyme and a transition state complex is formed, usually producing an enzyme bound intermediate; (c) The products successively leave the active site. Each of these steps has its own characteristic rate. Changing either the stability of any complex or the activation energy necessary for the reaction to proceed to the subsequent step can alter the reaction profile and hence, the overall rate of the observed reaction (Fersht, 1977). In the case of chymotrypsin, a protein hydrolase, relatively small changes in the substrate can effect whether the binding of the substrate or the hydrolysis of the intermediate is the rate limiting step (Walsh, 1979). In many cases reactions in which large substrates are hydrolyzed, the rate of the reaction is determined by the rate of recognition and binding of the substrate to the enzyme. In case of purine nucleotide degradation, the nucleotide is bound primarily by ionic and hydrogen binding interactions (Walsh, 1979). With proteins, and especially lipids, hydrophobic bonding becomes more important in determining the interaction with the hydrolytic enzyme. Hydrophobic bonding will become stronger with decreasing temperature; whereas ionic- and hydrogen-bonded interactions will be relatively independent of temperature over the narrow limits being discussed here (Tanford, 1980; Walsh, 1979). Thus, lowering the temperature in the presence of important hydrophobic interactions might increase the free energy of the enzyme-substrate complex which in turn would reduce the activation energy of the next step which was rate-limiting and increase the overall rate of the reaction. Over a larger temperature range the effects on rates of diffusion and the thermodynamics of overall enzyme structure would presumably become more important. If the changes in rates of enzyme catalyzed reactions seen at partially frozen temperatures were due to simple concentration effects resulting

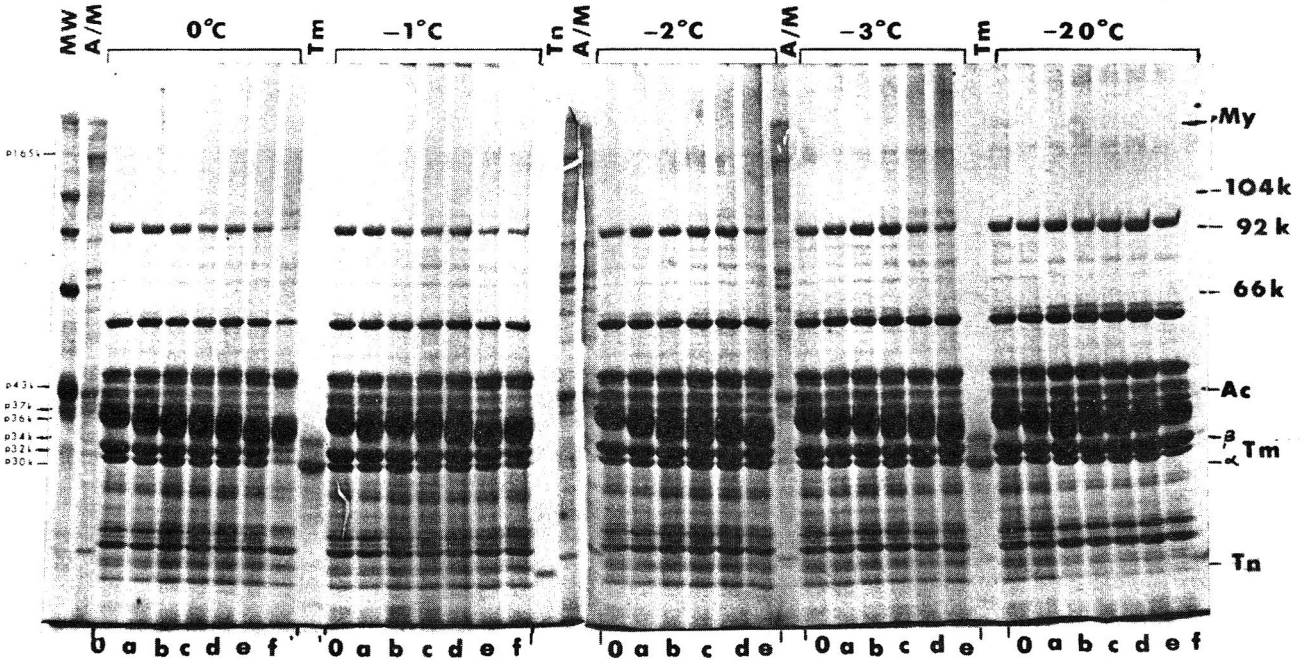

Fig. 7—SDS-PAGE profiles of sockeye salmon after holding in RSW. Each series presents the combined soluble protein extracts from each of three fish after double-staining with silver stain and Coomassie Blue. Samples 0 were controls frozen immediately after capture. The lanes contained standards as indicated.

Table 1—Relative rates of change in protein subunit concentrations in salmon held at various temperatures

	Holding temp (°C)	Protein subunit (kdaltons)								
		12	30	32	34	35	36	37	43	165
		Apparent half-times of formation (days)[a]								
COHO	0	< −50	>	>	NP	+13	−18	−20	−7	> +50
	−1	−40	>	>	+17	+5	−18	−25	−8	+35
						−12				
	−2	−40	>	>	+10	+5	−7	>	−10	+20
						−5				
	−3	−35	>	>	+18	+25	−18	>	−10	+35
	−20	−40	>	>	−12	NP	+20	>	>	> +50
SOCKEYE	0	< −50	−25	−21	+20	NP	−21	>	−16	> +50
	−1	−40	−25	−23	+11	NP	−13	>	−20	> +50
	−2	−35	>	>	+13	NP	−16	>	< −50	+35
	−3	−20	>	+14	+8	NP	−11	>	−30	+20
	−20	−35	>	+18	+4	NP	−23	>	>	+35
				−10	−6					

[a] Protein concentrations determined by amount of dye bound after SDS-PAGE. Rates of formation are given as the time for half the maximum change to take place. NP indicates polypeptide not present. Increasing and decreasing concentrations of specific polypeptides are indicated by + and −, respectively, with two values given when an increasing concentration was followed by a decrease. Samples which showed insignificant rates of change are indicated by > to indicate times greater than the confidence limit.

from sequestering water as ice, or due to mechanical damage caused by the ice itself, one would not expect to see the specificity in the effects on the reaction rates.

The differing effects of partially frozen temperatures on individual enzymes does not allow one to make generalized recommendations on the use of RSW systems or 0° to −3°C temperatures to hold fish. Clearly microbial growth was slower at the lower temperatures, and rapid chilling was important. After that point the relative advantages of partially frozen storage seemed very dependent on the fish being held and the nature of the ultimate product being made. In the case of salmon which is not prone to mushiness, sensory evaluation has shown that an adequate frozen product can be produced at practically any temperature in the partial freezing range. However, all the samples scored less than 8 on a scale of 10 after seven months frozen storage (Kramer et al., 1987). In contrast, Alaskan pollock become distinctly mushy, and functional properties necessary for making surimi are diminished after 5 days holding at −2°C in RSW (French, 1987). In the absence of further data on specific species, we should recognize the importance of RSW systems in rapidly chilling a large catch but not encourage the extra energy expenditures necessary to maintain temperatures below freezing unless its use on a specific product has been tested.

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Purification and Characterization of Cathepsin B from the Skeletal Muscle of Fresh Water Fish, *Tilapia mossambica*

S. V. SHEREKAR, M. S. GORE, and V. NINJOOR

ABSTRACT

Cathepsin B from the skeletal muscle of a fresh water fish *Tilapia mossambica* was purified 4280-fold with 9% recovery. The electrophoretic homogeneity of the preparation was established both under native and denatured conditions. The molecular weight of cathepsin B on the basis of its gel filtration profile was 23,500 daltons. The enzyme, an endopeptidase, hydrolysed Z-arg-arg-NNap and Bz-arg-NNap, with Km values of 0.57 and 3.23 mM, respectively. Cathepsin B did not display aminopeptidase activity, but cleaved Bz-arg-NH_2, exhibiting the specificity of a carboxypeptidase. Among protein substrates tested, only azocoll was hydrolyzed at lower pH values. Leupeptin, antipain and thiol blockers abolished the enzyme activity completely. The Kcat sec^{-1} value of fish cathepsin B seemed to be lower than that of mammalian enzyme.

INTRODUCTION

THE INVOLVEMENT of endo- and exopeptidases in the degradation of tissue proteins has been well documented (Goll et al., 1983; Etherington, 1984). Several proteolytic enzymes such as aspartic (Barrett, 1971), cysteine (Barrett and Kirschke, 1981; Katunuma and Kominami, 1983), serine (Katunuma and Kominami, 1977; Kay et al., 1982), metallo (Beynon et al., 1981), amino and carboxypeptidases (McDonald and Schwabe, 1977) involved in the scission of tissue proteins have been isolated and characterized from mammalian organ tissues. However, there is a paucity of information on the proteolysis and the proteinases of skeletal muscle especially of fish. Since muscle proteins are an important source of food, it is essential to gain insight into the nature of endogenous enzymes that lead to their degradation. In this context lysosomal enzymes in general, with their ability to degrade many food constituents (Ninjoor et al., 1969), and the cathepsins capable of causing proteolysis (Barrett and Kirschke, 1981) in particular, assume considerable significance.

The first proteinase to be detected in fish skeletal muscle (Siebert, 1958) was later identified as cathepsin D (Makinodon and Ikeda, 1969; Doke et al., 1980). Several other proteolytic enzymes such as a cysteine proteinase, calpain, from carp muscle (Taneda et al., 1983), a heat stable alkaline proteinase from carp and croaker muscle (Makinodan and Ikeda 1977, and Lin et al., 1980) and an alkaline proteinase from the shrimp muscle (Doke and Ninjoor, 1987) have been purified and their role in tissue proteolysis has been indicated.

Although cathepsin D has been believed to be a major proteinase that degraded muscle proteins (Schwartz and Bird, 1977; Makinodan et al., 1983; Matsumoto et al., 1983), recent investigations suggest increased involvement of cysteine proteinases such as cathepsin B, H, and L (Katunuma et al., 1981; Ouali et al., 1987) in the degradation of myofibrillar proteins. Our earlier study (Sherekar et al., 1986) also revealed that cathepsin B acted concertedly with cathepsin D to autolyse muscle extracts of *Tilapia mossambica* and Bombay duck (*Harpodon nehereus*). Further, *Tilapia* was also found to be rich in aminopeptidase(s). We have therefore proceeded to characterise these enzymes. The present paper relates to the purification and characterisation of cathepsin B from *Tilapia*.

Authors Sherekar, Gore, and Ninjoor are with the Food Technology & Enzyme Engineering Division, Bhabha Atomic Research Centre, Trombay, Bombay 400 085.

MATERIALS & METHODS

Chemicals

The following chemicals were obtained from Sigma Chemical Co. (St. Louis, MO): N-α-benzoyl-DL-arginine-β -naphthylamide (Bz-arg-NNap), N-α-benzoyl-L-arginine amide (Bz-arg-NH_2), N-α-benzoyl-L-arginine-4-methoxy-3-naphthylamide (Bz-arg-NNapOMe), N-α-benzoyl-DL-arginine-p-nitroanilide (Bz-arg-$PhNO_2$), L-arginine-β-naphthylamide (Arg-NNap), L-leucine-β-naphthylamide (Leu-NNap), N-benzoyl-L-tyrosine ethyl ester (Bz-tyr-EOt), N-α-benzoyl-L-arginine ethyl ester (Bz-arg-EOt), DL-dithiothreitol (DTT), L-cysteine, glutathione, casein, azocasein, bovine hemoglobin type II, bovine serum albumin (BSA), protamine sulfate, azocoll, leupeptin, antipain, pepstatin, chymostatin, dimethyl sulfoxide (DMSO), N-ethylmaleimide (NEM), iodoacetamide, soybean tyrpsin inhibitor type II (STI), phenylmethyl sulfonyl fluoride (PMSF), N-α-p-tosyl-L-lysinechloromethylketone (TLCK), ethylene-diaminetetraacetic acid, disodium salt (EDTA), mersalyl acid, Brij-35, Fast Garnet GBC salt, p-chloromercury benzoic acid (p-CMB). The chemicals for electrophoresis were procured from Eastman Kodak Co. (Rochester, NY). The N-CBZ-arginyl-arginine- -naphthylamide (Z-arg-arg-NNap) was purchased from Bachem Fine Chemicals (Torrance, Ca). The L-trans-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) was obtained from Peptide Institute Inc. (Osaka, Japan). The Ep-475 and Ep-459 were a gift from Dr. K. Hanada, Taisho Pharmaceutical Co. (Tokyo, Japan). The 2-mercaptoethanol was obtained from Fluka AG, Switzerland. Molecular weight markers, gel filtration media and DEAE-Sephacel were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden). The CM cellulose (CM-23) was the product of Whatman, W and R Balston Ltd., England. Organomercurial agarose (Affi-gel 501) was obtained from Bio-Rad (Richmond, Ca).

Purification of *Tilapia* muscle cathepsin B

Preparation of crude enzyme. Live fresh water fish *Tilapia mossambica* (referred to as 'Tilapia' hereafter) were obtained from commercial fish ponds. The fish were beheaded, eviscerated and cleaned with tap water. The muscle (400g), freed from bones and skin, was blotted, minced and homogenized in a Waring Blendor in 1600 mL ice-cold 0.2M KCl for three periods of 30 sec each at 'slow' speed. The homogenate, after six cycles of freeze-thawing was centrifuged at 12,000 × *g* for 15 min in a Sorvall RC-2 refrigerated centrifuge at 0–4°C. The supernatant was decanted through two layers of muslin cloth.

Ammonium sulfate fractionation

All steps of purification were carried out at 4°C unless otherwise stated. The crude extract was subjected to 30–70% ammonium sulfate fractionation and the precipitated protein was collected by centrifugation at 12,000 × *g* for 15 min. The precipitate, dissolved in the minimum amount of 50 mM acetate buffer, pH 5.2 containing 1 mM EDTA and 1 mM mercaptoethanol, was exhaustively dialysed against the same buffer. The preparation, freed from ammonium sulfate, was clarified by spinning at 12,000 × *g* for 15 min and the insoluble material discarded.

DEAE-Sephacel fractionation. The clear supernatant was applied on a DEAE-Sephacel column (2.6 × 33 cm) preequilibrated with 50 mM acetate buffer, pH 5.2 containing 1 mM EDTA (buffer A). The column bed was washed with the same buffer until the eluate showed an absorbance of less than 0.04 at 280 nm. A linear gradient (0-400 mM NaCl) in buffer A was then applied. The flow rate was 25 mL/hr and 5 mL fractions were collected. The active fractions were pooled, concentrated by ultrafiltration in an Amicon Diaflow cell (Amicon Instruments, Lexington, MA) using YM-10 membrane and dialyzed

against 50 mM acetate buffer, pH 5.2 containing 1 mM EDTA and 150 mM NaCl (Buffer B).

Gel filtration on Sephadex G-100. The concentrated sample from the DEAE-Sephacel step was applied on Sephadex G-100 column (2.5 × 60 cm) equilibrated with buffer B and eluted with the same buffer at a flow rate of 20 mL/hr in 5 mL fractions. The active fractions were pooled, concentrated and dialyzed against buffer B.

Affinity chromatography. The concentrated enzyme pool was applied on an organomercurial agarose column (1 × 15 cm) equilibrated with buffer B containing 200 mM NaCl instead of 150 mM (Scott et al., 1987). After complete recovery of unbound protein by washing with equilibration buffer the adsorbed protein was eluted with the same buffer containing 10 mM cysteine at a flow rate of 20 mL/hr in 2 mL fractions. The active fractions were pooled, concentrated and dialyzed against 20 mM acetate buffer, pH 4.5 containing 1mM EDTA (buffer C).

CM-cellulose chromatography. The post affinity chromatography preparation was applied on CM-23 column (1 × 22 cm) pre-equilibrated with buffer C. After washing the column with equilibration buffer for elution of unadsorbed protein, a linear gradient (0–400 mM NaCl) in the same buffer was applied. The flow rate was 25 mL/hr and the fraction size, 3 mL. The active fractions were pooled, concentrated and dialyzed against buffer B. The purified enzyme was stored at −15°C.

Molecular weight determination. Sephadex G-75 column (1.6 × 66 cm) was used for determination of molecular weight of purified enzyme. The column equilibrated with buffer B was calibrated using protein standards such as bovine serum albumin (Mr = 67 KD), ovalbumin (Mr = 43 KD), chymotrypsinogen (Mr = 25 KD) and ribonuclease (Mr = 13.7 KD). Blue dextran was employed for the determination of void volume. The enzyme was chromatographed under identical conditions as used for molecular weight standards.

Polyacrylamide gel electrophoresis (PAGE). Purity of enzyme preparations obtained at various stages was ascertained by PAGE in 7.5% slab gels according to Brewer and Ashworth (1969) at pH 8.3. The duration of electrophoresis was 90 min and the current applied was 2 mA/sample lane. Gels were stained with coomassie blue G and destained by 7% acetic acid.

Electrophoresis was also carried out at pH 4.5 employing acetic acid-β-alanine buffer. Activity staining (Barrett and Kirschke, 1981) was carried out employing Bz-arg-NNapOMe as the substrate. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the procedure of Laemmli (1970).

Assay of cathepsin B. The activity was assayed using Z-arg-arg-NNap as substrate (Barrett and Kirschke, 1981). The assay system (950 μL) containing 10–100 μL enzyme (corresponding to 0.3 μg–1.5 mg protein) 100 mM KH_2PO_4-Na_2HPO_4 buffer, pH 6.0 with 1.33 mM EDTA and 2 mM cysteine was preincubated at 40°C for 5 min for the activation of the enzyme. The preactivation was performed in all assays including those involving crude enzyme. The substrate (50 μL, 4 mM) was added and the reaction mixture, one mL, was incubated at 40°C for 30 min. Mersalyl acid reagent, 1.0 mL, was added to terminate the reaction and Fast Garnet GBC was used to couple released β-naphthylamine. After centrifugation the color intensity was read at 520 nm. β-naphthylamine was used as the standard. The unit of activity equalled μmole β-naphthylamine released/hr. Activity employing other naphthylamide substrates (dissolved in DMSO) such as Bz-arg-NNap, Arg-NNap, and Leu-NNap was measured according to the same procedure.

The method described by Schwert and Takenaka (1955) was followed to measure the esterolytic activity towards Bz-arg-EOt and Bz-tyr-EOt at 253 and 263 nm, respectively, at 25°C.

Hydrolysis of Bz-arg-$PhNO_2$ was carried out according to the method of Barrett (1972). The unit of activity corresponds to μmole substrate hydrolyzed/hr assuming that 0.1 μmole hydrolyzed substrate results in E_{410} of 0.293.

The method described by Sherekar et al. (1986) was followed to assay the carboxypeptidase activity using Bz-arg-NH_2 as the substrate. The unit of activity equals μmole ammonia released/hr.

Proteolytic activity of the enzyme. The activity towards azocoll, azocasein, protamine sulfate, hemoglobin and BSA was also determined (Ninjoor and Srivastava, 1985).

For proteolysis of azocoll the reaction mixture (2 mL) comprising of 10-20 μL enzyme (3–6 μg protein), 0.1M buffer in the range of pH 3.5–6.0, 2 mM EDTA and 2 mM DTT was incubated at 40°C for 5 min and 10 mg azocoll was added. After a further incubation for 60 min, the suspension was rapidly filtered and the absorbance of the filtrate at 520 nm was recorded. Suspensions without the enzyme served as controls. The unit of activity equals increase in the absorbance by 0.001 per min.

In the assay employing azocasein, hemoglobin, BSA and protamine sulfate as substrates the reaction mixture (2mL) included 10-20 μL (3–6 μg protein) enzyme, 0.1M acetate buffer pH 5.0, 2 mM EDTA and 2 mM DTT. The substrates at appropriate concentrations were added and the mixtures incubated for 1–4 hr at 40°C. The reaction was terminated by adding 1 mL 10% TCA. Absorbance of TCA filtrate at 366 nm was recorded for measuring azocasein hydrolysis. The products of proteolysis were monitored as tyrosine equivalents (Miller, 1959) in all other assays. The unit of activity was Δ OD 366×10^3/hr for azocasein hydrolysis and nmoles tyrosine released/min for the hydrolysis of other proteins.

Determination of optimum pH and temperature. An aliquot of the enzyme suspended in 0.1M activating buffer containing 1.33 mM EDTA and 2 mM cysteine of different pH was assayed as described earlier. The buffers used were acetate (pH 3.0 – 5.5) and phosphate (pH 6.0 – 8.0). For the determination of optimum temperature, the assay mixtures were incubated at varying temperatures.

Titration of E-64 with cathepsin B. The procedure described by Barrett and Kirschke (1981) was followed. The enzyme was incubated with varying concentrations of E-64 (0–200 nM) for 30 min at 30°C and the residual enzyme activity was measured. Experiments were also done after complete activation of the enzyme accomplished by preincubation with cysteine and EDTA in phosphate buffer, pH 6.0 for 3 hr at 25°C. The activated enzyme was then allowed to react with E-64 for 30 min at 30°C and the residual activity was determined.

Concanavalin A-Sepharose chromatography. The Con A-Sepharose column (1 × 5 cm) was equilibrated with 50 mM sodium phosphate buffer containing 0.2M NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, and 1 mM $MnCl_2$ (pH 6.0). Purified cathepsin B sample dialyzed against the same buffer was applied to the column and eluted with 7–8 bed volumes of the equilibration buffer. This was followed by elution with 0.2 M α-methyl-D-mannoside dissolved in the same buffer. Enzyme activity was monitored in all fractions. Miller's (1959) method was employed for protein estimation using BSA as standard.

RESULTS

Purification of cathepsin B

Tilapia cathepsin B was purified 4280-fold with 9% recovery as shown in Table 1. The enzyme could be distinctly separated from contaminating proteins in DEAE-Sephacel chromatography (Fig. 1). While the unbound protein exhibited both endo- and exopeptidase activities as evidenced by the hydrolysis of Bz-arg-NNap and Leu-NNap (not shown in the Fig.), the fractions eluting after the application of NaCl gradient showed activity towards Z-arg-arg-NNap, a specific substrate of cathepsin B. These fractions were inactive towards the aminopeptidase substrate, Leu-NNap. The peak of cathepsin B elution was at 0.2M NaCl concentration. Gel filtration of the DEAE pool resolved the proteins into a sharp peak followed by a broad shoulder (Fig. 2A). Cathepsin B activity was exclusively associated with the shoulder portion. The peak protein however, had low cathepsin B activity. Affinity chromatography (Fig. 2B) gave rise to 2990-fold purification of the enzyme but resulted in two bands following PAGE (Fig. 3a). Ion exchange chromatography on CM-cellulose (Fig. 2C) eliminated the contaminating protein (Fig. 3b) and a purification of 4280-fold was achieved. When this preparation was subjected to gel filtration on Sephadex G-75, a single peak of protein was obtained where the enzyme activity across the peak was uniform (results not shown). Electrophoresis at both pH 8.3 (Fig. 3b) and 4.5 yielded only a single band. Enzyme activity could be detected in the form of a diffused band with a crimson red color following electrophoresis at pH 4.5 employing Bz-arg-NNap-OMe as the substrate (Fig. 4). SDS-PAGE also revealed a single band (Fig. 5).

Molecular weight

The gel filtration of cathepsin B on sephadex G-75 showed an apparent molecular weight of 23500 daltons (Fig. 6).

Temperature and pH dependency

The optimum pH of cathepsin B for hydrolysis of Z-arg-arg-NNap was 6.0 (Fig. 7A). The activity of the enzyme showed

Table 1—Purification of Cathepsin B from Tilapia *muscle*

Fraction	Total protein (mg)	Total activity (units)	Specif c activity (units/mg)	Recovery (%)	Purifi-cation (fold)
Muscle homogenate	74590.0	2314.6	0.031	100	–
Supernatant (after freeze thaw and centrifugation)	15476.0	2064.7	0.13	92	4
30–70% $(NH_4)_2SO_4$ fraction	5154.0	1415.3	0.27	61	9
DEAE-Sephacel chromatography	259.2	887.4	3.42	38	110
Sephadex G-100 gel filtration	124.7	795.6	6.38	34	206
Affinity chromatography	6.3	587.2	92.73	25	2991
CM-cellulose chromatography	1.5	202.2	132.60	8.7	4277

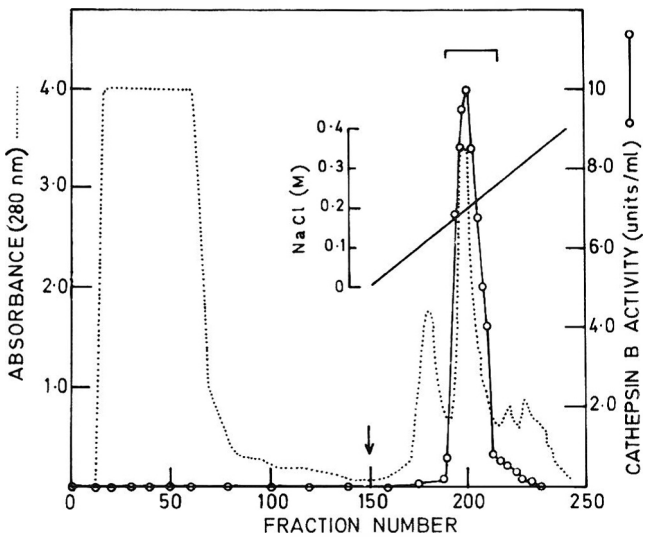

Fig. 1—Ion exchange chromatography of fish muscle cathepsin B. $(NH_4)_2SO_4$ fraction (30–70%) was applied on DEAE-Sephacel column. The arrow indicates the position where 0–400 mM NaCl gradient in the elution buffer was started. Fractions under the bar were pooled for further purification.

a rapid decrease at pH values above 6.0. On the acidic side also there was a decline in enzyme activity, the magnitude of which was less than that occurring at alkaline pH.

Maximum stability of the enzyme was observed in the pH range of 5.5–6.5 (Fig. 7B). The enzyme was maximally active at 42°C, and at 60°C it lost its activity completely.

Storage stability

The enzyme, in 50 mM acetate buffer containing 1 mM EDTA and 0.15M NaCl (pH 5.2), could be stored at −15°C for more than 2 months without appreciable loss in activity.

Effect of sulfhydryl reagents

The enzyme failed to show measurable hydrolysis of Z-arg-arg-NNap and other synthetic substrates in the absence of thiol compounds. Cysteine in the 2–5 mM range stimulated the enzyme. At concentrations above 5 mM, further elevation in the activity was only marginal. Concentrations lower than 2mM resulted in decreased activity. DTT appeared to be the most effective activator of the enzyme in view of its ability to stimulate the enzyme at low concentrations. Glutathione, however, was not an effective enzyme activator (Table 2).

Action on different substrates

The susceptibility of synthetic substrates to the action of cathepsin B is shown in Table 3. Substrates of aminopeptidases, trypsin and chymotrypsin were not hydrolyzed. A carboxypeptidase activity was evidenced by the hydrolysis of Bz-arg-NH_2. A low Km value obtained with Z-arg-arg-NNap suggests that this was a very sensitive substrate as compared to Bz-arg-NNap.

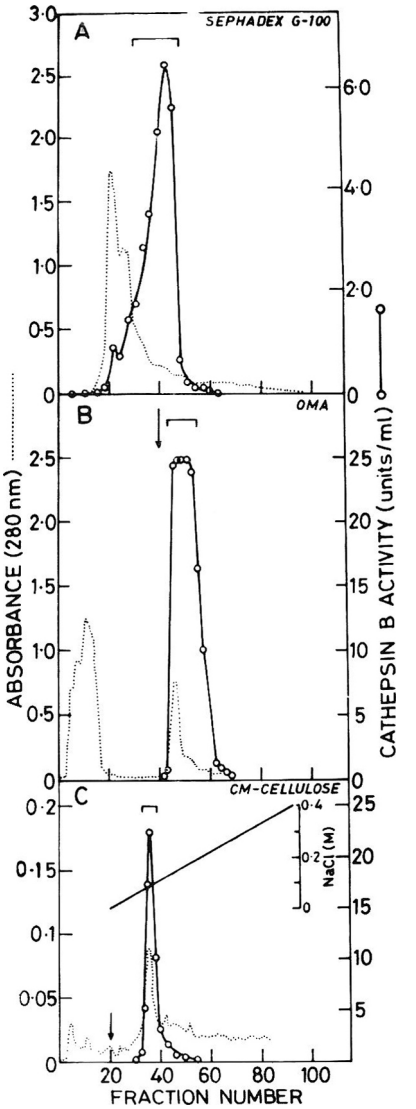

Fig. 2—Steps for the purification of cathepsin B. A: Gel filtration on Sephadex G-100. DEAE-Sephacel pool was chromatographed on Sephadex G-100 employing buffer B for equilibration and elution. B: Organomercurial agarose affinity chromatography of gel filtration pool. Arrow indicates the position where elution with 10 mM cysteine dissolved in the elution buffer was started. See Materials & Methods for details. C: CM-cellulose chromatography. Concentrated sample (5.8 mg) obtained after affinity chromatography was applied on CM-cellulose column and the enzyme was desorbed by 0–400 mM NaCl gradient (arrow) in buffer C. Fractions under the bar were pooled and concentrated.

Cathepsin B failed to degrade protein substrates such as hemoglobin, BSA, protamine sulfate and azocasein but hydrolysed azocoll at pH 4–4.5. The activity of the enzyme towards

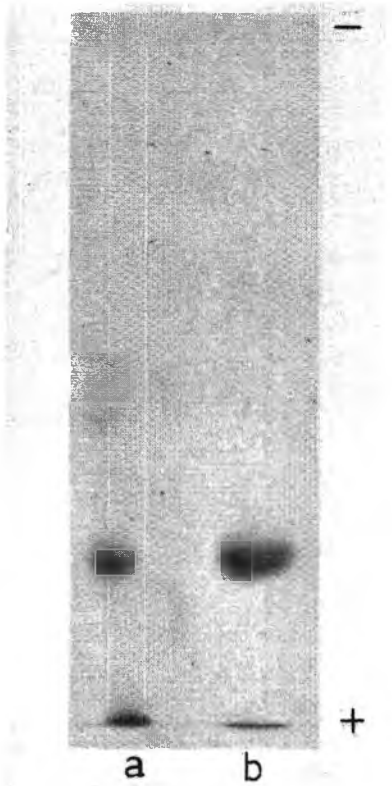

Fig. 3—PAGE of cathepsin B at pH 8.3: Organomercurial agarose (a) and CM-cellulose (b) sample.

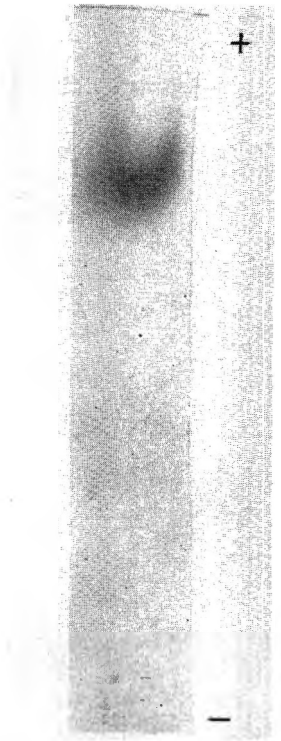

Fig. 4—Activity staining of cathepsin B following PAGE at pH 4.5.

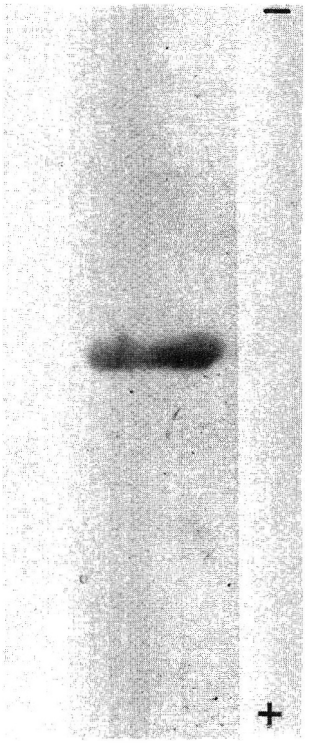

Fig. 5—SDS-PAGE of purified cathepsin B.

azocoll at pH 4.5 was 230 units/mg. At pH 3 and 6.0 the activity was 25.8 units/mg and 60 units/mg, respectively.

Effect of inhibitors

The data on the effect of various proteinase inhibitors on cathepsin B are presented in Table 4. At concentrations higher than usually employed, pepstatin, an aspartic proteinase inhibitor, and serine proteinase inhibitors such as STI and PMSF failed to suppress the enzyme activity. EDTA which inhibits metallo- and aminopeptidases has no inhibitory influence while leupeptin and antipain abolished the enzyme activity completely. Active site directed inhibitors of cysteine proteinases such as E-64, Ep-459 and Ep-475 also led to total loss of enzyme activity. NEM showed much less inhibition than expected. Na-tripolyphosphate and NaCl which improve muscle texture resulted in a decrease in the enzyme activity.

Active site titration of cathepsin B

The pattern of the curve obtained when the residual activity of cathepsin B was plotted against E-64 concentration is shown in Fig. 8. The linearity was maintained only up to 80% inhibition of the enzyme activity; therefore, the upper linear part of the curve was extrapolated to the abscissa. Kcat value of cathepsin B calculated on this basis was 5.23/sec while on the basis of protein measured, it was 3.14/sec.

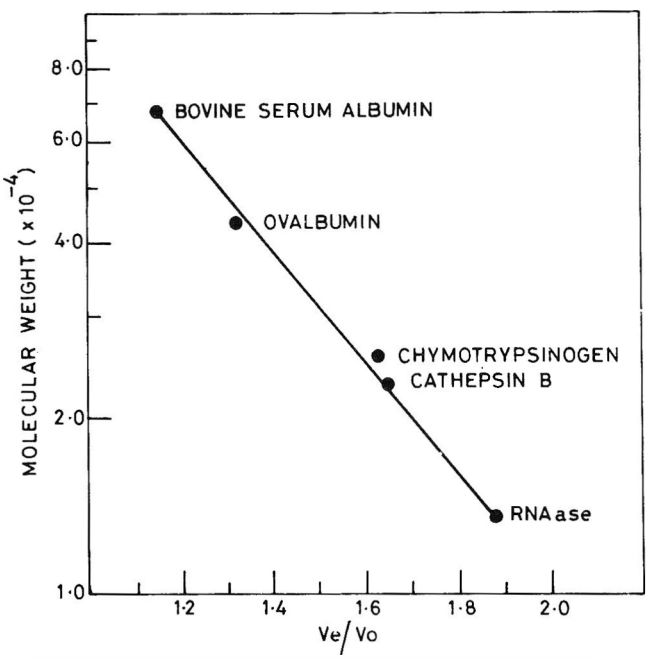

Fig. 6—Determination of molecular weight of cathepsin B. The procedure was identical to that of Fig. 2A except that Sephadex G-75 was used and the elution volumes (Ve) for cathepsin B and standard proteins were determined. Blue dextran was used for the estimation of void volume (Vo).

DISCUSSION

The foregoing results show that an enzyme hydrolysing N-blocked arginine derivative, Z-arg-arg-NNap has been purified 4280-fold from the muscle of fresh water fish, *Tilapia*. The endopeptidase nature of cathepsin B is apparent by the hydrolysis of this preferred substrate as well as of Bz-arg-NNap. However, the latter substrate is amenable to cleavage by cathepsin H also, which could be separated by the use of DEAE-chromatography (Barrett and Kirschke, 1981). A single band following electrophoresis of the purified enzyme and its positive response for activity staining points to the homogeneity of the preparation. Although the mammalian cathepsin B is among the best characterized cysteine proteinase, this communication is perhaps one of the very few describing its pu-

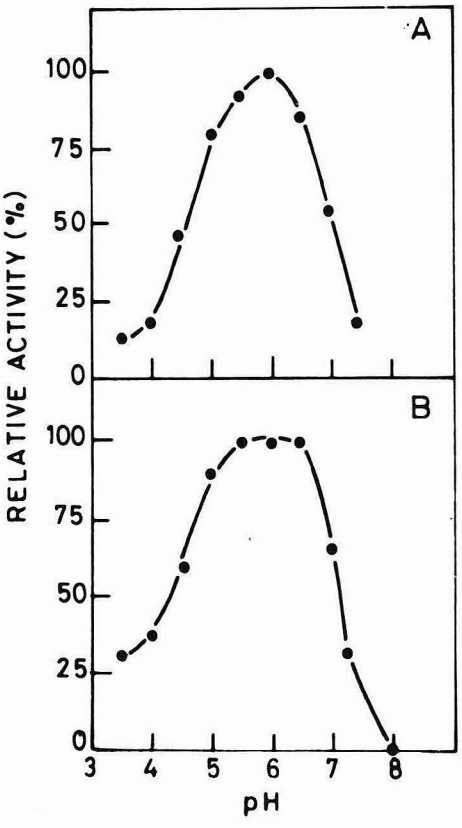

Fig. 7—Influence of pH on activity (A) and stability (B) of purified cathepsin B. A: The enzyme was assayed employing acetate (pH 3.5–5.5) and phosphate (pH 6.0–8.0) buffers of 100 mM containing cysteine and EDTA. B: The enzyme suspended in appropriate buffers was incubated at 40°C for 30 min. The retained activity was measured at pH 6.0 as stated in Materials & Methods.

Table 2—Effect of thiol compounds on cathepsin B activity

Compound	Final conc (mM)	Relative activity[a] (%)
Cysteine	0.5	76
	1.0	93
	2.0	100
	5.0	100
	10.0	102
	20.0	83
Dithiothreitol	0.5	100
	1.0	104
	2.0	133
Glutathione	1.0	28
	2.0	44
	4.0	67
	5.0	78
	10.0	57

[a] Activity determined in the presence of 2 mM cysteine was taken as 100% using Z-arg-arg-NNap as the substrate. Each thiol compound at the stated concentration was used in place of usual 2 mM cysteine.

Table 3—Action of cathepsin B on synthetic substrates[a]

Substrate	Specific activity[b] (μmole/mg/hr)
Z-arg-arg-NNap	132 (0.57)
Bz-arg-NNap	13.38 (3.23)
Bz-arg-$PhNO_2$	0.58
Bz-arg-NH_2	3.62
Leu-NNap	0
Arg-NNap	0
Bz-arg-EOt	0
Bz-tyr-EOt	0

[a] Details of the experimental procedure are provided in the text.
[b] Figures in parenthesis indicate the Km values (mM).

Table 4—Effect of inhibitors on cathepsin B activity[a]

Inhibitor	Final conc	Residual activity (%)
None	—	100
Iodoacetamide	1 mM	0
p-CMB	1 mM	37
NEM	1 mM	80
Ep-475	1 mM	0
Ep-459	1 mM	0
E-64	0.1 μM	0
Leupeptin	0.2 μM	0
Antipain	0.2 μM	0
Chymostatin	10 μg/mL	100
TLCK	1 mM	55
STI	1 mM	100
PMSF	1 mM	80
Pepstatin	10 μM	100
DMSO	5%	78
Na-tripolyphosphate	20 mM	60
Ca^{++}	5 mM	100
Cl^-	100 mM	83

[a] Enzyme was incubated with inhibitor at 30°C for 15 min and the remaining activity was measured as described in Materials & Methods. Z-arg-arg-NNap was used as the substrate.

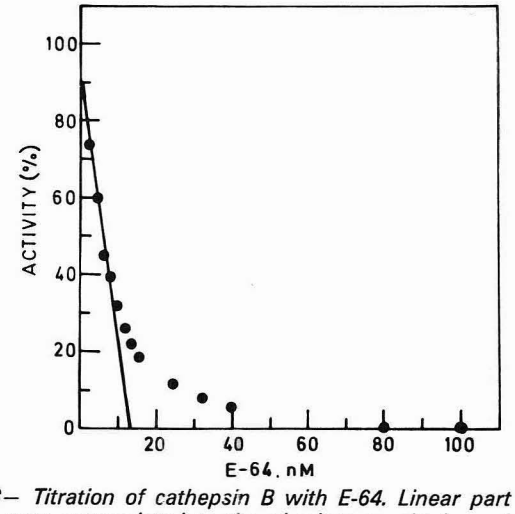

Fig. 8— Titration of cathepsin B with E-64. Linear part of the curve was extrapolated to the abscissa to obtain molarity of cathepsin B.

rification from fish muscle. Occurrence of this enzyme from various organs of three fish varieties was, however, reported by Bonete et al. (1984a) who also obtained an electrophoretically homogeneous preparation of cathepsin B from the muscle of grey mullet. Chen and Zall (1986) have reported the occurrence of a cathepsin B-like enzyme in surf clam viscera.

The molecular weight of *Tilapia* cathepsin B was 23.5 KD which is in close agreement with the value reported for grey mullet enzyme (Bonete et al., 1984b). In contrast, Chen and Zall (1986) reported a molecular weight of 17.5 KD for the cathepsin B-like enzyme from surf clam viscera. Molecular weights ranging between 24–29 KD have been ascribed to cathepsin B preparations isolated from various mammalian sources (Barrett and Kirschke, 1981: Towatari et al., 1979; Hirao et al., 1984; Fazili and Qasim, 1986; Scott et al., 1987) and a subunit structure for rat liver enzyme has been suggested (Towatari et al., 1979). Nevertheless, a single band with SDS-PAGE in our studies indicates that cathepsin B from *Tilapia* is a polypeptide of monomeric structure.

The chromatographic profile of the enzyme on Con A-Sepharose revealed that cathepsin B binds to the affinity gel only to the extent of 10%, which is consistent with earlier reports (Barrett, 1980; Takahashi et al., 1984a). Despite being a glycoprotein, cathepsin B seems to show poor affinity towards Con A-Sepharose (Takahashi et al., 1984b).

Failure of several inhibitors to suppress cathepsin B activity

and complete inhibition by active site directed inhibitors like E-64, Ep-459 and Ep-475 together with the absolute requirement for thiol groups for optimal activity unequivocally establishes that the fish enzyme is a cysteine proteinase. Lack of hydrolysis of Bz-tyr-EOt and Bz-arg-EOt together with absence of the inhibition by chymostatin, STI and PMSF indicate that the enzyme is devoid of chymotrypsin- and trypsin-type activity. It is significant that NaTPP and NaCl, chemicals used for fish preservation, suppress the enzyme activity.

Kcat sec^{-} value of muscle cathepsin B varied depending on the basis of calculation (Barrett and Kirschke, 1981). The lower value obtained on a protein basis could possibly be attributed to the presence of inactive material in the purified cathepsin B preparation. However, Kcat sec^{-} value of fish cathepsin B is lower than that reported for mammalian liver. While Kirschke et al. (1982) obtained a value of 158 for human liver enzyme, Katunuma et al. (1983) have reported, on a protein basis, a Kcat sec^{-} value of 8.01 for a rat liver preparation. Lower Kcat sec^{-} value of fish cathepsin B could be due to the intrinsic differences in the enzyme conformation, validity of which would be established only after values of muscle cathepsin B from different sources become available.

Like mammalian cathepsin B (Ninjoor et al., 1974), the fish enzyme displays both endopeptidase and carboxypeptidase activities. This is apparent by its ability to cleave both Z-arg-arg-NNap and Bz-arg-amide. However, neither the endo-aminopeptidase activity associated with cathepsin H, (Barrett and Kirschke, 1981) nor a true aminopeptidase activity was exhibited by fish cathepsin B because of its lack of hydrolytic response on Arg-NNap and Leu-NNap. It should be noted, however, that *Tilapia* muscle extract is rich in aminopeptidases. It also contains leupeptin-resistant Bz-arg-NNap hydrolase activity indicating the presence of cathepsin H. These activities were separated from cathepsin B by DEAE-Sephacel chromatography.

Elution of cathepsin B on Sephadex G-100 was preceded by the appearance of a small peak at 70 KD region which also hydrolysed Z-arg-arg-NNap. The similarity in the preferred substrate and high molecular weight seemed to suggest that this protein could be the enzyme described by Hirao et al. (1984) and Obled et al. (1984).

Protein substrates are not readily amenable to hydrolysis by cathepsin B (Hirao et al., 1984). The fish enzyme also displayed this property and attacked only azocoll, that too at low pH. Kirschke et al. (1980) have demonstrated higher hydrolysis of collagen at pH 3.0 than at pH 6.0. The inability of fish enzyme to cleave, proteolytically, hemoglobin, casein, azocasein or BSA leads us to conclude, that the preparation is indeed cathepsin B and not cathepsin L which is known to rapidly hydrolyze these proteins and also degrade Z-arg-arg-NNap (Kirschke et al., 1980; Barrett and Kirschke, 1981). It will be interesting, therefore, to study the action of cathepsin B and L individually and synergistically on the isolated muscle structural proteins.

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Effects of Storage Time and Temperature on the Microflora and Amine Development in Spanish Mackerel (*Scomberomorus maculatus*)

B.L. MIDDLEBROOKS, PAUL M. TOOM, WILLARD L. DOUGLAS, RICHARD E. HARRISON, and SANDRA MCDOWELL

ABSTRACT

Microbial content was characterized and levels of three amines (histamine, cadaverine, and putrescine) were determined in Spanish mackerel (*Scomberomorus maculatus*) decomposed at 0°C, 15°C, and 30°C for varying lengths of time. Correlations were shown (1) between the levels of the histamine, cadaverine, and putrescine and the time and temperature of decomposition, (2) between the ratios of cadaverine/histamine and putrescine/histamine levels and the temperature of decomposition, and (3) between increasing total microbial counts and rising amine levels. A total of 14 bacterial species with histidine decarboxylase activity were isolated from decomposing fish, including three species (*Acinetobacter lwoffi, Pseudomonas putrefaciens,* and *Aeromonas hydrophila*) not previously reported to have the potential to produce histamine.

INTRODUCTION

SCOMBROID POISONING caused by ingestion of seafood is one of the major causes of all food poisoning. Hughes et al. (1977) reported that nearly half of all foodborne illnesses of chemical origin which occurred between 1970 and 1974 were caused by either fish or shellfish poisoning. Almost half of these, 29 of 68 outbreaks, were due to scombroid poisoning. The group of fish most frequently implicated in scombroid poisoning is the family *Scombridae,* which includes the tunas, mackerels, and bonitos. Fish from other families have also been implicated in scombroid poisoning; for example, one of the fish species most recently found to be associated with the illness is the dolphin or mahi-mahi (*Coryphaena hippurus*) (Center for Disease Control, 1980).

Although the exact causative agent of scombroid poisoning is not known, histamine has been implicated as playing a major or perhaps primary role as potentiator of the illness. Histamine is formed by the decarboxylation of the amino acid histidine which is found in high levels in the tissues of scombroid fishes. When histamine is ingested it is detoxified primarily by the enzymes diamine oxidase and histamine-N-methyltransferase (Hui and Taylor, 1985). Pure histamine has been administered in relatively high doses to humans with no apparent ill effect (Arnold and Brown, 1978), leading to the suggestion that scombroid poisoning by spoiled fish is caused by histamine acting synergistically with other diamines, primarily putrescine and cadaverine, present in the fish (Bjeldanes et al. 1978). Putrescine is the decarboxylation product of the amino acid lysine and cadaverine arises from the decarboxylation of ornithine. Both putrescine and cadaverine may interfere with the normal histamine detoxification system of the intestine (by competing with histamine as substrates for diamine oxidase under certain conditions), leading to the suggestion that the combined effects of histamine and the diamines may be required for production of scombroid poisoning (Hui and Taylor, 1985).

Frank et al. (1981) reported the results of several studies on the distribution and classification of the types of bacteria present on many kinds of marine fish, both as natural microflora and as spoilage organisms. It was found that of those organisms which possess the ability to decarboxylate histidine to form histamine, the *Enterobacteriaceae* are primarily responsible for the decomposition of the scombroid fish. Taylor and Speckhard (1983) reported that *Morganella (Proteus) morganii, Klebsiella pneumoniae,* and *Hafnia alvei* are the only histamine-producing bacteria which have been isolated from fish causing scombroid poisoning, but indicated that these species are not normally associated with freshly caught marine fish. In addition to these species, *Proteus vulgaris, Proteus mirabilis, Clostridium perfringens, Enterobacter aerogenes,* and *Vibrio alginolyticus* have all been isolated from skipjack tuna (*Euthynnus pelamis*) and found to produce histamine (Arnold et al., 1980; Yoshinaga and Frank, 1982; Frank et al., 1983).

Edmunds and Eitenmiller (1975) undertook a study of the histamine and histidine decarboxylase levels in several species of marine fish, including the Spanish mackerel (*Scomberomorus maculatus*). This species was found to contain fairly high levels of histamine on spoilage. This study did not, however, include investigation of the diamines present in the decomposed fish or of the bacterial flora associated with these fish. The objectives of the present study were to examine the effects of storage temperature of Spanish mackerel on levels of histamine and other basic amines (specifically cadaverine and putrescine) in the fish, to determine the distribution of these amines in the fish, and to determine what correlation, if any, exists between the microbial flora developing in these fish and amine distribution and levels.

MATERIALS & METHODS

Source of fish used for study

All fish used for the study were Spanish mackerel caught by hook and line from a single area located approximately 3 miles south-southeast of the Chandeleur Island lighthouse in the northern Gulf of Mexico. Each fish was removed from the hook (without being touched by the handler if possible) and placed on ice until dead (10 to 15 min). It was then placed into a plastic bag containing several liters sea water (in all decomposition experiments, fish were held in sea water as it was felt that this procedure approximated the conditions under which fish are maintained in the holds of vessels after capture). Each bag and its contents were held temporarily on ice. Fishing continued until at least eight fish had been caught or until 6 hr had elapsed, at which time the fish were transported to the laboratory where incubation was begun at the appropriate temperature (timing of incubation was started at this point). The average time required to catch the fish was approximately 3 hr and transportation time to the laboratory was approximately 2 additional hr. A total of six groups of fish each were collected. The first three groups were collected within a 10-day period in June 1984, and the remaining three groups were collected during a 10-day period in August, 1984.

Author Middlebrooks is with the Dept. of Biological Sciences and Authors Harrison and McDowell are with the Dept. of Chemistry, Univ. of Southern Mississippi, Hattiesburg, MS 39406. Authors Toom and Douglas, formerly with the Univ. of Southern Mississippi, are now with the Center for Scientific Research, Southwest Missouri State Univ., Springfield, MO 65804.

Incubation of fish and preparation of fillets

A total of six decomposition experiments were performed during the source of this study, two at 0°C, two at 15°C, and two at 30°C. These temperatures were selected because they represent, respectively, the ideal temperature at which to store fresh fish prior to processing or sale to the consumer, a moderately cool temperature representative of conditions when refrigeration is employed but is inadequate, and a high temperature representative of conditions when no refrigeration is used. Three of the decomposition experiments, one at each temperature, were performed using groups of fish collected in June, 1984. The other three experiments were repeats of the first three and were performed using groups of fish collected in August, 1984. To perform each experiment, six fish were placed in separate plastic bags containing sea water and incubated at the desired temperature. At each of six sampling times, one fish was removed for analysis. For experiments conducted at 0°C, sampling times of 48 hr, 96 hr, 130 hr, 210 hr, 264 hr, and 380 hr were used. For experiments conducted at 15°C, sampling times of 22 hr, 44 hr, 68 hr, 92 hr, 120 hr were used. For experiments conducted at 30°C, sampling times of 6 hr, 12 hr, 24 hr, 32 hr, 40 hr, and 48 hr were used. Four fish, two collected in June and two collected in August, were used as zero time controls for all experiments. Anterior, median and posterior fillet sections were removed from each fish by placing the fish onto a clean, alcohol disinfected surface and removing three tissue sections from one side for bacterial analysis and three sections from the other side for analysis of amines. To remove each section, the skin was first peeled away and then a section of muscle tissue was cut out. As large a section as possible, depending upon the size of the fish, was removed, with a 1 to 2 cm space left between sections. Care was taken not to contaminate the sections with material from the gut cavity. Each individual section of fillet designated for amine analysis was placed into a small plastic bag, which was sealed, labeled, and stored at −20°C to await further processing (extraction of amines). Sections designated for microbial analysis were processed immediately.

Bacteriological Analysis

Total microbial counts. Total microbial counts were made from 10g tissue, homogenized in a sterile blender in 100 ml 0.1% peptone broth, using the aerobic plate count method (Gilliland, et al., 1976a). Duplicate plates were used at each dilution. Temperature of incubation was 7°C for fish spoiled at 0°C, 15°C for fish spoiled at 15°C, and 35°C for fish spoiled at 30°C. Incubation time varied according to temperature: 2 days at 35°C, 7–10 days at 15°C, 10–14 days at 7°C.

Characterization of bacterial populations. The same homogenates used for total microbial counts were used to inoculate plates for isolation/characterization studies of aerobic microorganisms present. The spread plate method was used to provide isolated colonies. For aerobic isolation/characterization studies, two sets of plates were inoculated, one incubated at 35°C to favor mesophiles, the other at 15°C to favor psychrotrophs (Gilliland, et al., 1976b). Each set of plates consisted of one trypticase soy agar plate and one MacConkey agar plate for each sample dilution (MacConkey agar plates were included to provide a degree of selectivity for Gram negative organisms should it have proven necessary, and because they provided a potential means of differentiating between colonies of similar appearance). For anaerobic studies, similar procedures were followed with the following exceptions. Homogenization of fish tissue samples was carried out in pre-reduced anaerobic broth (heated to remove dissolved oxygen) in a nitrogen atmosphere in an anaerobic glove box, and plates were placed in sealed anaerobic jars for incubation.

At the end of the incubation period, representative colonies from each set of plates were picked and inoculated into trypticase soy slants and broth (for aerobes) or solid and fluid thioglycollate medium (for anaerobes). All anaerobic isolations were conducted in an anaerobic glove box under nitrogen atmosphere. Gram stains were made of all isolates to determine purity and as a preliminary classification step. Isolates were subsequently identified using the appropriate Minitek Biochemical Differentiation System (BBL), following the manufacturer's directions. Additional morphological characterization and/or differential biochemical tests were employed as needed to complete tentative identification (Buchanan and Gibbons, 1974; MacFaddin, 1976). A total of 169 isolates were identified to the genus or species level and partial characterizations allowing tentative identification were carried out for an additional 200 isolates. All isolates showing evidence of amine formation were specifically designated for identification.

Identification of amine-forming organisms among isolates.

Isolates were screened for amine formation by inoculation of each organism into two types of media. The first was decarboxylase base medium supplemented with histidine, lysine, ornithine, or arginine, or with all four of these amino acids. All isolates were inoculated into histidine-supplemented medium to test for histidine decarboxylase activity and into medium supplemented with all four of the amino acids to test for decarboxylase activity specific for any of the amino acids. All isolates showing decarboxylase activity in the latter medium were inoculated into separate decarboxylase media supplemented with lysine, ornithine, or arginine to determine their specific decarboxylase activities. The second type of medium into which all isolates were inoculated was Spanish mackerel infusion broth (prepared by homogenizing muscle from fresh fish in twice its weight of water, steaming at 100°C for 1 hr, filtering and enriching with 1% glucose) (Omura et al., 1978). Bromcresol purple was included as pH indicator in all of these media. Inoculated tubes were overlaid with sterile mineral oil and each tube was incubated for 4 days at the temperature from which the organism was isolated. Tubes were examined daily for evidence of decarboxylase activity/amine formation as indicated by a change in color of the pH indicator. Cultures turning yellow and remaining yellow at the end of the incubation period were scored as negative (−), and those initially turning yellow, then changing back to purple were scored as positive (+).

Extraction of amines with methanol

Amines were extracted from the mackerel fillets using the methanolic extraction procedure of the AOAC (AOAC, 1980). Extracted samples were stored at 4°C until amine analyses were run.

Histamine Analysis

The procedure for histamine analysis was modification of the AOAC fluorometric method (AOAC, 1980). Anion exchange columns with a bed volume of about 4 mL packed gel were prepared from Dowex 1-X8 resin (Dow Chemical Co.) which had been converted to its −OH form with NaOH. Two hundred μL methanolic filtrate were placed onto the column and the sample eluted with about 7 mL deionized H_2O into a 10 mL volumetric flask containing 1 mL 0.1N HCl. The volume was adjusted to 10.0 mL with deionized H_2O.

The following constituents were then added sequentially to a 13 × 100 mm test tube: 500 μL column eluate or standard; 1.0 mL 0.0N HCl; 300 μL 1N NaOH; and 100 μL 0-phthalaldehyde (Sigma Chem. Co.) solution (0.1% in methanol). The mixture was vigorously stirred using a vortex mixer after addition of each component. It was incubated at room temperature for exactly 4 min following 0-phthalaldehyde addition and the reaction was then stopped with 300 μL 3.57N phosphoric acid. The fluorescence was determined within 1.5 hr on an Aminco-Bowman Spectrofluorometer using an excitation wavelength of 350 nm and an emission wavelength of 444 nm. The histamine concentration in the extract was determined by comparison to histamine standards prepared in 0.1N HCl.

For each batch of samples run, a set of three standards containing 0.5, 1.0, and 1.5 μg histamine per 5 mL were run, as well as a reagent blank. The histamine concentration in the sample was calculated by the following equation:

$$[(1/m) \times \text{Mean Sample Fluorescence} \times \text{Dilution/Net Weight}] \times \frac{}{100}$$

where m is the slope of the line created by plotting the relative fluorescence of the standards vs concentration.

Cadaverine and putrescine analysis

Cadaverine and putrescine levels in the methanolic extracts were determined by the method of Staruszkiewicz (1981) with modification. The instrument used for analysis was a Perkin-Elmer Sigma 1 gas chromatograph equipped with a 3% OV-225 column (1.8 m × 2 mm i.d.) and a nitrogen phosphorous detector set for nitrogen detection with a bead setting of 620. Operating conditions were as follows: (a) temperatures-injector, 230°C; detector, 230°C; column, 165°C; (b) gas flow rates—carrier gas (helium) 30 mL/min; hydrogen, 2.5 mL/min; air 100 mL/min. Hexanediamine (10μg/mL in 0.1N HCl) was used as internal standard.

For each analysis, 10 mL methanolic extract was placed into a round bottom flask. One mL internal standard and 0.5 mL 1N HCl

were added and the flask was swirled to ensure adequate mixing. The contents were then evaporated to dryness under a stream of nitrogen in a 50°C water bath. One mL ethyl acetate and 300 µL pentafluoropropionic anhydride (Pierce Chemical Co.) were added to the residue and the flask was stoppered, swirled to mix, and incubated at 50°C for 30 min. The contents were swirled once to mix the contents during the incubation. The stopper was removed and the contents were evaporated to dryness under nitrogen in the 50°C water bath. The residue was dissolved in 1 mL 30% ethyl acetate in toluene.

For each sample, a 1x8 cm column of neutral alumina (Fisher Scientific, Brockman Activity 1, 80/200 mesh) which had been equilibrated with water for 4 h prior to packing, was prepared and overlaid with 1 cm anhydrous sodium sulfate. Ten mL hexane was applied to the column and allowed to flow through. When the hexane level was about 1 mm above the surface of the bed, the dissolved residue was applied. The sample flask was rinsed twice with 3 mL portions of 30% ethyl acetate and the rinsings applied to the column, followed by two 9 ml portions of 30% ethyl acetate. All of the eluate was collected into a screw capped tube, placed at 50°C and concentrated to approximately 2 ml under nitrogen. Five µL of this sample was injected onto the gas chromatograph column.

A blank and a prepared standard containing known amounts of amines were run with each batch of samples. For the blank, 10 mL methanol was substituted for the sample. For the standard, 10 mL methanol was placed into the flask and 2 mL of a solution containing 10 µg/mL cadaverine and 5 g/mL putrescine in 0.1N HCl was added. The blank and standard were then assayed as described above for samples.

The cadaverine and putrescine concentrations in the samples were calculated as follows:

r = (*sample peak area*/hexaminediamine peak area)
− (*blank peak area*/hexaminediamine peak area)

r = ratio for standards and samples

The concentration of diamine in a sample is then given by:

C = S/r(standard) x r(sample) x 10/weight of sample x 100
[S is the concentration of the standard in mg/mL]

Table 1—Microbial isolates showing histidine decarboxylase activity[a]

Identification of isolate	Decomposition temperature(s) at which found
Acinetobacter lwoffi	0°C, 15°C
Aeromonas hydrophila	0°C, 15°C
Clostridium perfringens	15°C, 30°C
Enterobacter aerogenes	15°C, 30°C
Enterobacter sp.	30°C
Hafnia alvei	15°C, 30°C
Morganella morganii	0°C, 15°C, 30°C
Proteus mirabilis	15°C
Proteus vulgaris	30°C
Proteus sp.	15°C
Pseudomonas fluorescens/putida	15°C
Pseudomonas putrefaciens	0°C, 15°C, 30°C
Pseudomonas sp.	0°C, 15°C
Vibrio alginolyticus	0°C, 15°C, 30°C

[a]Activity determined using decarboxylase base broth supplemented with histamine

RESULTS & DISCUSSION

EIGHT BACTERIAL GENERA were represented among aerobic and facultatively anaerobic isolates from fish decomposed at 0°C. *Pseudomonas* species were the most frequently encountered, with 11/28 isolates identified as members of this genus. Nine of the isolates were of a single species, *Pseudomonas putrefaciens. Aeromonas hydrophila* was represented by six isolates and various *Proteus* species accounted for four isolates. Other species identified included *Citrobacter freundii, Vibrio alginolyticus, Acinetobacter lwoffi,* and *Morganella morganii.* A single isolate identified as *Staphylococcus aureus* may represent an airborne contaminant introduced at the time of plating since the colony from which it was taken was morphologically unique on the plate.

The 63 aerobic and facultatively anaerobic isolates from fish decomposed at 15°C included representatives of nine genera. Again *Pseudomonas* species were the most frequently encountered, with 16 representatives. *Aeromonas hydrophila, Vibrio alginolyticus,* and various *Proteus* species were also frequent isolates, with 14, 12, and 7 representatives, respectively. Other isolates included *Acinetobacter lwoffi, Hafnia alvei, Enterobacter aerogenes, Morganella morganii,* and *Staphylococcus sp.* Of these isolates only *Hafnia alvei* and one species of *Pseudomonas (Pseudomonas fluorescens/putida)* were not also represented among isolates from fish decomposed at 0°C.

Various *Pseudomonas* species accounted for 14 of the 32 aerobic and facultatively anaerobic isolates from fish decomposed at 30°C, with *Pseudomonas putrefaciens* the most frequently encountered species (5 isolates). Five *Enterobacter* isolates were identified, including three identified as *Enterobacter aerogenes.* Other genera found at 30°C but not at other temperatures include *Streptococcus* and *Alcaligenes* (specifically *Alcaligenes aquamarinum).*

Six anaerobic isolates from fish decomposed at 0°C were identified as *Peptococcus* species. Three anaerobic isolates from fish decomposed at 30°C were all members of genus *Clostridium,* with two identified as *Clostridium perfringens* and one as *Clostridium ramosum.* From fish decomposed at 15°C, 35 anaerobic isolates were identified, including 17 members of *Clostridium,* 6 of *Bacteroides,* 6 of *Eubacterium,* and 6 of *Peptococcus.* The single most frequently encountered species was *Clostridium perfringens* (12 isolates).

Most samples contained mixed populations of two or three different organisms. Many of the organisms identified might

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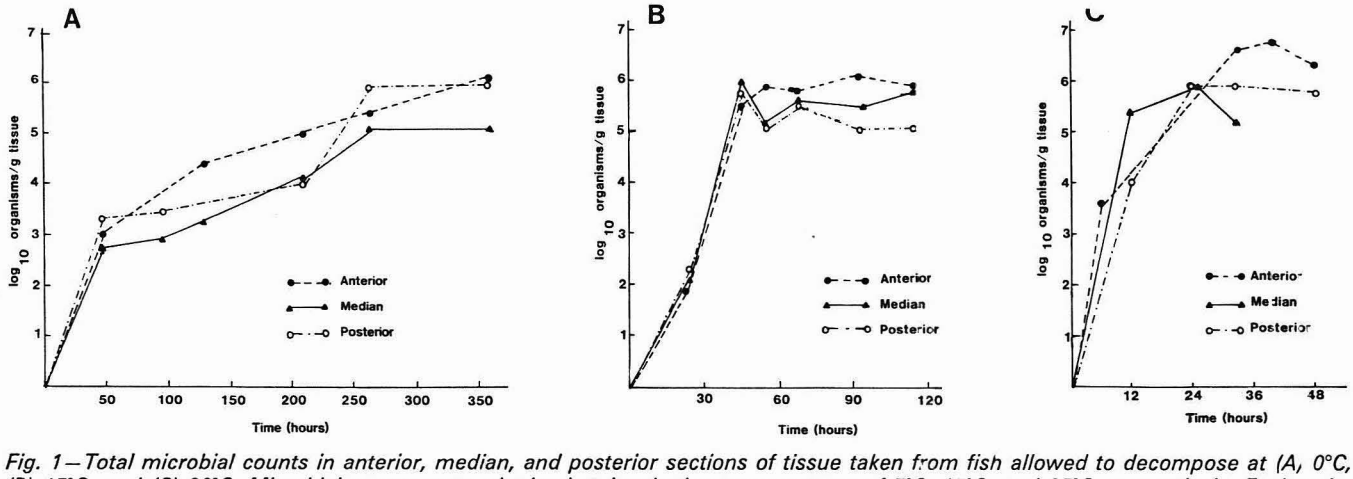

Fig. 1—Total microbial counts in anterior, median, and posterior sections of tissue taken from fish allowed to decompose at (A) 0°C, (B) 15°C, and (C) 30°C. Microbial counts were obtained at incubation temperatures of 7°C, 15°C, and 35°C, respectively. Each point represents the mean of two samples.

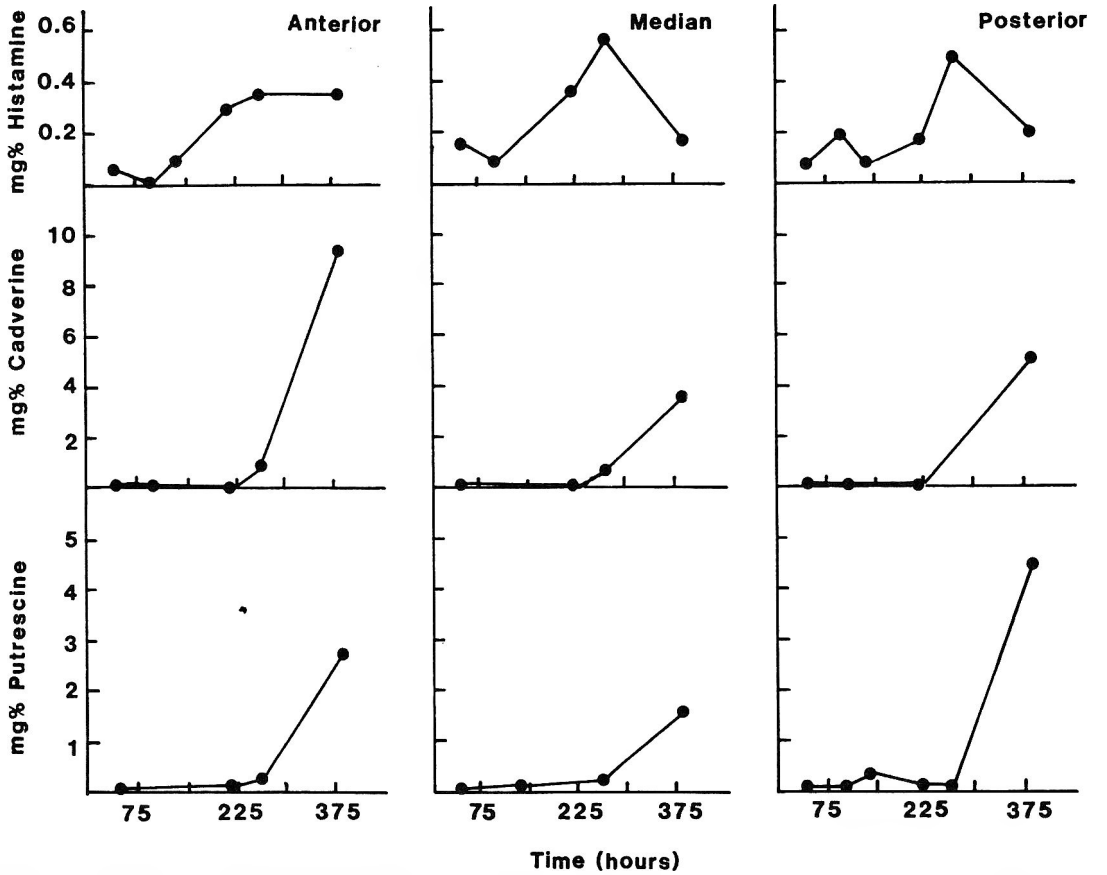

Fig. 2—Levels of histamine, cadaverine, and putrescine in anterior, median, and posterior sections of fish allowed to decompose at 0°C. Each point represents the mean of two samples.

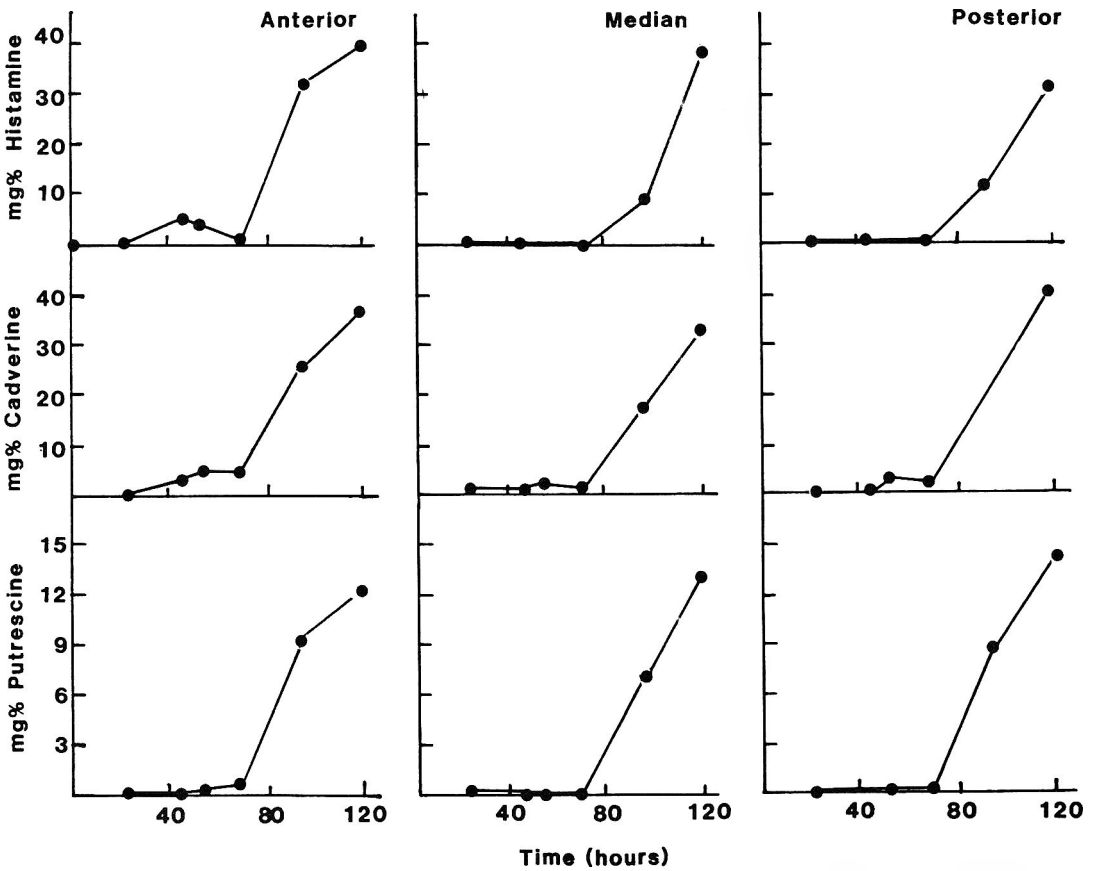

Fig. 3—Levels of histamine, cadaverine, and putrescine in anterior, median, and posterior sections of fish allowed to decompose at 15°C. Each point represents the mean of two samples.

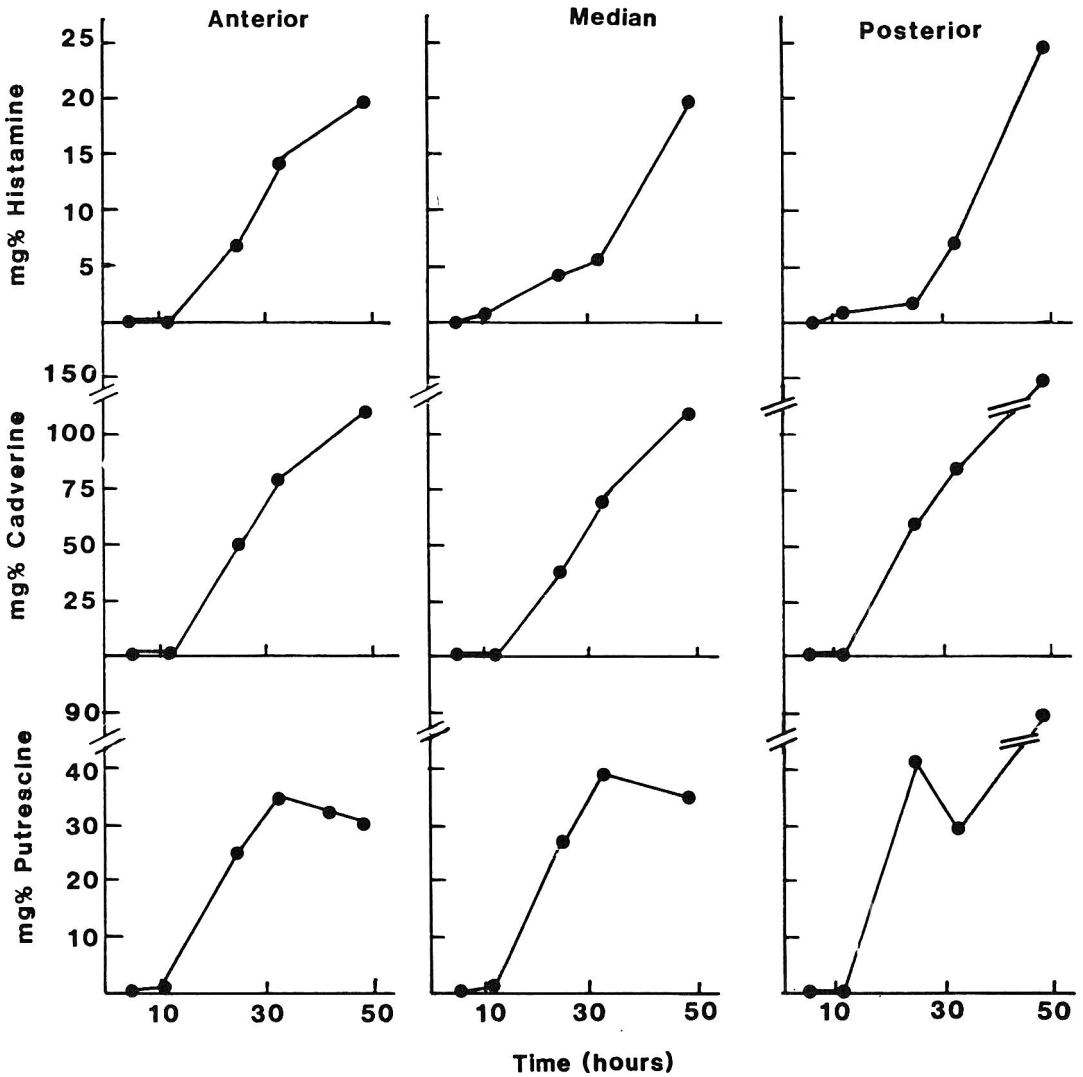

Fig. 4—Levels of histamine, cadaverine, and putrescine in anterior, median, and posterior sections of fish allowed to decompose at 30°C. Each point represents the mean of two samples.

be expected to be found as a result of human contamination of the fish during capture and handling (e.g. *Enterobacter sp.)* or because they are frequently associated with the marine environment and/or fish (e.g. *Vibro alginolyticus, Aeromonas hydrophila, Acinetobacter lwoffi).* Other organisms isolated are widely distributed in nature (e.g. *Clostridium sp., Pseudomonas sp.)* and may have already been associated with the fish at the time of capture or may have been introduced onto the fish during capture or subsequent handling prior to beginning the decomposition experiments.

Forty-nine out of approximately 400 bacterial isolates tested showed histidine decarboxylase activity. Among these isolates, 9 genera and 14 species were represented (Table 1). Some species were found only at one decomposition temperature, others at two, and still others at all three decomposition temperatures. The finding of potential histamine-formers in tissues of Spanish mackerel incubated at 0°C contrasts with the results of Arnold et al. (1980), who reported that no histamine-producing bacteria were isolated from tuna incubated at 1°C. Of isolates identified to species level, all except *Acinetobacter lwoffi, Pseudomonas putrefaciens,* and *Aeromonas hydrophila* were previously reported to form histamine in decomposing fish (Arnold et al., 1980; Yoshinaga and Frank, 1982; Frank et al., 1983; Taylor and Speckard, 1983). Tests for lysine, arginine, or ornithine decarboxylase activity were also performed for the same 400 isolates. Over 65% showed decarboxylase activity for one or more of these amino acids

Total microbial counts are summarized in Fig. 1. Maximum microbial counts were reached within 18–36 hr in fish decomposed at 30°C., but only after extended times in fish decomposed at 0°C. Counts were similar in tissue samples taken from anterior, median, and posterior sections of the fish. Increases in total microbial counts with time of incubation parallel increases in histamine levels in the same fish (Fig. 2 through 4).

Histamine levels in fish decomposed at 0°C remained low throughout the 380 hr (16-day) incubation period, with mean levels remaining less than 0.6 mg% (Fig. 2). The levels of both cadaverine and putrescine began to rise sharply at about 264 hr (11 days). Cadaverine levels of approximately 4–10 mg% and putrescine levels of approximately 2–4.5 mg% were found in the various sections at 380 hr.

The amine data for fish decomposed at 15°C are presented in Fig 3. Concentrations of all three amines remained low before 68 hr and then began to increase rapidly, reaching levels much higher than were observed for any of the samples from fish incubated at 0°C. Histamine and cadaverine levels approaching 40 mg%, and putrescine levels of over 12 mg% were found in all sections at 120 hr.

In fish decomposed at 30°C amine levels are remained almost undetectable at 12 hr (Fig. 4), but by 24 hr levels of all three amines had begun to increase. By 48 hr, levels as high as approximately 25 mg%, 150 mg%, and 90 mg% were observed for histamine, cadaverine, and putrescine, respectively. Levels of all amines were similar in all sections.

In studies on the distribution of histamine in decomposed tuna (Lerke *et al.* 1978, Frank *et al.* 1981) the highest levels of histamine were reported in the anterior muscle sections adjacent to the gills, presumably because of the presence of histamine-forming organisms in the microbial population of the gill cavity which colonized the anterior sections. In the present study, the bacterial populations isolated from the anterior, median, and posterior sections of individual Spanish mackerel were similar in all cases, and bacterial species with histamine decarboxylase activity were found with similar frequency in all sections. Amine levels in the various sections of individual fish were also similar. These findings suggest that under the conditions of the study (which as has been stated were similar to conditions under which fish are stored in vessels after capture), microorganisms entered muscle tissue via the skin from the water in which the fish were held. The source of these microorganisms could have been the gill cavity, the gut, or the environment.

Putrescine/histamine and cadaverine/putrescine ratios obtained in this study varied considerably at the various decomposition temperatures. In fish held at 15°C both ratios were less than 1/1 in all sections (Fig.3). In fish held at 30°C, a putrescine/histamine ratio of almost 8/1 was found in some samples (median fillet section, 24 hr sample and posterior fillet section, 32 hr sample); the putrescine/histamine ratio in the final (48 hr) samples from this experiment was 3/1 or less. The cadaverine/histamine ratio was over 5/1 in all fillet sections at all sampling times (Fig. 4).

In fish held at 0°C, a maximum cadaverine/histamine ratio of over 20/1 was observed (in anterior fillet sections at 380 hr and a maximum putrescine/histamine ratio of over 20/1 was observed (in posterior fillet sections at 380 hr) (Fig.2). The present study with Spanish mackerel thus indicates that not only the levels of amines, but also the ratios of these amines in a fish, reflect the temperature at which the fish was decomposed. Hui and Taylor (1985) found that potentiation of histamine by inhibition of histamine-metabolizing enyzmes occurs only at cadaverine/histamine or putrescine/histamine ratios of 5/1 or greater. In the present study, such ratios were found in some samples from fish held at 0°C and at 30°C, but in both cases histamine levels were well below the Food and Drug Administration's hazard level of 50 mg% for histamine in tuna fish. Indeed histamine levels in all fish examined in this study remained below the hazard level, although levels in fish held at 15°C approached it (Fig.3).

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Shelf-life of Sterile Yellow-eyed Mullet (*Aldrichetta forsteri*) at 4°C

GRAHAM C. FLETCHER and JO A. STATHAM

ABSTRACT

The relative effects of microbial and nonmicrobial spoilage on the shelf-life of yellow-eyed mullet were studied. Results of sensory and chemical analyses of sterile flesh stored at 4°C were compared with fillets which had either spoiled naturally while held at 4°C or frozen fillets held at −18°C. Inosine was produced rapidly in both treatments at 4°C, followed in sterile flesh by a slower breakdown to hypoxanthine. Hypoxanthine production from inosine was rapid in the presence of bacteria. Within 6 days sensory changes were observed in the frozen flesh and after 69 days, it was considered unacceptable. The development of off-odors and off-flavors in the absence of bacteria was not sufficiently slow to result in a significant extension in shelf-life for this species.

INTRODUCTION

IN RECENT YEARS many researchers have examined methods to extend the shelf-life of various chilled seafoods by inhibiting or preventing bacterial spoilage. Several successful methods have been devised including the use of ionizing radiation, carbon dioxide, and sorbates. For a given temperature, increases in the shelf-life of fish fillets of 1.5 to 5 times have been observed, but generally it is only the period of moderate to low quality which has been extended. The reason offered for the inability to preserve the initial high quality is that nonmicrobial factors such as autolysis or oxidation (free radicals) have contributed to product quality loss (Miyauchi, 1972; Fey and Regenstein, 1982; Statham, 1984).

Underlying much of the work is the assumption that, although autolysis results in an initial loss of quality, overt spoilage is due primarily to bacteria. For example, Shewan (1976) reported that, as volatile sulfur compounds and esters from amino acids are not produced in the absence of bacteria, sterile fish muscle can be kept at 0°C for 6 wk or more without serious sensory changes being discerned. However, little work has been reported on the respective effects of autolysis, oxidation and bacterial spoilage on the sensory qualitities of fish. It is, thus, important to examine spoilage in the absence of bacteria.

Seafoods have been sterilized by irradiation but the doses required to sterilize fish are so high that significant chemical changes have resulted. Hobbs and Hodgkiss (1982) reported that the flesh had sufficient unpleasant odors and flavors to mask any changes resulting from storage. Pasteurization by irradiation results in some shelf-life extension, but the products undergo a progressive loss in quality even in the absence of significant bacterial spoilage. Miyauchi (1960) found that storage of irradiated (100–200 krads) cod fillets for 6 wk at 0 to 1.7°C resulted in sensory ratings that were at the limit of acceptability. Fresh fish flavors were replaced by chalky and condensed milk flavors. In addition, slight sour odors; firmer, rubbery, chewy texture and a darkening of white meat developed. Although bacterial growth occurred, these changes appeared to result more from enzymatic and other chemical reactions than from bacterial spoilage. Hansen (1979) stated that the eating quality of irradiated trout decreased slowly but steadily at 0°C, until it became unacceptable after about 4 wk. In the absence of significant bacterial spoilage, Hansen (1979) attributed this to reactions such as nucleotide catabolism.

It has long been known that the flesh of healthy live fish is sterile (Liston, 1980). Several workers have isolated sterile flesh for use in various experiments (Ehira, 1976; Herbert, 1970; Lobben and Lee, 1968; Miller et al., 1973). However, only one report has been published where the shelf-life of naturally sterile flesh has been evaluated by sensory assessment. Herbert (1970) kept sterile cod at 2°C as a reference treatment when examining the effects of individual bacterial cultures on fish flesh. The Torry white fish panel, examining the sterile flesh over a period of 24 weeks, found that there was a loss of initial fresh fish odors and flavors and the development of the "cold-store" odors and slightly bitter flavors usually associated with frozen storage. However, "none of the odors and flavors associated with naturally spoiling cod fillets had developed." Herbert (1970) concluded that, "in white fish autolytic enzymes play a minor part in the development of characteristic spoilage type odors and flavors." This generalization cannot be extended to all types of fish because of species to species variation in fat and rates of nucleotide degradation. It would, therefore, be expected that nonmicrobial spoilage could influence the inherent shelf-life of the flesh from some species.

Yellow-eyed mullet (*Aldrichetta forsteri*) is common in estuarine waters of New Zealand and Australia. The annual catch in New Zealand is reported as 24 tons (Vlieg, 1984) while in Australia, 940 tons were caught during the 1983-84 season (Anon., 1985). Although in western countries, mullet are generally among the lesser-preferred species, the annual world catch is in the excess of 170,000 tons (FAO, 1984).

The shelf-life of members of the family Mugilidae is often limited due to rapid oxidation of lipids (Mendenhall, 1972; Sumner et al., 1984). The object of this study was to design a specific experiment to determine the shelf-life of sterile flesh from yellow-eyed mullet using sensory profiles and nucleotide analysis. Sterile flesh must be prepared from fish immediately after being killed; this species was chosen because of its availability and its ability to survive capture.

MATERIALS & METHODS

Mullet

Yellow-eyed mullet were line-caught from the Derwent Estuary on the south-east coast of Tasmania in March, 1985. Weights were between 174–290g (mean 233, s.d. = 48g). They were held live for 12 hr on the fishing vessel in seawater tanks that were continuously refilled from the sea, then transferred to laboratory tanks of recirculating seawater until processed.

Forty-three fish were used to prepare sterile flesh samples within 5 hr of arrival at the laboratory. They were killed by a blow on the head, immediately immersed in a Na_2CO_3 solution (5% w/w) and gently scrubbed to remove most of the surface slime (Herbert et al., 1971). After rinsing in tap water, the surface was sterilized by immersing for at least 5 min in 70% ethanol containing crystal violet (Ehira, 1976). The fish surface was then dried with sterile air in a laminar flow cabinet (Elephant CWS 1200, Gelman Sciences, Sydney,

Author Fletcher is affiliated with the Div. of Horticulture & Processing, DSIR, Mt. Albert Research Centre, Private Bag, Auckland, New Zealand. Author Statham is affiliated with CSIRO, Div. of Fisheries Research, Seafood Technology Group, "Stowell," Stowell Ave., Hobart, Tasmania, Australia, 7000.

Australia). Sterile flesh samples were obtained as follows: on one side of each fish a cut around the edge of the skin was made with a sterile scalpel; and the skin was rolled and pulled off with sterile forceps. Two to four sections of flesh (1–6g/section) were then excised from each fish, avoiding the gut cavity; areas were stained by crystal violet and hence also alcohol or areas heavily contaminated with blood. The flesh excised from each fish was placed in a sterile glass jar (100 mL) and sealed. The jars were chilled in ice then placed at 4 ± 0.5°C.

The other side of each fish was filleted and skinned. Five fillets were used as fresh samples (T=0) for taste panel analysis and the remaining fillets were vacuum-packaged in polyethylene (OTR >2400 mL/m^2/24 hr/atm) bags (5/bag), blast-frozen to −30°C and placed in −18 ± 0.5°C cold storage. This was the frozen treatment used as a standard of reference. Due to the time required to prepare sterile flesh, the 14 fish used to monitor normal microbial spoilage were held for 22 hr in the laboratory tanks and then killed. It was assumed that this extra time in captivity would have little effect on subsequent autolysis and oxidation. These were then filleted, skinned, placed in unsealed polyethylene bags (7/bag) and held at 4 ± 0.5°C to allow normal aerobic spoilage.

Sampling procedures

Concurrent samples were taken from the three treatments. However, as the naturally spoiling fillets were prepared 1 day after the others, the sterile and frozen samples were tested 2, 6, 9, 13, and 16 days after death while the naturally spoiling samples were tested 1, 5, and 8 days after death. A final sample of frozen mullet was examined after 69 days.

When possible, the sterility of the flesh in each jar was confirmed before its sensory and chemical qualities were assessed. The sterile jars were opened in a laminar flow cabinet and a small piece of flesh was excised and placed in 5 mL sterile nutrient broth with 0.5% added NaCl. This was incubated under aerobic conditions for 48 hr at 22°C and examined for turbidity. Growth was confirmed by microscopy of a wet mount. A loop of the broth was also streaked onto plates of Tryptone Soya Agar containing 0.2% yeast extract, 0.2% glucose and 0.5% NaCl (TSYG). Plates were examined for growth after 24 hr incubation at 22°C. If positive growth occurred in either the broth or the plates, the jar was rejected as contaminated. When it was not possible to test samples for sterility prior to quality assessment, they were tested subsequently and the results treated accordingly. After a week, most contaminated samples were obvious by the appearance and odor of the raw flesh in the jar.

On each sampling day, one bag of frozen mullet was placed at 4°C to thaw for 2.5 hr before sampling. One bag of naturally spoiling fillets and four to five jars of sterile flesh were used on each occasion. Two to five gram subsamples from each fillet of naturally spoiling flesh and from each jar of sterile flesh were held in liquid N_2 for later analysis of purine derivatives.

Analytical procedures

Chemical. pH was measured at five locations on the skeletal side of each naturally spoiling fillet and on at least one position of each piece of sterile flesh using an Orion pH meter equipped with a surface electrode. Total lipid was estimated on duplicate samples from five frozen fillets by the method of Hanson and Olley (1963).

Nucleotide derivatives were determined by high pressure liquid chromatography (HPLC) using the method of Ryder (1985).

Microbiological. Two fillet from the bag of naturally spoiling flesh were used to determine aerobic plate counts (APC). The fillets were individually homogenized in 100 mL sterile saline with a Colworth stomacher 400 (A.J. Seward, Suffolk, England), serial decimal dilutions prepared, and 0.1 mL aliquots of appropriate dilutions were spread on duplicate plates of TSYG. Colony forming units were counted after 4 days incubation at 22°C.

Sensory. For each treatment, the flesh to be evaluated was pooled into a single bowl. The whole fillets were cut into pieces of similar size to those of the sterile flesh (approximately 2.5 cm^2). Each bowl of flesh was cooked in a microwave oven to an internal temperature of 70°C and presented to the panel of 8–10 trained persons who were recruited from the CSIRO's staff. The score sheet used consisted of a profile containing optional and compulsory (typical odor, off-odor, typical flavor, salty, sweet, acid, bitter and off-flavor) terms descriptive of fresh and spoiling fish. The descriptors used were similar to those incorporated in a profile developed independently by Hogg and Scott (1984). Profile descriptors were scored on a 0–9 scale (0=absent, 9=strong); however, the panelists had not been instructed on how the scale should be structured, i.e., they were not given an example of the limit of intensity for each descriptor. Overall, texture, odor, and flavor acceptability were scored using the 7-point Smiley scale (Street and Carroll, 1972).

At each session the frozen sample (control) was evaluated first, then the other two treatments in random order. Initially, each person evaluated the odor and recorded his description and acceptability rating on the score sheet. A brief discussion was then chaired by the panel leader (author Fletcher), with panelists free to add descriptors which they had been unable to recognize, before proceeding to taste the sample. Panelists were allowed to omit tasting the sample if they considered it too objectionable. After the panel had recorded the description and acceptability ratings of the flavor and texture, a second discussion was held before the next sample was presented or the session was closed.

Data presentation

pH values and texture scores were subjected to analysis of variance. Results were compared from the same withdrawal day.

The individual scores for each profile descriptor were totalled and listed as an integer value on a 0–100 scale as a proportion of the total possible panel score (Bremner et al., 1988). Only those descriptors which were scored by more than 40% of the panel to give a total score of 16 or above (i.e., a mean score of approximately 4 on the 0–9 scale) and were common to all three treatments were listed. Descriptors which had significant scores, by the same criteria, but were not common to all treatments are discussed in the text. The complete profiles are not presented in this paper but have been presented in unpublished work (Fletcher and Statham, 1987).

RESULTS & DISCUSSION

Lipid content of mullet

The mean fat content of the fillets was 3.5% (s.d.=1.4%), a value higher than that reported by Vlieg (1984) who found that fillets of yellow-eyed mullet caught in New Zealand in February had a mean oil content of 1.8%.

Bacteriology

Of the 43 jars of flesh, only 19 proved to be sterile prior to sensory assessment, a far lower proportion that that obtained by the Torry laboratories (Burt, 1982) or this laboratory using the same techniques on white fish. This may be due either to mullet being a smaller fish, and hence, more difficult to sample successfully or it may indicate that the flesh of some species is not as uniformly sterile as that of others. Two of the jars examined 13 days after death were subsequently shown to have been contaminated, so results for this day were not presented.

The $\log_{10}$/g of the APCs from the naturally spoiling samples were 3.6 and 8.0 at 0 and 5 days, respectively, suggesting a shelf-life of less than 5 days (Shewan, 1938).

pH and textural changes

The typical pH fall and subsequent statistically significant rise (Amlacher, 1961) is shown (Table 1) for both naturally spoiling and sterile fish, indicating the autolytic production of alkaline compounds in both treatments. The increase in pH of the naturally spoiling fish at day 8 may be attributed to the production of bacterial metabolites of advanced spoilage.

None of the treatments showed consistent changes in sensory textural characteristics over their respective storage periods (Table 1). However, the sterile flesh was overall drier ($p<0.01$) and firmer ($p<0.01$) than the other two treatments and was tougher ($P<0.05$) than the frozen material. After 16 days, it had less ($p<0.01$) succulence than the frozen sample. It should be noted that wetness and firmness were significantly correlated with pH in the sterile flesh. The texture of the sterile flesh was less preferred ($P<0.01$) than that of the other two

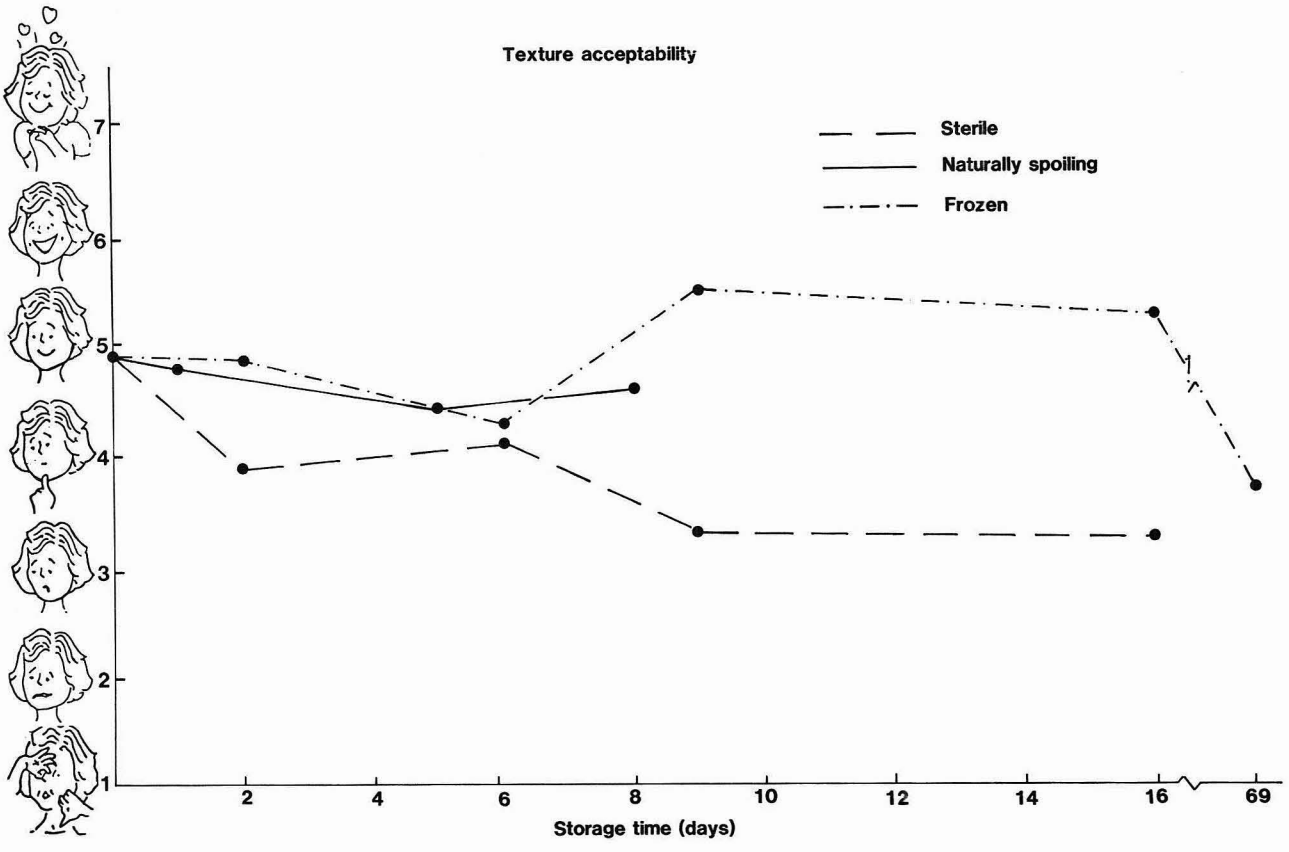

Fig 1.—Texture acceptability of sterile, naturally spoiling and frozen yellow-eyed mullet.

Table 1—Texture scores[a] and pH for stored mullet

Treatment	Attribute	Storage time (days)					Correlation with pH
		0 (Fresh)	1	5	8		
	pH	6.89	6.50	6.73	7.14		
	Wetness	2.67	3.62	2.00	3.50		−0.04
	Firmness	6.56	6.11	6.00	6.00		+0.04
Naturally	Springiness	6.00	6.13	5.67	6.00		−0.07
spoiling	Toughness	6.11	6.50	6.22	6.00		−0.97*
	Succulence	3.88	5.00	3.44	4.50		−0.20
	Fibrousness	6.78	6.63	6.33	5.00		−0.75
	Texture acceptability	4.78	4.70	4.33	4.50		−0.16
		0 (Fresh)	2	6	9	16	
	pH	6.89	6.55	6.78	6.75	6.82	
	Wetness	2.67	1.00	1.89	1.67	2.15	−0.97**
	Firmness	6.56	7.22	6.56	6.89	6.66	−0.93*
Sterile	Springiness	6.00	6.56	5.67	6.11	5.83	−0.78
	Toughness	6.11	7.33	6.78	7.22	6.17	−0.84
	Succulence	3.88	4.22	3.89	3.33	3.17	−0.50
	Fibrousness	6.78	6.89	5.89	6.44	5.83	−0.39
	Texture acceptability	4.78	3.75	4.00	3.22	3.16	0.32
		0 (Fresh)	2	6	9	16	
	Wetness	2.67	2.56	2.11	3.12	2.20	
	Firmness	6.56	6.33	6.78	5.75	6.80	
Frozen	Springiness	6.00	5.78	5.89	5.25	6.10	
−18°C	Toughness	6.11	5.56	6.11	5.13	6.20	
	Succulence	3.88	4.11	4.22	3.63	3.50	
	Fibrousness	6.78	6.11	5.89	5.50	5.76	
	Texture acceptability	4.78	4.75	4.17	5.38	5.13	

[a] Means of scores, using a 1-9 scale, where 9 is the anticipated maximum intensity for the attribute.
* Significant at the 5% level; **Significant at the 1% level.

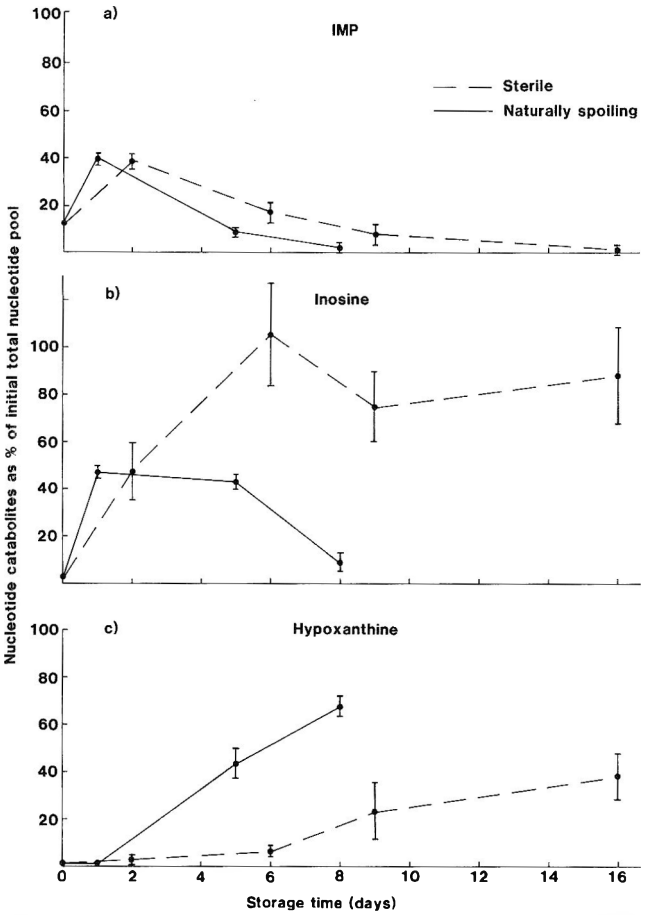

Fig. 2—Nucleotide breakdown in sterile and naturally spoiling mullet (a) IMP, (b) Inosine, (c) Hypoxanthine. Vertical bars represent standard errors.

treatments with mean scores below 4 on the Smiley scale after only 2 days (Fig. 1). After 69 days at −18°C, the texture acceptability of the frozen sample had dropped ($P<0.05$) to a mean of 3.6. If a score of 4 is considered marginal, this means the product is unacceptable.

Nucleotide catabolism

Results of nucleotide analyses are presented in Fig. 2. Amounts of catabolites are shown as a percent of the original total nucleotide pool (11.77 micromoles/g, S.E. ± 0.10). The results from the sterile samples showed greater variation than those from the naturally spoiling ones. This was possibly due to either the inability to sample from the same location on the fillet consistently or because of the small size of samples used. IMP was broken down in a similar manner for both treatments (Fig. 2a). Inosine (Fig. 2b) formed rapidly and accumulated as the major proportion of the total pool in the sterile flesh. The flesh of sea mullet (*Mugil cephalus*) has also been shown to accumulate inosine (Sumner et al., 1984). The breakdown of a small amount of inosine was reflected by a slow but steady increase in hypoxanthine in the sterile flesh (Fig. 2c). In the presence of bacteria, inosine did not accumulate but was rapidly broken down with a concomitant rise in hypoxanthine. The total nucleotide pool in the naturally spoiling fillets was reduced to approximately 70% of the original value after 8 days, suggesting that compounds produced during the later stages of nucleotide degradation were not detected at the wavelength used (254 nm). Murray et al. (1984) have also reported a loss of total nucleotides at this wavelength and have shown that considerable amounts of nucleotide catabolites were leached out in ice water during storage, but in the present experiment the fish was stored at 4°C with no significant drip loss.

Sensory evaluation

The descriptors common to all three treatments are listed in Tables 2 and 3. Typical odor declined in the frozen mullet between 16 and 69 days and decreased markedly between 9 and 16 days in the sterile mullet and between 5 and 8 days in the spoiling mullet. Off-odors increased after 16 days in the frozen mullet and continuously in the sterile and naturally spoiling mullet, with the naturally spoiling mullet reaching approximately the same score in 5 days as the sterile mullet did in 9 days. The sweet odor persisted throughout the frozen storage period but was lost rapidly in both the sterile and naturally spoiling samples. The acetic acid odor which developed in the sterile and naturally spoiling mullet was also detected in the frozen material at 69 days. A sour odor [17(5)] was also detected in the frozen material at 69 days. Ammonia and stale urine were terms used to describe the naturally spoiling [81(9)], [36(5)], and sterile [24(5)], [21(4)] mullet at their final sampling times. In addition sterile mullet was described as pungent [20(4)], [35(7)], and rancid oily [28(5)], [27(5)] at 9 and 16 days, respectively.

The typical flavor in the frozen mullet decreased between 16 and 69 days and declined continuously in the sterile and naturally spoiling samples. The off-flavor increased to [52(10)] during the 69-day storage period. A similar level was reached at 16 days in the sterile samples and between 5 and 8 days in the naturally spoiling material. The sweet flavor was lost in the naturally spoiling and sterile material but remained during the entire period of frozen storage. A cardboard flavor, suggestive of rancidity, increased to a score of [29(6)] in the sterile sample at 9 days and to [33(9)] in the naturally spoiling sample at 5 days, but appeared to be masked by the strong bitter flavor in these treatments at the last sampling times. The bitter flavor score (Y) of samples in both treatments was highly correlated ($r^2=78.4$) with the level of hypoxanthine (X, micromoles/100g) in the flesh ($Y=5.53+8.3X$). It should be noted that only two out of nine, and six out of eight, panelists were willing to taste the final samples of naturally spoiling and sterile mullet, respectively.

Creamy and fresh oily flavors were lost rapidly from the material held at 4°C but were lost only gradually from the frozen material. A waxy note persisted during frozen storage until 69 days, with scores ranging from [16(4)] to [18(6)]. This

Table 2—Odor changes in stored mullet[a]

	Treatments												
		Frozen					Sterile				Naturally spoiling		
	Fresh	Storage time (days)					Storage time (days)				Storage times (days)		
Odor descriptors	0	2	6	9	16	69	2	6	9	16	1	5	8
Typical odor[b]	62(9)[c]	70(9)	64(8)	67(8)	62(8)	45(10)	61(9)	46(8)	41(9)	21(8)	51(9)	52(8)	20(9)
Sweet	17(5)	25(6)	13(5)	16(4)	16(4)	18(7)	15(5)	–	–	–	20(5)	–	–
Acetic	–	–	15(7)	–	–	19(6)	–	18(5)	21(4)	14(3)	–	23(5)	33(7)
Off-odor	3(9)	2(9)	19(8)	9(8)	14(8)	48(10)	8(9)	37(8)	51(9)	62(8)	15(9)	46(8)	94(9)

[a] Only those descriptors which were scored by more than 40% of the panel, giving a total score of 16 or above, and were common to all treatments are listed.
[b] Those descriptors for which scoring was compulsory are shown in italics.
[c] Total panel scores (0-100 scale) for each descriptor are shown, followed, in parentheses, by the number of panelists who marked that particular descriptor.

Table 3—Flavor changes in stored mullet[a]

	Treatments												
		Frozen					Sterile				Naturally spoiling		
	Fresh	Storage time (days)					Storage time (days)				Storage time (days)		
Flavor descriptors	0	2	6	9	16	69	2	6	9	16	1	5	8
Typical flavor[b]	65(9)[c]	57(9)	54(9)	64(8)	56(8)	39(10)	45(9)	42(9)	30(9)	17(6)	61(9)	39(9)	23(2)
Sweet	17(9)	18(9)	10(9)	14(8)	13(8)	22(10)	15(9)	7(9)	4(9)	–	18(9)	2(9)	–
Bitter	3(9)	13(9)	12(9)	9(8)	9(8)	21(10)	10(9)	19(9)	19(9)	51(6)	14(9)	26(9)	84(2)
Cardboard	13(5)	15(4)	13(4)	13(4)	13(4)	21(5)	6(2)	19(5)	29(6)	–	5(2)	33(9)	–
Off-flavor	4(9)	5(9)	20(9)	10(8)	10(8)	52(10)	17(9)	30(9)	45(9)	51(6)	5(9)	35(9)	78(2)

[a] Only those descriptors which were scored by more than 40% of the panel, giving a total score of 16 or above, and were common to all treatments are listed.
[b] Those descriptors for which scoring was compulsory are shown in italics.
[c] Total panel scores (0-100 scale) for each descriptor are shown, followed, in parentheses, by the number of panelists who marked that particular descriptor.

flavor disappeared between 9 and 16 days in the sterile material and did not reach a significant intensity in the naturally spoiling material. Chicken and turkey were transient flavors in the frozen and sterile samples. These may be regarded as characteristic of non-microbial spoilage, either autolytic or oxidative. In the naturally spoiling material, these features may have either been prevented from developing or have been masked by bacterial spoilage, or the compounds responsible might have been degraded by the bacteria.

While the samples held at 4°C became rancid, the frozen sample developed a sour flavor at 69 days [27(9)]. There was a burnt flavor in the sterile sample at 9 [18(5)] and 16 [21(4)] days.

The mean Smiley scores from the odor, flavor, and overall acceptabilities were very similar; only the results for flavor acceptability are shown (Fig. 3). The low acceptability of the fresh fish at the beginning of the experiment was typical of the low scores normally given to steamed fish (Statham et al., 1985). The flavor acceptability of fish from the naturally spoiling and sterile treatments began to fall within the first two days of storage. In the case of naturally spoiling flesh, it was totally rejected after 8 days. The decline in acceptability of the sterile flesh was slightly slower, confirming that bacteria did play a significant role in the later stages of spoilage. The prediction of fish flavor changes with IMP degradation and hypoxanthine production has been discussed elsewhere (Fletcher et al., 1988). It should, however, be noted that the rapid breakdown of IMP in this species precludes a high acceptability rating for lengthy periods of storage.

The similarity in the sensory changes noted in the sterile and naturally spoiling mullet suggested that loss of quality could be attributed to the actions of nonmicrobial agents. Bacterial enzymes appeared to result in intensification of sensory characteristics similar to those of autolysis rather than the development of different ones.

The frozen material showed considerable changes during storage and was considered unacceptable after only 10 wk at −18°C. This showed that yellow-eyed mullet had a very short shelf-life during frozen storage and that fillets stored at −18°C were not a suitable reference for comparison with other treatments.

The effects of season, sex, feeding habits and catching methods result in great variability both within and between species

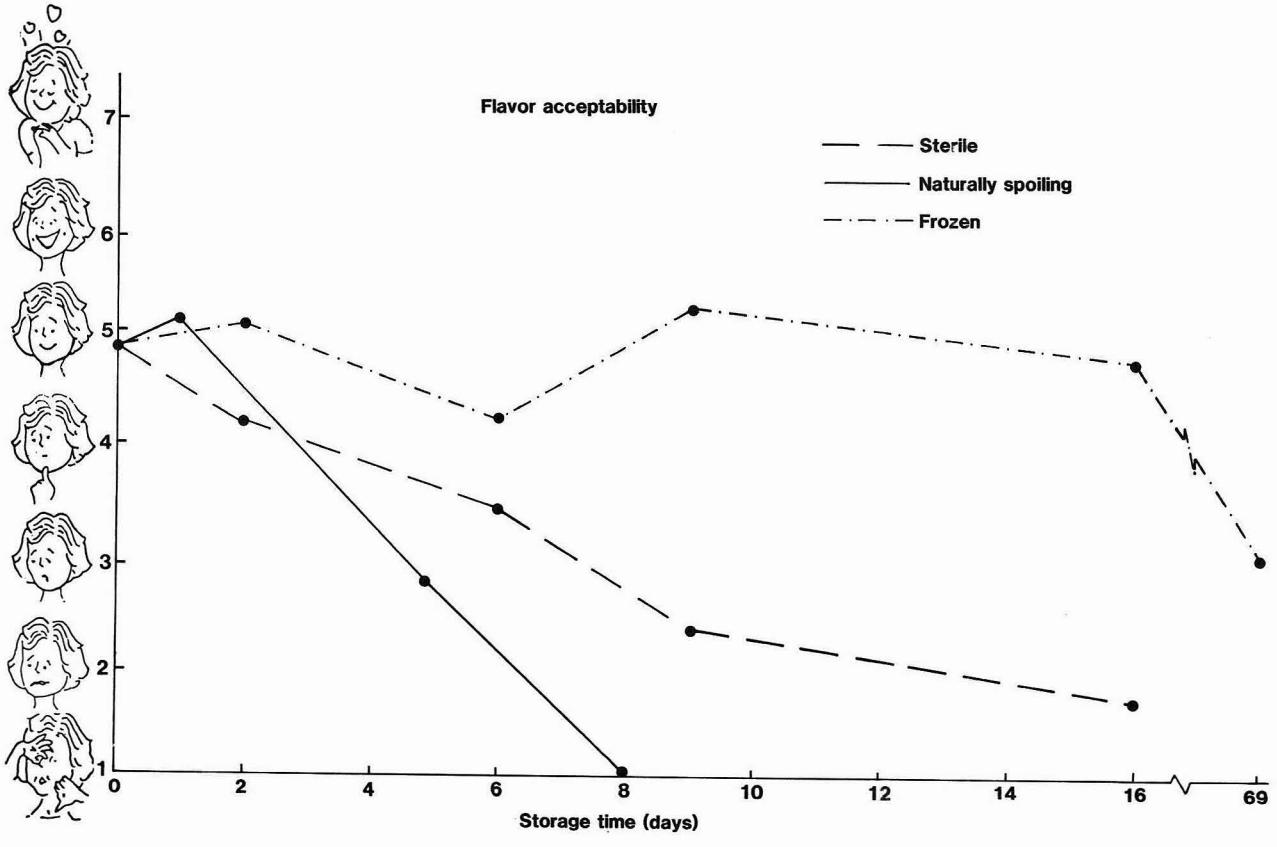

Fig. 3—Flavor acceptability of frozen, sterile and naturally spoiling yellow-eyed mullet.

of fish; therefore, the conclusions that can be drawn from these results are limited in their application to other situations. However, some significant implications should be considered.

First, to attempt to extend the high quality shelf-life of mullet stored in air at 4°C by inhibiting bacteria may not be as successful as would be expected. Although microbial spoilage did result in a more rapid development of some of the more offensive odors and flavors, the end of shelf-life, as determined by the current methods of sensory evaluation, was reached at a similar time whether bacteria were present or not. If results similar to the current work are found with other fatty species or species which are rapid producers of hypoxanthine, technologies depending on inhibiting microbial spoilage to extend shelf-life have very definite limitations. Oxidation may be prevented to some extent by combining the anti-bacterial treatment with storage under low oxygen tensions. Further extension in the shelf-life of fresh fish may only be accomplished by preventing autolytic spoilage; freezing is the only practical way of doing this.

Secondly, the fact that microbial and autolytic spoilage resulted in essentially the same sensory properties suggested that, at least for this species, bacteria generally only accelerated reactions that would have occurred by autolysis. This lessens the importance of describing the effects of particular bacteria in fish spoilage.

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Flavor Profiles of 17 Species of North Atlantic Fish

PATRICIA A. PRELL and F.M. SAWYER

ABSTRACT

A modified traditional Flavor Profile™ method was used by a trained panel to develop sensory profiles of the cooked muscle of 17 species of North Atlantic fish. Species were characterized for aroma and flavor total intensity, amplitude, order and intensity of character notes, and aftertaste. This information provides a data base for grouping species according to similarities in their characteristics. Flavor data were analyzed by cluster procedures and revealed four distinct groupings characterized by: (1) less than moderate total flavor intensity with a shellfish note; (2) less than moderate total flavor intensity with an earthy note; (3) a moderate flavor intensity with fish oil, gamey and sour notes; (4) high total flavor intensity with fish oil, sour, and stronger gamey notes.

INTRODUCTION

A NEW SEAFOOD nomenclature system, to be based on the sensory or "edibility" characteristics of fish, is under development by the National Marine Fisheries Service (NMFS) of the U.S. Department of Commerce. The goal of this new system is to enable consumers to make educated choices among novel species by providing the comparative sensory data necessary to select a desired flavor, texture, etc., of fish. Standardized methodology for evaluating the sensory properties of cooked fish has been developed (Kapsalis and Maller, 1980). Using this methodology, Cardello et al. (1982) evaluated texture and appearance of 17 species of North Atlantic fish, and Sawyer et al. (1984) evaluated and compared edibility characteristics of 18 species of snapper and rockfish. The objectives of the present report are to detail this methodology as it applies specifically to the evaluation of the aroma, flavor, and aftertaste of fish, and to present Flavor Profiles of the cooked muscle of the same 17 species of North Atlantic fish evaluated by Cardello et al. (1982).

MATERIALS & METHODS

Samples and preparation

The 17 species of fish were selected on the basis of commercial availability in the North Atlantic and on the basis of their possessing a wide range of flavor and texture characteristics. The texture study was reported by Cardello et al. (1982). Procurements were made over the time period the species are normally harvested. These species are shown in Table 1.

All samples of fish tested in this study, except Halibut and Swordfish, were from fish harvested by "day boats" and landed the night before or early in the morning of the day they were to be filleted. Halibut and Swordfish were purchased at local retail markets. On the same day on which the fish were filleted, boxes of fillets were packed with ice in insulated shipping containers and transported to the U.S. Army Natick Research, Development, and Engineering Center (Natick). Preparation of samples was done in a 10°C chill room. Fillets were trimmed by removing the skin, the belly flap, the edge of the nape, and the tail. The rest of each fillet was cut into 56g pieces. Most samples were approximately 5 cm square with thickness determined by the specific test species (heavily fleshed fillets such as swordfish were cut to approximately 2 cm thickness). Portions of the fillet trimmings, representing at least six different samples of the species, were taken to the Gloucester Laboratory, Northeast Fisheries Center, for species identification using thin-layer isoelectric focusing (Lundstrom, 1980; 1981).

Author Prell is with the Science & Advanced Technology Directorate, U.S. Army Natick Research, Development, and Engineering Center, Natick, MA 01760. Author Sawyer is with the Dept. of Food Science & Nutrition, Univ. of Massachusetts, Amherst, MA 01003.

Table 1 – Species of fish evaluated

Scientific name	Common name(s)
Anarhichas lupus	Wolffish, Atlantic Wolffish, Ocean catfish, Catfish
Brosme brosme	Cusk
Cynoscion regalis	Weakfish, weak
Gadus morhua	Cod, Atlantic Cod, Codfish, Scrod (1-1/2–2-1/2 lb), Market (2-1/2–10 lb), Whale (over 10 lb)
Hippoglossus hippoglossus	Halibut, Atlantic Halibut
Lophius americanus	Monkfish, Monk, Goosefish
Lopholatilus chamaeleonticeps	Tilefish, Tile
Melanogrammus aeglefinus	Haddock
Merluccius bilinearis	Whiting, Silver Hake
Morone saxatilis	Striped Bass, Bass
Mycteroperca microlepis	Grouper, Gag
Pollachius virens	Pollock
Pomatomus saltatrix	Bluefish, Blue
Pseudopleuronectes americanus	Blackback Flounder, Winter Flounder, Flounder, (fish under 4 lb)
Scomber scombrus	Mackerel, Atlantic Mackerel
Urophycis tenuis	White Hake, Hake
Xiphias gladius	Swordfish

Cooking method

To minimize effect of cooking method on the innate flavor characteristics of the fish species, a standardized method was developed which was a modification of the procedure recommended by the Association of Official Analytical Chemists (1980) for cooking seafood products. The fish samples were cooked in specially fabricated pouches, consisting of a laminate of 5.1×10^{-2} nm polyethylene and 2.5×10^{-2} nm polyester, and constructed to provide drainage of cooking juices through perforations into a lower compartment of the pouch, thus preventing the fish sample from cooking in its own liquids. Once heat-sealed, the pouches were suspended in a wire rack and immersed in 71°C water. Cooking time was determined from heat-penetration studies on samples of different thicknesses, using Cu-constantan thermocouples placed at the center of each sample. All fish samples were cooked, without added seasoning of any kind, to an internal temperature of 71°C.

Flavor Profile™ Method

Descriptive sensory analysis of the aroma, flavor-by-mouth, and aftertaste attributes of the cooked fish muscle was accomplished by the Natick trained flavor panel, using the traditional (with slight modification) Arthur D. Little Flavor Profile Method (Cairncross and Sjostrom, 1950; Caul, 1957). The 6- to 8-member panel was trained in the Flavor Profile method by A.D. Little, Inc., and has been continuously active on a wide range of products. The aroma profiles and the flavor-by-mouth profiles were developed in the following sequence: the total intensity evaluation was followed by the amplitude evaluation, then the character notes and their intensity were reported in the order perceived. Then, aftertaste was reported. *Total intensity of aroma* (TIA) and *total intensity of flavor* (TIF) indicate overall intensity of the initial impact of each. The *amplitude* is a numerical expression of the overall sensory impression of aroma or flavor, in terms of its fullness, and the appropriateness and balance (blending) of the individual notes. The perceptible *aroma, flavor and aftertaste*

notes are the odors or aromatics, the basic tastes and feeling factors which are defined in descriptive or associative terms.

The *order of appearance* of the notes refers to differences in the timing of the notes, which are usually large enough to be sensorially evident, particularly in an unblended flavor. The closer that notes appear to one another, the more blended is the sample. This information on product blend can best be presented in a discussion of the Profile. It is usually undesirable to have an inappropriate note as the first or last impression of a flavor. The *aftertaste* refers to flavor aromatics, tastes, or mouth sensations after the sample is swallowed. Intensities and persistence may be noted when appropriate.

TIA and TIF were rated on a 7-point scale (1 = slight; 4 = moderate; 7 = strong). Amplitude was rated on the traditional 4-point scale:)(= very low; 1 = low; 2 = medium; 3 = high. Intensity of aroma and flavor character notes were rated on the traditional Flavor Profile scale:)(= just recognizable or threshold; 1 = slight; 2 = moderate; 3 = strong; a designation of 1/2 was used to show an intermediate level of intensity, effectively making it a 7-point scale. For statistical cluster analysis, the scaled points were transformed to an equivalent 7-point scale)(= 1; 3 = 7.

At each testing session, a maximum of two samples was evaluated. Fish species were identified to panelists only at the session conclusion. Assembled panel members received a hot sample of the same fish species served on individual heated, deodorized, ceramic dishes covered with a coded glass petri dish to keep the sample warm and to concentrate the aroma. Unsalted crackers and distilled water were provided to reduce effects of residual flavor in the mouth. In analyzing aroma, the fork was used to break apart the fish flesh to expose a fresh surface for evaluation. In analyzing flavor, the petri dish was removed and a small bite (approximately 1.3 cm square) was chewed and rolled around the mouth with the tongue to ensure that all gustatory and olfactory receptors were stimulated. Then, 1 min after the sample was swallowed, the aftertaste was recorded. For fish species with light and dark flesh, the panel member's sample bite or sniff represented both kinds of flesh proportional to that naturally present. The corresponding Profile reported represents this integrated sample. If two samples or species of fish were to be tested during that session, the first evaluation was completed before the second was begun. The panel members independently reported their observations. Then the panel leader conducted an open discussion to clarify any aspects of the individual profiles, such as the definition or reference for a note, the order of appearance of a note, any relationships among notes, etc. Each final Profile reported is a composite of data determined by the panel after agreement that the tabulation represents the fish species under study. The Profiles are based on 3 to 18 replicates of each species, with the majority having 6 or more replications.

Preliminary sessions focused on the development of a vocabulary of aroma, flavor, and aftertaste terms (character notes) that could be perceived in a wide range of commercially available, edible fish species. For this purpose, approximately 25 different species of fish were examined by the trained flavor profile panel, and a set of descriptive aroma and flavor terms was developed. These terms, along with their definitions, and reference standards, are shown in Table 2. The subtleties of flavor differences prevent enumeration of a fixed set of flavor attributes to be encountered in all fish.

Using the above method, a descriptive analysis of the sensory aroma, flavor, and aftertaste attributes of each sample of fish was made and recorded for use in: (1) developing flavor profiles for the species of fish, (2) comparing these profiles with profiles of replicate evaluations of the same species of fish, (3) comparing profiles among different species of fish, (4) developing terminology for consumer testing, (5) monitoring the quality and freshness of all procured fish, and (6) correlating with and interpreting consumer test results.

Cluster analysis

The flavor data were analyzed by cluster analysis. The method was hierarchical and used the BMDP-P2M "Cluster Analysis of Cases" computer program (Engelman, 1977). The measure of distances among clusters was based on a standard Euclidean distance formula and the program normalized the data to give equal weight to all attributes. The latter procedure was used since there is no available empirical data on the relative perceptual importance of different flavor attributes in fish. However, cluster analysis puts heavier "standardization" weights on attribute data reported for each species (sweet, salty/briny, fresh fish) and lower "standardization" weights on attribute data that is not reported for each species (gamey, fish oil, etc.).

Table 2—Aroma and flavor character notes of cooked fish muscle as defined by the Flavor Profile Panel

Aroma	
Briny	The aromatics associated with the smell of clean seaweed and ocean air.
Sweet	The sweet fragrance, minus the identifying aromatics of many products, such as cooked fresh fish.
Fresh fish	The aromatics associated with cooked fresh fish that distinctively characterize it as fish, yet fresh.
Old fish	The aromatics associated with cooked fish with an off note related to trimethylamine.
Stale fish	The aromatics associated with cooked fish that is getting "off", but is not yet old.
Sour	The aromatics associated with vinegar or lemon. Sharp indicates a nostril irritating type of pungency. Dishrag indicates the sour aromatics of a wet, musk-stale dishrag.
Shellfish	The aromatics associated with any cooked shellfish such as lobster, clam, or scallop.
Gamey fish	The aromatics associated with the heavy, gamey, characteristics of some cooked fish such as Atlantic Mackerel, as opposed to a delicate aroma of fish such as sole. Analogous to the relationship of the heavy, gamey characteristics of fresh cooked venison compared to fresh cooked beef, or duck to chicken.
Fish oil	The aromatics associated with fish oil, such as found in Mackerel, canned sardines, or cod liver oil.
Earthy (slightly cooked potato)	The aromatics associated with slightly undercooked boiled potato, soil, or a slight musty note.
Nutty-buttery	The aromatics associated with the rich, full flavor of chopped nuts such as pecans and warm melted butter.
Musty	The aromatics associated with a moldy, dank cellar.
Scorched	The aromatics associated with the burnt character common to an over-heated ironing board, burnt sugar, or toasted popcorn, sometimes reminiscent of tar, phenol, or creosote.
Flavor	
Salty-briny	A combination of the taste sensations of sodium chloride and the other salt compounds found in ocean water.
Sour (sharp)	The taste sensation caused by acids. The taste of vinegar or lemon is a typical example. Sharp indicates a biting, stinging kind of sensation.
Sweet	The basic taste sensation of which the taste of sucrose is typical.
Fresh fish	As in aroma.
Old fish	As in aroma.
Stale fish	As in aroma.
Shellfish	As in aroma.
Gamey fish	As in aroma.
Fish oil	As in aroma.
Earthy (slightly cooked potato)	As in aroma.
Nutty-buttery	As in aroma.
Canned salmon	The flavor associated with the salmon character in canned salmon.
Bitter	The taste sensation of which the taste of a solution of caffeine or quinine are typical examples.
Metallic	The taste sensation suggesting the taste of a slightly oxidized metal such as tin or iron.
Mouth drying	The sensation of dry skin surfaces of the oral cavity; dry feeling in the mouth after swallowing; astringency.
Mouth filling	The sensation of a bloom or fullness of flavor dispersing throughout the mouth. Produced by compounds such as monosodium glutamate, chicken or beef broth.

RESULTS & DISCUSSION

DETAILED FLAVOR PROFILES of the cooked muscle of each of the 17 species of fish are presented in Table 3. A general description of distinctive sensory characteristics of each

Table 3—Flavor Profiles of the cooked muscle of 17 species of fish

Species: BLUEFISH (*Pomatomus saltatrix*)		COD (market) (*Gadus morhua*)		COD (scrod) (*Gadus morhua*)		CUSK (*Brosme brosme*)		FLOUNDER, Blackback (*Pseudopleuronectes americanus*)		GROUPER (*Mycteroperca microlepis*)	
Aroma		Aroma		Aroma		Aroma		Aroma		Aroma	
TIA[a]	6-7[a]	TIA	3	TIA	3	TIA	3	TIA	2-3	TIA	3
Amplitude	2-2 1/2[b]	Amplitude	2	Amplitude	1 1/2-2	Amplitude	1 1/2-2	Amplitude	1 1/2-2	Amplitude	1 1/2-2
Briny	1 1/2[c]	Briny	1 1/2	Briny	1-1 1/2	Sweet	1/2-1	Sweet	1	Sweet	1
Gamey fish	2	Fresh fish	1 1/2	Fresh fish	1 1/2	Fresh fish	1 1/2	Fresh fish	1-1 1/2	Fresh fish	1 1/2
Fresh fish	2	Sweet	1/2-1	Sweet	1/2-1	Briny	1	Briny	1	Briny	1
Fish oil	1	Sour	1/2-1	Sour	1/2-1	Sour	1	Sour	1/2	Sour	1/2
Sour	1	Shellfish (clam, scallop, lobster)	1/2-1	Shellfish (clam, scallop)	1/2	Shellfish (clam, lobster)	1/2-1	Other: shellfish, scorched		Shellfish (clam)	1
Sweet	1/2										
Flavor		Flavor		Flavor		Flavor		Flavor		Flavor	
TIF[a]	6[a]	TIF	2	TIF	2	TIF	3	TIF	2	TIF	2-3
Amplitude	2[b]	Amplitude	2	Amplitude	1 1/2-2	Amplitude	1 1/2-2	Amplitude	2	Amplitude	1 1/2
Sweet	1[c]	Sweet	1	Sweet	1/2-1	Sweet	1/2-1	Sweet	1	Sweet	1/2
Fresh fish	2	Fresh fish	1 1/2	Fresh fish	1-1 1/2	Fresh fish	1 1/2	Fresh fish	1 1/2	Fresh fish	1
Gamey fish	1 1/2	Salty-briny	1/2	Salty-briny	1/2	Shellfish (clam, lobster)	1/2-1	Salty-briny	1/2-1	Sour	1
Fish oil	1/2	Sour	1/2	Sour	1/2	Salty-briny	1	Sour	1/2-1	Salty-briny	1/2
Sour (sharp)	1 1/2	Mouth drying	1	Mouth drying	1	Sour	1/2	Mouth drying	1/2-1	Mouth drying	1 1/2
Salty-briny	1/2	Shellfish (clam, scallop, lobster)	1/2-1	Earthy (slight cooked potato)	1/2	Mouth drying	1	Other: shellfish		Shellfish (clam)	1
Mouth drying	1										
Other: nutty-buttery		Other: metallic bitter		Other: metallic, shellfish (clam, lobster, scallop)							
Aftertaste		Aftertaste		Aftertaste		Aftertaste		Aftertaste		Aftertaste	
Fresh, gamey fish		Fresh fish		Fresh fish		Fresh fish		Fresh fish		Sour	
Sour		Sour		Mouth drying		Sour		Mouth drying		Mouth drying	
Sweet				Sour		Mouth drying		Sweet		Fresh fish	

Species: HADDOCK (*Melanogrammus aeglefinus*)		HALIBUT (*Hippoglossus hippoglossus*)		MACKEREL (*Scomber scombrus*)		MONKFISH (*Lophius americanus*)		POLLOCK (*Pollachius virens*)		STRIPED BASS (*Morone saxatilis*)	
Aroma		Aroma		Aroma		Aroma		Aroma		Aroma	
TIA[a]	4[a]	TIA	4	TIA	6	TIA	3-4	TIA	3-4	TIA	5
Amplitude	1 1/2-2[b]	Amplitude	1 1/2-2	Amplitude	2	Amplitude	2	Amplitude	2	Amplitude	2
Briny	1 1/2-2[c]	Briny	1	Gamey fish	2	Briny	1-1 1/2	Sweet	1	Gamey fish	1 1/2
Sweet	1	Fresh fish	1 1/2	Fresh fish	2	Sweet	1	Briny	1	Fresh fish	1 1/2
Fresh fish	1 1/2	Sweet	1/2-1	Sweet	1/2-1	Fresh fish	1-1 1/2	Fresh fish	1 1/2	Sweet	1
Shellfish (lobster, scallop)	1	Sour	1/2-1	Briny	1	Shellfish (lobster, clam)	1-1 1/2	Sour (sharp)	1	Sour	1
Sour	1/2	Other: gamey fish, fish oil, old fish		Fish oil	1-1 1/2			Shellfish (clam)	1/2-1		
Other: old fish				Sour	1/2-1	Sour	1			Fish oil	1/2-1
Flavor		Flavor		Flavor		Flavor		Flavor		Flavor	
TIF[a]	3[a]	TIF	3-4	TIF	6	TIF	3	TIF	3	TIF	4
Amplitude	2[b]	Amplitude	1 1/2-2	Amplitude	2	Amplitude	2	Amplitude	2	Amplitude	2
Sweet	1[c]	Sweet	1/2	Gamey fish	2	Sweet	1	Sweet	1	Sweet	1
Fresh fish	1 1/2	Fresh fish	1 1/2-2	Fresh fish	2	Shellfish (lobster, clam)	1 1/2	Fresh fish	1 1/2	Fresh fish	1 1/2
Shellfish (lobster, scallop)	1	Sour	1-1 1/2	Sweet	1			Sour (sharp)	1	Gamey fish	1-1 1/2
Sour	1/2-1	Salty-briny	1	Fish oil	1-1 1/2	Fresh fish	1-1 1/2	Shellfish (clam)	1/2-1	Sour	1 1/2
Salty-briny	1/2	Mouth drying	1	Sour (sharp)	1	Sour	1	Salty-briny	1/2	Salty-briny	1
Mouth drying	1	Gamey fish	1/2-1	Salty-briny	1/2	Salty-briny	)(	Mouth drying	1	Fish oil	1/2-1
Other: earthy (slight cooked potato), metallic		Fish oil	1/2	Mouth drying	1/2	Mouth drying	1/2			Mouth drying	1
						Other: buttery					
Aftertaste		Aftertaste		Aftertaste		Aftertaste		Aftertaste		Aftertaste	
Fresh fish		Sour		Fresh, gamey fish		Sweet		Sour		Sweet	
Sour		Mouth drying		Fish oil		Fresh fish		Fresh fish		Sour	
Shellfish		Fresh fish		Sweet		Shellfish		Mouth drying		Fresh Fish	
		Fish oil-gamey		Sour				Sweet		Gamey fish	

Table 3—(Continued)

Species: SWORDFISH (*Xiphias gladius*)		TILEFISH (*Lopholatilus chameleonticeps*)		WEAKFISH (*Cynoscion regalis*)		WHITE HAKE (*Urophycis tenuis*)		WHITING (*Merluccius bilinearis*)		WOLFFISH (*Anarhichas lupus*)	
Aroma		Aroma		Aroma		Aroma		Aroma		Aroma	
TIA[a]	3-4[a]	TIA	3-4	TIA	4	TIA	3-4	TIA	4	TIA	3
Amplitude	2[b]	Amplitude	2	Amplitude	1 1/2	Amplitude	1 1/2	Amplitude	1 1/2-2	Amplitude	1 1/2-2
Sweet	1[c]	Fresh fish	1 1/2	Briny	1 1/2	Sour (dishrag)	1 1/2	Briny	1 1/2	Briny	1
Fresh fish	1 1/2-2	Sweet	1/2-1	Fresh fish	1	Fresh fish	1 1/2	Fresh fish	1 1/2	Fresh fish	1 1/2
Shellfish (clam)	1-1 1/2	Shellfish (clam lobster)	1	Gamey fish	1	Sweet	1/2-1	Sour	1	Sweet	1/2
				Sweet	1/2	Briny	1/2	Sweet	1/2	Sour	1
Briny	1	Briny	1	Sour	1	Other: old fish, earth (slight cooked potato),				Shellfish (clam, lobster)	1/2
Sour	1/2-1	Sour	1/2-1	Other: musty		Shellfish (clam)					
Nutty-buttery	1/2										
Flavor		Flavor		Flavor		Flavor		Flavor		Flavor	
TIF[a]	3[a]	TIF	3	TIF	3-4	TIF	3	TIF	3	TIF	3
Amplitude	2[b]	Amplitude	2	Amplitude	1 1/2-2	Amplitude	1 1/2	Amplitude	1 1/2-2	Amplitude	2
Sweet	1[c]	Sweet	1	Sweet	1	Sweet	1	Sweet	1	Sweet	1
Fresh fish	1 1/2-2	Fresh fish	1 1/2	Fresh fish	1 1/2	Fresh fish	1 1/2	Fresh fish	1 1/2	Fresh fish	1 1/2
Shellfish (clam)	1	Sour	1	Gamey fish	1/2-1	Sour (sharp)	1	Sour	1/2-1	Shellfish (clam, lobster)	1/2-1
		Salty-briny	1/2	Salty-briny	1/2-1	Salty-briny	1/2	Salty-briny	1/2	Salty-briny	1/2
Canned salmon	1/2	Shellfish		Sour	1	Mouth drying	1	Mouth drying	1		
Sour (sharp)	1 1/2	(lobster, scallop)	1	Mouth drying	1-1 1/2	Earthy (slight cooked potato)	1/2-1	Earthy (slight cooked potato)	1	Mouth drying	1
Salty-briny	1/2										
Mouth drying	1	Mouth drying	1	Fish oil	1/2	Other: old fish, shellfish		Other: shellfish		Sour	1/2-1
		Other: fish oil								Other: fish oil	
Fish oil	1/2										
Mouth filling	1										
Other: nutty-buttery											
Aftertaste		Aftertaste		Aftertaste		Aftertaste		Aftertaste		Aftertaste	
Fresh fish		Fresh fish		Mouth drying		Fresh fish		Fresh fish		Fresh fish	
Sour		Sweet		Fresh fish		Mouth drying		Sour		Mouth drying	
Sweet				Gamey		Sweet		Mouth drying		Sweet	
Shellfish				Sweet		Earthy (slight cooked potato)		Sweet			
				Sour		Sour					

[a] TIA = Total Intensity Aroma; TIF = Total Intensity Flavor; 7-Point Scale: 1 = Slight, 7-Strong

[b] Amplitude Scale:)(= Very Low, 3 = High

[c] Character Note Scale:)(= Just Recognizable or Threshold, 3 = Strong

species is provided in the discussion. The Profiles reported represent the panel's perception of each species at the freshest quality available in the study. Sample variability occurred for some species from session to session and sometimes within a session. The listing of aroma and flavor notes as "other" indicates they were reported by less than half of the panel members in at least half of the panel sessions. The discussions indicate variability in sample quality when it occured.

Bluefish (*Pomatomus saltatrix*)

Bluefish has a strong total aroma and flavor impact, with a highly aromatic distinctive gamey, fresh fish, sour, fish oil character. The amplitudes (overall impressions) are moderate, with character notes blending together to give a very full aroma and flavor. The aroma of Bluefish has an early impact of a slight to moderate briny, moderate fresh and gamey fish notes, slight fish oil and sour notes, and a very slight sweet. The flavor has an early impact of slight sweet, moderate fresh fish, slight to moderate gamey fish and sour (sharp), very slight fish oil and salty-briny, and slight mouth drying. This fish has a distinctive sour (sharp) at a higher intensity than in most fish. In both aroma and flavor, the gamey and fish oil notes seem to be related. They are found at higher intensities in the dark flesh (especially near the skin), but are also present in the lighter flesh. The aftertaste is strong and persistent. The reported Profile integrates proportionate light and dark flesh. The most common sample variation was in the intensity of flavor of the fish oil and sour notes, and sometimes the fresh and gamey notes.

Cod (market) (*Gadus morhua*)

Cod (market) has a moderately low total intensity aroma and flavor, with a low sweet, fresh fish character. The aroma and flavor amplitudes are moderate, as the overall impression of Cod is of a mild fish with an uncomplex aroma and flavor. The aroma is slight to moderate briny and fresh fish, and below slight sweet, sour, and shellfish. The flavor is slight sweet and mouth drying, slight to moderate fresh fish, very slight salty-briny and sour, and below slight shellfish. The aftertaste is fresh fish and sour. Occasionally, samples of market Cod had a stale fish character note.

Cod (scrod) (*Gadus morhua*)

Cod (scrod) has a moderately low total intensity aroma and flavor, with a low sweet, fresh fish character. The aroma and

flavor amplitudes are below moderate, as the overall impression of Cod is of a mild fish with an uncomplex aroma and flavor. The aroma is slight to moderate briny and fresh fish, and below slight sweet and sour, with a very slight shellfish. The flavor is below slight sweet, slight to moderate fresh fish, very slight salty-briny, sour, and earthy (slight cooked potato), and slight mouth drying. The aftertaste is fresh fish, mouth drying, and sour. Some samples in many sessions had a stale fish character note and some had a metallic note.

Cusk (*Brosme brosme*).

Cusk has a moderately low total intensity aroma and flavor, with a low sweet, fresh fish character. The aroma and flavor amplitudes are below moderate, as this fish lacks fullness and blending, especially in flavor. The aroma is below slight sweet, slight to moderate fresh fish, slight briny and sour, and below slight shellfish. The flavor is below slight sweet and shellfish, slight to moderate fresh fish, slight salty-briny and mouth drying, and very slight sour. The flavor is flat, washes out quickly, and is affected by the chewy, slightly tough texture. The aftertaste is fresh fish, sour, and mouth drying. Approximately 1/3 of the samples in each delivery were slightly stale or very slightly old fish.

Blackback flounder (*Pseudopleuronectes americanus*)

Blackback Flounder has a low total intensity aroma and flavor with a delicate, fresh fish character. The aroma amplitude is just below moderate and the flavor amplitude is moderate. The aroma is slight sweet and briny, slight to moderate fresh fish and very slight sour. The flavor is slight sweet, slight to moderate fresh fish, below slight salt-briny, sour and mouth drying. The aftertaste is a low level fresh, mouth drying and sweet. Sample variation was common, with stale fish and scorched notes present in some samples.

Grouper (*Mycteroperca microlepis*)

Grouper has a moderately low total intensity aroma and flavor, with a low level of aromatics. The aroma and flavor amplitudes lack full bodied characteristics, have a low impact of blended character, and low fresh fish. The aroma of Grouper has an early impact of slight sweet, and briny notes, and slight to moderate fresh fish, followed by a very slight sour (pungent), and a shellfish note. The flavor is very slight sweet, salty-briny, slight fresh fish, sour (sharp), and shellfish, and slight to moderate mouth drying (the highest intensity for the fish tested). The aroma and flavor shellfish note has a clam character. There are confusing overtones to the sour and shellfish notes that are difficult to describe. The aftertaste is persistent and consists of a moderate level mouth drying and sour, and a low level of fresh fish. The dry tough texture is difficult to ignore. The samples seemed relatively consistent within a session, and the greatest variations between sessions seemed to be in the sour, shellfish notes, with confusing overtones.

Haddock (*Melanogrammus aeglefinus*)

Haddock has a moderate total intensity aroma and a moderately low total intensity flavor, with a sweet, fresh fish, shellfish character. The aroma amplitude is just below moderate and the flavor amplitude is moderate, as this fish has a fullness of flavor and an interesting shellfish characteristic. The aroma is slight to moderate briny, and fresh fish, slight sweet and shellfish, and very slight sour. The flavor is slight sweet, shellfish, and mouth drying, fresh fish at slight to moderate, sour at below slight, and salty-briny at very slight. The sweet, fresh fish, and shellfish flavors seem to be related, and the sour is fleeting. Some of the panelists mentioned earthy (slight cooked potato) and metallic notes, particularly in the dark flesh portion. The aftertaste is fresh fish, sour, and shellfish. There was some variation within a session and between sessions in the Haddock samples, particularly in aroma, and was evidence as stale fish character notes.

Halibut (*Hippoglossus hippoglossus*)

Halibut has a moderate total intensity aroma and flavor, with a sweet, fresh fish character,and a very slight gamey fish flavor note. The aroma and flavor amplitudes are below moderate, as some samples lack a blended flavor. The aroma is slight briny, slight to moderate fresh fish, below slight sweet and sharp, sour (pungent). The flavor is very slight sweet, slight to moderate fresh fish, above slight sour, slight salty-briny and mouth drying, below slight gamey and fish oil. The definite sour flavor note is higher than in most fish. The aftertaste is predominantly sour and mouth drying, with some fresh fish, and another hard-to-describe note that seems to be a combination of fish oil and gamey. The Halibut samples examined varied considerably from one session to another with some variation within a session, as some samples had a distinctive gamey, fish oil flavor.

Mackerel (*Scomber scombrus*)

Mackerel has a strong total aroma and flavor impact, with a highly aromatic distinctive gamey, fresh fish, fish oil character. The amplitudes are moderate, with character notes blending together to give a very full aroma and flavor. The aroma of Mackerel has an early impact of moderate gamey and fresh fish notes, a slight to moderate fish oil, and slight sweet, briny, and sour notes. The flavor has an early impact of moderate gamey and fresh fish notes, a slight to moderate fish oil, slight sweet and sour (sharp), and a very slight salty-briny and mouth drying. The mouth drying characteristic seems largely overpowered by the oiliness. In both aroma and flavor, the gamey and fish oil notes seem to be related. They are found at higher intensities in the dark flesh, but are also present in the lighter flesh. The reported Profile integrates proportionate light and dark flesh. The gamey fish note of Mackerel is heavy and sometimes associated with a burnt or scorched characteristic. Despite the intensity and heaviness of this fish characteristic, it is typical of fresh Mackerel and not indicative of aged fish. The aftertaste is strong and persistent. Samples of Mackerel varied from session to session and sample to sample within a session, with the most common variation being the intensity of the fish oil, fresh, and gamey notes.

Monkfish (*Lophius americanus*)

Monkfish has a moderately low total intensity aroma and flavor. Its most distinguishing characteristic is a distinctive shellfish aromatic note similar to lobster and clam. The aroma and flavor amplitudes are moderate as this fish is full, complex and blended with interesting notes. The aroma is slight to moderate briny (fresh, seaweed), fresh fish and shellfish, and slight sweet and sour. The dark flesh and gelatinous areas near the skin have more intense briny-seaweed and shellfish-clam character than the white flesh. The flavor is slight sweet and sour, slight to moderate shellfish and fresh fish, threshold salty-briny, and very slight mouth drying. The shellfish note adds interest and complexity to the aroma and flavor. The sweet, shellfish, fresh fish and buttery notes seem closely related and give a full flavor. The aftertaste is sweet, fresh fish, and shellfish. Some Monkfish samples had stale notes which appeared early in the Profile. There was some variability in Monkfish samples, particularly in the amount of dark flesh and gelatinous layer.

Pollock (*Pollachius virens*)

Pollock has a moderately low total intensity aroma and flavor with sweet, fresh fish, low shellfish character. The aroma

and flavor amplitudes are moderate, as they are blended with some interest notes. The aroma is slight sweet, briny and sour (sharp), slight to moderate fresh fish, and below slight shellfish-clam. The flavor is slight sweet, sour (sharp) and mouth drying, slight to moderate fresh fish, below slight shellfish-clam, and very slight salty-briny. Some of the samples had a small amount of dark flesh which had a hard-to-define character related to gamey and fish oil. The aftertaste is sour, fresh fish, and sweet, with a persistent mouth drying. Some samples had a metallic note and some had a stale fish character.

Striped bass (*Morone saxatilis*)

Striped Bass has a total intensity of aroma above moderate and total intensity of flavor at moderate. It is a moderately strong flavored fish with a fresh, gamey, fish oil character. The aroma and flavor amplitudes are moderate, with character notes blended to give a full aroma and flavor. The aroma has an early impact of fresh and gamey fish at slight to moderate, slight sweet, sour and briny, and below slight fish oil. The flavor also has an early impact of fresh, gamey fish, and sour notes at slight to moderate, sweet, salty-briny, and mouth drying at slight, and fish oil at below slight. The sour flavor note is stronger than in most fish. Particularly in flavor, the gamey and fish oil vary, being more intense in the dark flesh. The aftertaste is sweet, sour and fresh fish, with some gamey fish character.

Swordfish (*Xiphias gladius*)

Swordfish has a moderately low total intensity aroma and flavor, with a sweet, fresh fish, shell fish character. The aroma and flavor amplitudes are moderate, complex, tightly blended, and the fish has full flavor, with complex first notes, sour (sharp), and mouthfilling characteristics. The aroma is just below moderate fresh fish, slight to moderate shellfish, slight sweet and briny, below slight sour, and very slight nutty-buttery. The flavor is just below moderate fresh fish, slight sweet, shellfish, mouth drying, and mouth filling, slight to moderate sour (sharp, citric), very slight canned salmon, salty-briny, and fish oil. The fish oil appears late in the flavor. In aroma and flavor, the fresh fish, shellfish, and nutty-buttery seem rich, closely related, and to have a salmon or tuna character. The canned salmon flavor note is also related and hard to define. The sour (citric) flavor is almost lemon and is stronger than in most of the fish tested. The aftertaste is sour, fresh fish, sweet, and shellfish.

Tilefish (*Lopholatilus chamaeleonticeps*)

Tilefish has a moderately low total intensity aroma and flavor, with a sweet, fresh fish, shellfish character. The aroma and flavor amplitudes are moderate, as this fish is rich and full, complex, and blended. The aroma is slight to moderate fresh fish, below slight sweet and sour (pungent), and slight shellfish and briny. The flavor is slight to moderate fresh fish, slight sweet, sour, shellfish and mouth drying, and very slight salty-briny. The sweet, fresh fish, and shellfish notes seem closely related and associated with a rich character like butter, lobster or scallop. The aftertaste is fresh fish and sweet. A few samples tested had a fish oil note.

Weakfish (*Cynoscion regalis*)

Weakfish has a moderate total intensity aroma and flavor, with a fresh fish, gamey, briny (seaweed) character, and a late, low level fish oil flavor. The aroma and flavor amplitudes are below moderate, as the character notes are not fully blended. The aroma is slight to moderate briny (stronger than in most fish, with a seaweed character), slight fresh fish, gamey and sour, and very slight sweet. The flavor is slight to moderate fresh fish, slight sweet and sour, below slight gamey, and salty-briny, above slight mouth drying, and very slight fish oil. Gamey and fish oil are more intense in the dark flesh. The aftertaste is mouth drying, fresh, gamey fish, with sweet, and sour notes. Compared to other fish, this aftertaste is stronger and more persistent. The character notes of individual samples varied more than in most fish, especially in the levels of gamey and fish oil notes, which depended largely on the amount of dark flesh present.

White hake (*Urophycis tenuis*)

White Hake has a moderately low total intensity aroma and flavor, with sour (dishrag) aroma, and an earthy note in flavor. The aroma and flavor amplitudes are low to moderate due to the old fish, sour (dishrag), and earthy notes, which indicate a lack of blended characters. The aroma of White Hake is a slight to moderate sour (dishrag) and fresh fish and below slight sweet and briny. Aroma notes mentioned by panelists are old fish, earthy (slight cooked potato), and shellfish (clam). The flavor is slight sweet, sour (sharp), and mouth drying, slight to moderate fresh fish, below slight salty-briny and earthy (slight cooked potato). There was an unblended quality to the flavor, and when both earthy and old fish occurred, they seemed related. The aftertaste is fresh fish, mouth drying, sweet, earthy, and sour. This fish often showed great variation in the fresh fish and old or stale fish characteristics.

Whiting (*Merluccius bilinearis*)

Whiting has a moderate total intensity aroma and a moderately low total intensity flavor, with a mild, slightly bland aroma and flavor, and a late earthy flavor note. The aroma and flavor amplitudes are just below moderate, as the fish is uncomplex. The aroma is below moderate briny and fresh fish, slight sour, and very slight sweet. The flavor is slight sweet, mouth drying and earthy (slight cooked potato), below moderate fresh fish, below slight sour and salty-briny. The aftertaste is predominantly fresh fish with sour, mouth drying, and sweet notes. Variations were common and usually appeared as stale fish, earthy, and shellfish notes.

Wolffish (*Anarhichas lupus*)

Wolffish has a below moderate total intensity aroma and flavor, with a fresh fish, shellfish character. The aroma and flavor amplitudes are moderate. The aroma is slight briny and sour, slight to moderate fresh fish, and very slight sweet and shellfish. The flavor is slight sweet and mouth drying, below moderate fresh fish, and below slight shellfish, salty-briny and sour. The sour seemed more pronounced near the gelatinous layer. The aftertaste is fresh fish, mouth drying, and sweet. Sample variations were common, with low levels of bitter, metallic, gamey, and fish oil present in some samples. These were more evident in dark flesh or near the gelatinous layer.

Cluster analysis

To identify groups of similar and dissimilar species, the flavor data were analyzed by cluster analysis. In the resulting tree-diagram data (Fig. 1), Haddock and Wolffish were grouped together first. Tilefish and Pollock were grouped together second and all four fish were then grouped together in the third step. The largest branch of the tree-diagram consists of seven species that are similar in flavor: Haddock, Wolffish, Tilefish, Pollock, Cod (market), Flounder, and Cusk. All of these species are less than moderate in total flavor intensity and in fresh fish note, and have slight sweet, salty-briny, sour, and mouth drying notes. In addition, all of them, except Flounder, have

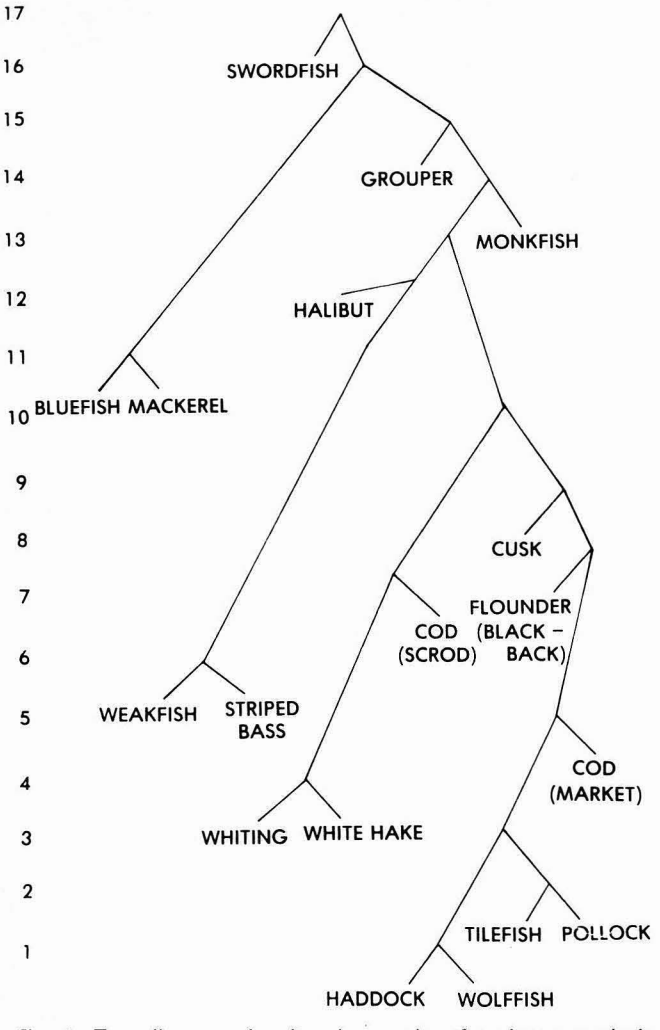

Fig. 1—Tree-diagram showing the results of a cluster analysis applied to the trained panel Flavor Profile data for the cooked muscle of 17 species of North Atlantic fish.

a slight shellfish flavor, but the type of shellfish flavor differed. A second branch consists of Whiting, White Hake, and Cod (scrod). These fish have a less than moderate total flavor intensity and are distinguished from the seven in the largest group because they have an earthy note, and none have shellfish notes. A third branch consists of Halibut, Weakfish, and Striped Bass. These three fish have moderate or below moderate total flavor intensity, and have slight to moderate, but distinctive, fish oil, gamey, and sour notes. A fourth branch consists of Bluefish and Mackerel. These fish are both high in total flavor intensity, possess slight to moderate fish oil and sour notes, and a moderate or less than moderate gamey note. Lastly, Monkfish, Grouper, and Swordfish form weak links to the remainder of the tree, suggesting independent flavor groups for these species.

The odor and flavor data presented here, combined with corresponding texture and appearance data on the same species (Cardello et al., 1982), provides a starting base for the development of a comprehensive data bank of similar information on all species having importance to the U.S. consumer and fishing industry. Such a comprehensive data bank would facilitate development of a market-oriented nomenclature system for fin fish based on relationships of sensory ("edibility") characteristics among species (Cardello et al., 1983). This, in conjunction with utilization of descriptive attributes having demonstrated usefulness to consumers (Sawyer et al., 1988), would aid in developing tools for education in the marketplace.

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Oxygen Transmission Rate of Packaging Films and Light Exposure Effects on the Color Stability of Vacuum-Packaged Dry Salami

J.R. YEN, R.B. BROWN, R.L. DICK, and J.C. ACTON

ABSTRACT

Color and nitrosoheme pigment stabilities of dry salami packaged in films differing in oxygen transmission rate (OTR) were studied. Product exposed to light for 8 wk had greater ($p<0.05$) retention of redness properties (Judd-Hunter +a coordinates, a/b ratios, hue angles (θ), and saturation (S) index values) and greater ($p<0.05$) nitrosoheme pigment stability as the OTR of the packaging film decreased from 90 cc $O_2/m^2/24$ hr to $\leq$11 cc $O_2/m^2/24$hr. Redness properties and percent nitrosoheme pigment progressively decreased ($p<0.05$) during light exposure. Significant redness fading occurred in light-exposed product when compared to dark-stored product. Oxygen transmission to the dry salami surface was a major factor in color loss, primarily if light energy was also present.

INTRODUCTION

THE DEVELOPMENT of cured meat color, a pink to red appearance, is dependent on reaction conditions involving the muscle pigment myoglobin, added sodium nitrite (or potassium nitrite) and reductants, tissue pH (Kelly and Watts, 1957; Siedler and Schweigert, 1959; Fox and Thomson, 1963; Fox and Ackerman, 1968; Lee and Cassens, 1976; Giddings, 1977) and the time-temperature schedule used in product heating (Fox et al., 1967; Monagle et al., 1974; Acton and Dick, 1977; Acton et al., 1979). The nitrosoheme pigment formed is sensitive to the effects of light and oxygen which interact to promote color fading or color loss at the exposed surface of packaged cured meat products placed in lighted displays (Mandigo and Kunert, 1973; Lin and Sebranek, 1979; Lin et al., 1980; Wesley et al., 1982; Acton et al., 1986). Color fading, which results in a tan, brown or gray appearance, is thought to occur in a two-step sequence. The first is a light-accelerated dissociation of nitric oxide from the pigment that is catalyzed by the presence of oxygen (Tarladgis, 1962), followed by the oxidation of the dissociated nitric oxide (Tarladgis, 1962; Fox, 1966). Light also serves as an energy source for the oxidation reaction. There is some evidence that the pyrrole structure of the heme moiety may be oxidized (Erdman and Watts, 1957; Tarladgis, 1962). The light-induced oxidation of the nitrosoheme pigment has also been studied by Bailey et al. (1964) and Reith and Szakaly (1967a, b).

Color fading or color loss also occurs in the interior of a cured product (absence of light), although the rate is extremely slow compared to that at the product surface. Visually, discoloration in light-displayed, packaged products starts near the edge of the product surface where there is less package adhesion and greater residual air space (Lin and Sebranek, 1979). Packaging films of relatively low oxygen permeability are used with vacuum-packaging systems to exclude oxygen from the product. Vacuum level and film oxygen barrier combinations are critical to maintenance of an acceptable color in cured meats (Lin et al., 1980; Amundson et al., 1982; Sebranek, 1985). Color and/or pigment stabilities are measured by several techniques. Nitrosoheme pigment concentrations, surface reflectance ratios, Judd-Hunter tristimulus colorimetry values (+a values and a/b ratios) and visual color ratings decrease during storage of cured meats at rates dependent on vacuum level, length and intensity of light display and initial nitrite level (Barton, 1967; Froning et al., 1971; Mandigo and Kunert, 1973; Lin and Sebranek, 1979; Wesley et al., 1982; Acton et al., 1986).

Fox (1966) stated that use of an oxygen-impermeable packaging material will prevent color loss in cured meats and Tarladgis (1962) suggested that avoiding exposure to light will delay nitric oxide dissociation from the nitrosoheme pigment. The latter condition is difficult in practice when products must be displayed for consumers. Lin and Sebranek (1979), Lin et al. (1980), Rizvi (1981) and Acton et al. (1986) generally recommended barrier films of $\leq$15 cc $O_2/m^2/24$ hr in oxygen transmission rate (OTR at test conditions of approximately 23°C, 0% RH, 1 atm on a total film basis and not per mil thickness) for minimal loss of cured-meat color and greater retention of the nitrosoheme pigment. Terlizzi et al. (1984), in describing cook-in-the-film packaging systems, stated that higher OTR films ($\leq$70 cc $O_2/m^2/24$hr) provide adequate color protection for large deli-style cured meats that are generally stored in the dark prior to slicing.

The present study was conducted to determine the effect of packaging film OTR on color stability and nitrosoheme pigment retention in fermented, dry salami during an 8-wk light display period. The effects of light exposure or storage in the dark were also determined after 8 wk for all packaged salami samples.

MATERIALS & METHODS

Salami preparation and packaging

Thirty-two unsliced, fermented, dry salami chubs (Monarch Brand) from four separate dates of manufacture were purchased from PYA Distributors, Greenville, SC. Each salami chub had a diameter of approximately 10 cm and weighed approximately 2.3 kg. The salami chubs were sliced 5 cm in thickness and each slice (approximately 150 g) served as a sample. Individual samples were vacuum-packaged at 72.4 cm Hg in each of five packaging films which differed in oxygen transmission rate (OTR). Vacuum packaging was conducted using a Multivac, Type AG 500 vacuum packaging machine (Cryovac Division of W.R. Grace and Co., Duncan, SC).

The five packaging films (from Cryovac Division of W.R. Grace and Co., Duncan, SC) had the following respective average OTRs: 1, 11, 30, 72 or 90 cc $O_2/m^2/24$ hr at 23°C, 0% RH and 1 atm. These film OTRs are given on a total film basis and not on a per mil of thickness basis. Film thickness was not constant and varied slightly depending on type of film material.

As a group, the eight salami chubs within each date of manufacture served as replicates and the five packaging films served as treatments in this study.

Light display and sampling methods

Packaged salami samples were displayed at 5 ± 1°C under 1880 ± 260 lux of continuous lighting (Deluxe White fluorescent lights) for 8 wk. The product display area consisted of a white formica surface

The authors are affiliated with the Dept. of Food Science, South Carolina Agricultural Experiment Station, Clemson Univ., Clemson, SC 29634-0371.

centered under the fluorescent lights. Samples were rotated periodically to assure uniform exposure to the light. A duplicate set of the initial samples packaged in each type of film was stored in the dark at 5 ± 1°C for 8 wk.

Duplicate samples of the salami packaged in each of the films were removed for color evaluations after light display periods of 0,1, 2, 4, 6, and 8 wk. The samples maintained in the dark were evaluated after 8 wk storage.

Compositional analyses

Compositional analyses were conducted to provide information on the type of dry salami used in this study.

Moisture, fat, protein, and ash contents (AOAC, 1984) were determined in duplicate or triplicate for each sample at 0 and 8 weeks of storage. The Kjeldahl nitrogen method (N × 6.25) was used for protein (AOAC, 1984). The procedure of Acton and Keller (1974) was used to measure sample pH and lactic acid content. Duplicate samples were analyzed.

Color evaluations

Tristimulus color coordinates (L, a, b) were determined on the packaged salami sample surface which had been directly exposed to light during the display period. The color coordinates were measured using a Gardner Color and Color Difference Meter, Model C-4 (L). The colorimeter was standardized with a standard white ceramic tile No. R85046 (L = 94.5; a = −1.1; b = +2.7). Due to surface variations caused by the coarse-cut fat and lean particle dispersion typical of this type of sausage, a series of five random surface measurements were made for each of the packaged samples. The samples which had been stored in the dark for 8 wk were evaluated using the same procedure.

In addition to reporting tristimulus color coordinates, additional color characteristics were calculated. The a/b ratio and hue angle (0) were used as indices of redness change (Francis, 1975). The hue angle is the angle whose cotangent is a/b in the Judd-Hunter Color Space. The saturation index (S) expressed the degree of hue departure from gray of the same lightness and is calculated using the following equation:

$$S = (a^2 + b^2)^{\frac{1}{2}} \quad (1)$$

Total color difference (ΔE) was measured between the initial (0 week) salami sample and each packaging treatment sample that had been exposed to the light (1–8 wk) or stored in the dark (8 weeks). Total color difference was calculated using the following equation (Francis and Clydesdale, 1975):

$$\Delta E = [(L_x - L_y)^2 + (a_x - a_y)^2 + (b_x - b_y)^2]^{\frac{1}{2}} \quad (2)$$

where x = L, a or b coordinate of initial salami sample, and y = L, a or b coordinate of displayed or stored salami sample.

Heme pigment analyses

Homogenized salami samples were used for heme pigment analyses. The methods utilized for nitrosoheme pigment and total heme pigment extractions were described by Hornsey (1956) with modifications outlined by Acton and Dick (1977). The results, reported as percent pigment conversion, are the percent of total heme pigment converted to nitrosoheme pigment.

Statistical analyses

Statistical analyses were conducted using analysis of variance with the general linear model procedure (SAS, 1985). Separation of means was conducted using the Least Significant Difference Test at The 5% level of probability.

RESULTS & DISCUSSION

Composition of dry salami lots

Fermented, dry sausages have been classified primarily by moisture content or weight loss measured after drying (Wilson, 1960; Acton and Dick, 1976). The moisture of the salami obtained for this study ranged from 35.5% to 39.2% (Table 1). In the classification procedure proposed by Acton and Dick (1976), fermented sausages having a moisture content between 32.5% and 40% are designated "medium dry." Although there are some significant ($p<0.05$) differences in other constituent concentrations among the salami replicates (Table 1), the overall compositional profile is similar to results reported by other researchers (Acton and Dick, 1976; Terrell, 1977; Ziegler et al., 1987).

Lactic acid of the salami in this study ranged from 1.65% to 2.31% and the pH ranged from 4.65 to 4.89 (Table 1). No "correction factor" for total acidities could be used in this study. However, salami of replicates I and IV had the same pH (4.81) yet differed significantly ($p<0.05$) in lactic acid as calculated from total acidity determinations. This difference may have resulted from differences in the inherent buffering capacity of each initial meat mix (Honikel and Hamm, 1974) prior to fermentation. These ranges indicated typical production variations that might occur in the fermentation phase for different batches of dry sausage. However, these values are within the expected ranges of lactic acid concentration and pH reported in previous studies (Acton and Dick, 1976; Terrell, 1977).

Dry salami color properties

Oxygen transmission rate (OTR) of packaging films. The effects of different packaging film OTRs on color properties of fermented, dry salami exposed to continuous lighting for all time periods are given in Table 2. The average initial L coordinate (lightness) of samples from the four replicate lots of salami was 42.5 ± 0.2. Although there were significant ($p<0.05$) differences observed from salami L coordinates among the various packaging treatments, the change in visual product lightness was very small and would not be noticeable to consumers.

Tristimulus +a coordinates represent the red hue in the Judd-Hunter Color Solid. There was no significant ($p>0.05$) decrease in +a coordinates until the OTR of the packaging film was ≤30 cc O_2/m^2/24 hr. Higher OTRs (72 and 90 cc O_2/m^2/24 hr)resulted in even greater decreases ($p<0.05$) of redness (Table 2). Tristimulus +b coordinates (yellow hue compo-

Table 1—Composition, lactic acid content and pH of fermented, dry salami

Constituent or pH	Replication			
	I	II	III	IV
Moisture (%)	37.58^{b}	38.95^{a}	39.16^{a}	35.54^{c}
Fat (%)	39.01^{a}	37.30^{b}	34.39^{c}	37.52^{b}
Protein (%)	21.88^{a}	20.95^{ab}	20.03^{bc}	19.42^{c}
Ash (%)	5.19^{a}	5.10^{ab}	4.87^{c}	4.91^{bc}
Lactic acid (%)	2.31^{a}	2.10^{b}	2.29^{a}	1.65^{c}
pH	4.81^{b}	4.65^{c}	4.89^{a}	4.81^{b}

^{a,b,c} Means in a row with the same or one of the same letters are not significantly ($p>0.05$) different.

Table 2—Effect of oxygen transmission rate (OTR) of packaging films on color properties of fermented, dry salami displayed under continuous lighting over all time periods

Color property	Film OTR (cc O_2 /m^2/24 hr)				
	1	11	30	72	90
L	42.6^{b}	43.2^{a}	42.7^{b}	42.5^{b}	43.6^{a}
+a	13.7^{a}	13.2^{a}	11.3^{b}	10.0^{c}	9.3^{d}
+b	6.6^{c}	7.2^{b}	7.5^{a}	7.7^{a}	7.5^{a}
a/b ratio	2.09^{a}	1.90^{b}	1.55^{c}	1.34^{d}	1.27^{d}
θ	0.46^{a}	0.50^{d}	0.60^{c}	0.67^{b}	0.70^{a}
S	15.2^{a}	15.1^{a}	13.7^{b}	12.8^{c}	12.2^{d}
ΔE	3.52^{c}	3.85^{c}	4.92^{b}	5.65^{a}	5.88^{a}
% pigment conversion	91.4^{a}	89.1^{b}	86.8^{c}	85.3^{d}	83.4^{e}

^{a,b,c,d,e} For each color parameter, means in a row with the same or one of the same letters are not significantly ($p>0.05$) different.

nent) signficantly ($p<0.05$) increased for each increase in film OTR up to 30 cc $0_2/m^2/24$ hr. Further increases in film OTR did not signficantly alter +b coordinates.

The a/b ratio, a redness index, signficantly ($p<0.05$) decreased with each increase of film OTR from 1 to 72 cc $0_2/m^2/24$ hr. Parallel reverse results were found for S, another index of redness based on the a and b coordinates. S values significantly ($p<0.05$) increased as film OTR decreased from 90 to 11 cc $0_2/m^2/24$ hr (Table 2). The hue angle θ, significantly ($p<0.05$) increased from 0.46 to 0.70 radian with each increase of OTR for the five films evaluated.

Salami surface redness characteristics were definitely altered by oxygen penetration through the packaging films since the +a coordinates, a/b ratios and S values decreased and θ increased as film OTR increased. Increased oxygen penetration also affected pigment conversion (Table 2). Each increase of film OTR resulted in a significant ($p>0.05$) step-wise decrease in the percent of nitrosoheme pigment in the samples. Total color difference, ΔE, which indicates the magnitude of difference between locations in the Judd-Hunter Color Solid, was not significantly ($p>0.05$) different between salami samples packaged in the "low" OTR films (1 versus 11 cc $0_2/m^2/24$ hr) or between those packaged in the "high" OTR films (72 vs 90 cc $0_2/m^2/24$ hr) (Table 2). However, salami surface ΔE values significantly ($p<0.05$) increased as the packaging film OTR increased from "low" to "intermediate" (30 cc $0_2/m^2/24$ hr) to "high" for the films used in this study.

Time of light exposure. The effects of time (weeks) of light exposure on color properties of the salami samples are given in Table 3. The results are reported as means for salami packaged in all films. The L and +b coordinates significantly ($p<0.05$) increased and the +a coordinate significantly ($p<0.05$) decreased during the 8 wk of light display. The a/b ratio, S and θ also changed significantly ($p<0.05$) over time. Total color difference, ΔE, significantly ($p<0.05$) increased from the first week to the sixth and eighth weeks (Table 3). The primary hue component change which affected changes in ΔE values was redness (+a) since the lightness (L) and yellowness (+b) components had maximum changes of +3.3% and +14.7%, respectively, during the period of 1–8 wk. The +a values decreased ($p<0.05$) by 24.8% in the same time period.

Visual changes in salami surface color properties from a red-maroon to a tan-brown appearance, which take place when sufficient oxygen is present under light display conditions, have been termed "fading" or "discoloration" (Lin et al., 1980; Acton et al., 1986). In this study, the initial visual red-maroon color (0 week) became maroon-brown to completely tan-brown at the final evaluation period (8 wk) for salami packaged in the "intermediate" and "high" OTR films, respectively. These visual observations agreed with the major changes which occurred in salami redness components (+a, a/b ratio, θ and S) that were instrumentally measured. All of the salami samples remained approximately the same in visual lightness, as previously noted. It is important to note that sample lightness (L coordinate) in the Judd-Hunter Color Solid does not have to change when hue changes occur (Hunter, 1975).

The effects of oxygen penetration to the salami surface and interior must be combined with the effects of light since the means at each period of display included results for all packaging films. Oxygen penetration over time of light display is best shown by the significant ($p<0.05$) decrease in the percent pigment conversion (Table 3), which is related to film OTR (Table 2). Fox (1966) stated that the nitrosoheme pigment is sensitive to light-induced dissociation of nitric oxide from the heme moiety. Following dissociation, nitric oxide is oxidized by oxygen. In this two-step sequence, light energy accelerates nitric oxide dissociation and the dissociation is catalyzed by the presence of oxygen which is then consumed in the oxidation step (Tarladgis, 1962). Thus, OTR of the packaging film, as previously discussed (results in Table 2), is extremely important to the effects of light on salami color stability during extended periods of light exposure.

Light display vs dark storage (at 8 wk). After 8 wk in the light display, every color property of the packaged dry, fermented salami except lightness (L coordinate) was signficantly ($p<0.05$) different from that of the packaged salami stored in the dark (Table 4). All redness characteristics (+a, a/b ratio, θ, S, and % pigment conversion) indicated significant ($p<0.05$) product fading or discoloration after 8 wk of light display as compared to dark storage. There was a 41% difference in the total color difference, ΔE, between salami samples stored in the dark as compared to those displayed in the light. These results confirm that light serves as an energy source for the reactions that promote cured meat discoloration (Tarladgis, 1962; Reith and Szakaly, 1967b; Mandigo and Kunert, 1973).

The effects of oxygen penetration to the surface (and interior) of the packaged salami are shown in Table 5, based on the OTR of the packaging film. In general, the majority of the significant ($p<0.05$) differences in color properties between salami samples exposed to light or stored in the dark for 8 wk occurred for films with high OTRs. There were no significant ($p>0.05$) differences due to light or dark storage for any color property for samples packaged in the film with an OTR of 1 cc $0_2/m^2/24$ hr. When the OTR of the packaging film was 11 cc $0_2/m^2/24$ hr, the +b coordinate was significantly ($p<0.05$) higher for the light-displayed sample as compared to that stored in the dark. With the increase in +b, significant ($p<0.05$) effects were also noted for the a/b ratio and hue angle (θ). With greater oxygen penetration for the films with OTRs of 30, 72, and 90 cc $0_2/m^2/24$ hr, the majority of the color characteristics were significantly ($p<0.05$) different between the light-displayed and dark-stored salami samples.

The nitrosoheme pigment conversion data were obtained by analyses of representative sample homogenates which included subsurface as well as surface pigment levels. Methodology for determining surface pigment concentration of cured meats is not presently available. Therefore, the results for salami in each packaging film indicate the overall effects of oxygen penetration to the surface and interior. Conversion values after 8 wk of light display or dark storage (Table 5) ranged from

Table 3—Effect of time on color properties of fermented, dry salami displayed under continuous lighting

Color property	Week of light exposure					
	0	1	2	4	6	8
L	41.7[c]	42.0[c]	42.5[b]	42.5[b]	43.4[a]	43.4[a]
+a	14.1[a]	12.9[b]	11.8[c]	11.5[c]	10.4[d]	9.7[e]
+b	6.3[d]	6.8[c]	7.7[a]	7.8[a]	7.3[a]	7.5[ab]
a/b ratio	2.26[a]	1.96[b]	1.57[c]	1.51[cd]	1.45[d]	1.32[e]
θ	0.43[e]	0.50[d]	0.58[c]	0.61[c]	0.64[b]	0.69[a]
S	15.5[a]	14.7[b]	14.2[bc]	14.0[c]	12.9[d]	12.4[d]
ΔE	-	3.83[c]	4.43[b]	4.51[b]	5.78[a]	6.04[a]
% pigment conversion	92.8[a]	89.3[b]	87.7[c]	85.5[e]	86.4[d]	85.0[e]

[a,b,c,d,e] For each color parameter, means in a row with the same or one of the same letters are not significantly ($p>0.05$) different.

Table 4—Effect of light display or dark storage after 8 weeks on color properties of fermented, dry salami

Color property	Light display	Dark storage
L	43.4[a]	43.7[a]
+a	9.7[a]	12.6[b]
+b	7.5[a]	6.4[b]
a/b ratio	1.32[a]	2.00[b]
θ	0.69[a]	0.48[b]
S	12.4[a]	14.1[b]
ΔE	6.04[a]	4.27[b]
% pigment conversion	85.3[a]	87.6[b]

[a,b] For each color property, means in a row with the same letter are not significantly ($p>0.05$) different.

Table 5—Effect of light display or dark storage after eight weeks on color properties of fermented, dry salami packaged in films with different oxygen transmission rates

Color property	Film OTR (cc O_2 /m²/24 hr)									
	1		11		30		72		90	
	Light	Dark	Light	Dark	Light	Dark	Light	Dark	Light	Dark
L	43.6	44.3	42.9[a]	45.2[b]	42.6	43.1	43.6	43.5	45.0	43.9
+a	12.6	12.9	12.0	12.9	9.2[a]	11.9[b]	7.6[a]	11.5[b]	6.2[a]	11.8[b]
+b	7.0	6.6	7.8[a]	6.6[b]	8.1[a]	6.4[b]	8.1[a]	6.6[b]	7.3	6.8
a/b ratio	1.83	1.99	1.55[a]	1.96[b]	1.11[a]	1.90[b]	0.93[a]	1.75[b]	0.83[a]	1.76[b]
θ	0.51	0.48	0.58[a]	0.48[b]	0.73[a]	0.51[b]	0.82[a]	0.53[b]	0.88[a]	0.54[b]
S	14.4	14.5	14.4	14.5	12.3	13.7	11.1[a]	13.3[b]	9.7[a]	13.7[b]
ΔE	3.6	4.2	4.5	4.6	5.7[a]	4.2[b]	6.8[a]	4.8[b]	8.4[a]	4.3[b]
% pigment conversion	89.3	90.0	88.3	87.7	83.9[a]	88.0[b]	82.5[a]	86.6[b]	82.4	85.7

a,b For these color properties within a film OTR, the mean value of the light-displayed sample is significantly ($p<0.05$) different from the mean value of the sample stored in the dark.

90.0% to 82.4%. From our observations of the packaged salami samples in this study, samples having at least 87% to 88% pigment conversion were visually red-maroon whereas those of less than 87% to 88% conversion had a maroon-brown to tan-brown hue. However, the nitrosoheme pigment conversion values at the actual product surface would be expected to be considerably less than 80% when visual surface discoloration or fading occurred.

For samples maintained in the light for 8 wk, the results generally agree with those reported for surface color fading of chicken bologna (Acton et al., 1986) and beef-pork bologna (Lin et al., 1980). Acton et al. (1986) suggested that loss of surface color redness characteristics occurred when the packaging film OTR exceeded approximately 17 cc $0_2/m^2/24$ hr. Lin and Sebranek (1979) and Lin et al. (1980) reported excellent maintenance of color characteristics when the OTR of the film was 7 cc $0_2/m^2/24$ hr, whereas an OTR of 60 cc $0_2/m^2/24$ hr was completely unsatisfactory.

Salami displayed in the light for 8 wk had 51% and 55% reductions in the +a coordinates and a/b ratios, respectively, between the extremes in packaging film OTRs (Table 5). Similar comparisons between the extremes in packaging film OTRs were 8.5% and 12% reductions for the +a coordinates and a/b ratios, respectively, of the salami samples held in dark storage. These comparisons suggested that at 8 weeks of display or storage, light had a greater effect on discoloration than oxygen penetration. In the two-step sequence of cured meat discoloration (Tarladgis, 1962; Fox, 1966), oxygen penetration or presence becomes a significant factor primarily if light energy is also available.

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Oxidative Stability of Batter-Breaded Restructured Nuggets Processed from Prerigor Pork

K.S. RHEE, J.T. KEETON, Y.A. ZIPRIN, R. LEU, and J.J. BOHAC

ABSTRACT

Restructured pork slices made with either prerigor or postrigor meat, with or without 1.5% of defatted glandless cottonseed flour (GCF), were batter-breaded with or without GCF in predust and batter-mix. Prerigor products had less cooking losses than postrigor products; GCF in the meat or coating reduced cooking losses. Prefried prerigor products were more susceptible to lipid oxidation and warmed-over flavor development than prefried postrigor products. GCF in the meat or coating markedly reduced the oxidative deterioration in nuggets stored at 4°C or −20°C, regardless of the rigor state of meat used for restructuring. Little lipid oxidation occurred in vacuum-packaged raw nuggets stored at −20°C.

INTRODUCTION

BATTER-BREADED MUSCLE FOOD products have been popular among people of all ages. When frozen in ready-to-cook form or precooked, they offer convenience to the consumer. When processed through restructuring/reforming from less valuable muscles of the carcass, such as the shoulder, they become value-added items. However, precooked, uncured, restructured meat products are very susceptible to lipid oxidation that leads to flavor deterioration. It was previously reported (Rhee et al., 1988) that the oxidized ("warmed-over") flavor development in prefried, batter-breaded restructured pork nuggets can be minimized by incorporating an antioxidant food ingredient, defatted glandless cottonseed flour (GCF), into either the meat or the coating system. The cottonseed flour, as used in that study, did not have adverse effects on the coating pick-up or palatability.

Hot-boning—the prerigor excision of muscle or muscle systems from the meat animal carcass—and further processing of prerigor meat have economic advantages through reductions in refrigeration costs, space requirements, processing delays and product turn-over time (Cuthbertson, 1980). The fat- and water-binding capacity of prerigor muscles is known to be greater than that of postrigor muscles (Kastner, 1982). In addition, prerigor grinding of pork semitendinosus muscles was shown to reduce the rate of lipid oxidation during refrigerated storage (Judge and Aberle, 1980). Drerup et al. (1981) also reported that prerigor grinding and salting reduced lipid oxidation in fresh pork sausage during frozen storage.

The present study was undertaken to determine various quality traits of batter-breaded restructured pork nuggets manufactured from prerigor meat, with or without GCF in the meat or in the batter-breading system. The variables that were included in the present study were the rigor state (prerigor vs postrigor) of meat used to produce restructured pork, storage temperature (4°C vs −20°C), processing state (prefried vs raw) of the nuggets prior to frozen storage, and packaging method (polyvinyl chloride, PVC, vs vacuum packaging) for frozen storage of raw nuggets.

Authors Rhee, Keeton, and Ziprin are with the Meats & Muscle Biology Section, Dept. of Animal Science, Texas A&M Univ., College Station, TX 77843. At present, author Leu is with Minh Food Corp., Pasadena, TX 77506, and author Bohac is with Longmont Turkey Processors, Inc., Longmont, CO 80502-1479.

MATERIALS & METHODS

Processing

Two gilts (111 and 108 kg) were slaughtered at the Texas A&M Univ. Rosenthal Meat Science & Technology Center. Immediately after slaughter, left sides were deboned and desinewed prerigor, and the warm muscles were restructured. Right sides (for postrigor processing) were chilled at 5°C for 2 days before they were submitted to the same processing sequence. The procedures and materials for restructuring meat included grinding the pork trim through a 1.27 cm grinder plate; blending with 0.5% NaCl, 0.25% polyphosphate and 3.0% water as crushed ice (with or without 1.5% GCF); coating frozen (−25°C) and tempered (7 min at room temperature) pork slices (1.2 cm-thick half-circle slices; 9.7% fat for prerigor product with no GCF, 9.2% fat for postrigor product with no GCF) with batter-breading (with or without GCF in predust and batter mix); and prefrying and reheating (15 min in a 218°C preheated gas oven) batter-breaded nuggets as described previously (Rhee et al., 1988). The prefrying time from the previous study was changed to 1 min frying–1 min resting–30 sec frying. The overall experimental design and abbreviations used for different products are shown in Fig. 1.

Determination of cooking losses, fat, and moisture

Batter-breaded nuggets were weighed in the raw state and after prefrying and reheating. The weight loss during prefrying was calculated as percent weight of raw product and expressed as precooking loss. Weight loss after reheating was computed as percent weight of prefried product and reported as reheating loss. Precooking loss and reheating loss were combined for total cooking losses.

Samples were finely ground before fat and moisture analyses by AOAC (1980) procedures.

Determination of lipid oxidation

Raw samples were either wrapped with an oxygen-permeable PVC film (6,500 cc O_2/m^2/24 hr) or vacuum-packaged in high-oxygen barrier bags (30–40 cc O_2/m^2/24 hr) before storage at −20°C. Prefried samples were wrapped in PVC film and stored at 4°C and −20°C; then, lipid oxidation in the samples was determined with or without reheating.

The extent of lipid oxidation was determined by the distillation 2-thiobarbituric acid (TBA) method of Tarladgis et al. (1960) as modified by Rhee (1978) with propyl gallate and EDTA added before the blending step to protect the sample from further lipid oxidation during the assay process. To minimize the sampling error, the coating or the meat from 12 nuggets of each product category per storage period was finely chopped and pooled, and an aliquot of the pooled sample was used for the TBA test. Results were expressed as TBA value (mg malonaldehyde/kg sample).

Sensory evaluation

Prefried nuggets stored at 4°C for 0 or 9 days were reheated and immediately evaluated by a 6-member trained sensory panel as described previously (Rhee et al., 1988).

Statistical analysis

The SAS package (SAS, 1982) was used to analyze data by analysis of variance, mean separation by the Student-Newman-Keuls test, and correlation analysis, where appropriate, using Pearson simple correlation technique.

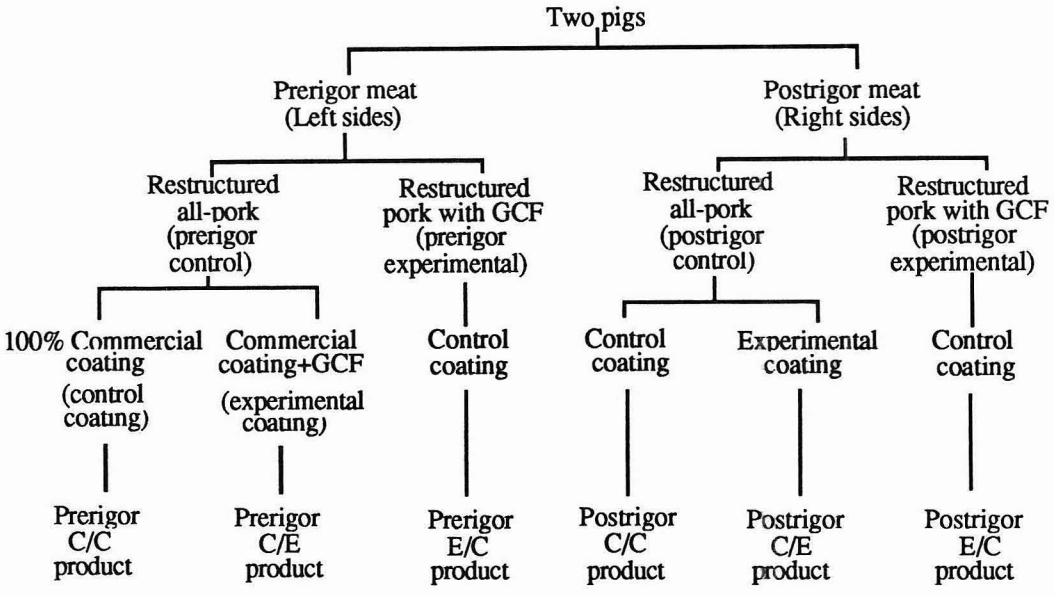

Fig. 1—Overall experimental design (GCF: defatted glandless cottonseed flour).

Table 1—Cooking losses stratified by the rigor state of meat used and product formulation

Category	Cooking losses (%)		
	Prefrying loss	Reheating loss	Total losses
Rigor state of meat			
Prerigor	3.3[b]	11.3[a]	14.7[b]
Postrigor	6.7[a]	12.3[a]	18.8[a]
Product (meat/coating)			
Control/control (C/C)	6.8[a]	13.9[a]	20.8[a]
Control/experimental (C/E)	3.9[b]	11.4[b]	15.3[b]
Experimental/control (E/C)	4.1[b]	10.6[b]	14.2[b]

[a,b] Means in the same column within the same data set (category) with different superscript letters are significantly different ($P<0.05$).

Table 2—Fat and moisture content of prefried nuggets stratified by the rigor state of meat used, product formulation, and reheating status

Category	Fat (%)		Moisture (%)	
	Coating	Meat	Coating	Meat
Rigor state of meat				
Prerigor	16.9[a]	16.7[a]	37.0[a]	56.8[a]
Postrigor	16.1[a]	14.0[b]	37.2[a]	56.5[a]
Product (meat/coating)				
Control/control (C/C)	16.1[a]	16.3[a]	35.7[a]	55.4[a]
Control/experimental (C/E)	17.7[a]	15.8[a]	38.5[a]	56.4[a]
Experimental/control (E/C)	15.9[a]	13.9[a]	36.7[a]	58.1[a]
Reheating status				
Without reheating	16.2[a]	13.9[b]	38.6[a]	59.3[a]
With reheating	17.1[a]	16.9[a]	35.4[b]	53.8[b]

[a,b] Means in the same column within the same data set (category) with different superscript letters are significantly different ($P<0.05$).

RESULTS & DISCUSSION

DATA FOR COOKING LOSSES are presented in Table 1. Total cooking losses were significantly higher for nuggets made with postrigor meat than prerigor meat, due to a higher prefrying loss for nuggets made with postrigor meat. Reheating loss was not significantly different between the two. Incorporation of GCF into the coating or meat substantially decreased both precooking and reheating losses; the losses were similar between C/E (control meat/experimental coating) and E/C (experimental meat/control coating).

Fat and moisture data for prefried nuggets are shown in Table 2. The fat content of the coating (crust) from prefried or prefried-reheated nuggets was not significantly affected by the rigor state of meat used, product formulation (absence or presence of GCF in the meat or coating) or reheating. In contrast, the fat content of the meat was higher for prerigor products than for postrigor products and also higher for prefried-reheated nuggets than for prefried nuggets. Moisture of both the coating and meat was higher for prefried nuggets than for prefried-reheated nuggets but was not significantly affected by either the rigor state of meat used or product formulation.

Table 3—Thiobarbituric acid (TBA) values of prefried nuggets[a] stored at 4°C stratified by the rigor state of meat, product formulation, reheating status, and storage time

Category	TBA value (mg malonaldehyde/kg)	
	Coating	Meat
Rigor state of meat		
Prerigor	1.32[b]	3.83[b]
Postrigor	1.16[c]	1.86[c]
Product (meat/coating)		
Control/control (C/C)	1.50[b]	4.70[b]
Control/experimental (C/E)	1.23[c]	2.42[c]
Experimental/control(E/C)	1.00[d]	1.42[d]
Reheating status		
Without reheating	1.08[c]	2.72[b]
With reheating	1.41[b]	2.98[b]
Storage time (days)		
0	1.12[c]	0.97[d]
3	1.19[b,c]	2.75[c]
6	1.32[b]	3.71[b]
9	1.34[b]	3.96[b]

[a] Packaged in O_2 permeable polyvinyl chloride film.
[b-d] Means in the same column within the same data set (category) with different superscript letters are significantly different ($P<0.05$).

TBA values of prefried nuggets stored at 4°C are shown in Table 3. Meat as well as coating TBA values were higher for prerigor products than postrigor products. The reason for the coating of prerigor products having somewhat higher ($P<0.05$) TBA values than that of postrigor products was not clear, but it was likely that, when the crust was removed from nuggets for analyses, very small amounts of the meat surface in contact with the crust might have also been removed. For both the coating and meat, TBA values of those products with GCF in either the coating or meat were lower than TBA values of C/C, while C/E values were slightly higher than E/C values. Similar observations were made in a previous study (Rhee et al., 1988) where nuggets were only made with postrigor pork shoulders. Reheating prior to TBA assays (or after storage) did

Table 4—Sensory scores of prefried nuggets[a] stored at 4°C stratified by the rigor state, products formulation and storage time

Overall category	Sensory score[d]					
	Crust color	Crust crispiness	Juiciness	Greasiness	Warmed-over flavor	Overall palatability
Rigor state of meat						
Prerigor	5.5[b]	6.0[b]	5.8[b]	1.9[b]	3.1[c]	5.5[c]
Postrigor	5.7[b]	6.0[b]	5.8[b]	2.1[b]	3.5[b]	5.9[b]
Product (meat/coating)						
Control/control (C/C)	5.6[b]	6.0[b]	5.7[c]	2.0[b]	2.9[c]	5.2[c]
Control/experimental (C/E)	5.9[b]	5.9[b]	6.1[b]	2.1[b]	3.6[b]	6.0[b]
Experimental/control (E/C)	5.2[c]	6.2[b]	5.5[c]	1.9[b]	3.5[b]	5.9[b]
Storage time (days)						
0	5.6[b]	6.1[b]	5.9[b]	2.0[b]	3.8[b]	6.3[b]
9	5.5[b]	5.9[b]	5.7[b]	2.0[b]	2.8[c]	5.1[c]

[a] Packaged in O_2 permeable polyvinyl chloride film.
[b,c] Means in the same column within the same data set (category) with different superscript letters are significantly different ($P<0.05$).
[d] Based on the following scales: crust color (1 = extremely light tan, 8 = very dark brown); crust crispiness (1 = extremely soggy, 8 = extremely crispy); warmed-over flavor (1 = very pronounced warmed-over flavor, 5 = no warmed-over flavor); overall palatability (1 = extremely unpalatable, 8 = extremely palatable).

Table 5—Thiobarbituric acid (TBA) values of prefried nuggets[a] stored at −20°C

Category	TBA value (mg malonaldehyde/kg)	
	Coating	Meat
Rigor state of meat		
Prerigor	1.27[b]	1.46[b]
Postrigor	1.19[b]	0.93[c]
Product (meat/coating)		
Control/control (C/C)	1.24[bc]	1.68[b]
Control/experimental (C/E)	1.32[b]	0.99[c]
Experimental/control (E/C)	1.13[c]	0.91[c]
Reheating status		
Without reheating	1.07[c]	0.98[c]
With reheating	1.39[b]	1.50[b]
Storage time (mo)		
0	1.12[c]	0.97[c]
2	1.30[b]	1.05[c]
4	1.08[c]	1.30[b]
6	1.42[b]	1.47[b]

[a] Packaged in O_2 permeable polyvinyl chloride film.
[b,c] Means in the same column within the same data set (category) with different superscript letters are significantly different ($P<0.05$).

Table 6—Thiobarbituric acid (TBA) values of raw nuggets stored at −20°C

Category	TBA value (mg malonaldhyde/kg)	
	Coating	Meat
Rigor state of meat		
Prerigor	0.56[b]	0.68[b]
Postrigor	0.51[b]	0.57[b]
Product (meat/coating)		
Control/control (C/C)	0.64[b]	0.82[b]
Control/experimental (C/E)	0.45[c]	0.53[c]
Experimental/control (E/C)	0.51[c]	0.53[c]
Packaging		
PVC-wrapped[a]	0.66[b]	0.99[b]
Vacuum-packaged	0.41[c]	0.27[c]
Storage time (mo)		
2	0.60[b]	0.59[b]
4	0.53[bc]	0.62[b]
6	0.47[c]	0.67[b]

[a] PVC: polyvinyl chloride film.
[b,c] Means in the same column within the same data set (category) with different superscript letters are significantly different ($P<0.05$).

not significantly affect TBA values of the meat although the mean value was numerically higher for reheated meat, but coating TBA values were higher ($P<0.05$) for prefried-reheated nuggets than prefried nuggets, probably reflecting the moisture loss from the crust (the outer part of nuggets) during reheating. Meat TBA values increased steadily up to day 6 but no significant changes were noted between day 6 and day 9. Increases in TBA values for the coating up to day 6 were very small from the practical viewpoint.

Sensory scores of prefried nuggets stored at 4°C and reheated prior to evaluation are shown in Table 4. Of the various sensory attributes evaluated, only warmed-over flavor (WOF) and overall palatability were significantly affected by the rigor state of meat used, with postrigor products having less WOF and being more palatable. Crust color was lighter for E/C than for C/C or C/E. Nuggets were more juicy when made with GCF in the coating than without. C/E and E/C had less WOF and were more palatable than C/C; no significant differences were found between C/E and E/C. Only WOF and overall palatability scores were significantly different between day 0 and day 9. Nuggets at day 9 had more WOF and were less palatable than those at day 0. Sensory WOF scores for prefried-refrigerated-reheated nuggets correlated well with meat TBA values of the nuggets, with a correlation coefficient of −0.86 ($P<0.01$). The same correlation coefficient was found in previous study by Rhee et al. (1988) where nuggets were made only with postrigor meat. Poste et al. (1986) also reported high correlations between sensory warmed-over aroma scores and TBA values for ground lean pork stored at 4°C for up to 16 days.

TBA values of prefried nuggets stored at −20°C are shown in Table 5. Coating TBA values of nuggets stored at −20°C were not noticeably different ($P>0.05$) from the values of nuggets stored at 4°C (Table 3), whereas meat TBA values were significantly lower for nuggets stored at −20°C. As was the case for prefried samples stored at 4°C, meat TBA values of samples stored at −20°C were also significantly higher for prerigor products than for postrigor products. Meat TBA values of C/E and E/C were similar ($P>0.05$); C/E and E/C values were substantially lower ($P<0.05$) than C/C values. Meat TBA values increased markedly upon reheating prefried nuggets stored at −20°C. Meat TBA values also increased ($P<0.05$) after 4 or 6 months of storage, but the increases were much smaller than those observed when prefried nuggets were stored at 4°C (Table 3). Coating TBA values were affected ($P<0.05$) by product formulation, reheating status and storage time; nevertheless, the TBA value differences were very small and of minor significance from the practical standpoint.

TBA values of raw nuggets stored at −20°C are shown in Table 6. Raw nuggets stored at −20°C had markedly low TBA values than prefried nuggets stored at the same temperature (Table 5). Raw-nugget meat TBA values did not differ significantly between 2, 4, and 6 months of frozen storage. Vacuum packaging, instead of PVC film packaging, kept TBA values of the meat extremely low. Even for frozen raw nuggets, GCF present in the coating or meat significantly reduced coating and meat TBA values. The rigor state of meat used did not show any significant effect on TBA values of the meat or coating ($P>0.05$), although the values tended to be higher for prerigor products.

There have been few reported research studies pertaining to

batter-breaded restructured red meat products. Huffman et al. (1987) addressed the use of polyphosphate in restructured beef and pork nuggets to increase the oxidative stability of frozen nuggets. That study reported that incorporation of 0.25% or 0.50% polyphosphate into the meat protected batter-breaded restructured beef and pork nuggets from lipid oxidation during 20 wk of storage at −23°C. On the other hand, the present study indicates that addition of 0.25% polyphosphate alone may not be effective for control of lipid oxidation in precooked batter-breaded restructured pork nuggets during an extended period of frozen storage and may be even less effective when precooked nuggets are refrigerated (Tables 3 and 4). Incorporation of GCF into the coating or meat, in addition to the polyphosphate used for restructuring of meat, was certainly more effective than the use of phosphate alone for minimization of lipid oxidation and WOF development in precooked pork nuggets during extended frozen storage (Table 5) or refrigerated storage (Tables 3 and 4; Rhee et al., 1988).

The propensity for precooked, prerigor pork nuggets to undergo oxidative flavor deterioration during storage more than precooked, postrigor nuggets (Tables 3 and 4) was not anticipated. Drerup et al. (1981) reported that prerigor grinding and salting in the production of fresh pork sausage reduced lipid oxidation during 0°C storage as compared with postrigor grinding and salting. It is possible that prerigor meat, when processed as was done in the present study, would still be warm during the restructuring process and the product temperature was not reduced in sufficient time to avoid an increased reaction rate at the initial stage (initial free radical formation) of lipid oxidation.

SUMMARY & CONCLUSIONS

BATTER-BREADED RESTRUCTURED PORK nuggets made with prerigor meat had less cooking losses than nuggets made with postrigor meat. Incorporation of GCF into either the meat or coating reduced cooking losses. When prefried, the meat from prerigor products had higher fat than postrigor products.

Prefried nuggets made with prerigor meat were more susceptible to lipid oxidation and WOF development than the counterparts made with postrigor meat; the presence of GCF in the coating or meat reduced lipid oxidation or WOF development in stored nuggets, regardless of the rigor state of meat used and the temperature of storage. Reheating of prefried nuggets tended to increase lipid oxidation.

When stored raw at −20°C, nuggets showed little lipid oxidation even after 6 months of storage, especially when vacuum-packaged, irrespective of whether nuggets were made with prerigor or postrigor meat.

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Evaluation of an Accelerated Processing System for Precooked Beef Products

S.T. WEIFFENBACH, D.D. JOHNSON, and R.L. WEST

ABSTRACT

Twelve beef carcasses were used to determine the effects of accelerated processing (hot-boning) on cooking, palatability and storage characteristics of precooked beef products. The conventional (CS) and the accelerated processed (AS) sides were fabricated into rib roast, strip loin, chuck roll, and pizza topping and products were precooked by convection oven, broiling, water bath or in a steam kettle, respectively. Products from the AS sides had lower cooking losses for strip loin steaks and chuck rolls and shorter cooking times for loin steaks and rib roasts than did product from CS sides. Rib roasts and chuck rolls from both processing systems were very acceptable in tenderness, but AS loin steaks were unacceptable in tenderness. Loin steaks and rib roasts from AS had less off-flavor than roasts from CS sides.

INTRODUCTION

SMALLER HOUSEHOLDS with greater disposable income and more active lifestyles have resulted in consumers seeking high quality, convenient food items at competitive prices. Precooked meat entree items that take less than 20 min to prepare can fill the needs of consumers for convenient food items. However, recent market introductions of precooked entree items have been less than successful due principally to the high prices associated with these products. An evaluation of the processing systems used to produce these products would suggest many inefficient components (several heat and chill cycles, the inclusion of inedible bone and fat and high cooking losses) that contribute to these high prices.

Kastner (1983) reported that beef processing efficiency could be improved by fabricating carcasses prior to chilling which would (1) reduce refrigeration demands by 40% to 50%; (2) reduce cooler space by 50% to 55%; (3) eliminate the need for shrouding, neckpinning, scribing, and operations to support those functions; (4) reduce labor utilization in the fabrication operation by as much as 25%; (5) decrease cooler shrinkage by up to 2%; and (6) reduce product in-plant residence time. Cooking muscle prior to chilling results in additional savings in time and reduced cooking loss (Ray et al., 1980; Griffin et al., 1981; Loucks et al., 1984). These same researchers reported that beef cooked prior to chilling resulted in increased shape distortions, decreased sensory panel tenderness scores and increased Warner-Bratzler shear force values when compared to conventionally chilled beef.

Zaglul (1981) reported that multiple electrical stimulation and boning based on muscle pH could prevent the negative effects of accelerated processing without excessive holding times and heat dissipation. This system could be feasible for items that are cooked prior to chilling or freezing. Precooking incorporated into a hot boning operation would capitalize on body heat remaining for cooking and on the efficiencies associated with chilling only the edible portions.

This study was undertaken to evaluate several processing systems that could produce a quality product and reduce processing costs. The objectives of this study were to compare the cooking characteristics, storage stability and palatability attributes of precooked beef items produced by accelerated processes with those produced by conventional processes.

Authors Johnson and West are with the Inst. of Food & Agricultural Sciences, Univ. of Florida, Gainesville, FL 32611. Author Weiffenbach is with the Burger King Corp., Miami, FL 33152.

MATERIALS & METHODS

Slaughter

Twelve crossbred steers were slaughtered at the Univ. of Florida Meats Laboratory following standard slaughter procedures. During exsanguination, all carcasses were subjected to low voltage stimulation (Koch-Britton Stimulator 75-LV, 60 volts, 18 sec continuous impulse, square wave, alternating polarity). After hide removal, all carcasses were subjected to high voltage stimulation (Koch-Britton Stimulator 350, 550 volts, 30 1-sec impulses). Alternating sides were assigned to either an accelerated fabrication treatment (AS) or a conventional fabrication treatment (CS). The AS sides were held in the slaughter area at approximately 24°C until the longissimus muscle pH reached 5.8. The pH was monitored with an Orion pH meter (Model 211) using an Ingold combination pH electrode (Model 18513) inserted into the longissimus muscle at the 12th rib. The average time required for pH decline was 69 min (after the second stimulation) and ranged from 35 to 130 min. The control sides were held in a cooler at 1 ± 1°C for 48 hr prior to grading and subsequent fabrication.

Fabrication and cooking

After the appropriate holding times, all sides were fabricated into Institutional Meat Purchase Specifications (IMPS) 180 strip loin, IMPS 107 rib roast, IMPS 116A chuck roll and trimmings (approximately 75% lean). Strip loins and rib roasts were cut into two equal portions and alternating halves were passed once through a Ross TC 700 Mechanical Tenderizer. Strip loins were cut into 2.54 cm steaks and broiled on Farberware Open-Hearth electric broilers to an internal temperature of 60°C. Rib roasts were netted and cooked (Vortron 500 smokehouse) to an internal temperature of 60°C using the following cooking schedule: for the first 25% cooking time, the dry bulb was set at 55°C with the wet bulb set at 52°C to produce 90% relative humidity, and for the remaining 75% of cooking time, the smokehouse was maintained at 71°C with low relative humidity. The chuck rolls were netted and placed into Cryovac CN510 cooking material which was vacuumed and clip-sealed (Tipper Clipper, Tipper Tie Div., Rheem Mfg. Co. Union, NJ). They were then placed in a 71°C water tank, heated by live steam, and cooked to an internal temperature of 60°C. Trimmings were formulated into a 25% fat raw material, coarse-ground (0.95 cm plate) and mixed with rehydrated textured vegetable protein (TVP) (Heller's #70) and pizza sausage seasoning (Heller's GC-3027) in the following proportions: 67% meat, 10% TVP, 20% water and 3% seasoning. The product was finely ground (0.32 cm plate) and cooked with continuous agitation for 10 min in a steam-jacked kettle.

Cooking times for loin steaks, rib roasts, and chuck rolls were monitored by the insertion of copper-constantan thermocouples in the geometric center of each cut. The thermocouples were attached to either a Speedomax W (Leeds and Northrup) or Honeywell (Model 153 × 64-PIO-III-141) temperature recorder. Cooking losses for loin steaks, rib roasts and chuck rolls were determined by weight differences between the raw and cooked product.

Packaging and storage

Precooked loin steaks were individually placed in cryovac B 620 bags and heat-sealed with a vacuum-packaging machine (Reiser VM-31). All but one of the steaks from each tenderization treatment were randomly assigned to three storage treatment groups. The first group was cooled to 22°C and immediately used for shear force determina-

tions. The second group was held for 21 days at 1±1°C before shear force and palatability determinations were made. Steaks in the third group were stored frozen (−33°C) for 60 days and used for palatability evaluations.

Precooked rib roasts were placed in Cryovac B 620 bags and heat-sealed with a vacuum-packaging machine (Reiser VM-31). They were then chilled for 30 min in a prototype propylene glycol chiller (Meyer Metal Craft, Kansas City, MO) at −11°C. Rib roasts were held overnight at 1±1°C to allow temperature equilibration. The packaged roasts were opened and two 2.54 cm steaks were removed for shear force determinations and then vacuum-packaged. After 21 days storage at 1±1°C, rib roasts were cut into 2.54 cm steaks for shear force and palatability determinations.

Precooked chuck rolls were cut into two equal portions which were individually placed into Cryovac B 620 bags and heat-sealed with a vacuum-packager. Chuck roasts were chilled in the prototype propylene glycol chiller for 30 min at −11°C and then held overnight at 1±1°C for temperature equilibration. Two 2.54 cm steaks were removed for shear force determinations. Chuck rolls were repackaged and stored for 21 days at 1±1°C. After storage, two 2.54 cm steaks were removed for shear force determinations.

Precooked pizza toppings were placed into plastic nonvacuum bags and stored frozen (−33°C) for 30, 60, and 90 days. Palatability evaluations were conducted on days 0, 30, 60, and 90.

Shear force determinations

Shear force values were used to obtain quantitative values for the tenderness of strip loin steaks, rib roasts, and chuck rolls. A Warner-Bratzler shear device was used to obtain peak shear force values used for the tenderness score. Prior to shearing, all samples were allowed to equilibrate to room temperature (22°C). As many cores (1.27 cm in diameter) as possible were removed from the longissimus muscle of the strip loin steaks and rib roasts and from the Serratus ventralis and Rhomboideus muscles of the chuck rolls. Each core was sheared once, perpendicular to the muscle fibers (AMSA, 1978).

Fat and moisture

Fat and moisture of pizza toppings were monitored before and after cooking. Fat was determined in duplicate using the modified Babcock procedure (Koniecko, 1979). Moisture was determined using the oven-drying technique (AOAC, 1980).

Sensory evaluations

A sensory panel was trained to evaluate strip loin steaks and rib roasts for flavor intensity, juiciness, tenderness, sensorially detectable connective tissue and off-flavor (AMSA, 1978). Pizza toppings were evaluated by a sensory panel trained to discriminate for spice flavor intensity, textured vegetable protein flavor intensity, and off-flavor. Prior to sensory panel evaluations, all samples were warmed in a microwave oven (Sharp Model R8310). Strip loin steaks and pizza topping samples were warmed for 3 min on the high setting and rib roast samples were warmed for 6 min on the medium high setting.

Microbial analysis

Loin steak and rib roast samples were aseptically collected before and after cooking and after 10 and 19 days of vacuum storage at 1±1°C. Samples were placed in sterile Stomacher 400 bags and diluted with sterile Butterfield's phosphate diluent in the ratio of 9g diluent to 1g sample. They were then homogenized in a Stomacher 400 (Model BA6021, Tekmar, Cincinnati, OH) for 1 min and serially diluted. Aerobic standard plate counts (APC) were determined in quadruplicate by placing 0.5 mL diluent on pre-poured standard plate count agar (Difco Laboratories). Mesophilic APC were determined after incubation at 35°C for 48 hr. Psychrotrophic APC were determined after incubation at 20°C for 5 days. The data were converted to common logarithms (base 10) of the counts.

Statistical analyses

Data were analyzed using the General Linear Models procedure of the Statistical Analysis System (SAS, 1982). Data were analyzed by product. Rib data were analyzed by fabrication method, tenderization treatment, and storage period (2×2×2 factorial). Warner-Bratzler shear force data for strip loin steaks were analyzed by fabrication method and tenderization treatment within storage period (2×2×2 factorial). All other loin steak data were analyzed by fabrication method and tenderization treatment within storage period (2×2 factorial). Chuck roll and pizza topping data were analyzed by fabrication method and storage period (2×2 factorial). Microbial data were reported by bacterial type and tested by fabrication method, tenderization treatment and storage period. Means for microbiological sampling period were separated using Duncan's multiple range test (Steel and Torrie, 1980). Means with two levels were separated using F-tests derived from analysis of variance.

RESULTS & DISCUSSION

Cooking times

Means and standard errors for cooking times of strip loin steaks, rib roasts and chuck rolls by fabrication method and tenderization treatment are shown in Table 1. The accelerated process decreased ($P<0.05$) the cooking time for loin steaks and rib roasts by approximately 27%. Similar results were reported by Ray et al. (1980) who found hot-boned beef roasts cooked 22% faster than their conventionally boned counterparts. No differences ($P>0.05$) in cooking times were observed for chuck rolls; however, there was a tendency for the accelerated (AS) chuck rolls to cook faster than conventional (CS) chuck rolls. Figure 1 shows typical plots of internal temperature changes by time for AS and CS chuck rolls. The greater temperature difference between the CS chuck rolls and the water than between the AS chuck rolls and the water could have been responsible for the different cooking rates. The CS chuck rolls' cooking rate was much greater in the 4°C to 38°C temperature range than in the 38°C to 60°C temperature range. At 38°C, the initial temperature of the AS chuck rolls, the cooking rate of the CS chuck rolls was markedly reduced. Myosin and collagen denaturation at 40°C to 60°C (Martens et al., 1982) may have reduced the rate of heat transfer. More research is needed to further understand the differences in cooking rates of hot-boned versus conventionally boned beef roasts with various cooking methods.

Mechanical tenderization had no effect ($P>0.05$) on the cooking times of loin steaks or rib roasts (Table 1). This is not in agreement with the findings of Bowling et al. (1976), Goldner et al. (1974), Glover et al. (1977) or Schwartz and Mandigo (1974). These researchers found mechanically tenderized, normally processed meat cooked faster than nontenderized meat. However, Savell et al. (1977) reported no difference in cooking time between mechanically tenderized and control steaks. No significant interaction between fabrication method and tenderization treatment was detected.

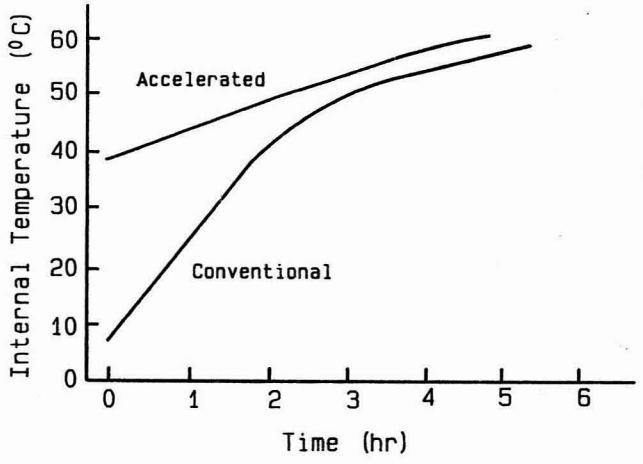

Fig. 1—Typical internal temperature changes by time for accelerated and conventionally fabricated chuck rolls in a 71°C water bath.

Table 1—Means and standard errors for the effects of fabrication method and tenderization treatment on the cooking times and cooking losses of loin steaks, rib roasts and chuck rolls

Trait	Product	Fabrication method[a]		Tenderization treatment[b]		SE
		AS	CS	T	NT	
Cooking time[c]	Loin steaks	17.0[x]	23.4[y]	20.5	19.9	0.5
	Rib roasts	131.8[x]	180.6[y]	154.4	157.9	5.0
	Chuck rolls	40.3	45.1	–	–	1.7
Cooking loss[d]	Loin steaks	17.14[x]	21.05[y]	19.92	18.27	0.94
	Rib roasts	9.01	8.02	9.34[x]	7.70[y]	0.53
	Chuck rolls	9.90[x]	11.89[y]	–	–	0.60

[a] AS = Accelerated process; CS = Conventional process.
[b] T = Blade tenderized; NT = Not tenderized.
[c] Cooking times of loin steaks expressed in min/steak and of rib roasts and chuck rolls expressed in min/kg.
[d] Cooking losses of all products are expressed as percent of raw weight.
[xy] Means on a row within a main effect with different superscripts are different ($P<0.05$).

Cooking losses

Cooking losses for loin steaks, rib roasts, and chuck rolls by fabrication method and tenderization treatment are also presented in Table 1. The AS system resulted in 19% and 17% reductions ($P<0.05$) in cooking losses for loin steaks and chuck rolls, respectively, when compared to their conventionally boned counterparts. These findings are in agreement with those reported by Paul et al. (1952), Cia and Marsh (1976), Kastner et al. (1976), Marsh (1977), Ray et al. (1980) and Griffin et al. (1981) who reported reduced cooking losses for hot-boned meat products when compared to conventionally boned controls. No difference ($P>0.05$) in cooking loss due to fabrication method was observed for rib roasts. The relatively mild heat treatment and high relative humidity during the first 25% of the cooking process for rib roasts may have been responsible for the observed results.

Mechanical tenderization increased the cooking loss of rib roasts by 28% (Table 1). Davis et al. (1975), Glover et al. (1977), and Savell et al. (1977) also have reported increased cooking losses due to mechanical tenderization. Cooking losses for loin steaks were not affected ($P>0.05$) by mechanical tenderization. Boyd et al. (1978), Schwartz and Mandigo (1974), Goldner and Mandigo (1974), Bowling et al. (1976) and Loucks et al. (1984) also reported that cooking losses were not affected by mechanical tenderization.

Fat loss (0.54 vs 0.50%) and moisture loss (3.6 vs 3.2%) for AS and CS, respectively, during cooking of pizza toppings were not affected by the fabrication method. Similar processing characteristics of the trim from both the AS and CS sides would be expected due to rigor development induced by the electrical stimulation treatment, therefore, water-holding capacities should be similar. These findings were substantiated by West (1983) who found electrically stimulated hot-boned meat had similar functional properties to meat boned after chilling.

Shear force

Means for Warner-Bratzler shear force values for loin steaks, rib roasts, and chuck rolls sampled after cooking (day 1) and after 21 days of storage, are presented in Table 2, segregated by fabrication method and tenderization treatment. The AS loin steaks and rib roasts had significantly higher ($P<0.05$) shear values at both sampling periods than their CS counterparts. The AS loin steaks had average shear values over 5.0 kg/1.27 cm core which indicated an unacceptably tough product. While the AS rib roasts had higher shear values than the CS rib roasts, both values were very low and indicated acceptable products. Fabrication method had no effect ($P>0.05$) on the shear force values of the chuck rolls. Shear force values for loin steaks and rib roasts were not affected ($P>0.05$) by mechanical tenderization. These results are not in agreement with those reported by Goldner et al. (1974), Goldner and Mandigo (1974), Schwartz and Mandigo (1974), Davis et al. (1975), Glover et al. (1977), Hinnergardt et al. (1975) and Bowling et al. (1976) who reported increased tenderness due to mechanical tenderization.

Rib roasts aged 21 days in-vacuo had lower ($P<0.05$) shear force values than unaged rib roasts (2.36 kg vs 2.79 kg/1.27 cm, respectively). The tenderness of precooked loin steaks and chuck rolls was not affected ($P>0.05$) by vacuum aging for 21 days.

Sensory characteristics

Mean palatability scores for rib roasts by the fabrication method, tenderization treatment and storage period are presented in Table 3. Fabrication method had no effect ($P>0.05$) on flavor, juiciness or connective tissue scores for the rib roasts. The AS rib roasts were scored as being less ($P<0.01$) tender than the CS rib roasts. However, the scores indicate that both were very tender. The AS rib roasts had less ($P<0.001$) off-flavor than the CS rib roasts.

Mechanical tenderization had no effect ($P>0.05$) on any palatability attributes for rib roasts (Table 3). Savell et al. (1977) demonstrated that two and three passes through the mechanical tenderizer could lower shear force values without affecting sensory panel juiciness scores; however, sensory panel tenderness scores were not improved with multiple passes in that study.

After 21 days of storage at 1 ± 1°C, sensory panel scores for flavor and tenderness did not differ ($P>0.05$) from the 1 day scores. There was a tendency for the rib roasts stored 21 days to be less tender; however, this tendency did not reach significance. The 21 day storage rib roasts were scored as being less ($P<0.01$) juicy and as having more ($P<0.05$) detectable connective tissue than the 1 day storage rib roasts. The 21 day rib roasts had lower ($P<0.001$) off-flavor scores than the 1 day rib roasts. The magnitude of the scores indicated only slight off-flavors were detected and that the products were acceptable after 21 day storage.

Mean values for the effects of fabrication method and tenderization treatment on palatability scores of precooked loin steaks stored 21 days at 1°C and 60 days at −33°C are presented in Table 4. Fabrication method had no effect ($P>0.05$) on the flavor intensity scores of precooked loin steaks stored 21 days. The AS loin steaks were scored as being less ($P<0.001$) tender and as having more ($P<0.05$) detectable connective tissue. However, after the 21 day storage period, the AS loin steaks showed a distinct advantage in off-flavor scores. The panel scored the AS loin steaks as having less ($P<0.05$) off-flavor. Tenderization treatment exerted no effect ($P>0.05$) on any of the palatability attributes evaluated for loin steaks stored 21 days. These data agree with Davis et al. (1975) who reported mechanical tenderization did not affect sensory panel juiciness, tenderness or flavor scores of beef.

Sensory panel results for loin steaks stored frozen (−33°C)

Table 2—Mean Warner-Bratzler shear force values for loin steaks, rib roasts and chuck rolls by fabrication method and tenderization treatment within storage period

	Shear values (kg/1.27 cm)				
	Fabrication method[a]		Tenderization treatments[b]		
Product and time	AS	CS	T	NT	SE
Loin steaks, day 1	5.33[x]	4.43[y]	4.82	4.93	0.23
Loin steaks, day 21	5.72[x]	3.85[y]	4.55	5.01	0.25
Rib roasts, day 1	3.02[x]	2.56[y]	2.74	2.84	0.12
Rib roasts, day 21	2.53[x]	2.19[y]	2.32	2.40	0.11
Chuck rolls, day 1	3.60	3.33	–	–	0.20
Chuck rolls, day 21	3.64	3.30	–	–	0.15

[a] AS = Accelerated process; CS = Conventional process.
[b] T = Blade tenderized; NT = Not tenderized.
[xy] Means on a row within a main effect with different superscripts are different (P<0.05).

Table 3—Mean palatability scores for rib roasts by fabrication method, tenderization treatment and storage period

	Fabrication method[a]		Tenderization treatment[b]		Storage (days)		
Trait	AS	CS	T	NT	1	21	SE
Flavor intensity[c]	5.8	5.6	5.8	5.6	5.8	5.6	0.1
Juiciness[d]	6.2	6.0	6.0	6.2	6.3[x]	5.9[y]	0.1
Tenderness[e]	7.2[x]	7.4[y]	7.2	7.4	7.4	7.2	0.1
Connective tissue[f]	7.0	7.1	7.0	7.1	7.2[x]	6.9[y]	0.1
Off flavor[g]	3.9[x]	3.6[y]	3.7	3.8	3.9[x]	3.6[y]	0.1

[a] AS = Accelerated process; CS = Conventional process.
[b] T = Blade tenderized; NT = Not tenderized.
[c] 8 = extremely intense; 1 = extremely bland.
[d] 8 = extremely juicy; 1 = extremely dry.
[e] 8 = extremely tender; 1 = extremely tough.
[f] 8 = none detected; 1 = abundant amount.
[g] 4 = no off-flavor; 1 = extreme off-flavor.
[xy] Means on a row within a main effect with different superscripts are different (P<0.05).

Table 4—Means and standard errors for the effects of fabrication method and tenderization treatment of palatability scores of precooked loin steaks stored 21 days at 1°C or 60 days at −33°C

		Fabrication method[a]		Tenderization treatment[b]		
Storage	Trait	AS	CS	T	NT	SE
21 days/1°C	Flavor intensity[c]	6.2	6.0	6.1	6.0	0.11
	Juiciness[d]	5.5[x]	4.8[y]	5.2	5.2	0.17
	Tenderness[e]	4.4[x]	5.5[y]	5.1	4.8	0.17
	Connective tissue[f]	4.6[x]	5.2[y]	4.9	4.9	0.16
	Off-flavor[g]	3.6[x]	3.1[y]	3.3	3.4	0.12
60 days/−33°C	Flavor intensity	5.3[x]	5.8[y]	5.5	5.6	0.11
	Juiciness	4.8	5.1	4.7	5.3	0.21
	Tenderness	5.2[x]	6.0[y]	5.5	5.7	0.17
	Connective tissue	5.7[x]	6.3[y]	6.0	6.0	0.12
	Off-flavor	3.9[x]	3.8[y]	3.9	3.9	0.03

[a] AS = Accelerated process; CS = Conventional process.
[b] T = Blade tenderized; NT = Not tenderized.
[c] 8 = extremely intense; 1 = extremely bland.
[d] 8 = extremely juicy; 1 = extremely dry.
[e] 8 = extremely tender; 1 = extremely tough.
[f] 8 = none detected; 1 = abundant amount.
[g] 4 = no off-flavor; 1 = extreme off-flavor.
[xy] Means on a row within a main effect with different superscripts are different (P<0.05).

Table 5—Means and standard errors of mesophilic and psychrotrophic standard plate counts (log_{10}/g) for loin steaks and rib roasts by fabrication method

	Bacterial	Fabrication method	
Product	type	Accelerated	Conventional
Loin steaks	Mesophilic	2.37[x] ± 0.19	3.16[y] ± 0.24
	Psychrotrophic	2.71[x] ± 0.20	3.39[y] ± 0.24
Rib roasts	Mesophilic	4.17[x] ± 0.22	3.68[y] ± 0.25
	Psychrotrophic	4.15[x] ± 0.23	3.84[y] ± 0.25

[xy] Means on a row with different superscripts are different (P<0.05).

for 60 days (Table 4) were similar to the results for the steaks stored fresh (1°C) for 21 days. The AS loin steaks had lower (P<0.01) flavor intensity scores than the CS loin steaks; however, steaks from both fabrication methods were scored as being slightly intense in flavor. No differences (P>0.05) in juiciness scores were detected between fabrication methods. Both tenderness and connective tissue scores were lower (P<0.01) for the AS loin steaks than for the CS loin steaks, indicating that the AS steaks were less tender, possibly due to the greater amount of detectable connective tissue. The AS loin steaks received higher (P<0.05) off-flavor scores than the CS loin steaks. Though the differences were small, the accelerated system tended, once again, to have suppressed off-flavor development. Perhaps, 48 hr chilling was enough time to initiate the lipid oxidation process in the control side. Once begun, the oxidation process was self-propagating and, because it was a chemical reaction, it might have been accelerated during cooking. Mechanical tenderization had no effect (P>0.05) on any of the palatability attributes evaluated for precooked loin steaks stored frozen for 60 days.

Table 6—Means and standard errors of mesophilic and psychrotrophic standard plate counts (log_{10}/g) for loin steaks and rib roasts by storage period[a]

Product	Bacterial type	Storage period 0	1	10	19
Loin steaks	Mesophilic	3.39[x] ± 0.17	1.35[y] ± 0.18	2.40[z] ± 0.34	3.92[x] ± 0.36
	Psychrotrophic	3.76[xy] ± 0.19	1.37[z] ± 0.16	3.14[y] ± 0.31	3.94[x] ± 0.38
Fib roasts	Mesophilic	3.60[y] ± 0.14	1.53[z] ± 0.21	3.39[y] ± 0.20	7.18[x] ± 0.06
	Psychrotrophic	3.85[y] ± 0.13	1.33[z] ± 0.19	3.81[y] ± 0.21	7.24[x] ± 0.07

[a] 0 = raw; 1 = after cooking; 10 = after 10 days at 1°C; 19 = after 19 days at 1°C.
[xyz] Means on a row with different superscripts are different (P<0.05).

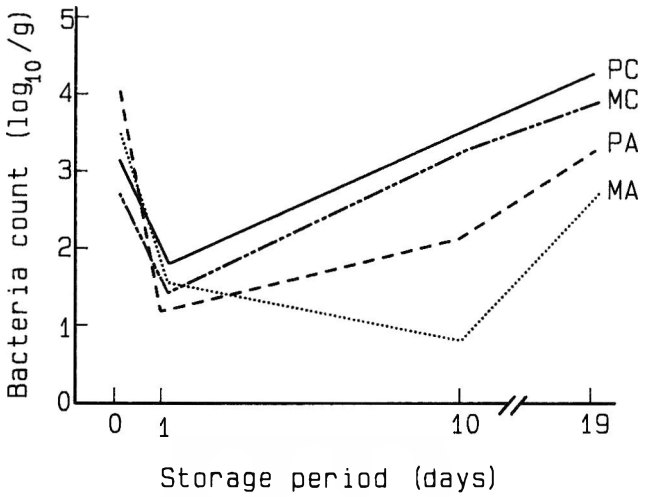

Fig. 2—Mean mesophilic (M) and psychrotrophic (P) standard plate counts (log_{10}/g) of loin steaks for accelerated (A) and conventional (C) fabrication methods with different storage periods. Samples were collected at fabrication (0), after cooking (1), after 10 days at 1°C (10), and after 19 days at 1°C (19).

The mean sensory panel scores for pizza toppings (data not shown in tabular form) were interpreted to show that the fabrication method had no effect (P>0.05) on spice flavor intensity, textured vegetable protein (TVP) flavor intensity, or off-flavor development. Younathan et al. (1980) reported that extracts of vegetables reduced lipid oxidation when mixed with ground turkey meat. Possibly, the high level (30%) of rehydrated textured vegetable protein incorporated into the pizza toppings was effective in controlling the development of oxidative rancidity during frozen storage. Also, if off-flavors had developed, they may have been masked by the strong spice flavors. Storage period had an effect (P<0.001) on spice flavor intensity; however, the differences were small (0.6 on an 8-point scale) and erratic. No explanation for those findings was apparent. Storage period had no effect (P>0.05) on TVP flavor intensity or off-flavor scores.

These results indicated that highly seasoned cooked ground beef products could be produced through the use of an accelerated processing system with sensory characteristics equivalent to products that were conventionally produced. Also, they indicated that such a product could be stored frozen in inexpensive packaging materials (i.e., without vacuum packaging) with little deterioration in palatability for at least 90 days.

Microbiological findings

Mesophilic and psychrotrophic standard plate counts (APC) for loin steak and rib roast samples collected at fabrication (0 day), immediately after cooking (1 day), and after 10 days and 19 days of storage, were analyzed for the effects of the fabrication method, tenderization treatment, sample period, and the interactions. Mechanical tenderization had no effect (P>0.05) on APC for loin steak or rib roast samples.

The mean values for the effects of the fabrication method on the mesophilic and psychrotrophic APC for loin steaks and rib roasts are shown in Table 5. Both APC values for the CS loin steaks were higher (mesophilic, P<0.01; psychrotrophic, P<0.05) than for the AS loin steaks. The AS rib roasts had higher (mesophilic, P<0.01; psychrotrophic, P<0.05) APC than the CS rib roasts. The results for rib roast APC are in agreement with those reported by Fung et al. (1980) and Kennedy et al. (1982) who reported higher mesophilic and psychrotrophic APC on hot-boned beef cuts than on conventionally boned beef cuts during fresh vacuum storage.

Mesophilic and psychrotrophic APC for loin steaks and rib roasts were affected (P<0.001) by storage period (Table 6). As was expected, cooking reduced all mesophilic and psychrotrophic counts. Both APC increased during 10 and 19 days of vacuum storage. Standard plate counts for rib roasts stored 10 days increased to levels that were not different from those at the initial storage period. After 19 days, however, both APC for rib roasts were higher than they were for all other sample periods. A band saw, used to remove sections of the rib roasts for day 1 shear force determinations, might have been the source of contamination. Furthermore, when the vacuum packages were aseptically opened on day 10 to collect microbiological samples, the exposure to air prior to re-vacuum packaging might have assisted bacterial proliferation.

Figure 2 shows the interaction effect of the fabrication method and storage period on mesophilic (P<0.001) and psychrotrophic (P<0.05) APC for strip loin steaks. Both APC were initially higher on the AS strip loin steaks than on the CS strip loin steaks. While both APC were higher on the AS strip loin steaks than they were on the CS strip loin steaks initially, they were lower after cooking. Possibly, the bacteria that were present on the AS strip loin steaks were not as well established in the tissue as were those on the CS strip loin steaks. The surface desiccation and low temperature of chilling during the 48 hr holding time probably were more favorable for the more viable bacteria and allowed sufficient time to adjust to their environment. During 10 days vacuum storage, the APC for the CS strip loin steaks increased more rapidly than did the psychotrophic APC for the AS strip loin steaks, while the mesophilic counts for AS steaks declined during 10 days of vacuum storage. Perhaps, those bacteria were less viable after cooking and were suppressed by the low temperature and lack of oxygen during vacuum storage than were the remaining psychotrophic bacteria. During the remaining 9 days of vacuum storage, the growth rates for the mesophilic and pychrotrophic bacteria on the CS strip loin steaks decreased; however, the growth rate for the psychrotrophs on the AS strip loin steaks remained the same and the mesophiles increased.

Boyd et al. (1978) reported increased APC on fresh vacuum-packaged beef due to mechanical tenderization; however, results of this study do not indicate increased bacterial proliferation due to mechanical tenderization to be a problem with precooked beef. Precooked beef products that have acceptable shelf-life and palatability can be produced with accelerated processing; however, attention must be given to various details to ensure the quality of the final product.

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—Continued on page 1102

A Procedure for Measuring Excised Muscle Contraction

J.A. DICKENS and C.M. PAPA

ABSTRACT

A procedure was developed to accurately measure the contraction of excised Pectoralis major muscles (along the longitudinal axis) from broilers during the development of rigor. A linear variable differential transformer (LVDT) and computer equipment were used to continuously monitor contraction through the course of rigor. A holding tank was designed and constructed and in conjunction with the LVDT allowed cooling of the muscle while contraction was being monitored over a predetermined time period. Applying the procedure to broiler Pectoralis major muscles, the results indicated much less variation using the LVDT in comparison with a technique of manual measurement; however, mean values were not significantly different.

INTRODUCTION

Recent developments in poultry processing technology, such as hot deboning (Hamm, 1982), have established the need for basic information relating to the effects of different process parameters on product quality. One area that needs to be investigated is the effect of chilling on muscle contraction which has been shown to affect meat tenderness (Welbourn et al., 1968).

Studies to determine the effect of temperature on the contraction of excised muscles historically have been accomplished using manual measuring techniques on narrow muscle strips. Lee and Rickansrud (1978) placed narrow strips of breast and thigh muscle on filter paper and observed the amount of contraction at different temperatures. Their work described the contraction at low temperatures to range from 13–20% of original length. Klose et al. (1970) photographed muscle strips on a graduated scale at predetermined times and measured contraction by noting changes in dimensions on successive photographs.

Smith et al. (1969) reported a method using the whole muscle which entailed marking the intact muscle with indelible ink at the ends of an imaginary line parallel to the direction of the muscle fibers. The marks were made at a set distance from each other and then measured after excision and again at predetermined times with a vernier micrometer to determine contraction.

The variation in reported results relating to the effect of chilling on muscle contraction existed because equipment for accurately measuring muscle contraction was not available. The objective of this study was to develop the equipment and procedure for accurately measuring the postmortem contraction of the whole Pectoralis major muscle.

MATERIALS & METHODS

EQUIPMENT was developed to record continuous measurements of the magnitude of muscle contraction over a given period of time. The equipment (Fig. 1) consisted of an acrylic tank assembly (#3), 29 cm x 30 cm x 10 cm deep, a water bath and a Linear Variable Differential Transformer (LVDT) with a range of ± 5 cm (#1). The tank incorporated a rigid hold-down (#4) device and input and output lines for water circulation so that measurements could be made at different temperatures. There was a slot in one side of the tank for the core extension of the LVDT to slide through. The LVDT (Fig. 1, #1) consisted of a primary coil and two secondary coils symmetrically spaced on a cylindrical moveable core. To make a measurement, the anterior end of the muscle (Fig. 2, #2) was attached to the rigid hold-down device (#3) and the posterior end to the moveable core extension (#1) of the LVDT at its null position. As the muscle contracted it moved the core away from the null position and created an electrical output proportional to its displacement (Herceg, 1976). This electrical output was converted to a physical measurement and used to determine muscle contraction as a percentage of initial length. The physical make up of the LVDT minimized the friction between the core and coil which enhanced the response capabilities for the dynamic measurements needed for this study.

The LVDT had a nominal input voltage of 3 volts root mean squared (rms) at a frequency of 2500 cycles per second. The input and output voltages were fed directly into a scanner so both voltages could be monitored. The scanner was coupled to a digital voltmeter (DVM)

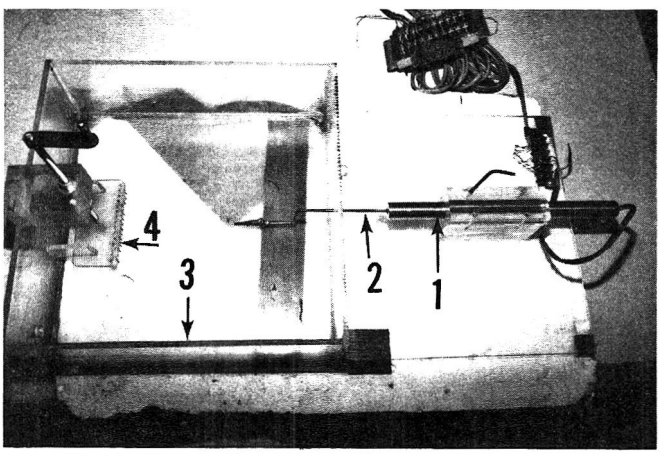

Fig. 1—LVDT and Tank Assembly: (1) Linear variable differential transformer; (2) Core extension; (3) Acrylic tank; (4) Rigid hold-down.

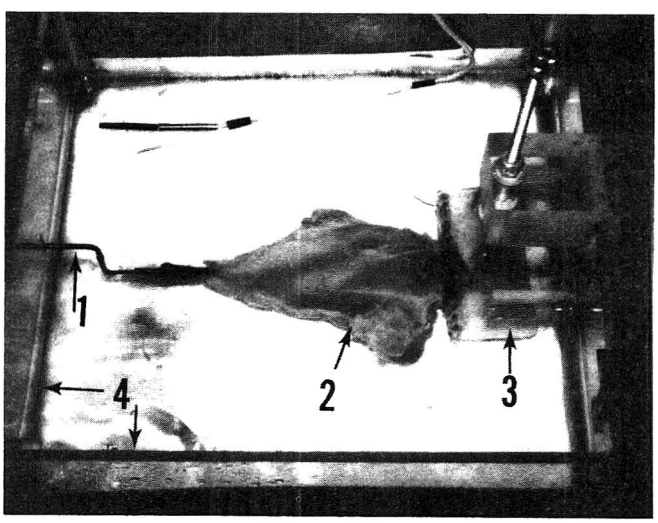

Fig. 2—Muscle mounted during trial run: (1) Core extension; (2) Pectoralis major; (3) Rigid hold-down; (4) Acrylic tank.

Authors Dickens and Papa are with the USDA-ARS, Richard B. Russell Agricultural Research Center, P.O. Box 5677, Athens, GA 30613.

with a General Purpose Interface Buss (GPIB) (International standard, I EEE-488 Interface) and in turn the DVM was coupled to a personal computer (PC) by a second GPIB interface (Fig. 3).

Fluctuations in line voltage caused the input voltage to the LVDT to vary. This variation caused an error in the computer calculation of the length change. To overcome this problem, the equation for changing the output voltage of the LVDT into a physical measurement was modified. Since the input/output ratio of the LVDT was linear, the ratio of these voltages was used to modify the equation so that for each input voltage the corresponding output voltage was changed and corrected measurements were generated. To check the LVDT for linearity and to ensure that the voltage fluctuations did not affect the output, a dial caliper was used to measure the movement of the center core of the LVDT at several different voltages. The caliper was incremented at 0.000254 cm. All measurements were correct to ± 0.02% with the input voltage having no effect.

Seven to 8 week old broilers were obtained form a local processor. The birds were stunned at 50 volts AC for 10 sec, killed by outside neck cut, scalded at 52°C and feathers removed in the pilot plant. The Pectoralis major muscles were immediately removed as described by Hamm (1982). The whole Pectoralis major muscles were then measured (maximum longitudinal length) with a dial caliper and the posterior end was connected to the LVDT and the anterior end to the rigid hold-down device. (Fig. 2). Water in the tank was maintained at approximately 1°C. The time from electrical stunning to placing the muscle in the bath was less than 20 min. The LVDT was positioned to the null position, locked down and the computer initialized. Care was taken not to put any tension on the muscle while connecting it to the LVDT or while positioning the LVDT. The first reading was used as the null or zero contraction point.

The LVDT monitored the contraction of the Pectoralis major along the longitudinal axis of the whole muscle and sent the data to the PC for calculations and storage. Ten readings were made every 15 min, averaged, recorded, and printed out for the duration of the experiment. Each run lasted until muscle contraction ceased. The data were stored by the computer to allow analysis at a later date and a hard copy of the data was printed by the internal printer of the computer.

In a separate trial, 28 broilers were used to determine muscle contraction by a modification of the more conventional method of manually measuring the distance between indelibly dyed points on the muscle surface (Smith et al., 1969). Indelible ink spots were placed at extreme points on the Pectoralis major, one near the muscular origin of the shoulder joint, and the other at the caudal end of the sternum. Intact length was based on a tracing of the muscle made on plastic film. Immediately after excision of the muscle a second trace was made and again after chilling in an ice water slush. Measurements were made with a pair of outside calipers. The manual measurements and the measurement of the tracings were both used to calculate the percentage of muscle contraction.

Data were statistically analyzed using the Analysis of Variance (SAS, 1985). Since data were reported in percent contraction of total length and the recorded values were below 30%, statistical analysis based on the arcsine transformation (Sokal and Rohlf, 1969) of the data was also performed. This analysis was done to prevent the variance from being a function of the mean.

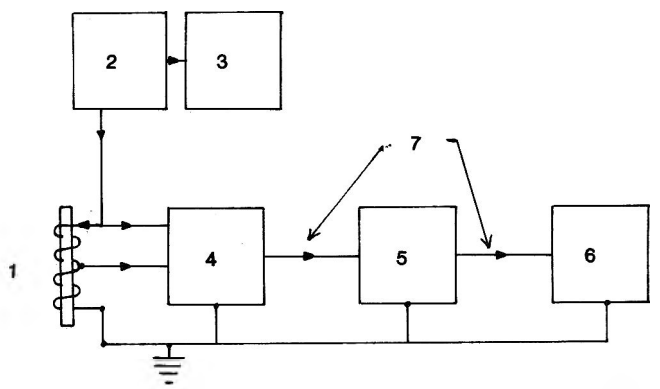

Fig. 3—Block diagram of component wiring: (1) Linear variable differential transformer; (2) 2500 Hz-3 VAC power supply; (3) Frequency meter; (4) Scanner; (5) Digital volt meter; (6) Personal computer; (7) General purpose interface buss.

RESULTS & DISCUSSION

THE CONTRACTION of whole Pectoralis major muscles "hot stripped" from 7—8 wk old broiler type chickens with a postmortem time of less than 20 min, varied from carcass to carcass. The results (Table 1) indicated that whole muscles did not exhibit the same contraction characteristics that have been reported for muscle strips. Data for individual intact muscle contractions using the LVDT ranged from 3 to 6% of the original length, while the manual method values ranged from 0.5 to 12%. Although contraction was measured for longer postmortem times, only the first 4 hr were analyzed since contraction was essentially completed by that time.

Average initial lengths, both of which were manual measurements, for the excised Pectoralis major muscles were 16.1 cm for the LVDT trial and 17.2 cm for the manual measuring trial. Preliminary observations with the manual method indicated that shortening of the overall length due to excision was approximately 6%.

There were no significant differences in the mean values of the two measuring methods (Table 1). The sample standard deviation obtained by use of the LVDT was approximately one-fifth that of the manual method. The arcsine transformation of the data did not change the statistical significance of the results, but the coefficient of variation was reduced to 8^{+} for the LVDT and 39^{+} for the manual measurements.

Previously published results have shown average muscle contraction or shortening for poultry muscles ranging from 13% when muscle strips were used (Lee and Rickansrud, 1978) to 22% of the original length for whole muscles (Smith et al., 1969). Results from this study indicated an average value of 6% after excision regardless of the measuring method (manual or the LVDT).

Use of the described LVDT procedure for determining muscle contraction should help standardize data in experimental applications because it will eliminate almost all of the human error associated with physical measurements. The only manual measurement required was the initial length of the muscle. Also, the use of the computer equipment permited continuous monitoring of muscle contraction for an extended period of time and storing the data for future analysis. The increased number of measurements that can be obtained in a given time period should provide a more accurate profile of the contraction over time for prerigor and inrigor muscles. Selection of the LVDT to be used in any specified case is dependent upon the environmental parameters of the study and the type of muscle (length and anticipated contraction) to be monitored.

The LVDT described here does not measure extensibility or the loss of extensibility of muscle strips as does the rigormeter developed by Briskey in 1964 (Briskey et al., 1966). The LVDT does, however, allow for the monitoring of the contraction of a whole muscle during the development of rigor and seems especially well-suited for the application when a complex muscle, such as the broiler Pectoralis major, is being investigated.

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—*Continued on page 1061*

Table 1—Contraction of hot boned pectoralis major muscle in ice/water slush (1°C)

Measurement technique	Initial length (cm)	% Contraction after excision	Standard deviation	Coefficient of variation
LVDT[a] (N = 14)	16.11	5.314[b]	0.74	13.93
Manual (N = 28)	17.2	4.661[b]	3.74	80.24

[a] Linear Variable Differential Transformer.

[b] Means in the same column with like superscripts are not significantly different ($P < 0.05$).

TBA Values and 7-Ketocholesterol in Refrigerated Raw and Cooked Ground Beef

V. R. DE VORE

ABSTRACT

Thiobarbituric acid (TBA) values and 7-ketocholesterol were determined on raw and cooked ground beef patties after 0, 2, and 4 days of storage at 4°C. The mean 7-ketocholesterol at days 0, 2, and 4 was 9.65, 23.8, and 42.3 μg for 100g raw patties and 6.33, 277.9, and 484.7 μg for 100g cooked patties. The TBA values increased after 2 and 4 days for the raw and cooked patties. TBA values and 7-ketocholesterol were correlated with each other for raw ($r^2 = 0.82$) and cooked ($r^2 = 0.98$) patties. The peak suspected to be 7-ketocholesterol was confirmed by mass spectral analysis. The 7-ketocholesterol in the cooked patties stored two days represented a 0.3% oxidation of the free cholesterol.

INTRODUCTION

CRYSTALLINE CHOLESTEROL and aqueous cholesterol dispersions are susceptible to oxidation at relatively mild temperatures when exposed to air (Smith, 1981). Foods are exposed to elevated temperatures and air during the cooking process, making the cholesterol in foods susceptible to oxidation. Evidence of the possible health implications of dietary cholesterol oxides has stimulated the need for analysis of cholesterol oxides in foods (Pearson et al., 1983; Addis, 1986).

The 7-ketone derivative of cholesterol, 7-ketocholesterol, is one of the cholesterol oxides reported in food. Recently, 7-ketocholesterol has been suggested in heated egg yolk (Naber and Biggert, 1985), powdered scrambled egg mix (Missler et al., 1985), commercial dehydrated egg products (Tsai and Hudson, 1985; Nourooz-Zadeh and Appelqvist, 1987), dehydrated organ meats (Park and Addis, 1985) and heated tallow (Park and Addis, 1986). In a previous study, Tu et al. (1967) did not detect 7-ketocholesterol in freshly cooked ground chuck. Beef purchased from a local market and cooked hamburger from a fast food restaurant did not have 7-ketocholesterol (Park and Addis, 1985). However, Higley et al. (1986) detected trace amounts of 7-ketocholesterol in raw and cooked hamburger.

In addition to cholesterol, the structural membranes of muscle foods contain appreciable quantities of polyunsaturated fatty acids (PUFA). The causes of PUFA oxidation and the possible health implications have been reviewed by Pearson et al. (1983) and Addis (1986). The thiobarbituric acid (TBA) test has been used to estimate the oxidation of PUFA in meat. It is believed to measure one (or more) of the breakdown products of PUFA oxidation. TBA values increased in ground raw beef stored at refrigerated temperatures, indicating more oxidation (Keskinel et al., 1964; Greene, 1969; Govindarajan et al., 1977; Caldironi and Bazan, 1982). Cooked meat stored under similiar conditions undergoes a rapid oxidation of tissue PUFA (Rhee, 1978; Igene et al., 1979).

No studies were found concerning the effect of storage on the oxidation of cholesterol in meat. This study was undertaken to determine if the oxidation of cholesterol, as measured by 7-ketocholesterol, occurred under the cooking and storage conditions that were known to cause increased TBA values.

Author DeVore is with the Dept. of Human Nutrition & Food Management, The Ohio State Univ., 1787 Neil Ave., Columbus, OH 43210, and The Ohio Agricultural Research & Development Center, Wooster, OH 44691.

MATERIALS & METHODS

Reagents

The 5-cholesten-3β-ol-7-one (7-ketocholesterol) was obtained from Steraloids, Inc. (Wilton, NH). Cholesterol and cholesteryl palmitate were purchased from Sigma Chemical Co. (St. Louis, MO). All organic solvents were HPLC grade. Chloroform, methanol and ethyl acetate were obtained from J. T. Baker Chemical Co. (Phillipsburg, NJ) and all other solvents were obtained from Burdick and Jackson (Muskegon, MI).

Beef muscle preparation and pattie storage

Beef semitendinosus muscles were purchased from a local supermarket with no knowledge of their previous history. Each muscle was trimmed of exterior fat and 0.25–0.5 in (0.63–1.25 cm) of surface tissue was removed. The lean tissue that was removed is referred to as surface muscle. The remaining muscle is referred to as trimmed muscle. The trimmed muscle was ground in a food processor with a stainless steel blade (General Electric, Bridgeport, CT) to the consistency of ground beef. The ground meat was made into 50 g uniformly shaped patties (8.5 cm diameter) and wrapped in oxygen permeable PVC film. Raw and cooked patties were stored for 2 and 4 days at 4°C in a home-style refrigerator. The cooked patties were cooked for 30 sec on each side in a microwave (Frigidaire) at the high power setting. The initial TBA and 7-ketocholesterol analyses were done immediately after preparation of the patties.

Extraction of lipids and sample clean-up

Lipids were extracted from 1 g of ground muscle into 20 mL of 2:1 (v/v) chloroform/methanol (Folch et al., 1957). The 7-ketocholesterol was concentrated and separated from the lipid extract by the sample clean-up procedure of Park and Addis (1985). However, the present study employed the disposable Bondelute columns and a Vac Elut processing station (Analytichem International, Harbor City, CA) to replace the hand-packed glass column and vacuum system described by Park and Addis (1985). A 2.8 mL silica column was preconditioned slowly with hexane (15-30 mL), charged with lipid extract redissolved in 4 mL of hexane/ethyl acetate (90:10, v/v) and washed with 2.0 mL of the 90:10 hexane/ethyl acetate. After a final wash with 2 mL of hexane/ethyl acetate (80:20, v/v), the column was dried for 2 min and then eluted with four 0.5 mL aliquots of acetone. The acetone eluate containing the 7-ketocholesterol was evaporated under nitrogen in a Reacti-Block heating unit equipped with Reacti-Vap evaporator (Pierce Chemical Co., Rockford, IL). The residue was redissolved in 1 mL hexane/2-propanol (93:7, v/v). To minimize cholesterol oxidation during sample preparation, the lipid extracts were dried on a vacuum rotary evaporator and the evaporation step was performed under nitrogen.

The eluates from the disposable Bondelute silica column were examined by thin-layer chromatography (TLC) to determine the separation efficiency of 7-ketocholesterol from the major lipid components in the lipid extract. A mixture of cholesteryl palmitate, cholesterol and 7-ketocholesterol was applied to the disposable silica column and eluted with solvents of increasing polarity. The nonpolar cholesteryl palmitate was eluted in the hexane/ethyl acetate (90:10). The 2 mL wash of hexane/ethyl acetate (80:20) eluted the cholesterol while the more polar 7-ketocholesterol was eluted in the acetone. The 80:20 hexane/ethyl acetate and the acetone elutions from a meat extract were chromatographed on the HPLC and monitored at the wavelength max-

imum for cholesterol (209 nm). Most of the cholesterol from the meat sample was in the 80:20 hexane/ethyl acetate eluate. Triglycerides eluted in the nonpolar eluate and the phospholipids remained on the column. The disposable silica column is an alternative to the hand-packed glass column for effectively separating 7-ketocholesterol from the major tissue lipids.

High pressure liquid chromatography (HPLC)

High pressure liquid chromatography (HPLC) was used for quantitation of 7-ketocholesterol. The HPLC was conducted with a normal-phase column procedure similar to that of Park and Addis (1985). HPLC was carried out on a Beckman system equipped with a 421A controller, 114M pump, 164 variable wavelength detector and a SP4290 integrator (Spectra-Physics, San Jose, CA). A 20 μL sample was injected onto an Econosphere column (150 mm × 4.6 mm), packed with a 3 micron silica (Alltech Applied Associates, Inc., Deerfield, IL). The mobile phase was hexane/2-propanol (93:7). Column effluent was monitored at 233 nm, with 0.05 absorbance units full scale (AUFS) and recorded at a 0.5 cm/min chart speed. The flow rate was 1.4 mL/min and a pressure of 1000 to 1120 psi.

The 7-ketocholesterol standards were prepared in hexane/2-propanol (93:7) at concentrations of 0.3-6.3 μg/mL. This corresponded to 6-126 ηg of 7-ketocholesterol injected onto the column. The concentration of 7-ketocholesterol in the meat was calculated from a standard curve for peak area vs concentration.

Recovery of 7-ketocholesterol added to the chloroform/methanol meat extract was determined for three replications. The recovery from the chloroform/methanol meat extracts spiked with 6.3, 3.2 and 1.5 μg/g 7-ketocholesterol was 96.7%, 91.1% and 95.5%, respectively. The lipid extraction and sample clean-up technique were effective for isolating 7-ketocholesterol from meat samples. Park and Addis (1985) reported 100-104% recovery of 7-ketocholesterol when the amount of 7-ketocholesterol added ranged from 100 to 20 μg/g.

TBA and cholesterol analyses

Lipid oxidation was assessed by the 2-thiobarbituric acid method of Tarladgis et al. (1960). Absorbance values were multiplied by the correction factor determined by Tarladgis et al. (1960) and were expressed as TBA value: mg malonaldehyde/kg tissue. The total and free cholesterol concentrations in each muscle were determined in triplicate from a chloroform/methanol (2:1, v/v) muscle extract (Carlson and Goldfarb, 1977).

Mass spectral analysis

The peak suspected as 7-ketocholesterol after co-chromatography with the 7-ketocholesterol standard was collected repeatedly from the HPLC. The pooled fraction was made free of solvent by evaporation under nitrogen and redissolved in minimal amounts of hexane/2-propanol (93/7). The sample was stored under nitrogen and subjected to a mass spectral identification within 24 hr. Mass spectra were obtained with a V.G. Mass Spectrometer (model 70-250S; V.G. Analylical Limited, Manchester, England). Samples were introduced directly on a solid probe. The initial temperature was 22°C and increased 2°C/sec to a final temperature of 350°C. The ionizing voltage was 70 eV. The mass spectrum for the standard 7-ketocholesterol and meat sample were normalized to the most intense peak (M/Z 400) (Fig. 1).

Statistical analysis

The correlation between TBA values and 7-ketocholesterol was evaluated using the Statistical Analysis System (SAS, 1982).

RESULTS & DISCUSSION

Lipid oxidation in raw and cooked beef patties

The TBA values and 7-ketocholesterol increased upon storage of raw ground beef patties at 4°C for 2 and 4 days (Table 1). 7-Ketocholesterol was not detected in muscle sample No. 3 and only a trace in muscle sample No. 2 on the initial day. Park and Addis (1985) reported no detection of 7-ketocholesterol in raw beef muscle purchased from a local supermarket. This is in agreement with the initial values for muscle samples 2 and 3 in the present study (Table 1). Raw patties prepared from the trimmed muscles had low initial TBA values (Table 1). After 2 and 4 days storage, the TBA values were higher compared to initial values and were similar to the TBA values for the surface layer of raw beef muscles (Table 3). Ground and cubed raw meat stored at 4°C for 2, 8, and 10 days had significantly higher TBA values compared to the initial values (Keskinel et al., 1964; Greene, 1969; Govindarajan et al., 1977; Caldironi and Bazan, 1982).

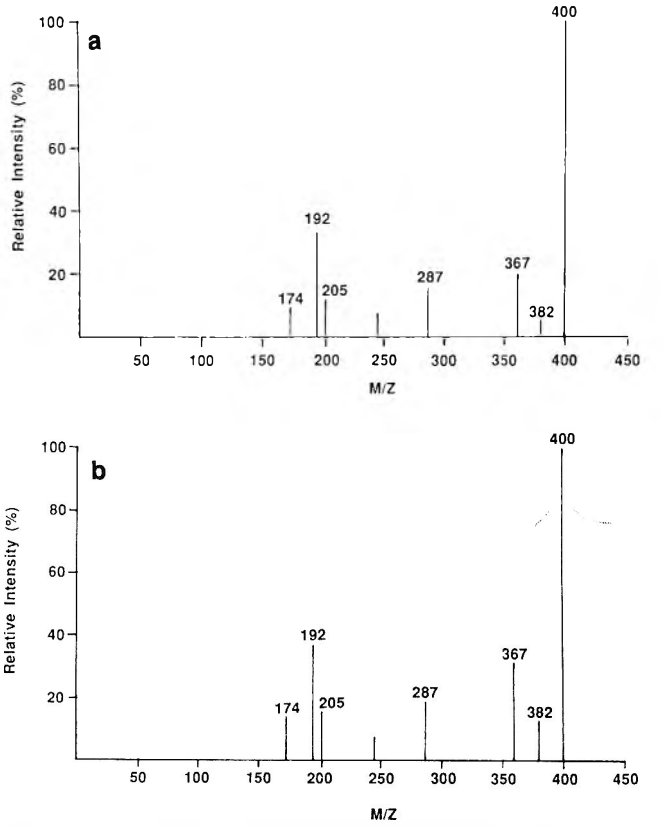

Fig. 1.—Mass spectra of (A) standard 7-ketocholesterol; (B) HPLC fractions of cooked meat stored two days.

The initial TBA values and 7-ketocholesterol for raw and cooked ground beef patties are similar (Tables 1 and 2). Cooking the patties did not cause an immediate increase in either TBA values or 7-ketocholesterol. The patties were prepared from trimmed muscle, cooked for 1 min and analyzed immediately. Thus, the patties may have been analyzed before the end of the induction period. Siu and Draper (1978) reported a correlation between cooking time and malonaldehyde content of beef roasts. They observed that a low malonaldehyde content of cooked meat was related to short cooking times (<30 min).

TBA values and 7-ketocholesterol of cooked beef increased markedly after two days of storage and doubled from day 2 to day 4 (Table 2). The peak suspected to be 7-ketocholesterol in cooked meat stored 2 days was collected from the HPLC and subjected to mass spectral analysis. The mass spectrum for the suspected peak collected from the HPLC and the standard 7-ketocholesterol were similar to each other and corresponded to the mass spectrum for 7-ketocholesterol reported by Park and Addis (1985).

Total and free cholesterol

Total and free cholesterol concentrations in raw and cooked ground beef are presented in Tables 1 and 2. In this study, total and free cholesterol were determined by the enzymatic cholesterol assay (Carlson and Goldfarb 1977), rather than by the classical colorimetric assay. The total cholesterol content of raw ground beef (Table 1) was higher than the reported

Table 1—TBA values and 7-ketocholesterol of raw ground beef patties stored at 4°C for 0, 2 and 4 days and 0 day cholesterol

Muscle sample	TBA value[a] (mg/kg) Storage days			7-ketocholesterol[b] (μg/100g) Storage days			Cholesterol[c] (mg/100g)		% Cholesterol ester
	0	2	4	0	2	4	Total	Free	
1	0.1	1.3	1.9	25.6	37.5	60.4	57.6	54.8	4.90
2	0.2	0.6	1.1	trace	13.1	46.0	86.6	77.6	10.4
3	0.2	0.7	1.4	ND[d]	32.5	—	98.6	87.4	11.4
4	0.2	0.5	0.9	11.3	12.1	20.6	91.5	82.5	9.80
Overall Means	0.2	0.8	1.3	9.65	23.8	42.3	83.6	75.6	9.10

[a] mg malonaldehyde/kg tissue
[b] Each value represents duplicate HPLC determinations on two lipid extracts of each muscle.
[c] Each value represents triplicate determinations.
[d] Not detected

Table 2—TBA values and 7-ketocholesterol of cooked ground beef patties stored at 4°C for 0, 2 and 4 days and 0 day cholesterol

Muscle sample	TBA value[a] (mg/kg) Storage days			7-ketocholesterol[b] (μg/100g) Storage days			Cholesterol[c] (mg/100g)		% Cholesterol ester
	0	2	4	0	2	4	Total	Free	
1	0.1	4.9	8.6	18.2	275.0	392.0	101.5	95.7	5.70
2	0.1	5.8	10.7	trace	264.7	622.2	113.9	107.0	6.10
3	0.2	7.4	17.4	ND[d]	390.6	—	112.7	101.0	10.4
4	0.1	5.1	9.2	ND	181.1	440.0	124.0	114.0	8.10
Overall Means	0.1	5.8	9.5	6.33	277.9	484.7	113.0	104.4	7.60

[a] mg Malonaldehyde/kg tissue.
[b] Each value represents duplicate HPLC determinations on two lipid extracts of each muscle.
[c] Each value represents triplicate determinations.
[d] Not detected

Table 3—TBA values and 7-ketocholesterol for the surface layer of raw beef muscle

Muscle sample	TBA value[a] (mg/kg)	7-ketocholesterol[b] (μg/100g)
1	0.2	18.0
2	1.0	23.0
3	1.3	47.3
4	0.5	21.6
Overall Mean	0.8	27.5

[a] mg malonaldehyde/kg tissue
[b] Each value represents duplicate HPLC determinations of two lipid extracts from each muscle.

values for the semitendinosus muscle (54.8 mg/100g) by Tu et al. (1967) and (63.9 mg/100 g) by Eichhorn et al. (1986). The overall mean of the total cholesterol for cooked ground beef (Table 2) was higher than reported for cooked rib steak (101.9 mg/100 g) by Rhee et al. (1982).

The cholesterol in raw and cooked beef muscle was primarily free cholesterol (Tables 1 and 2). Only 7.6% to 9.1% was esterified cholesterol. Tu et al. (1967) reported 6.0% cholesterol ester for raw beef semitendinosus muscle. Smith (1981) proposed that, in tissues, cholesterol oxidation may be linked to the free radicals produced from oxidation of the PUFA esterified to cholesterol. In this study, cholesterol esters were removed in the hexane/ethyl acetate (90:10) elution of the sample clean-up procedure. Thus, it is unlikely that the 7-ketocholesterol detected in raw and cooked beef stored 2 and 4 days was from the oxidation of esterified cholesterol. The 7-ketocholesterol in cooked patties stored for 2 days represented a 0.3% oxidation of the free cholesterol. This compares closely to values of 0.4% in heated egg yolk reported by Naber and Biggert (1985).

Oxidation of surface lipids

TBA values and 7-ketocholesterol were determined for the surface tissue trimmed from four beef semitendinosus muscle samples (Table 3). The TBA values and 7-ketocholesterol were higher on the surface of each muscle sample than the initial values for the respective trimmed muscle sample (Table 1). Muscle samples 2 and 3 were in the display case at the supermarket for an unknown length of time prior to purchase. Muscle samples 1 and 4 were removed from the round immediately prior to purchase. Exposure of the muscle to light and air in the display case may promote the oxidation of the cholesterol on the surface.

Correlations between TBA values and 7-ketocholesterol

The correlation coefficients between 7-ketocholesterol and TBA values were 0.82 ($p \leq 0.0018$) and 0.98 ($p \leq 0.0001$) for raw and cooked ground beef patties, respectively (Fig. 2 and 3). A relationship between the oxidation of these two lipid components of meat has not been previously reported. Polyunsaturated lipids are susceptible to oxidation and the formation of highly reactive free radicals. Igene et al. (1980) established that membrane phospholipids are the major source of polyunsaturated fatty acids. The structural membranes in lean beef muscle contain the major share of the cholesterol

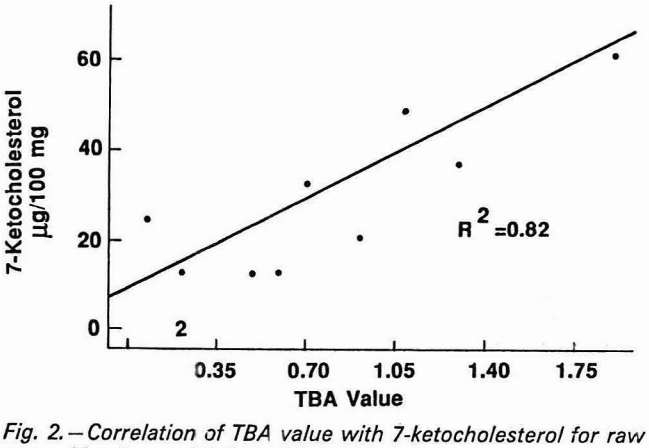

Fig. 2.—Correlation of TBA value with 7-ketocholesterol for raw ground beef stored 0, 2, and 4 days at 4°C.

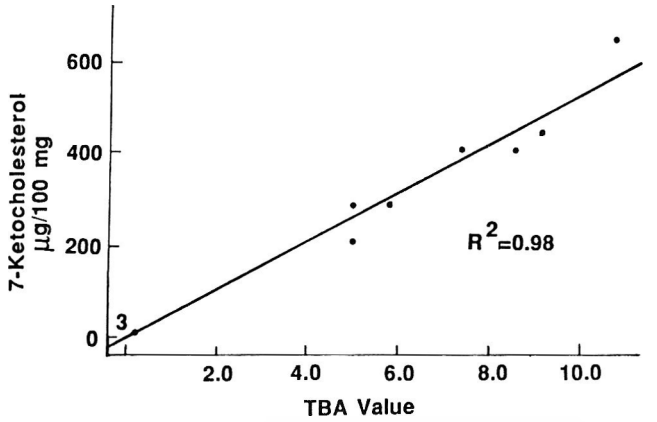

Fig. 3.—Correlation of TBA value with 7-ketocholesterol for cooked ground beef stored 0, 2, and 4 days at 4°C.

(Rhee et al., 1982). PUFA and cholesterol are integral components of membrane structure and are susceptible to autoxidation. Free radicals from phospholipid oxidation may initiate cholesterol oxidation in the tissue membranes of ground beef. Further studies are needed to elucidate the cause of cholesterol oxidation in meat and to determine if other cholesterol oxides are present in stored meat.

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Effects of Blade Tenderization and Proteolytic Enzymes on Restructured Steaks from Beef Bullock Chucks

T.L. ROLAN, G.W. DAVIS, S.C. SEIDEMAN, T.L. WHEELER, and M.F. MILLER

ABSTRACT

Three studies were conducted to determine the effects of blade tenderization (BT), fungal protease, ficin and papain on sensory ratings, textural properties and frozen storage life of restructured steaks from beef bullock chuck. Neither blade tenderizing of chuck muscles prior to incorporating it into restructured steaks nor the use of 0.05% or 0.10% fungal protease had an effect on the amount of connective tissue or textural properties of the cooked product. The addition of 0.10% fungal protease to restructured steaks increased the frozen storage life of the restructured steaks from 82 days (0%) to 149 days and over 157 days for 0.05%. The addition of ficin and papain had a detrimental effect on texture, flavor and overall satisfaction ratings. The methods tested in the present study are not suitable treatments for reducing the amount of detectable connective tissue in restructured steaks from beef bullock chucks.

INTRODUCTION

RECENTLY, much emphasis has been placed on decreasing the amount of connective tissue in restructured beef products (Cohen et al., 1982). A task force designated to develop intermediate-value beef products identified the detrimental effect of connective tissue in restructured steaks as a research problem requiring considerable attention (Breidenstein, 1982). The purpose of producing restructured beef products is to effectively market less valuable carcasses (bullocks) and carcass components (chucks). Since these sources of meat contain excessive insoluble connective tissue after cooking, some method to reduce significantly the amount of detectable connective tissue without degrading the myofibrillar proteins must be identified.

The use of proteolytic plant enzymes and fungal proteases has been widely studied (El-Gharbawi and Whitaker, 1963; Tsen and Tappel, 1959). Miyada and Tappel (1956) reported that papain, ficin and fungal enzymes readily hydrolyzed the soluble fraction of the proteins of muscle fibers and that all of them hydrolyzed collagen to some extent. In contrast, Wang et al. (1957) reported that the proteolytic enzymes of plant origin act preferentially against connective tissue. Moreover, Wang et al. (1957) also found that ficin and papain were the only proteolytic enzymes that degraded elastin.

Booren et al. (1981) stated that blade tenderization (BT) is necessary when producing sectioned and formed steaks from the less tender muscles of the beef carcass. According to Bowling et al. (1976), it may be advantageous to pass the less tender cuts through a blade tenderizer more than once.

The objective of this experiment was to determine the effects of blade tenderization and application of proteolytic enzymes on restructured beef bullock steaks.

Authors Davis and Wheeler are with the Animal Science Dept., College of Agricultural Sciences, Texas Tech Univ., Lubbock, TX 79409. Author Rolan, formerly of Texas Tech Univ., is now at Oscar Mayer Foods Corporation, P.O. Box 7188, Madison, WI 53707. Author Seideman, formerly of U.S. Meat Animal Res. Center, ARS, P.O. Box 166, Clay Center, NE 68933, is now at Bryan Foods, West Point, MS 11177. Author Miller, formerly of Texas Tech Univ., is now with the Meats & Muscle Biology Section, The Univ. of Georgia, Athens, GA 30602.

MATERIALS & METHODS

Processing

Thirteen beef blends (6.81 kg raw meat) were formulated into restructured beef steaks at the Roman L. Hruska U.S. Meat Animal Research Center. Beef lean was obtained from the chucks of four 254-kg bullock carcasses. Visible connective tissue was removed from the chuck muscles and fat was trimmed to yield 10 ± 2% fat in the finished product. In Study I, three blends of boneless bullock chuck meat were passed through a Ross TC 700 blade tenderizer either none (control), one (1×) or two (2×) times. The blends were coarsely ground (1.9-cm plate) twice and mixed in a paddle mixer for 20 min with 0.375% NaCl and 0.5% sodium tripolyphosphate (STP). Each blend was stuffed into 16-cm cellulose casings and frozen to an internal temperature of −20°C. The logs were tempered to 0°C, pressed at 40.5 MNm^{-2} in a Ross Model 720 press, refrozen and cut into 2.5-cm steaks.

The three blends of boneless bullock chuck meat in Study II had 0, 0.05% or 0.10% fungal protease added. In Study III, ficin or papain was added to seven blends at the 0, 0.00375%, 0.0075% or 0.015%. Meat for all 10 blends in Studies II and III were coarsely ground twice (1.9-cm plate), the enzymes were added and each blend was mixed, stuffed, pressed and cut into steaks as was described for samples in Study I.

Two 2.54-cm thick steaks for sensory panel analysis and one 2.54-cm thick steak for 2-thiobarbituric acid (TBA) analysis were removed from each meat log. Each steak was individually wrapped in polyethylene-coated freezer paper and stored at −20°C for later evaluation.

Cooking characteristics

Representative steaks from each treatment group were tempered to approximately −3°C before being broiled to an internal temperature of 70°C on Farberware Open Hearth broilers. Internal temperature was monitored by copper/constantan thermocouples placed in the geometric center of each steak; cooking time required to reach 70°C was recorded. Steaks were weighed frozen, thawed, and after cooking to determine thaw and cooking losses. After cooking, steaks were cut and visually scored by two evaluators for internal degree of doneness using photographic standards (6 = very rare, 1 = very well done) obtained from the National Live Stock and Meat Board (Chicago, IL).

Sensory evaluation

Samples (1 cm^2) of the cooked steaks were served warm to a trained eight-member sensory panel. Each member independently evaluated each sample for juiciness (8 = extremely juicy, 1 = extremely dry), tenderness (8 = extremely tender, 1 = extremely tough), connective tissue amount (8 = none, 1 = abundant), flavor desirability and overall satisfaction (8 = extremely desirable, 1 = extremely undesirable), and defect description [10 = none, 9 = other, 8 = too salty, 7 = too chunky, 6 = too crumbly, 5 = too coarse, 4 = too fine, 3 = too mushy, 2 = off flavor (fishy, liver, blood, rancid, cereal, metallic), 1 = gritty].

Textural properties

Steaks were cooked on Farberware Open Hearth broilers to an internal temperature of 70°C as monitored by copper/constantan thermocouples. Steaks were tempered overnight to an internal temperature of 2°C, cut into six 1 × 1 × 1 cm cubes and sheared four times each on a Universal Testing Machine (Instron, Model 1132) using a Warner-Bratzler shear device.

Storage life

TBA analysis (Tarladgis et al., 1960) for the measurement of oxidation products of lipids was conducted at 15, 55, 85, 125, and 157 days of frozen storage in Studies II and III.

Statistical analysis

A one-way analysis of variance (Steel and Torrie, 1980) using blend as the main effect was used to analyze the data in Studies I and II. Study III data were analyzed as a complete randomized block design. Mean separations were accomplished using Tukey's Studentized Range Test (SAS, 1982) in all three studies. The predetermined probability was 5% for all analyses and was used throughout this discussion.

RESULTS & DISCUSSION

Study I

Application of blade tenderization (BT) to boneless bullock chuck meat did not affect cooking traits of the restructured steaks (Table 1). Other researchers (Davis et al., 1977; Goldner and Mandigo, 1974; Schwartz and Mandigo, 1974) determined that cooking losses of pork chops or beef steaks were not affected by BT. Moreover, BT of the chuck meat did not affect sensory panel ratings for amount of connective tissue, juiciness, tenderness, or flavor in the cooked steaks (Table 1). Bowling et al. (1976) reported that BT of intact muscle reduced the amount of connective tissue detected by sensory panelists in chops from ram lamb and kid goats. Overall satisfaction ratings were higher for 1× than for either control or 2× BT treatments (Table 1); this result was probably due to steaks from the 1× BT treatment having less connective tissue as evaluated by the sensory panel and being rated higher for tenderness and juiciness.

Means for Instron values (Table 1) indicate BT had no effect upon certain measures of muscle tenderness. Fail elongation values were greater for the control than for either 1× or 2× BT steaks (Table 1), which indicates that upon cooking, restructured steaks have a high degree of distortion and lack of uniformity (Berry et al., 1985).

The process of grinding the chuck muscle prior to mixing apparently served the same purpose with regard to tenderization as BT. Thus, since the lean was ground prior to its incorporation into restructured steaks, these data indicated application of BT was not necessary to improve eating quality or decrease detectable connective tissue amount in the cooked product.

Study II

Addition of fungal protease at 0.05% and 0.10% had no effect on cooking traits or sensory attributes of restructured steaks (Table 1). A trend was shown for steaks treated with fungal protease to contain less connective tissue detectable to sensory panelists than the control (Table 1), thus the enzyme may have degraded some stromal proteins. Restructured steaks, treated with 0.05% and 0.10% fungal protease, were rated as "too mushy" by about 47% and 43%, respectively, of the panel members (Table 2); however, this result was not supported by the Instron data (Table 1) which showed no textural differences. Moreover, 21% of the members of the trained panel rated steaks with 0.10% fungal protease as having an off-flavor.

TBA analysis showed that the addition of fungal protease increased the frozen storage-life of restructured steaks (Table 3). Sixty-six days were added to the storage life of steaks treated with 0.10% fungal protease and steaks with 0.05% of the enzyme had a frozen storage-life beyond 157 days.

Study III

Thaw and cooking losses, cooking time and degree of doneness scores show no significant differences for restructured steaks treated with ficin or papain (Table 4). Data for sensory properties of cooked restructured steaks in Table 4 indicated that all enzyme-treated steaks were rated more tender than were control steaks. As concentration of ficin or papain increased, detectable connective tissue amount decreased in the cooked steaks. These results agree with those reported (Anonymous, 1969) that enzymes derived from tropical plants act primarily on the connective tissue fiber but that they also attack the muscle fiber proteins. However, at these levels, steaks treated with ficin or papain-treated steaks were rated "moderately undesirable" to "extremely undesirable" in overall satisfaction ratings by the trained panel (Table 4). As ficin or papain increased, flavor desirability decreased; at the highest concentrations of ficin or papain, the cooked steaks were rated as "moderately undesirable." The mushy texture and off-flavors (Table 2) caused by the activity of these enzymes at high temperatures made them undesirable and may cause consumer rejection of restructured steaks formulated with these enzymes.

Means for textural properties of the restructured steaks are presented in Table 4. No significant differences were found for fail elongation measurements, since all steaks were precut to the same thickness. No data were collected on steaks treated

Table 1—Cooking, sensory, and textural traits of restructured steaks from Studies I and II

	Type and level of tenderization method							
	Study I				Study II			
	BT				Fungal protease, %			
Trait	0	1×	2×	SE	0	0.05	0.10	SE
Cooking trait								
Thaw loss, %	1.09[d]	1.66[d]	0.38[d]	2.55	0.16[d]	0.20[d]	0.09[d]	0.32
Cooking loss, %	32.40[d]	31.15[d]	30.92[d]	4.04	33.37[d]	35.79[d]	29.80[d]	4.44
Degree of doneness[a]	1.0[d]	1.5[d]	1.0[d]	0.71	1.5[d]	1.0[d]	2.0[d]	0.71
Cooking time, min	27.5[d]	32.0[d]	29.0[d]	3.81	30.0[d]	31.5[d]	28.0[d]	3.87
Sensory rating[b]								
Juiciness	5.62[d]	6.19[d]	5.26[d]	0.60	5.67[d]	5.47[d]	5.19[d]	0.35
Tenderness	6.02[d]	6.27[d]	5.73[d]	0.31	5.81[d]	6.92[d]	6.54[d]	0.79
Connective tissue amount	5.61[d]	6.16[d]	4.86[d]	0.63	5.66[d]	5.16[d]	4.50[d]	0.98
Flavor desirability	6.06[d]	5.90[d]	5.80[d]	0.92	6.10[d]	5.52[d]	5.19[d]	0.93
Overall satisfaction	5.47[e]	5.80[d]	5.27[e]	0.13	5.78[d]	4.57[d]	4.07[d]	1.00
Textural measures[c]								
Peak load, kg	5.27[d]	4.40[d]	5.31[d]	3.06	4.20[d]	2.71[d]	3.27[d]	5.13
Fail energy, cm-kg	14.24[d]	10.98[d]	14.35[d]	5.09	13.25[d]	7.64[d]	8.38[d]	14.31
Fail elongation, cm	6.72[d]	6.01[e]	6.01[e]	0.32	6.01[d]	5.10[d]	6.01[d]	2.22

[a] Means based on a 6-point scale (6 = very rare, 1 = very well-done).

[b] 6 = moderately juicy, 4 = slightly dry; 7 = very tender, 5 = slightly tender; 6 = traces, 4 = moderate; 6 = moderately desirable, 3 = moderately undesirable; 5 = slightly desirable, 4 = slightly undesirable.

[c] Peak load = maximum force required to shear the sample; fail energy = amount of work required to shear the sample; fail elongation distance required to shear the sample.

[d e] Means in a row within a study, with a common superscript, are not different (P > 0.05).

Table 2—Sensory panel members' evaluation of defects in enzyme-treated restructured steaks from Studies II and III

Defect description, %	Type and level of enzyme									
	Study II				Study III					
	Fungal protease, %				Ficin, %			Papain, %		
	0	0.05	0.10	Control	0.00375	0.0075	0.015	0.00375	0.0075	0.015
Gritty	–	–	–	–	–	7.1	–	–	–	–
Off-flavor	6.7	13.3	21.4	12.5	6.2	7.1	40.0	–	14.3	13.3
Too mushy	–	46.7	42.8	–	75.0	100.0	80.0	57.1	64.3	93.3
Too fine	–	13.3	7.1	–	6.2	7.1	6.7	7.1	–	6.7
Too coarse	–	–	–	–	–	–	–	–	–	–
Too crumbly	–	20.0	–	–	–	–	6.7	14.3	7.1	–
Too chunky	–	–	–	–	–	–	–	–	–	–
Too salty	–	–	–	–	–	–	–	–	–	–
Other	–	–	–	–	–	7.1	–	–	21.4	–
None	93.3	33.3	42.8	87.5	25.0	–	6.7	28.6	–	–

Table 3—TBA values of enzyme-treated restructured steaks

Days of Storage	Type and level of enzyme									
	Study II				Study III					
	Fungal protease, %				Ficin, %			Papain, %		
	0	0.05	0.10	Control	0.00375	0.0075	0.0015	0.00375	0.0075	0.015
15	0.15^{b}	0.34^{ab}	0.28^{b}	0.11^{b}	0.29^{b}	0.63^{a}	0.42^{a}	0.44^{a}	0.55^{a}	0.50^{a}
55	0.37^{c}	0.27^{c}	0.37^{c}	0.20^{c}	0.45^{bc}	0.70^{b}	0.26^{c}	0.89^{b}	1.62^{a}	1.33^{ab}
85	1.20^{bc}	0.35^{d}	0.43^{d}	1.30^{bc}	1.97^{b}	0.78^{d}	0.51^{d}	2.83^{a}	2.40^{ab}	1.70^{b}
125	2.00^{c}	0.53^{d}	0.68^{d}	2.64^{c}	3.49^{b}	1.02^{d}	0.88^{d}	4.75^{a}	4.48^{a}	3.43^{b}
157	–	0.77^{b}	1.12^{b}	–	–	–	3.90^{a}	–	–	–

^{a,b,c,d} Means in a row with a common superscript are not different (P > 0.05).

Table 4—Cooking, sensory and textural traits of restructured steaks from Study III

Trait	Control	Type and level of enzyme						SE
		Ficin, %			Papain, %			
		0.00375	0.0075	0.015	0.00375	0.0075	0.015	
Cooking trait								
Thaw loss, %	0.30^{d}	0.08^{d}	0.20^{d}	0.24^{d}	0.18^{d}	0.24^{d}	0.29^{d}	0.64
Cooking loss, %	34.52^{d}	31.74^{d}	39.85^{d}	–	35.73^{d}	31.17^{d}	30.90^{d}	4.44
Degree of doneness[a]	1.0^{d}	1.0^{d}	1.0^{d}	–	1.0^{d}	1.5^{d}	1.5^{d}	1.00
Cooking time, min	31.5^{d}	26.5^{d}	33.5^{d}	–	31.5^{d}	31.5^{d}	27.5^{d}	10.91
Sensory rating[b]								
Juiciness	5.69^{d}	5.32^{d}	4.63^{d}	4.04^{d}	5.08^{d}	4.86^{d}	5.06^{d}	0.35
Tenderness	5.25^{e}	7.69^{d}	7.69^{d}	7.94^{d}	7.50^{d}	7.42^{d}	7.81^{d}	0.42
Connective tissue amount	4.76^{e}	6.25^{de}	6.84^{d}	6.94^{d}	6.00^{de}	5.82^{de}	6.51^{d}	1.08
Flavor desirability	5.63^{d}	4.94^{d}	4.79^{de}	3.51^{e}	5.22^{d}	4.84^{de}	3.84^{e}	1.51
Overall satisfaction	4.82^{d}	3.38^{def}	2.50^{efg}	1.34^{g}	3.86^{de}	3.13^{ef}	1.78^{fg}	1.13
Textural measures[c]								
Peak load, kg	5.38^{d}	1.12^{e}	–	–	1.87^{e}	1.71^{e}	1.73^{e}	2.53
Fail energy, cm-kg	15.21^{d}	4.39^{e}	–	–	4.83^{e}	6.48^{de}	3.97^{e}	6.70
Fail elongation, cm	6.16^{d}	6.12^{d}	–	–	5.81^{d}	6.16^{d}	6.16^{d}	0.72

[a] Means based on a 6 point scale (6 = very rare, 1 = very well done).

[b] 6 = moderately juicy, 4 = slightly dry; 8 = extremely tender, 5 = slightly tender; 6 = traces, 4 = moderate; 6 = moderately desirable, 3 = moderately undesirable; 5 = slightly desirable, 1 = extremely undesirable.

[c] Peak load = maximum force required to shear the sample; fail energy = amount of work required to shear the sample; fail elongation = distance required to shear the sample.

^{d,e,f,g} Means in a row with a common superscript are not different (P > 0.05).

with 0.0075% or 0.015% ficin since the physical properties of these restructured steaks were unacceptable after cooking at elevated temperatures. Force and work required to shear each steak were much less for the enzyme-treated steaks which supported the taste panel's conclusions that these steaks contained much less connective tissue and were much more tender than the control steaks.

TBA analysis (Table 3) showed that as level of ficin increased, frozen storage-life increased to over 120 days for the two highest concentrations. Conversely, with the addition of papain, the steaks were rancid (Table 3) at the end of a 57-day storage period, which was well below the point (78 days) at which control steaks were considered rancid. Thus, ficin added to restructured steaks had an antioxidant effect while the addition of papain tended to promote rancidity.

CONCLUSIONS

THESE DATA INDICATED that the use of BT and the addition of fungal proteases had little effect on sensorially detectable connective tissue or textural traits and were not suitable methods for softening connective tissue or for tenderizing restructured steaks made from bullock muscles. Although ficin and papain greatly decreased the amount of detectable connective tissue, acceptable restructured steaks cannot be formulated using ficin or papain at these levels without a concomitant detrimental effect on flavor, overall satisfaction and texture of the cooked product. Though ficin seemed to retard rancidity, its nonselective degradation of the myofibrillar proteins justified little or no further study as to its use in restructured beef products. These results justify further experimentation at lower levels of these enzymes to try and reduce the mushy "too tender" textural problems.

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Reduction of Metmyoglobin by Extracts of Bovine Liver and Cardiac Muscle

C. FAUSTMAN, R.G. CASSENS, and M.L. GREASER

ABSTRACT

Enzymatic reduction of metmyoglobin (*in vitro*) was investigated using a partially purified metmyoglobin reductase from bovine cardiac muscle. Greater substrate reduction ($P<0.05$) occurred at pH 6.3 versus 7.0 or 7.3 and at 37.5°C compared to 22°C using either partially purified cytochrome b_5 or potassium ferrocyanide as reaction mediators. Differences in effectiveness between potassium ferrocyanide and the cytochrome b_5 preparation were dependent on specific pH/temperature conditions. The cytochrome preparation alone (i.e. without metmyoglobin reductase) reduced metmyoglobin at a rate comparable to that of the reductase. At 22°C, the cytochrome b_5 preparation assay displayed much slower re-oxidation than the cardiac reductase/ferrocyanide assay (48 vs 2 hr, respectively).

INTRODUCTION

THE COLOR of fresh meat is probably the most important characteristic by which consumers judge freshness and quality for making purchase decisions. The appeal of fresh meat decreases as myoglobin is oxidized from its cherry red oxy- form to the brown red met- form (Giddings, 1974). Consequently, maintenance of the ferrous state, or reduction of ferric myoglobin if formed, is important for extending the shelf-life of fresh meat. Giddings (1974) has reviewed enzymatic reduction of metmyoglobin in meat. Subsequent investigations of metmyoglobin reduction in meat systems have provided evidence for the phenomenon but without explanation of a mechanism (Lanier et al., 1978, O'Keefe and Hood, 1982, Ledward, 1985, Renerre and Labas, 1987). Evidence for nonenzymatic reduction was presented by Brown and Snyder (1969). NADH and NADPH were active reductants and were enhanced in their metmyoglobin-reducing ability by the addition of flavins and methylene blue. Several metmyoglobin reductases have been described which require NADH and an appropriate mediator to facilitate conversion of ferric myoglobin to its ferrous form (Matsui et al., 1975; Al-Shaibani et al., 1977; Hagler et al., 1979; Levy et al., 1985).

The primary objective of this study was to partially purify a metmyoglobin reductase from bovine cardiac muscle and investigate the effects of mediator type, pH and temperature on *in vitro* metmyoglobin reduction.

MATERIALS & METHODS

Metmyoglobin substrate

Horse heart myoglobin was obtained from Sigma Chemical Company and the following procedure was adopted to remove denatured protein. A 50 mg/mL myoglobin solution in 50 mM Tris-HCl (pH 7.0) was centrifuged at 150,000 × *g* for 30 min. The supernatant was applied to a Sephacryl S-200 column (105 × 2.5 cm) and fractions were collected. The resulting myoglobin solution was found to be 95% or greater in the met- form by the methods of Krzywicki (1982).

The authors are with the Muscle Biology and Meat Science Laboratory, Univ. of Wisconsin-Madison, Madison, WI 53706.

Cardiac muscle metmyoglobin reductase preparation

A partial purification of the enzyme, essentially following the methods of Hagler et al. (1979), was carried out at 4°C. Deviations from the original procedure included use of Tris-HCl buffer instead of sodium phosphate, a 2-60 mM gradient instead of 2-50 mM gradient on the DEAE cellulose column, and substitution of horse heart metmyoglobin for bovine heart metmyoglobin in the activity assay. One kg bovine cardiac muscle was homogenized in a Waring blender with 2L cold, distilled water at high speed for 1 min. The homogenate pH was adjusted to 7.5 with 2N NH_4OH and centrifuged at 13,700 × *g* for 20 min. The supernatant was brought to 40% $(NH_4)_2SO_4$ saturation and centrifuged at 13,000 × *g* for 20 min. The resulting supernatant was made 70% saturated with $(NH_4)_2SO_4$ and centrifuged at 13,000 × *g* for 20 min. The pellet was suspended in distilled H_2O and dialyzed against 20mM sodium phosphate buffer (pH 6.0) with 3 changes of 10 volumes each. The solution was centrifuged at 26,000 × *g* for 30 min and the resulting supernatant was applied to a CM-cellulose ion exchange column (4 × 5 cm) which had been previously equilibrated with 20mM sodium phosphate buffer (pH 6.0). The enzyme moved through the column unimpeded while the colored heme proteins adhered to the matrix and were thus removed. Fractions were collected and those with absorbance at 280 nm were pooled. The solution was made 70% saturated with $(NH_4)_2SO_4$ and centrifuged at 13,700 × g for 20 min. The pellet was suspended in, and dialyzed against, 2 mM Tris-HCl (pH 7.0) — 1mM EDTA with three changes of 10 volumes each. The sample solution was centrifuged at 26,000 × *g* and the supernatant applied to a DEAE cellulose column which had been previously equilibrated with 2 mM Tris-HCl (pH 7.0) - 1 mM EDTA. Elution was carried out by a gradient formed from 600 mL 2 mM Tris-HCl (pH 7.0) - 1 mM EDTA and 600 mL 60 mM Tris-HCl (pH 7.0)- 1 mM EDTA at a flow rate of 120 mL/hr. Fractions were assayed (Hagler et al., 1979) and those with metmyoglobin reductase activity were found to have a conductivity between 1.20 and 2.15 mmho. Fractions with activity were concentrated with a 70% $(NH_4)_2SO_4$ precipitation. The pellet was dissolved in distilled water and dialyzed against 50 mM Tris-HCl (pH 7.0)-1mM EDTA to obtain the reductase solution. Aliquots of 1 mL each were stored at −22°C until needed.

Cytochrome b_5 preparation

A partial purification of cytochrome b_5 was accomplished using the method of Strittmater et al. (1978) scaled down to accommodate 1 kg beef liver. The supernatant obtained after Step 3, Triton X-100 solubilization, was concentrated and frozen in 1 mL aliquots.

Assay for metmyoglobin reducing activity

Assays of metmyoglobin reduction were carried out at 22°C and 37.5°C in 1 mL cuvettes. Reaction mixtures contained: 0.50 μmol EDTA, 25 μL buffer (0.1M citrate, pH 4.7, 0.1M citrate, pH 5.7, or 0.1M phosphate, pH 7.0), 50 μL cytochrome b_5 preparation or 0.30 μmol potassium ferrocyanide, 400 μL metmyoglobin solution (0.36mM), 60 μL enzyme solution, 0.15 μmol NADH and enough distilled water to make a total volume of 1 mL. The reaction was initiated with the addition of NADH and followed at 580 nm (the wavelength at which the difference in absorption between oxymyoglobin and metmyoglobin is maximal). The millimolar absorptive difference for horse heart oxymyoglobin and metmyoglobin at 580 nm was calculated with assistance from D. Livingston (priv. comm.) as follows and determined as 11.6 at pH 6.3, 11.5 at pH 7.0 and 11.3 at pH 7.3. At pH 7.0, the ϵ_{mM} for horse heart oxymyoglobin at 580 nm is 14.4 while at the same pH, horse heart metmyoglobin has an ϵ_{mM} of 9.7 at 500 nm (Antonini, 1965). Metmyoglobin spectra unlike oxymyoglobin spectra are pH dependent (Livingston priv. comm.).

To normalize the ϵ_{mM} for metmyoglobin at 500 nm, for pH values other than 7.0 (i.e. × = 6.3 or 7.3):

$(A_{500}$ @ pH X / A_{500} @ pH 7.0) * 9.7 = ϵ_{mM} (normalized at pH X)

To obtain the ϵ_{mM} for metmyoglobin at 580 nm, a metmyoglobin solution at the appropriate assay pH was made up and the following calculation used:

(A_{580} / A_{500} (normalized at pH X) × 9.7 = ϵ_{mM} metmyoglobin @ 580 nm

The millimolar absorptive difference at 580 nm for horse heart metmyoglobin and oxymyoglobin was then calculated as,

$$\Delta\epsilon_{580} = 14.4 - \epsilon_{mM} \text{ metmyoglobin @ 580 nm}$$

Statistical analysis

For each combination of mediator, temperature, and pH, initial reaction velocities were recorded in duplicate for three different trials. A least significant difference test for mean effects of the treatment variables was used to analyze data under a general linear models procedure (SAS, 1982).

RESULTS

THE RESULTS for assays with metmyoglobin reductase preparation are presented in Table 1. Initial rates of reduction were significantly greater ($P<0.001$) at 37.5°C vs 22°C for all pH/mediator conditions. For each of the two mediators, metmyoglobin reduction was greatest at pH 6.3 with a decrease in initial velocities occurring at pH 7.0 and 7.3. This trend occurred at both 22°C and 37.5°C.

The relative effectiveness of the cytochrome b_5 preparation versus that of potassium ferrocyanide was dependent on the specific reaction conditions. The cytochrome b_5 preparation was significantly ($P<0.05$) more effective than ferrocyanide in enhancing initial velocity at pH 6.3 for both the 22°C and 37.5°C trials and at pH 7.0 for 37.5°C. At all other temperature/pH conditions, differences between the two mediators were not significant ($P<0.05$).

In the course of running control experiments, it was observed that the cytochrome b_5 preparation was capable of reducing metmyoglobin in the absence of the metmyoglobin reductase preparation. Results for these assays run at pH 7.0 are shown in Table 2. In the absence of reductase preparation, the cytochrome b_5 preparation was as effective in reducing metmyoglobin at 22°C and actually more effective at 37.5°C ($P<0.05$). Potassium ferrocyanide did not demonstrate a similar ability to reduce metmyoglobin in the absence of reductase preparation. It appears that the crude cytochrome b_5 preparation from liver contains components which interact with cytochrome b_5 to promote metmyoglobin reduction. Non-enzymatic reduction by NADH at both temperatures was minimal and not significant ($P<0.05$).

When the mixtures were allowed to stand at 22°C, it was observed that assays with reductase and potassium ferrocyanide re-oxidized more quickly than those with reductase and cytochrome b_5 preparation, or cytochrome b_5 preparation alone. Spectral evidence for the observed re-oxidation behaviors are given in Fig. 1 and 2. The assay, which included cardiac metmyoglobin reductase preparation and potassium ferrocyanide, reached a maximum of reduced pigment at 0.5 hr and quickly returned to the oxidized, met- state at 2 hr (Fig. 1). Scans for the assay with cytochrome b_5 preparation alone (Fig. 2) also showed maximum reduced substrate at 0.5 hr, but a full 48 hr was required to return to the brown, met- form. The cardiac reductase extract with cytochrome b_5 preparation as mediator showed a similar time course/spectrum relation to that in Fig. 2.

Table 1—Initial velocities for metmyoglobin reduction at two temperatures

pH	Mediator	Initial Velocity (nmol/min) 22°C	37.5°C
6.3	Cyt b_5	17.4[a]	33.2[a]
	Ferrocyanide	11.3[b]	20.7[b]
7.0	Cyt b_5	9.9[bc]	17.4[c]
	Ferrocyanide	8.6[bc]	14.7[d]
7.3	Cyt b_5	8.6[bc]	13.7[d]
	Ferrocyanide	7.6[c]	14.3[d]

[a-d] Mean values in the same column with different superscripts differ significantly ($P<0.05$).

Table 2—Initial velocities of metmyoglobin reduction at various assay conditions (pH 7.0)

Assay Condition				Initial Velocity (nmol/min)	
MetMb reductase	Cyt b_5	FKeCN	NADH	22°C	37.5°C
+	+	−	+	9.9[a]	17.4[a]
+	−	+	+	8.6[a]	14.7[b]
−	+	−	+	10.2[a]	20.2[c]
−	−	+	+	0.0[b]	0.0[d]
−	−	−	+	0.6[b]	0.3[d]

[a-d] Mean values in the same column with different superscripts differ significantly ($P<0.05$).

DISCUSSION

A SCHEMATIC DIAGRAM of the metmyoglobin reduction pathways observed in these experiments is shown in Fig. 3. NADH is the ultimate source of reducing equivalents for all

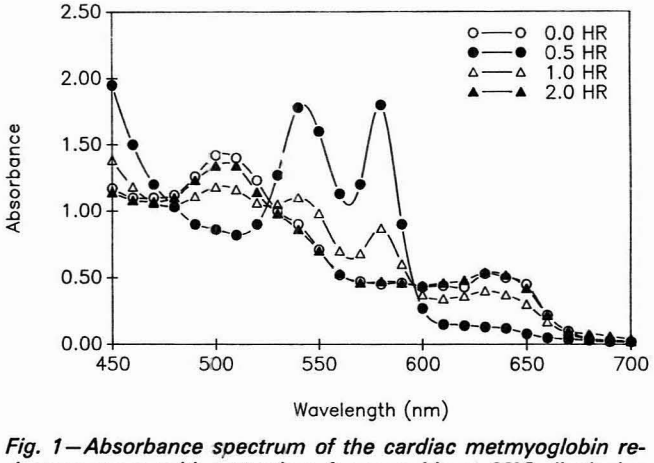

Fig. 1—Absorbance spectrum of the cardiac metmyoglobin reductase assay with potassium ferrocyanide at 22°C displaying the time course of reduction and subsequent oxidation.

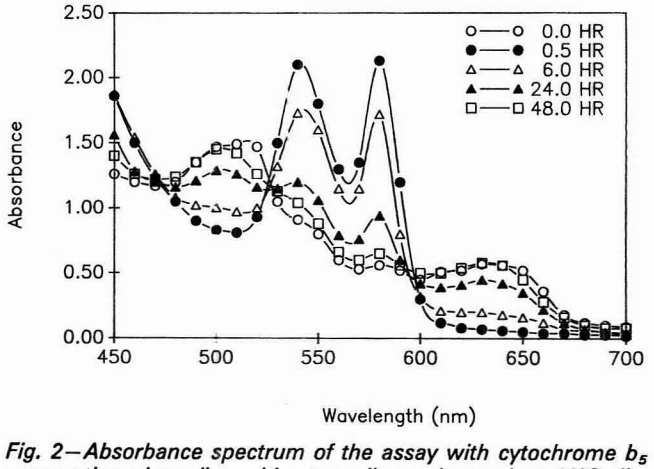

Fig. 2—Absorbance spectrum of the assay with cytochrome b_5 preparation alone (i.e. without cardiac reductase) at 22°C displaying the time course of reduction and subsequent oxidation.

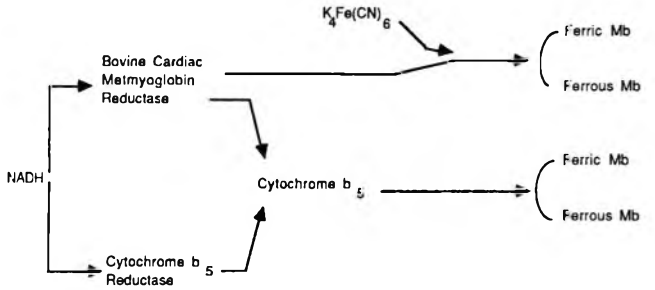

Fig. 3—Proposed pathways for observed metmyoglobin reduction.

pathways. Potassium ferrocyanide is believed to facilitate metmyoglobin reduction in a manner similar to its role in methemoglobin reduction (Hegesh and Avron, 1967; Hagler et al., 1979). The ferrocyanide is not a simple electron carrier but binds to the heme protein in such a way as to make the heme group accessible to the metmyoglobin reductase.

Livingston et al. (1985) have demonstrated that purified metmyoglobin reductase enzymatically reduces cytochrome b_5, and subsequently, the reduced cytochrome b_5 then reduces ferric myoglobin to ferrous myoglobin. This pathway would occur in assays which included metmyoglobin reductase and the cytochrome b_5 preparation as mediator.

Strittmater et al. (1978) indicated that cytochrome b_5 reductase was a component of the liver extract at the step through which we completed our partial purification of cytochrome b_5. Cytochrome b_5 reductase is a flavoprotein which reduces cytochrome b_5 by transferring electrons to it from NADH (Strittmater, 1965). Thus, our liver extract was able to reduce metmyoglobin in the absence of metmyoglobin reductase via enzymatic reduction of cytochrome b_5 by cytochrome b_5 reductase with subsequent reduction of ferric myoglobin to ferrous myoglobin by the reduced cytochrome b_5.

It may be possible that our bovine liver/cytochrome b_5 preparation contained metmyoglobin reductase. Work on the mechanism of reduction by Livingston et al. (1985) resulted in cardiac metmyoglobin reductase being classified as an NADH:cytochrome b_5 reductase. This classification also applies to liver microsomal cytochrome b_5 reductase and so these two proteins perform similar functions albeit in different anatomical locations. Further work is necessary to define the exact proteins responsible for reduction by the liver preparation.

A comparatively rapid re-oxidation was observed for the cardiac reductase/potassium ferrocyanide assay (Fig. 1) compared to that for the assay with cytochrome b_5 preparation alone (Fig. 2). It is likely that in the assays which included ferrocyanide, the ferrocyanide is oxidized to ferricyanide over the time course of metmyoglobin reduction. When the NADH is exhausted, the ferricyanide acts to oxidize directly the reduced pigment. This would explain the more rapid re-oxidation observed.

Several workers have used ferricyanide as an oxidant in studies of metmyoglobin reduction in meat (Lanier et al., 1978, Stewart et al., 1965, O'Keefe and Hood, 1982). In such cases, ferricyanide itself would likely be reduced to ferrocyanide. If the metmyoglobin-reducing system of meat is similar in mode of action to the action of bovine cardiac metmyoglobin reductase, rate results generated from these studies must be questioned because of ferrocyanide action on enhancing reduction. Ledward (1972) used low pO_2 to generate ferrimyoglobin. This procedure is unlikely to influence the meat's native reducing system and thus provides an advantage over using ferricyanide as an oxidant.

CONCLUSION

EXTRACTS of bovine cardiac muscle and liver were shown to reduce metmyoglobin. The liver extract provides an excellent model for studying metmyoglobin reduction as it is relatively easy to obtain and does not require addition of any nonphysiologic mediators (i.e. potassium ferrocyanide). Reduction of metmyoglobin by either or both of these two systems may provide a means of improving meat color by utilizing components of low commercial value variety meats.

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Color Characteristics and Functional Properties of Flaked Turkey Dark Meat as Influenced by Washing Treatments

E.A. ELKHALIFA, P.P. GRAHAM, N.G. MARRIOTT, and S.K. PHELPS

ABSTRACT

Effects of potassium phosphate and sodium acetate washing procedures on the composition, color characteristics, and functional properties of turkey dark meat were determined. All evaluations were compared to control thigh and breast tissues. Higher moisture and lower protein, fat and ash ($P<0.05$) were found in tissues subjected to washing. Concentration of sarcoplasmic protein was reduced ($P<0.05$) by washing. Hunter L, a, and b color values were changed ($P<0.05$) to a color similar to breast meat by washing. Washed tissues, due to higher moisture, had less cooking loss in water and their protein solubility values increased with salt concentration. Emulsifying capacity (EC) values were not reduced ($P>0.05$) due to washing. Phosphate-washed tissue stabilized a test emulsion better than the control and acetate/phosphate-washed thigh tissues.

INTRODUCTION

A COLOR MODIFICATION process has received recent attention from the poultry industry as a potential process for formulating the over-supply of poultry dark meat into muscle food products. Washing of dark meat results in bleaching and loss of flavor providing a modified muscle food than can be incorporated more readily into popular poultry products made with white meat and/or can be used in other products to potentially improve the sensorial properties over what is achieved with dark meat as a raw material.

The principal pigments responsible for the color of dark meat are myoglobin and hemoglobin. Other pigments are also found in muscle and meat but are present in such small amounts that their contribution to meat color is minor. Myoglobin and hemoglobin have been shown to be extractable by water (Fleming et al., 1960; Fox, 1966; Saffle, 1973), a 4:1 acetone to water mixture (Hornsey, 1956), 0.1N acetate buffer at a pH of 4.5 (DeDuve, 1948), 0.5% sodium bicarbonate solution (Ball and Montejano, 1984) or 0.04M sodium phosphate buffer (Warris, 1976, 1979; Hernandez et al., 1986).

One of the important problems encountered in washing is the loss of component substances, e.g., proteins which affect the yield and functional behavior of the meat. The objectives of this study were to lighten the color of turkey dark meat by preferentially extracting the water soluble components, in particular myoglobin and hemoglobin, and to assess the effects of washing on composition and functional properties of the meat.

MATERIALS & METHODS

Materials

Turkey thigh and breast muscles were supplied by a commercial turkey processing plant and transported to the Virginia Polytechnic Institute & State Univ. Muscle Food Products Research Laboratory. All chemicals used were analytical reagent grade.

Author Elkhalifa is affiliated with the Faculty of Science & Technology, Univ. of Gezira, Medani, Sudan. Authors Graham, Marriott, and Phelps are with the Dept. of Food Science & Technology, Virginia Polytechnic Institute & State Univ., Blacksburg, VA 24061.

Preparation of meat tissues

The meat tissues were obtained in unfrozen, chilled condition. Upon arrival, the raw product was reduced in size to approximately 3.5 × 5.5 × 2-cm chunks. The chunked material was stored in a −20°C environment overnight, tempered to 4°C and flaked with an Urschel Comitrol Model 3600 using a head opening of 6.1 mm × 17 mm. Portions of approximately 500g of each type of tissue were placed in polyethylene bags and frozen at −20°C until used. For replication of the tests, fresh samples were thawed at 4°C for 24 hr before use.

Washing treatments

After several preliminary trials, two washing media were selected. The washing media included: (A) 0.03M potassium phosphate buffer (pH 5.8, 7.4 and 8.0); (B) 0.02M sodium acetate buffer (pH 5.2) and 0.03M potassium phosphate buffer (pH 7.4).

For medium (A), the washing treatment consisted of mixing in duplicate 75 g of thigh tissue with 375 mL cold buffer (pH 5.8) in a mixer (Kitchen-Aid, Model 4-C, Hobart Mfg. Co., Troy, OH) for 5 min. The slurry was centrifuged at 1000 × *g* for 15 min at 4°C. The supernatant was filtered through a double-layer cheese cloth. The residue was washed twice with the buffer at pH 7.4 and 8.0, respectively. The supernatants were combined and retained for protein analysis. The washed meat was pressed manually to remove excess moisture.

For medium (B), the meat tissues were washed twice in 200 mL and 120 mL acetate buffer for 5 min and 3 min, respectively. The slurry was centrifuged and filtered; the extract was retained. The meat residue was washed in phosphate buffer (pH 7.4) in a similar manner. The supernatants and washed meat were treated as in medium A. All washed meat was ground, packed and stored at 4°C for 2–3 days for subsequent analyses.

Proximate analysis and pH

Moisture, protein and crude fat determinations were made on different raw samples following AOAC (1984) methods. The pH of raw tissue was determined. A 10 g sample of tissue was homogenized in a Polytron (Brinkmann Instruments, Westbury, NY) with 100 mL distilled water for 60 sec. The pH of the homogenized sample was measured using a pH Meter. The pH of the supernatants from washing treatments was also recorded.

Color measurements of raw tissue

Color measurements were made with a Hunterlab Model D25 Color and Color-difference meter with a 51 mm diameter aperture (illuminated area) and a white color standard (C20-1651). A technique similar to that of Young and Whittle (1985) was used. The samples were finely ground, spread out on a tray to a depth of 15 mm and leveled off. The sample tray was raised toward the specimen area to express liquid from the mince around the entire edge of the specimen area. Further readings were taken on the opposite surface of the samples.

Protein fractions and amino acid analyses

The extraction buffer consisted of a low ionic strength buffer of 0.03 M potassium phosphate (pH 7.4), and a high ionic strength buffer of 1.1 M potassium iodide (KI) in 0.1 M potassium phosphate (pH 7.4), (Helander, 1957).

Samples (ca 2g) were placed in 25 mL pre-cooled 0.03M phosphate buffer and homogenized at 40,000 rpm in a Brinkman Polytron for 5 sec at 22°C to separate the sarcoplasmic proteins. The homogenate was transferred to a centrifuge tube. The homogenizer blades were rinsed in 10 mL buffer which was added to the homogenate. The homogenate was centrifuged at 27,000 X *g* for 15 min at 4°C.

The residue was then similarly extracted with pre-cooled 1.1M KI in 0.1M potassium phosphate buffer to separate myofibrillar proteins. After this extraction, the residue was washed thoroughly with deionized water and centrifuged. Samples of the residue were treated with chloroform-methanol solvent (3:1 v/v) to remove lipids associated with connective tissue. The residue was then drained, dried at 105°C for 24 hr and weighed. The dried residue has been referred to as connective tissue (Asghar and Yeates, 1974). Protein concentration of all the extracts was determined by the biuret method (Gornall et al., 1949).

The myofibrillar protein fractions were freeze-dried and stored at −20°C until analyzed for amino acids. One mg samples were saturated with nitrogen and then hydrolyzed with 6N HCl at 110°C for 22 hr. Amino acid analyses were performed on a Beckman HPLC amino acid analyzer with a Shimadzu CR2AX data system. Tryptophan and cystine were not determined.

Functional properties evaluation

Water-holding capacity (WHC). A method for determination of cooking loss as a measure for WHC was carried out as described by Honikel et al. (1981). Unsalted and salted (2% NaCl) meat homogenates were prepared in a Brinkman Polytron using 22g ground meat, 11g ice water or 11g ice-cold 2% NaCl solution, respectively, and homogenized four times for 3 sec. The unsalted samples were used at once, but the salted homogenate was held for 30 min to allow the NaCl to reach an equilibrium. Approximately 10 g of the unsalted or salted meat homogenate was weighed into a preweighed centrifuge tube. The tube was covered with aluminum foil and placed in a boiling water bath for 20 min. The tube and contents were then allowed to cool, and the juice, released by heating, was drained off. The cooked meat was put on a filter paper, blotted with a dry filter paper to absorb all excess moisture and then placed back into the tube which was reweighed for determining the moisture loss during cooking to an endpoint temperature of 80°C.

Protein solubility. The soluble protein was determined by the method of Hwang et al. (1977) except that the homogenate was centrifuged at 27,000 x *g* for 20 min. Protein solubility tests were determined for NaCl concentrations of 0, 2, 3, and 5%. The results were calculated as percentage of soluble protein in the total protein.

Emulsifying capacity (EC). The emulsifying capacity of the meat homogenate was determined in triplicate according to the method of Swift et al. (1961). Ten grams ground meat were homogenized with 40 mL 3% NaCl solution in a Brinkman Polytron for 1 min. Twelve and one-half grams of the resulting slurry was placed in a 250 mL beaker and 12.5 mL of 3% NaCl was added. The homogenate was pre-blended with 50 mL vegetable oil for 60 sec to form an initial emulsion. Then, the sample was titrated with oil at a rate of approximately 1 mL/sec until the emulsion collapsed as determined by the electrical resistance method of Webb et al. (1970). The mean value of eighteen determinations was calculated.

Emulsion stability (ES). Emulsion stability characteristics were evaluated as described by Inklaar and Fortuin (1969) except that 15g ground meat was used. In this procedure, 15g ground meat was blended with 45 mL 3% NaCl for 1 min. The, 25g of vegetable oil was slowly added to the meat homogenate over a 5 min period while blending for emulsion formation. When all of the oil was added, the blending was continued for an additional 1 min. Duplicate 35g emulsion samples were stuffed into 50 mL graduated centrifuge tubes, covered with aluminum foil and placed into a water bath at 85°C for 15 min. After heating, the tubes were cooled under running tap water for 15 min and then centrifuged at 3000 rpm (800 × *g*) for 15 min. The amount of oil separated was read, and the centrifuging was repeated until the volume of separated oil did not change any further. The separated oil was calculated as a percentage of the total amount of the oil added. Average of twelve determinations was calculated for each treatment.

Statistical analyses

All results were analyzed by analysis of variance using the statistical analysis system (SAS, 1982). When the F-test was significant, differences between treatment means were determined using least significant difference (LSD) values (Steel and Torrie, 1980).

RESULTS & DISCUSSION

Proximate analysis and pH

The compositional analyses and pH values of raw control and washed tissues are given in Table 1 and 2. The washing procedures significantly ($P<0.05$) increased moisture over the control tissues and consequently, reduced the protein and fat. The amount of moisture in the washed tissues increased (Table 1) as pH increased (Table 2). The influence of pH effects on muscle hydration is well documented because of the pH effects on protein net charges (Hamm, 1986). An increase of pH at the basic side of the isoelectric point of fibrillar protein (pH 5.0–5.1) caused an increase of the negative charge and, therefore, resulted in repulsion between the filaments and was thought to be responsible for the creation of larger spaces for entrapment of water molecules.

The loss in protein and ash (Table 1) was likely due to the removal of water-soluble fractions from the tissues. Losses of water-soluble proteins due to washing were 24.6% and 21.7% for phosphate and the acetate/phosphate treatments, respectively (Table 2). Since fat has a lower density than the buffer solution, part of the fat floated off during the extraction process (Table 1).

Table 1—Chemical composition of control and washed turkey thigh tissues

Component (%)	Raw material type: Unaltered breast	Unaltered thigh	Phosphate-washed thigh	A/P-washed thigh[a]
Moisture	74.3[e]	74.6[e]	85.6[c]	84.8[d]
Fat	1.0[e]	5.6[c]	2.4[d]	2.2[d]
Ash	1.1[c]	1.0[d]	0.6[e]	0.5[f]
Protein[b]	23.6[c]	18.5[d]	11.7[e]	11.0[e]

[a] Acetate/phosphate washed thigh.
[b] Total N × 6.25.
[c-f] Means in the same row with identical superscripts are not different ($P>0.05$).

Table 2—Mean pH values of extract and turkey thigh tissues and protein loss due to washing

Treatment	pH: Tissue	pH: Extract	Protein loss (%)[a]
Breast (unaltered)	6.06 ± 0.05	----	----
Thigh (unaltered)	6.47 ± 0.06	----	----
Phosphate washed thigh	7.61 ± 0.11	6.93 ± 0.06	24.6[b]
Acetate/Phosphate washed thigh	7.36 ± 0.10	6.73 ± 0.09	21.7[c]

[a] Calculated as percent total protein of unaltered thigh tissues.
[bc] Percent means in the same column with identical superscripts are not different ($P>0.05$).

Color measurements of raw tissues

Color characteristics of the control and washed turkey tissues are presented in Table 3. Unwashed thigh tissues had the lowest "L" (lightness) and "b" (yellowness) values and the highest "a" (redness) value, thus indicating that they were darker and more red than the breast or washed thigh tissues. On the other hand, the washed thigh tissues signficantly increased in lightness compared to both control thigh and breast tissues. The increase of L-values due to washing was indicative of color lightening since the L-value represents a darkness to lightness characteristic in sample appearance. The tissue washed with phosphate buffer presented the highest L-values. The washing procedures applied to the thigh tissues also produced a significant reduction in redness (a-values) compared to values of control thigh and breast tissues which was attributable to the reduction of color pigmentation and the color dilution effect of increased moisture. Tissues washed with phosphate had a-values than were numerically lower than the acetate/phosphate washed tissues but not signficantly lower ($P>0.05$). Both the washing treatments produced an increase in the yellowness (b-value) of the tissues to values higher ($P<0.05$) than the unwashed thigh tissues. The difference was not observed in the b-values of the washed tissues when compared to the b-value of the breast tissue.

Also shown in Table 3 are a/b ratio, color saturation, hue and total color difference values. These values were calculated

Table 3—Color characteristics of control and washed turkey thigh tissues

Tissue sample	L	a	b	a/b[a]	Saturation[b]	Hue[c]	Total color difference ΔE[c]
Control							
Breast	50.9[f]	9.17[e]	11.15[e]	0.82	14.44	50.57	5.45
Thigh	44.85[g]	9.72[e]	9.75[f]	1.00	13.77	45.09	–
Washing treatment							
Phosphate	55.87[e]	4.62[f]	11.11[e]	0.42	12.03	67.42	12.22
Acetate/Phosphate	54.78[e]	5.58[f]	10.88[e]	0.51	12.23	62.85	10.82

[a] Calculated as a ratio from individual "a" and "b" observations.
[b] Saturation is obtained by the equation $(a^2 + b^2)^{1/2}$ and is proportional to the strength of color.
[c] Angle whose inverse tangent is b/a.
[d] $\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$, all color differences were compared to unaltered thigh tissue.
[e-g] Means in the same column with identical superscripts are not different (P>0.05).

from observed L, a, b values. The b-value when used jointly with a-value, as ratio (a/b), indicate intensity of the red (+a) color characteristic (Francis and Clydesdale, 1975). In this study, the a/b ratio indicated the red color change in tissue as a function of the two washing media. The ratios decreased from the original ratio of 1.00 found in the unwashed thigh tissue to 0.42 in the phosphate-washed tissue. The total color difference (ΔE) is a measure of the distance between two sample points in the L, a and b coordinates of the Judd-Hunter Color Space. The ΔE values of the washed tissues (12.22 and 10.82) were the most distant from the thigh control tissue. This indicated that the washing process changed the color of the raw thigh tissue. Saturation can be thought of as a measure of how different the color is from gray and is conveyed by words such as depth, vividness, and purity. Breast and thigh control samples had higher saturation values than the washed thigh samples. There was a noticeable decrease in the saturation values due to washing treatments. This further indicated that the purity of color decreased with washing. A possible explanation for these saturation values is that breast and thigh control samples having a dominant red color would achieve a higher saturation value than those with a more homogeneous white color balance (e.g., washed samples). Hue (color shade) values increased due to washing procedures. Phosphate-washed tissue presented a higher value. As the red color was removed, samples decreased in darkness. Thus, hues other than red would tend to predominate.

Protein fractions and amino acid analyses

Percentages of sarcoplasmic, myofibrillar and stroma proteins are presented in Table 4. The washing treatments, applied to the thigh tissue, significantly (p<0.05) reduced total protein when compared to unwashed tissue.

Both washing treatments reduced (P<0.05) the sarcoplasmic protein concentration. This result is in disagreement with Bowie (1985) who reported that washing thigh tissue with water, ascorbic acid, peroxide and bisulfite did not significantly reduce sarcoplasmic protein concentration; but these washes did greatly reduce the nonprotein N (NPN) content. Since the sarcoplasmic protein and NPN fractions are water soluble, the phosphate and acetate buffers do not selectively extract NPN and hence greater reduction in sarcoplasmic protein concentration would be expected due to the leaching effect of the buffer solutions.

Table 4—Protein fraction of control and washed turkey thigh tissues

Tissue sample	Protein fraction (%)			Total protein (%)
	Sarcoplasmic	Myofibrillar	Stroma	
Control				
Breast	7.12[a]	13.79[a]	2.69[a]	23.60[a]
Thigh	4.50[b]	11.44[b]	2.56[a]	18.50[b]
Washing treatments				
Phosphate	0.49[c]	9.32[b]	1.89[a]	11.70[c]
Acetate/Phosphate	0.51[c]	8.59[b]	1.89[a]	11.00[c]

[a-c] Values within a column with identical superscripts are not different (P>0.05), (N=6).

Myofibrillar proteins of thigh tissues washed with phosphate or acetate/phosphate buffers were not significantly (P>0.05) different from unwashed thigh tissue. However, a numerical reduction in myofibrillar protein occurred in the washed tissue. Because myofibrillar proteins are not extracted at low ionic strengths (μ<0.2) but are solubilized at high ionic strengths (μ>0.5), a significant reduction in myofibrillar proteins was not expected in washed tissues in this study. These observations agree with those of Bowie (1985) who found no significant differences in myofibrillar protein of washed and unwashed thigh tissue. Stroma proteins (insoluble proteins) of washed tissues were not different (P<0.05) from control tissues. Washing treatments do not likely affect such proteins.

Myofibrillar protein fractions, collected in extraction and fractionation procedure, were also analyzed for amino acids (Table 5). Of the essential amino acids, only the amounts of methionine, threonine and valine were significantly reduced (>20%) by phosphate washing. Isoleucine, lysine, and phenylalanine were reduced by more than 10%. The nonessential amino acids proline, serine and tyrosine were greatly reduced by 45.5%, 35.5%, and 54.5%, respectively. However, glutamic acid increased by 42.5%. The acetate/phosphate treatment generally increased the amount of essential and nonessential amino acids except the amount of serine and tyrosine decreased by 3.9% and 2.2%, respectively. Histidine, isoleucine, lysine, threonine and valine concentrations increased by over 15%. Of the nonessential amino acids, glycine was greatly increased

Table 5—Amino acid composition of the myofibrillar protein fraction of control and washed turkey thigh tissues[a]

Amino acid	Control[b]		Washing treatment[b]	
	Breast	Thigh	Phosphate	Acetate/Phosphate
Essential				
Histidine	1.96	1.02	0.93(– 8.8)[c]	1.22(+19.6)[c]
Isoleucine	3.54	2.15	1.91(–11.2)	2.65(+23.3)
Leucine	6.81	4.53	4.18(– 7.7)	5.04(+11.3)
Lysine	8.12	5.29	4.55(–14.0)	6.10(+15.3)
Methionine	–	0.49	0.39(–20.4)	0.53(+ 8.2)
Phenylalanine	3.26	1.94	1.64(–15.5)	2.16(+11.3)
Threonine	4.13	1.50	1.17(–22.0)	1.83(+22.0)
Tryptophan[d]	N.D.	N.D.	N.D.	N.D.
Valine	3.50	2.08	1.64(–21.2)	2.44(+17.3)
Nonessential				
Alanine	4.59	3.54	3.62(+ 2.3)	3.78(+ 6.8)
Arginine	5.70	3.70	2.94(–20.5)	3.85(+ 4.1)
Aspartic acid	7.39	5.48	5.26(– 4.0)	5.49
Cystine[d]	N.D.	N.D.	N.D.	N.D.
Glutamic acid	11.82	5.48	7.80(+42.3)	5.82(+ 6.2)
Glycine	2.60	2.20	2.25(+ 2.3)	2.70(+22.7)
Proline	3.18	2.24	1.22(–45.5)	2.35(+ 4.9)
Serine	3.77	0.76	0.49(–35.5)	0.73(– 3.9)
Tyrosine	–	1.34	0.61(–54.4)	1.31(– 2.2)

[a] Expressed as grams of amino acid residue per 100g protein.
[b] Means of triplicate samples.
[c] Values between brackets are percent difference of individual amino acids calculated from untreated thigh.
[d] N.D. = not determined.

(22.7%) in concentration. Alanine, arginine, glutamic acid and proline were higher (<10%) in concentration than the control.

Functional properties evaluation

Water-holding capacity (WHC). Cooking loss is often used to measure the WHC of meat (Table 6). The amount of fluid released by the cooking of ground or unground muscle can be determined without centrifugation (Lee et al., 1978; Honikel et al., 1981). The differences in WHC between the washed and control tissues were significant ($P<0.05$) when the tissues were cooked in water. Phosphate-washed tissue exhibited the least cooking loss (30.85%) in water, where as untreated thigh tissue experienced the highest cooking loss (45.67%). This result can be explained by the fact that an increase in pH produced by phosphate improves WHC in meat (Hamm, 1986) and the potential loss of lipids and certain solids during washing. This trend was reversed when the tissues were cooked in 2% NaCl. The presence of NaCl ions appeared to increase the amount of water lost by the washed tissues and substantially improved the WHC of the control tissues. This means that under the conditions of the test, the washed tissues were losing their "gained" water, the amount of lost water being higher for the acetate/phosphate washed tissue.

Protein solubility. The values for protein solubility are give in Table 7. The protein solubility values of the control tissues were significantly ($P<0.05$) higher than those values of the washed tissues at different NaCl concentrations. This observation is logical since water soluble proteins were extracted due to the washing procedures. For the control tissues, the protein solubility index was salt concentration dependent and increased substantially with the increase of NaCl concentration from 0 to 5%. The protein solubility of washed tissues increased with salt concentration except at 2% NaCl. It is noteworthy to mention that protein extractability of washed tissues increased in water.

Emulsifying capacity (EC) and emulsion stability (ES). The emulsifying characteristics of control and washed tissues were determined by EC and ES measurements (Table 8). The EC of the washed tissues were not ($p>0.05$) different from control thigh tissue. These results are in disagreement with those of Jimenez-Colmenero and Garcia (1983) who reported that the EC of mechanically deboned pork meat was reduced due to water washing. Although a great reduction in the amount of sarcoplasmic protein was observed in our study, the non-significant decrease in the amount of myofibrillar protein accounts for the insignificant differences in EC among the control and washed thigh tissues. According to Gaska and Regenstein (1982a, b), chicken breast muscle exhaustively washed by low and high-salt and resuspended in 0.15 and 0.6 NaCl exhibited good emulsion properties. In addition, sarcoplasmic proteins had little emulsifying effect in the presence or absence of myofibrillar proteins.

There is a qualitative difference between the EC and ES. Emulsion stability is not a measure of maximum oil addition but rather of the ability of the emulsion products to retain its properties. Breast tissue can more effectively stabilize the emulsion than the unwashed and washed thigh tissues (Table 8). Phosphate-washed tissue had a significant influence on stabilizing the emulsion when compared to unwashed thigh and phosphate/acetate washed-tissues.

Table 6—Cooking loss[a] values of control and washed turkey thigh tissues

Tissue sample	% Cooking loss	
	H_2O	2% NaCl
Control		
Breast	41.42^{Bb}	35.11^{Cc}
Thigh	45.67^{Ab}	40.82^{Bc}
Washing treatments		
Phosphate	30.85^{Dc}	46.09^{Ab}
Acetate/Phosphate	35.72^{Cc}	48.03^{Ab}

[a] Percent cooking loss was determined as a measure for water-holding capacity, (N = 12).

[bc] Means in the same row with identical lower case superscripts are not different ($P>0.05$).

[A-D] Means in the same column with identical upper case superscripts are not different ($P>0.05$).

Table 7—Protein solubility[a] of control and washed turkey thigh tissues

Tissue sample	NaCl concentration (%)			
	0	2	3	5
Control				
Breast	13.9^{Ce}	36.6^{Ad}	40.1^{Ac}	43.5^{Ab}
Thigh	17.0^{Be}	32.4^{Bd}	39.1^{Ac}	43.0^{Ab}
Washing treatment				
Phosphate	20.9^{Ad}	16.6^{De}	24.0^{Bc}	27.0^{Cb}
Acetate/Phosphate	19.6^{Ad}	18.8^{Cd}	27.1^{Bc}	30.8^{Bb}

[a] Protein solubility or Nitrogen Solutility Index (NSI) expressed as percent total protein (N = 12).

[b-e] Means in the same row with identical lower case superscripts are not different ($P>0.05$).

[A-D] Means in the same column with identical upper case superscripts are not different ($P>0.05$).

Table 8—Emulsifying capacity (EC) and emulsion stability (ES) of control and washed turkey thigh tissues

Tissue sample	Emulsifying capacity (mL oil/2.5g tissue)	Emulsion stability (% oil released)
Control		
Breast	81.97^{a}	1.92^{c}
Thigh	75.38^{b}	54.64^{a}
Washing treatments		
Phosphate	79.15^{ab}	26.40^{b}
Acetate/Phosphate	77.16^{b}	51.92^{ab}

a-c Means in the same column with identical superscripts are not different ($P>0.05$).

CONCLUSIONS

COMPOSITIONAL CHANGES in washed tissues included a lowering of protein and an increase in moisture that could not be removed by the pressing step. The amount of moisture gain increased as the pH of washed tissues increased. Differences in total protein were attributed primarily to losses of sarcoplasmic proteins. Both washing treatments appeared to be effective in altering the color characteristics and provided thigh tissue with color values similar to those of control breast tissue. The functional properties of thigh tissue examined in the present work were affected by the washing treatments. The apparent differences in functionality data for the washed tissue might have been due to the primary characteristics of the protein remaining in the washed tissues and/or the compositional differences, particularly the much higher moisture content relative to the protein content. Changes in meat characteristics due to washing and its effects on product quality will require further study.

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Comparison of Broiler Tissues for Oxidative Changes After Cooking and Refrigerated Storage

C.Y.W. ANG

ABSTRACT

Broiler parts were cooked in water as: split breast without bone, leg without bone, skin, liver, and depot fat. Cooked tissues from one side of the birds were analyzed for initial thiobarbituric acid (TBA) numbers while tissues from the other side were ground and analyzed after 5 days storage at 4°C. The greatest increases in TBA numbers were found in liver and breast meat followed by leg meat, skin and depot fat. The oxidation rate during storage was positively correlated with protein, water, phospholipids and nonheme iron content and negatively correlated with total lipids or neutral lipids. Major constituents might affect the tissue stability by influencing the physical or environmental conditions for oxidation process.

INTRODUCTION

COOKED MEATS may develop oxidative off-flavor characteristics upon refrigerated storage and subsequent reheating (Tims and Watts, 1958; Pearson et al., 1977). Poultry lipids generally exhibit a higher degree of unsaturation compared to red meats, thus poultry meat is more susceptible to oxidation. Wilson et al. (1976) reported that oxidative changes, as measured by the thiobarbituric acid (TBA) numbers, occurred most readily in turkey meat, followed by chicken, pork, beef and mutton. Within the same species of poultry, dark meat was found to generate higher TBA numbers than white meat. The authors (Wilson et al., 1976) suggested that the relative content of phospholipid (PL) in meat was at least partially responsible for these observations.

Igene and Pearson (1979) and Igene et al. (1981) demonstrated in model meat systems that both triglyceride (TG) and PL contributed to the off-flavor development, and that PL made the greatest contribution. Pikul et al. (1984a and 1984b) pointed out that fat from chicken breast meat contained two times more malonaldehyde than fat from leg meat, due to a larger PL fraction in fat from breast meat. However, the TBA numbers were higher in leg meat because the breast meat contained much less total fat.

Heme compounds have been suggested as another factor related to oxidative changes in animal tissues (Tappel, 1953). Love (1983) reviewed a number of studies on the role of heme and nonheme iron in the warmed-over flavor of cooked meats. Some results showed that heme iron was an effective catalyst (Wills, 1966; Liu and Watts, 1970), while others found that nonheme iron was responsible for the oxidative reaction in cooked meats (Sato and Hegarty, 1971; Love and Pearson, 1974; and Igene et al., 1979). Chen et al. (1984) speculated that meat cooked by slow heat might be more susceptible to off-flavor development based on their findings that more nonheme iron was released by slow heating. However, Godber et al. (1985) reported that faster heating promoted oxidation in steaks and suggested that nonheme iron had no influence in the warmed-over flavor (WOF) formation.

While no information is available to show that either protein or water is directly relevant to the generation of TBA reactive products, Tappel (1955) and Marayan et al. (1964) reported the occurrence of complex reactions between proteins and oxidized lipids. On the other hand, water is one of the most important environmental factors in many chemical reactions. Water activity of a food product may influence the rate of lipid oxidation (Leung, 1986). Hence, it is of importance to investigate the influence of protein and water content on oxidative stability of different tissues.

The objectives of the present study were to compare the rate of oxidation of various cooked broiler tissues during refrigerated storage and to correlate the relevant constituents, including PL, total lipids (TL), nonheme iron, protein and water, with the oxidative changes.

Author Ang is with the USDA-ARS, Richard B. Russell Agricultural Research Center, P. O. Box 5677, Athens, GA 30613.

MATERIALS & METHODS

Broiler samples

Four trials were conducted, each in a different week. In each trial, six (6) commercially processed broilers were obtained from a local processing plant prior to chilling (within 1 hr postmortem). Approximately 1 kg pooled, unchilled livers and 1 kg depot fat were also collected from more than 20 broilers from the same processing line, but not necessarily from the same broilers, for cooking, storage, and chemical analyses. All samples were packed with crushed ice in insulated containers and stored overnight at 4°C.

Each of the six carcasses was separated by circular saw into two halves (left and right sides). Each half was then deskinned and cut into parts. Skin from breast and leg was obtained. Breast meat and leg meat (thigh and drumstick) were freed of bones. Wings were not used. Each of the 1-kg portion of liver or depot fat was arbitrarily divided into two equal portions.

Cooking methods

In each trial, one type of tissue from one side of each of three broilers was grouped into one composite. Thus, two composites were made per tissue per broiler side. Breast meat, leg meat and skin were cooked in 88°C water in a stainless steel steam jacketed kettle until internal temperature of the parts reached 80°C. Liver and depot fat were cooked separately in a household electric pot with cooking water set at 88°C, and were considered "cooked" when the internal temperatures reached 68° and 54°C, respectively.

Preliminary tests were conducted to determine the cooking time for each part to reach the desired internal temperature. Cooking times were approximately 20 min for breast, leg and fat, 15 min for liver and 10 min for skin.

The internal temperature of a part was measured with a hand-held electronic digital thermometer with a tubular point metal probe (Fisher Scientific Co., Norcross, GA) inserted into the center of a part at the end of cooking time. Several readings with probes inserted into different pieces of a part were averaged. When the chicken skin was cooked, several layers of skin folded together and it was possible to measure the skin temperature by inserting the probe in the center of skin lumps.

The selections of the end-point internal temperatures and cooking times were based on common practices for cooking these products for human consumption. Liver, due to its unique texture property, is easily "cooked," i.e., its proteins coagulate at lower temperature than that of the breast or leg tissues. On the other hand, depot fat, with negligible protein content, is commonly cooked for the same length of time at the same cooking temperature as the muscle parts while the end point temperature may be lower than that of muscles, as determined in the present experiment.

After cooking, parts were cooled in ice water for 15 min (internal

temperature 10–15°C). Surface water was drained from the cooled parts for 10 min. Fat was cooled overnight at room temperature in the original cooking kettle. Each composite tissue from one side of the broilers was blended in a food processor, placed in screw-capped glass jars (125g per jar filled to capacity) and stored at 4°C for 5 days. At day 5, the jars were flushed with N_2 and stored at −30°C until analyzed. Each composite of tissues from the other side was blended in the food processor under N_2, filled in jars under N_2 and stored at −30°C. These samples were labeled as 0 day. Preliminary tests showed that TBA numbers of samples prepared and stored under these conditions did not change if analyzed within 30 days, and all TBA tests were performed within the same week of preparation. In the case of liver and depot fat, only one composite was made for each of the two portions ("sides"). Several replicate jars for each composite were prepared for different chemical analyses so that no jar was thawed or opened unnecessarily before the other tests.

In trials 1 and 3, left sides of the broilers were used for 0-day analyses and right sides for 5-days storage tests. In trials 2 and 4. The left and right sides were exchanged for 5-day and 0-day analyses.

Chemical analyses

Each composite was analyzed in duplicate. In the case of liver and depot fat, four samples were analyzed per composite (portion). Cooked 0-day samples were analyzed for protein, moisture, total iron, nonheme iron, TL, and lipid phosphorus. Water content was determined by vacuum oven drying at 95°C and protein content was by the Kjeldahl nitrogen method (AOAC, 1980). Total iron content was determined by a wet digestion method (McHard et al., 1976) followed by atomic absorption spectroscopy (AOAC, 1980). Nonheme iron was analyzed by the method of Schricker et al. (1982) and heme iron content was calculated as the difference between total iron and nonheme iron.

Total lipid of each tissue was extracted with a combination of sodium dodecylsulfate, ethanol and n-heptane as reported by Burton et al. (1985). Lipid phosphorus was determined according to the procedure of Morrison (1964). Phospholipids content was estimated by multiplying the phosphorus values by a factor of 25.5. Neutral lipids content was calculated by difference between TL and PL.

The state of oxidation was measured as TBA numbers using a modified version of the method of Tarladgis et al., (1964). An antioxidant, BHT (butylated hydroxytoluene), was added to the meat samples (0.03% by weight) during blending in an effort to reduce sample auto-oxidation during final preparation and distillation (Pikul et al., 1983). TBA numbers were calculated as mg malonaldehyde per kg meat. Both the 0-day and 5-day samples were analyzed for TBA numbers and were designated as TBA-0 and TBA-5, respectively. The difference in TBA numbers (TBA-D) between 0-day and 5-day samples were calculated by substrating TBA-0 from TBA-5. The TBA increase ratio (TBA-IR) were calculated by dividing TBA-D by TBA-0.

Statistical analyses

Data were subjected to various statistical analyses, including Duncan's Multiple Range test to compare treatment means, simple correlations between TBA numbers and tissue constituents and the General Linear Model (GLM) procedures. All computations were based on the programs of Statistical Analysis System (SAS, 1984).

Preliminary statistical calculation showed no significant difference between the two composites per trial. Thus, data of individual analyses (4 samples per trial; total 16 samples) were used in computing the mean and standard deviation of a tissue and that would show the range of variation in a wider scope than if four trial-means were used.

RESULTS & DISCUSSION

ANALYTICAL RESULTS describing the major tissue constituents are presented in Table 1. Breast meat contained the highest amount of protein followed by leg meat, liver, skin, and depot fat, which had only a trace amount of protein. Water content was highest in liver, followed by breast meat, leg meat, skin and depot fat, which contained very little moisture. Other than depot fat, skin contained the highest amount of TL followed by leg meat, liver and breast meat.

Lipid composition, i.e., PL and NL, expressed as percentage of TL as well as of total tissue, is presented in Table 2. Among the five tissues tested, liver had the highest amount of PL, almost equivalent to NL. Breast total lipids contained a higher percentage of PL than the leg lipids. However, since leg meat had a much higher amount of TL, the actual concentration of PL in leg meat was still higher than that in breast meat. Skin and depot fat were composed almost entirely of NL.

Table 1 – Water, protein and total lipids content of freshly cooked broiler tissues (0-day samples)

Tissue	Water (%)	Protein (%)	Total lipids (%)
Breast meat	70.50 ± 0.55[a]	26.08 ± 1.23	1.54 ± 0.23
Leg meat	67.21 ± 0.72	23.55 ± 0.85	7.85 ± 0.84
Skin	55.78 ± 1.86	11.22 ± 0.75	31.49 ± 2.93
Liver	72.78 ± 1.15	18.80 ± 2.22	5.44 ± 0.47
Depot fat	2.92 ± 1.67	0.05 ± 0.02	97.03 ± 1.66

[a] Values are mean ± standard deviation of 16 samples prepared in 4 trials.

Data on lipid composition are important because PL usually contain more unsaturated fatty acids, especially arachidonic acid (20:4), than NL (Pikul et al., 1984b). Results of the present study on the distribution of PL and NL in various tissues were in general agreement with earlier reports (Katz et al., 1966; Igene et al., 1985; Pikul et al., 1985a, b). However, the absolute values reported in different studies were not the same, due to the variation in cooking status (raw or cooked), in biological status (sex, age and species) of birds and in sampling techniques.

Total iron and nonheme iron content are also shown in Table 2. Liver contained the highest amount of each type of iron followed by leg meat, skin, and breast meat. Relative to liver, all other tissues had very low heme iron values (not shown in Table 2). The iron content in depot fat was too low to be determined as nonheme or heme iron.

Five tissues were used to follow oxidation after cooking. These tissues were selected because they exhibit differences in lipid constituents associated with oxidation (Katz et al., 1966; Pikul et al., 1985a, b). In the present study, more complete compositional profiles of all the five tissues were obtained and extensive replication was included in the experimental design. Thus the influence of compositional factors on the oxidation of cooked meat could be evaluated more accurately by statistical methods.

The effect of the type of tissue, trial, storage time and the interactions between those factors on the TBA numbers are shown in Tables 3a and 3b. For calculations of correlations and GLM procedures, only the average values of each composite (average of duplicate analyses) were used because only Composite-1 of one side of the broilers could be compared with Composite-1 of the other side, whereas Sample-1 of one composite could not be compared with Sample-1 of another composite. Therefore, for TBA analyses, sample numbers (n, or analytical data units) were 80 for two storage times (n = 80 or df = 79 for calculations in Table 3a and n = 40 for Table 3b). Analyses indicated that there were significant interactions between tissue and trial and between tissue and storage time.

The influence of trial variation was not an important concern because paired tissue parts were used in each trial for 0-day TBA test (TBA-0) and the 5-day storage test (TBA-5). The TBA-D values of the same trial and the TBA increase ratios (TBA-IR) were of more significance rather than the absolute TBA numbers. There were no tissues-by-trial interactions for TBA-D and additionally, no trial variations for TBA-IR values (Table 3b).

All TBA numbers, determined and derived, are presented in Table 4. When tissues were freshly cooked, the initial TBA numbers were highest in leg meat, followed by liver, breast and skin. After 5-days storage at 4°C, however, liver had the highest TBA numbers, followed by leg meat, breast meat and skin. Fat had the lowest TBA numbers regardless of storage time. The TBA-0 numbers of breast meat were not as high as leg meat or liver, but the increase ratios (TBA-IR) in breast

Table 2—Phospholipids (PL), neutral lipids (NL), total iron and nonheme iron content of freshly cooked broiler tissues (0-day samples)

Tissue	In tissue		In total lipids	Iron (ppm)	
	% PL	% NL	% PL	Total	Nonheme
Breast meat	0.22 ± 0.05[a]	1.32 ± 0.22	14.44 ± 3.00	4.64 ± 0.73	5.13 ± 0.64
Leg meat	0.42 ± 0.05	7.43 ± 0.83	5.42 ± 0.80	11.27 ± 1.21	9.68 ± 1.22
Skin	0.11 ± 0.03	31.37 ± 2.93	0.36 ± 0.11	8.10 ± 0.82	6.71 ± 0.69
Liver	2.69 ± 0.32	2.76 ± 0.33	49.41 ± 4.13	99.77 ± 12.28	86.54 ± 13.08
Depot fat	<0.20[b]	96.78 ± 1.65	<0.20[b]	0.85 ± 0.18	<1.00[b]

[a] Values are mean ± standard deviation of 16 samples prepared in 4 trials.
[b] Below detection limit.

Table 3a—General Linear Model analysis of TBA numbers

Source of variation (S.V.)	df[a]	F value	PR > F[b]
Tissue (TI)	4	249.38	0.0001
Trial (TR)	3	7.84	0.0002
TI × TR	12	2.58	0.0086
Storage (ST)	1	1354.57	0.0001
TI × ST	4	128.29	0.0001
Error	55	-	
Total	79	-	

Table 3b—General Linear Model analysis of individual TBA numbers[c]

S.V.	PR > F			
	TBA-0	TBA-5	TBA-D	TBA-IR
Tissue (TI)	0.0001	0.0001	0.0001	0.0001
Trial (TR)	0.0027	0.0015	0.0325	0.0548
TI × TR	0.1629	0.0117	0.0594	0.1710

[a] Degree of freedom.
[b] Probability of getting a larger value of F due to chance.
[c] TBA-0 = TBA at 0 day; TBA-5 = TBA at 5 day (cooked and stored 5 days at 4°C); TBA-D = difference between TBA-5 and TBA-0; TBA-IR = TBA increase ratio (TBA-D/TBA-0).

meat during storage were comparable to liver. These results imply a particularly strong oxidation rate for breast meat.

It has been reported that dark meat exhibits higher initial TBA values than white meat due to the higher levels of PL in the dark tissues (Dawson and Schierholz, 1976; Igene et al., 1979, 1985; Pikul et al., 1985a). The present data confirmed these findings. On the other hand, however, only limited data are available on TBA numbers of other tissues. Pikul et al. (1985a) showed that raw skin had a higher TBA number (0.46) than leg (0.36) or breast meat (0.31). Another study (Pikul et al., 1985b) reported that liver had a high TBA number (0.90) and adipose tissue had a low value (0.20) but there were no data for cooked tissues. If the absolute values from these two studies can be compared directly, then the TBA number of liver was highest among the five raw tissues. Results of the present study on cooked tissues, generally agreed with this trend, except that skin was found to have lower TBA values than leg and breast meat.

Low TBA numbers for skin noted in this study could be explained by the fact that skin PL contains only about one-half the polyunsaturated fatty acids with 20- and 22-carbon atoms compared to leg and breast tissues (Pikul et al., 1985a). Dawson and Schierholz (1976) reported that roasted turkey skin had higher TBA numbers than the turkey breast or leg meat. In that case, the high TBA numbers of skin might be due to the intense heat applied to the turkey surface during roasting.

Some earlier data showed significant correlations between TBA numbers and sensory scores of oxidized flavor (Zipser et al., 1964; Jacobson and Koehler, 1970; Fooladi *et al.*, 1979; Melton, 1983; Igene et al., 1985). However, absolute TBA numbers of various tissues may not represent the status of oxidation nor can they accurately indicate sensory assessment. Instead, the rate of change in TBA numbers may be a more accurate measure of oxidation rate. In the present study, therefore, not only the absolute TBA numbers of freshly cooked and cooked-stored samples, but also the net changes of TBA numbers during storage were evaluated. Consequently, the susceptibility to oxication of various tissues could be assessed accurately and compositional factors relating to the oxidative changes could be determined. The increase in TBA numbers may provide a more accurate index than the absolute TBA numbers for tissue oxidation as well as flavor changes in various tissues.

All data obtained were subjected to statistical correlation analyses. Simple correlations between TBA numbers and tissue constituents are shown in Table 5. Almost all values of the correlation coefficients (r) were significant at the 0.01 level. However, relatively higher coefficients were found between TBA numbers and the major constituents, i.e., protein, water and TL or NL in tissue (NLIT). TBA-0 numbers were not correlated with PL, PLIT (% phospholipids in tissue), iron, nonheme or heme iron. For TBA-5, high positive correlations were found with protein, moisture, PL and negative correlation with TL and NLIT. TBA-D and TBA-IR followed the same pattern as TBA-5 in correlations with tissue components except that TBA-IR was not correlated with heme iron ($P > 0.01$).

The correlation data (Table 5) are particularly interesting regarding the findings on protein, water, TL and NLIT in relation to TBA numbers. It appeared to be rational to speculate that these constituents played important roles in the physical or environmental conditions for the oxidation process within the meat matrix. The positive influence of water on the TBA numbers could be in part due to its function as a solvent for the dissolved oxygen and any metal catalysts. The high protein content probably was responsible for the crumbly form of ground cooked breast tissue, allowing more surface area in contact with air, whereas the high fat content seemed to be associated with the cohesively compact form of the depot fat and skin, that in turn reduced the surface areas of these tissues. Further studies are needed in relating tissue texture characteristics and environmental conditions to oxidative stability.

Different tissues exhibited different characteristics in oxi-

Table 4—Oxidative changes of cooked broiler tissues as measured by thiobarbituric acid numbers (TBA)[a]

Tissue	TBA-0	TBA-5 (mg malonaldehyde/kg tissue)	TBA-D	TBA-IR %
Breast meat	2.68 ± 0.53BC	15.78 ± 1.56B	13.60 ± 1.37A	506 ± 106A
Leg meat	4.13 ± 1.03A	17.33 ± 1.26AB	13.21 ± 1.82A	352 ± 144B
Skin	2.26 ± 0.43C	7.85 ± 0.89C	5.59 ± 0.73B	256 ± 60C
Liver	3.19 ± 0.71B	18.95 ± 3.73A	15.76 ± 3.22A	503 ± 83A
Depot fat	0.32 ± 0.08D	0.30 ± 0.10D	–[b]	–[b]

[a] Values are mean ± standard deviation of 16 samples prepared in 4 trials. Means within the same column with different letters are significantly different ($P \leq 0.05$). See Table 3b for designations of TBA terms.
[b] Negligible.

Table 5—Correlations between tissue constituents and TBA numbers (n=40)

Tissue constituents[a]	TBA numbers 0-day (TBA-0)	5-day (TBA-5)	Difference (TBA-D)	Increase ratio (TBA-IR)
	Correlation coefficients (r)[b]			
Protein	0.79	0.89	0.88	0.82
Water	0.81	0.90	0.90	0.86
TL	−0.82	−0.92	−0.90	−0.87
PL	NS	0.64	0.68	0.61
PLIT	NS	0.53	0.56	0.44
NLIT	−0.82	−0.92	−0.91	−0.87
Iron	NS	0.53	0.56	0.48
Nonheme iron	NS	0.54	0.57	0.49
Heme iron	NS	0.46	0.48	NS

[a]Abbreviations: TL = total lipids; PL = % phospholipids in TL; PLIT = % phospholipids in tissue; NLIT = % neutral lipids in tissue. See Table 3b for designations of TBA terms.

[b] All values of coefficients as shown are significant ($P<0.01$). NS denotes not significant ($P>0.01$).

dative stability because of the compositional variations among tissues. While some components (such as PL and nonheme iron) apparently influenced the oxidation rate by direct involvement in the reactions, other major constituents (such as protein, water, TL and NLIT) were also highly correlated with the tissue stability, possibility due to their roles as environmental factors for the oxidative process.

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Relation Between Gelation Behavior of Ground Chicken Muscle and Soybean Proteins and Their Differential Scanning Calorimetric Studies

KATSUJI SHIGA, TAKAYASU KAMI, and MASAYA FUJII

ABSTRACT

The thermal denaturation of ground chicken muscle, soybean protein, and the mixture of both were investigated by differential scanning calorimetry (DSC) under different salt concentration. The relationship between DSC analysis and the properties of heat-induced gels from these samples were determined. The DSC thermograms of ground chicken muscle showed three endothermic peaks and those of soybean protein showed two peaks. The DSC thermogram of their mixture showed three peaks. When NaCl was added to ground chicken muscle the endothermic peaks shifted to lower temperatures and the rheological values of heat-induced gels increased. When NaCl was added to soybean protein, the endothermic peaks shifted to higher temperatures and the rheological values of heat-induced gels decreased.

INTRODUCTION

DIFFERENTIAL SCANNING CALORIMETRY (DSC) is being used increasingly in the study of thermal denaturation of proteins in food such as meat, egg, and soybean protein (Karmas and Dimarco, 1970; Wright et al., 1977; Quinn et al., 1980; Stabursvik and Martens, 1980; Samejima et al., 1983; Stabursvik et al., 1984; Wright and Wilding, 1984; Hermansson, 1978; Grozav et al., 1985). DSC can detect the heat denaturation of a protein in complex protein systems as an endothermic peak in its thermogram. Moreover, DSC technique has the advantage that it can be used to observe thermal changes and denaturation of proteins in solution as well as in insoluble suspensions or pastes. Thus, DSC is a useful technique in the study of the heat denaturation of proteins in foods such as ground meat and paste-state soybean protein which are complex and concentrated protein systems.

The purpose of this study was to investigate, by DSC, the heat denaturation of ground chicken muscle, soybean protein and their mixture, and to elucidate the relationship between the results of DSC analysis and the rheological properties and water-holding capacity of heat-induced gels from these samples.

MATERIALS & METHODS

Materials

Ground muscle was prepared from chicken breast muscle and acid-precipitated soybean protein was prepared from defated soybean meal as described previously (Shiga et al., 1985). Composition of ground chicken muscle was as follow: total solids, 25–27%, crude protein, 22–23%; fat, 2–3%. Total solids of the wet acid-precipitated soybean protein was 30–38% (crude protein content in dry matter was about 97%).

Total solids of ground chicken muscle was adjusted to 20% by adding the appropriate quantity of water. The soybean protein was ground in mortar in the presence of the appropriate quantity of water and 1N NaOH. The resulting paste was finally adjusted to 20% total solids and adjusted pH 7.0 with additional water and 1N NaOH, respectively. Mixed ground chicken muscle and soybean protein was prepared by mixing equal amounts of the two components. To samples of each of the three preparations were added NaCl in concentrations ranging from 0 to 3%, and the samples were then allowed to stand overnight at 5°C.

Authors Shiga, Kami, and Fujii are with the Faculty of Applied Biological Science, Hiroshima Univ., 2-17 Midori-machi, Fukuyama 720 Japan.

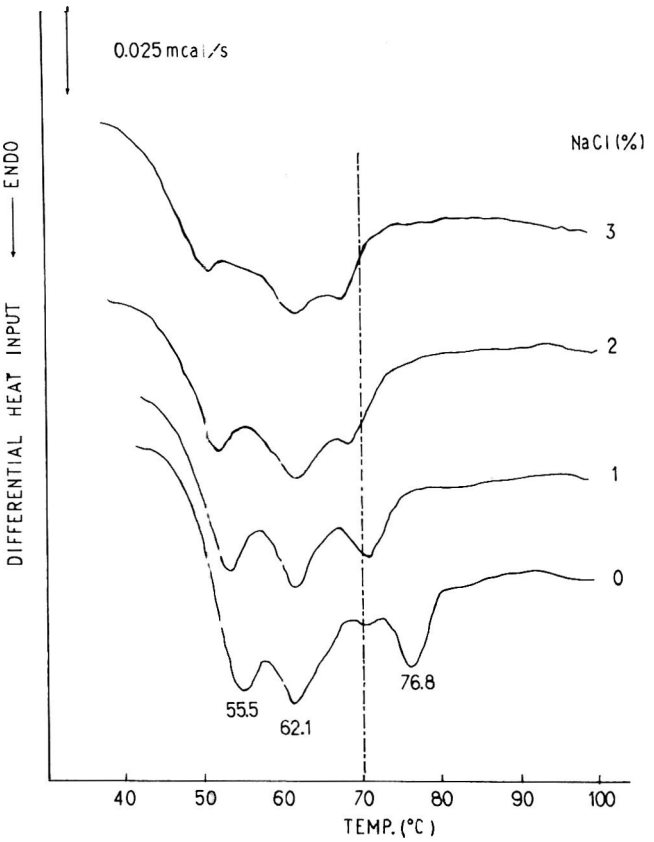

Fig. 1—DSC thermograms of ground chicken ground muscle with various concentration of NaCl. Vertical bar represents 0.025 mcal/sec.

Measurements of rheological properties

The heat-induced gels for investigation of rheological properties were prepared by packing the ground chicken muscle, soybean paste or mixed sample into a pair of specially designed aluminum cases (10 × 18 × 7 mm). The two cases containing the samples were put together and carefully sealed with polyvinylidenechloride film and a rubber ring. The sealed cases were heated in a water bath (70° or 100°C) for 20 min and, after heating, cooled to room temperature (25°C) in tap water. The gels thus formed were carefully taken out of the cases (Shiga and Kushida, 1983).

The rheological properties (breaking stress and strain) of the heat-induced gels were measured as described previously (Shiga and Kushida, 1983).

Water-holding capacity (WHC) was measured by the method of

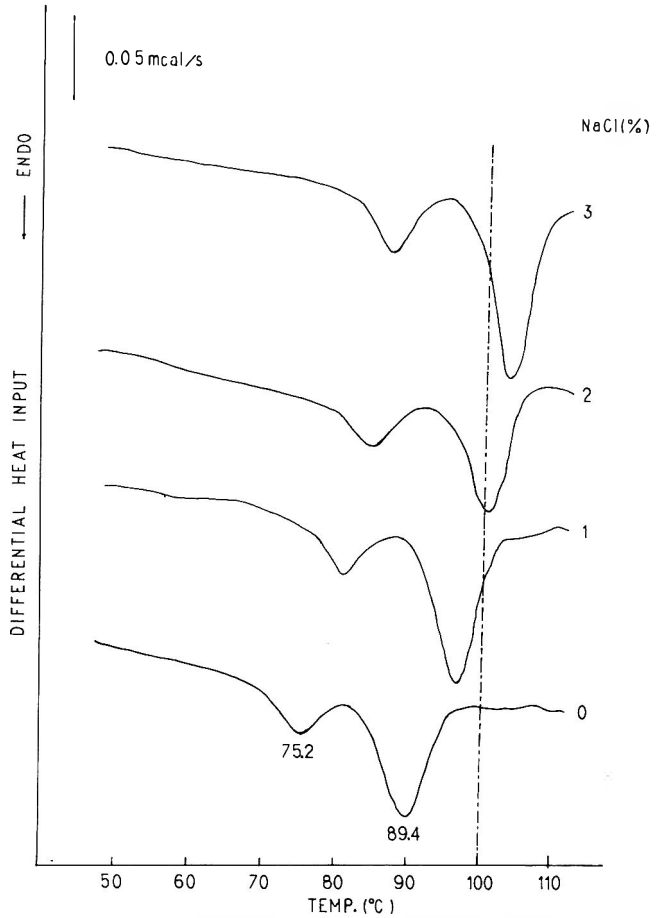

Fig. 2—DSC thermograms of soybean protein with various concentration of NaCl. Vertical bar represents 0.05 mcal/sec.

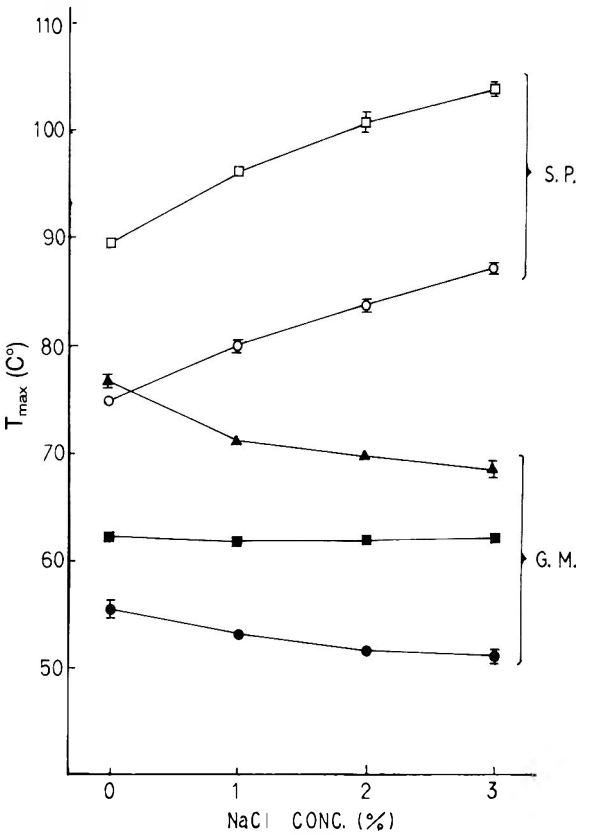

Fig. 3—Effects of NaCl concentration of T_{max} of ground chicken muscle and soybean protein. S.P.: Soybean protein; G.M.: ground chicken muscle, ○ and □ indicate first and second peaks of soy protein thermograms, ●, ■ and ▲ indicate first, second, and third peaks of ground chicken muscle thermograms, respectively.

Ikeda et al. (1968). The values were calculated by the following equation:

$$\text{WHC (\%)} = \frac{\text{Water content of sample} - \text{Separated water}}{\text{Water content of sample}} \times 100$$

Differential scanning calorimetry

Differential scanning calorimetry (DSC) was done with a Rigaku Denki High Sensitive DSC-10 A (8230). Samples (10–16 mg) were sealed in Rigaku Denki aluminum pans. An equal weight of water or NaCl solution was sealed in another pan and used as reference. The heating rate was 5°C/min over a temperature range of 20–112°C. The instrumental sensitivity was 0.25 or 0.5 mcal/sec. The temperature at which the maximum rate of heat input occurred in an endothermic peak was expressed by T_{max}. Each DSC analysis was repeated four times.

Protein extraction from heat-induced gel

Five gram samples were placed in glass test tubes (20 mm diameter) which were loosely sealed with aluminum foil and heated in a water bath (70°C, ground muscle; 100°C, soybean protein; 70° and 100°C for the mixtures) for 20 min. The heated samples were cooled in tap water (about 25°C). Heat-induced gels were ground in a mortar and homogenized with a 20 mL 0.6M NaCl solution (containing 0.04M $NaHCO_3$ and 0.01M Na_2CO_3, pH 9.0). This homogenate was allowed to stand overnight at 5°C and centrifuged at 10,000 × *g* for 20 min at 5°C. The supernatant was decanted and used for SDS-polyacrylamide gel electrophoresis.

SDS-polyacrlyamide gel electrophoresis

The protein extract obtained from heat-induced gels was subjected to SDS-polyacrylamide gel electrophoresis (PAGE) according to the method of Laemmli (1970). Electrophoresis was carried out in 10% acrylamide slab gels (13.5 × 15 × 0.2 cm) containing 0.1% SDS, at 30 mA for 5-6 hr. The protein extract was mixed with an equal volume of 0.02M Tris-HCl buffer containing 2% SDS and 2% 2-mercaptethanol, and 1 mL glycerol was added to 4 mL diluted extracts. The diluted extracts (40, 20, and 10 μL of the extracts from the ground chicken muscle, soybean protein and mixed sample, respectively), about 50 μg native soybean protein and crude actomyosin were applied to each slot of the gel. The gel was stained with Coomassie Brilliant Blue.

The crude protein concentration of diluted extracts were as follows: Ground muscle: 0.18% (N), 0.17% (S); Soybean protein: 0.12% (N), 0.15% (S); Mixture (70°C): 0.36% (N), 0.38% (S); Mixture (100°C): 0.23% (N), 0.30% (S); (N) = nonsalted; (S) = salted.

RESULTS

DSC analysis of ground chicken muscle, soybean protein, and their mixture

DSC thermograms of ground chicken muscle showed three major endothermic peaks (Fig. 1). It has been suggested that the first peak was due to myosin, the middle peak might be due to connective tissue, sarcoplasmic protein and myosin, and the third peak was ascribed to actin (Stabursvik and Martens, 1980). The T_{max} of these peaks shifted to lower temperatures when NaCl was added (Fig. 1 and 3). A greater effect of NaCl on T_{max} was observed for actin and its T_{max} decreased from 76.8 to 68.3°C upon addition of 3% NaCl. Quinn et al. (1980) reported that when NaCl was added to ground beef muscle, significant changes were observed in DSC thermograms. At the low salt level (0.23M), the peak at 84°C disappeared and the peak at 76°C increased in size. At the high salt level (0.67M), all original peaks disappeared and a single new peak occurred at 71–72°C. They assumed that the single large peak probably

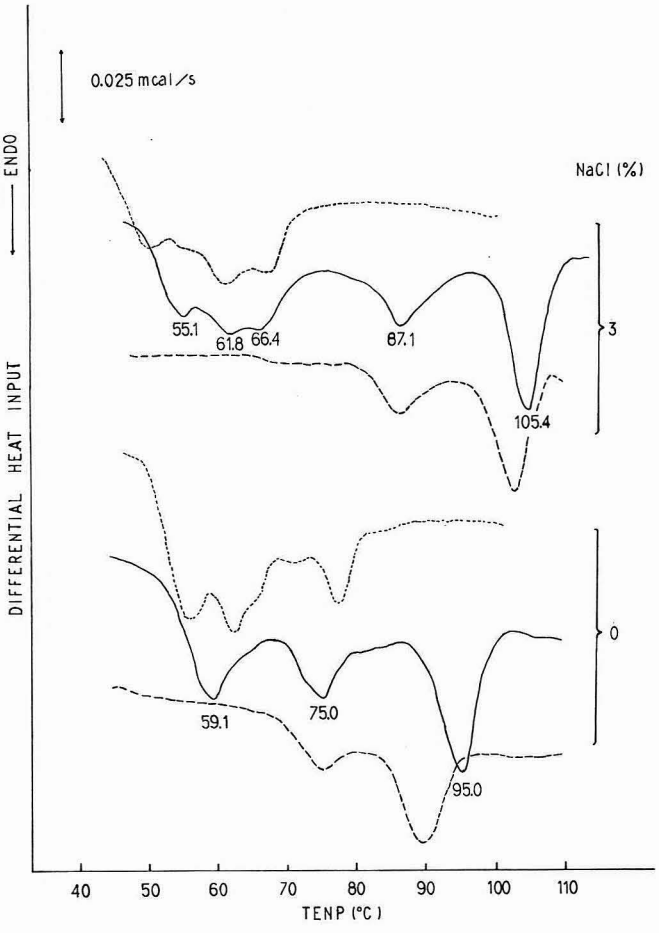

Fig. 4-A—DSC thermograms of mixed sample of ground chicken muscle and soybean protein with and without 3% NaCl. Vertical bar represents 0.025 mcal/sec. (———): mixed sample; (·····): ground meat; (---): soybean protein.

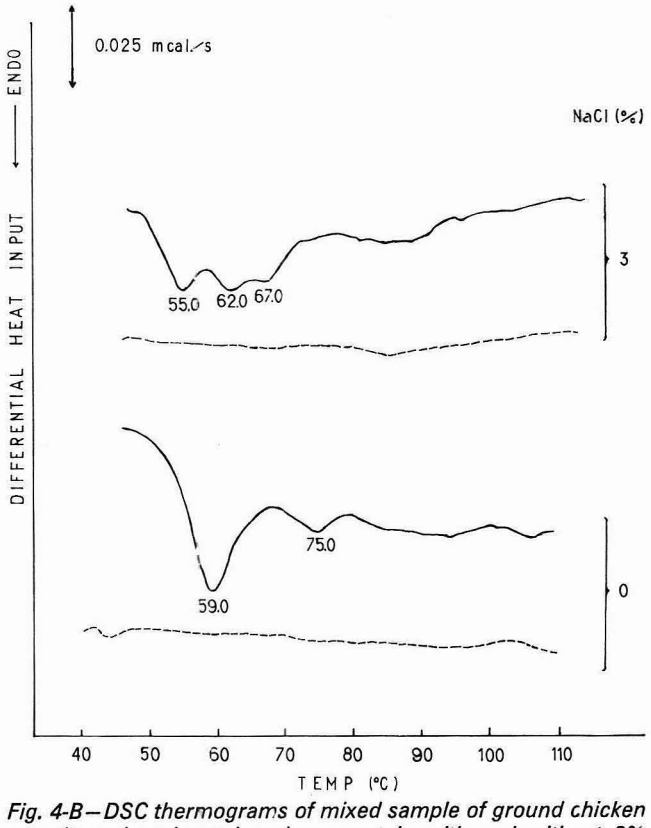

Fig. 4-B—DSC thermograms of mixed sample of ground chicken muscle and preheated soybean protein with and without 3% NaCl. Vertical bar represents 0.025 mcal/sec. (———): mixed sample; (---): preheated soybean protein.

consisted mainly of actin. But a clear explanation about other protein components was not given.

DSC thermograms of soybean protein (Fig. 2) showed two endthermal peaks. It has been suggested that the first peak and the second peak were due to 7S component and 11S component of soybean protein, respectively (Hermannson, 1978). The T_{max} of these peaks shifted to higher temperature due to the addition of NaCl, in contrast with muscle protein. For example, when the NaCl concentration increased from 0 to 3%, T_{max} of the second peak shifted from 89.4 to 103.8°C (Fig. 3). This result is similar to that of Hermannson (1978).

The DSC thermogram of a mixture of ground chicken muscle and soybean protein showed three major endothermic peaks (Fig. 4-A). Stabursvik and Marten (1980) suggested that when the pH of white muscles such as chicken muscle changed from 5.6 to 6.5, the first and middle peaks merged into one single peak. Since the pH value of ground chicken muscle was about 5.6 and those of the mixture was about 6.2 (Shiga et al. 1984), the first peak probably corresponded to a complex of the first peak and the middle peak of ground muscle. The DSC thermogram of a mixture of ground chicken muscle and preheated soybean protein (100°C for 8 min) showed two majar peaks (Fig. 4-B). Since the preheated soybean protein showed no dedectable peak, the two peaks were due to the chicken muscle. Thus, the first and middle peaks of the mixture containing native soybean protein were due to the chicken muscle and the third peak was due to soybean protein (Fig. 4-A). The T_{max} for peak of soybean protein increased by about 5°C when the pH decreased from 7.0 to 6.0 (Harmansson, 1978). Since the pH of soybean protein paste was 7.0 and that of the mixture was about 6.2, the third peak (95.0 °C) of the mixture probably corresponded to the second peak of soybean protein. The middle peak (75.0°C) might involve the first peak of soybean protein; however, any direct proof was absent.

The DSC thermogram of the salted mixture (Fig. 4-A) showed five endothermic peaks. Since the DSC thermogram of the salted mixture containing preheated soybean protein (Fig. 4-B) showed three major peaks at lower temperatures region, the three peaks that were distributed in the lower temperature region (Fig. 4-A) seem to correspond to the three peaks of salted ground muscle protein, and the remaining two peaks (87.1° and 105.4°C) appeared to correspond to the two peaks of salted soybean protein. Thus, this thermogram was similar to the combined thermograms of the salted ground muscle and soybean protein. Further investigations are needed to confirm these results of the mixed sample.

Properties of heat-induced gel from ground chicken muscle, soybean protein and their mixture

The rheorogical properties (breaking stress and strain) and water-holding capacity of heat-induced gels from ground chicken muscle, soybean protein and their mixture are shown in Table 1.

The breaking stress, strains and water-holding capacity values of the heat-induced gel from nonsalted ground chicken muscle were low, but all values increased due to the addition of 3% NaCl. Thus, the heat-induced gelling properties of ground chicken muscle were improved on the addition of the salt.

When soybean protein was heated at 100°C in the absence of NaCl, soybean protein formed firm and elastic gel. But the breaking stress and strain of heat-induced soybean protein gel decreased from 419 to 315 (g/cm) and from 1.02 to 0.76, respectively, in the presence of 3% NaCl. When soybean protein was heated at 70°C in the absence of salt, a soft gel was obtained, but in the presence of salt, a stable gel was not obtained.

Table 1—Effects of the presence of NaCl on the properties of heat-induced gels of ground meat, soy protein and their mixture

Samples	NaCl (%)	Breaking stress (g/cm²) 70°C	Breaking stress (g/cm²) 100°C	Breaking strain 70°C	Breaking strain 100°C	Water-holding capacity (%) 70°C	Water-holding capacity (%) 100°C
Ground meat	0	187	156	0.29	0.14	64.8	52.4
	3	718	550	0.36	0.29	92.5	82.4
Soy protein	0	109	419	0.77	1.02	–	95.9
	3	–	315	–	0.76	–	94.2
Mixture	0	184	177	0.33	0.25	90.3	82.6
	3	284	305	0.39	0.40	92.1	85.6

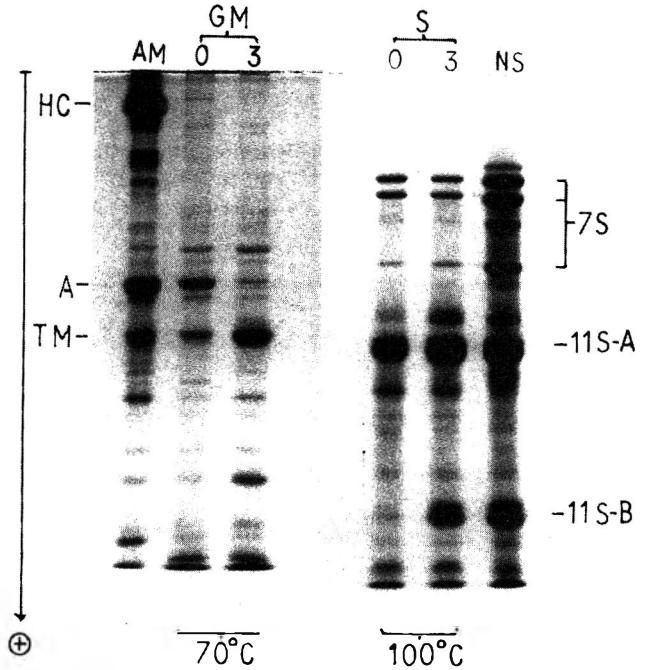

Fig. 5—SDS-PAGE of extracts from heated ground chicken muscle (70° C) and soybean protein (100°C). Electrophoresis was carried out in 10% polyacryolamide gel containing 0.1% SDS. AM: crude actomyosin; GM: extracts from ground meat; S:extracts from soybean protein; 0 and 3: without and with 3% NaCl, respectively; NS: unheated soybean protein; Sample load: 40 μL for GM, 20 μL for S; HC: heavy chain of myosin; A: actin; TM: tropomyosin; 7S: 7S component; 11S-A: acidic polypeptide of 11S component; 11S-B: basic polypeptide of 11S component.

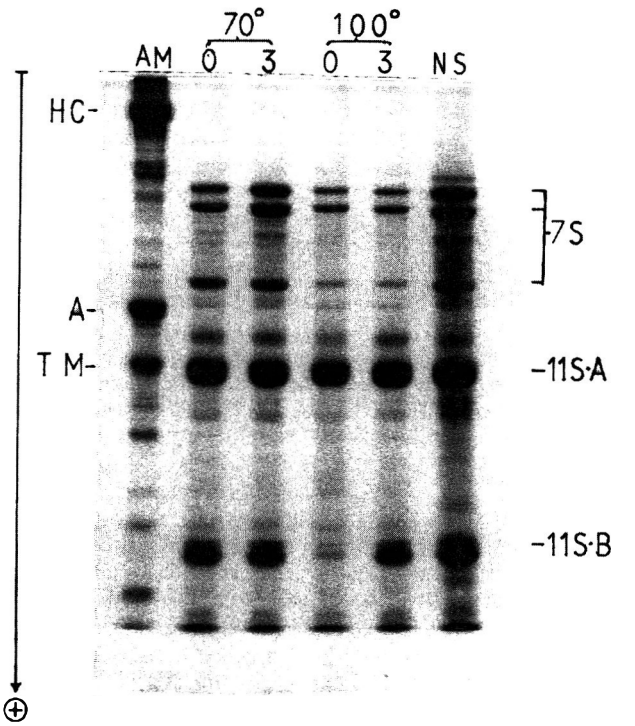

Fig. 6—SDS-PAGE of extracts from heated mixed sample ground chicken muscle and soybean protein. Electrophoresis was carried out in 10% polyacrylamide gel containing 0.1% SDS. AM: crude actomyosin; 70 and 100: extracts from mixed samples heated at 70 and 100°C, respectively; 0 and 3: without and with 3% NaCl, respectively; NS: unheated soybean protein; Sample load: 10 μL; HC: heavy chain of myosin; A: actin; TM: tropomyosin; 7S: 7S component; 11S-A: acidic polypeptide of 11S component; 11S-B: basic polypeptide of 11S component.

The rheological values and water-holding capacity of the heat-induced gels from mixed sample were intermediate between ground chicken muscle and soybean protein. The rheological measurements of the mixed sample increased slightly due to the addition of 3% NaCl.

SDS-PAGE of extracted proteins from heat-induced gels

SDS-PAGE patterns of extracts obtained from ground chicken muscle gels heated at 70°C are shown in Fig. 5. The extract from the gel heated in the absence of NaCl (GM-0) revealed decreased levels of actin and tropomyosin bands, and the absence of myosin (heavy-chain) band. In contrast to this, the extract from the gel heated in the presence of 3% NaCl showed disappearance of myosin and actin band, and an increase of intensity in the tropomyosin band. This suggested that when ground chicken muscle was heated at 70°C in the absence of NaCl myosin was denatured almost completely, but actin was not completely denatured at this temperature. However, in the presence of 3% NaCl, actin was already denatured at 70°C. Since tropomyosin is highly heat stable and does not participate in the gel formation of actomyosin system, this protein was extracted readily from the gel (Samejima et al. 1982).

SDS-PAGE patterns of extracted proteins from soybean protein gel heated at 100°C are also shown in Fig. 5. The proportion of 7S component bands in the extract from the gel heated in the absence of NaCl decreased in comparison with native soybean protein and 11-S basic polypeptide band was absent. In contrast, the extract of the sample heated in the presence of 3% NaCl exhibited an 11S-basic polypeptide band of high intensity. An 11S-acidic polypeptide band was observed clearly in both cases. This suggested that when soybean protein was heated at 100°C in the absence of NaCl, 7S and 11S components were denatured at 100°C, but in the presence of 3% NaCl, the 11S component was not denatured completely at the same temperature. It is assumed that since the 11S-acidic polypeptide did not participate very much in the formation of gel, this protein was extracted readily from the gel (Hashizume and Watanabe, 1979).

SDS-PAGE patterns of extracted proteins from mixed sample gels heated at 70°C or 100°C are shown in Fig. 6. The proportion of muscle protein in the extracted proteins was low and most of the protein bands belong to soybean protein. In the samples heated at 70°C, the proportion of each soybean protein component was similar to that of the native soybean protein, whether or not salt was added. This suggested that most of soybean protein was left in its native state, but most

of the muscle protein was denatured at 70 °C. SDS-PAGE patterns for the mixed samples heated at 100 °C were similar to those of soybean protein analysed separately (S-O and −3 in Fig. 5).

DISCUSSION

DSC THERMOGRAM of ground chicken muscle showed three endothermic peaks and that of soybean protein showed two endothermic peaks. In the presence of increasing concentration of NaCl, the peaks for ground chicken muscle shifted to lower temperatures and those for soybean protein shifted to higher temperatures. These results were basically in agreement with those reported by other workers (Wright et al., 1977; Hermansson et al., 1978; Quinn et al., 1980; Stabursvik et al., 1984).

In general, the heat treatment of meat products such as sausage is carried out at about 70°C. When ground chicken muscle was heated at 70°C in the absence of NaCl, actin should not be denatured, but if it was, the denaturation should not be complete, because the denaturation temperature (T_{max}) of actin was 76.8°C (Fig. 1). However, when ground chicken muscle was heated at 70°C in the presence of 3% NaCl, actin should be denatured, because the denaturation temperature (T_{max}) of actin was decreased to 68.3 °C. This behavior was consistent with the SDS-PAGE data of extracted proteins from ground chicken muscle gels heated at 70 °C. These results indicate that one of the reasons for the improvement in rheological properties and water-holding capacity of heat-induced ground chicken muscle gel (at 70 °C) due to salting (Table 1) might be the decrease of denaturation temperature for actin.

When soybean protein was heated at 100 °C, the 11S component should be denatured in the absence of NaCl, but incompletely denatured in the presence of 3% NaCl, because the heat denaturation temperature (T_{max}) of 11S component increased from 89.4°C to 103.8°C on addition of 3% NaCl. This was also supported by the SDS-PAGE of extracted proteins from soybean protein gels heated at 100 °C. This indicates that one of the reasons for a decrease in rheological values of heat-induced soybean protein gel due to salting might be the increase of the denaturation temperature for 11S component.

A little improvement of rheological properties for the mixed sample due to addition of NaCl might be attributed to the opposite effect of salting on the individual two proteins. The mixed samples involved many problems that are difficult to explain because their DSC thermogram profiles and heat-induced properties were rather complex. Further investigation is needed to elucidate the relationship between the DSC profiles and heat-induced properties of the mixed samples.

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Effect of Enzymatic Digestion, pH and Molecular Weight on the Iron Solubilizing Properties of Chicken Muscle

M. L. POLITZ and F. M. CLYDESDALE

ABSTRACT

The effects of a simulated gastrointestinal pH, enzymatic digestion and molecular weight (MW) on the iron solubilizing properties of a heated dilute salt insoluble fraction of chicken muscle were examined. The solubility of 50 ppm added $FeCl_3$ increased linearly from 0–260 min during pepsin digestion. The total soluble iron reached a maximum concentration following a 120 min pepsin-30 min pancreatin digestion, with pepsin digestion products ranging in MW from 6200–2500. Solubilization capacity, defined as an *in vitro* measure of total iron bioavailability, did not correlate to binding by free sulfhydryl groups. The soluble low molecular weight iron chelates found may explain, in part, the mechanism by which the "meat factor" enhances iron bioavailability.

INTRODUCTION

MEAT, POULTRY AND FISH not only increase the bioavailability of heme iron but also potentiate the absorption of nonheme iron from vegetable foods. Hence, much of the iron deficiency in the world might be ascribed to the virtual lack of these products in many developing countries (Bothwell and Charlton, 1981).

The enhancement of animal proteins on iron absorption has been referred to as the "meat factor." The plant kingdom also contributes an equally important enhancer for increased nonheme iron absorption in the form of ascorbic acid. However, meat is the only factor known to increase heme iron absorption (Hallberg et al., 1979). This phenomenon results from a variety of meat products, but eggs, milk, cheese, and ovalbumin either have no effect or an inhibitory one on iron absorption (Cook and Monsen, 1976). A recent review of the effects of dietary proteins, in general, on iron bioavailability has been published by Berner and Miller (1985).

Slatkavitz and Clydesdale (1988) showed that iron solubility was significantly affected by an acid-insoluble fraction of chicken muscle, whereas neither a water-soluble extract nor an acid-soluble extract had any effect. Pepsin digestion products from the acid-insoluble fraction with MW, 10,000 solubilized significantly more iron than those with MW 10,000. Slatkavitz and Clydesdale (1988) concluded that the influence of chicken breast muscle on iron solubility was related to the production of digestion intermediates which act as ligands in the formation of soluble iron complexes.

The exact mechanism for the meat factor has not been clearly shown but may be related to the release of amino acids and/or polypeptides during proteolytic digestion which chelate dietary nonheme iron, thereby, facilitating its absorption (Martinez-Torres et al., 1981; Morck and Cook, 1981; Bothwell and Charlton, 1981; Kane and Miller, 1984; Slatkavitz and Clydesdale, 1988). It was the intent of this study to further investigate the mechanism responsible for the meat factor using solubility as an index of bioavailability since the solubility of iron in the lumen of the gastrointestinal tract is the first prerequisite for its absorption (Clydesdale, 1983; Forth and Rummel, 1973). The elucidation of this mechanism is extremely important since it would provide not only a better understanding of iron absorption but also the potential means to produce stable iron absorption enhancers for the addition to food.

Author Clydesdale is with the Dept. of Food Science & Nutrition, Massachusetts Agricultural Experiment Station, Univ. of Massachusetts, Amherst, MA 01003. Author Politz is presently affiliated with General Food Corporation, Technical Center, Prospect Plains Road, Cranbury, NJ 08512.

MATERIALS & METHODS

Materials

Boneless chicken breasts were obtained from a local supermarket. Ferric chloride anhydrous (Fisher Scientific Co., NJ) was dissolved, and brought to 100 mL with, pH 2, double-distilled-deionized (DDD) water prepared with 6N HCl. Iron was added to the protein solutions at a concentration of 50 ppm. Prep tyrode buffer contained the following ingredients, in grams, made up to 6L with DDD water: NaCl (48.0); HCl (1.2); $MgSO_4.7H_2O$ (1.56); $NaH_2PO_4.H_2O$ (0.345); glucose (6.0); $NaHCO_3$ (6.0); $CaCl_2$ (1.2); NaN_3 (1.2). The pH of the prep tyrode buffer was adjusted to 5 with 6N HCl. Azocoll (Sigma Chemical Co., St. Louis, MO) was used to determine the inhibition of pepsin in solution. Sephadex G-25 fine gel (Pharmacia Fine Chemicals, NJ) was used to separate the protein digestion products following pepsin digestion. The molecular weight (MW) standards used for the gel filtration standard curve were: trypsin inhibitor, MW = 6,309; insulin chain A, MW = 2,532; glutathione (Sigma Chemical Co., St. Louis, MO), MW = 631; dl-leucine (Merck and Co., NJ), MW = 131.17. The 5, 5'-dithiobis-(2-nitrobenzoic acid) reagent (DTNB) (Sigma Chemical Co., St. Louis, MO) was used for the determination of free sulfhydryl groups. The 2,4,6-trinitrobenzenesulfonic acid (TBNS) method (Snyder and Sobocinski, 1975) was used to determine which fractions contained the various molecular weight standards during column calibration.

Apparatus

All atomic absorption analyses were conducted using a Perkin-Elmer Model 372 atomic absorption spectrophotometer. Iron concentrations were read directly at 248.3 nm against 1.0, 2.5, and 5.0 ppm iron standards, which were prepared using a certified atomic absorption reference solution from Fisher Scientific Company. A Lambda 3 Perkin-Elmer dual beam UV-VIS spectrophotometer was used to measure the absorbance of the iron-bathophenanthroline complex at 533 nm, the total soluble protein at 280 nm, the TBNS reaction products at 420 nm and the DTNB reaction products at 412 nm in 1 cm cuvettes. A recorder, Omega Engineering (Budmess) Model 199-TC-X-X-DSS, was used to record the temperature inside the chicken pellet and the water bath during sample preparation.

Sample preparation

All glassware was acid-washed in concentrated HCl and rinsed in DDD water to remove contaminant iron.

Slatkavitz and Clydesdale (1988) found that a factor which increases iron solubility was present in the insoluble fraction of chicken muscle but not the water or acid soluble fraction. They postulated that this was responsible, in part, for the "meat factor." To further investigate this hypothesis, a 10-g sample of chicken breast muscle, containing approximately 2.34g protein (Watt and Merrill, 1975) and with low endogenous iron, was cut up and homogenized for 1.5 min with 100 mL of 0.15N NaCl. The resulting slurry was centrifuged for 15 min at 2,235 X *g* and the total soluble protein in the supernatant measured using the Lowry method (Cooper, 1977). The supernatant was discarded and the insoluble pellet washed once with 100 mL 0.15N NaCl. After homogenization, centrifugation and protein deter-

mination, the supernatant was discarded. The precipitate represented the dilute salt insoluble chicken fraction used in this study. This extraction procedure removed approximately 99.5% of the water soluble proteins, calculated on the basis of lean muscle tissue containing 73.7% water (Hultin, 1984) for a total of 588.5mg.

Since chicken muscle is always cooked prior to consumption, the insoluble fraction was subjected to a heat treatment (98°C water bath for 15 min). It is true that heating only the insoluble fraction does not duplicate normal cooking procedures. However, normal cooking would render the soluble proteins insoluble. These additional insoluble proteins, as well as any small molecular weight compounds, would dilute the insoluble proteins of interest. The temperature inside the chicken pellet reached 86°C after 5 min in the boiling water bath and 97°C after 10 min. The heated protein pellet was homogenized with 100 mL of 0.15N NaCl for 1.5 min. The resultant suspensions of the heated, dilute salt insoluble protein are referred to as DSIP. Twenty millileters, rather than 100, of 0.15 M NaCl were used to prepare more concentrated DSIP fractions for gel chromatography. This was necessary because of the dilution which occurs during column fractionation. Samples were frozen at −20°C until use.

Iron analysis

All samples were analyzed in duplicate for total soluble, soluble-complexed, total ionic, ferric, and ferrous iron according to a modification of the method of Lee and Clydesdale (1979), as described by Rizk and Clydesdale (1983). The only exception was the use of HNO_3 rather than HCl. The ionic iron is actually bathophenanthroline (batho) reactive iron and may include some soluble complexed iron (Clydesdale and Nadeau, 1985) but it is constant since all analyses were timed.

Timed digestion studies

The timed digestion studies simulated gastric and/or gastrointestinal digestions using pepsin-HCl (pH 2) or pepsin - HCl (pH 2) followed by pancreatin-prep tyrode buffer (pH 5). A pepsin:protein ratio of 1:100 (w/w) and a pancreatin:protein ratio of 1:50 (w/w) were chosen based on the studies of previous investigators (Akeson and Stahman, 1964; Saunders et al., 1973; Nelson and Potter, 1980; Slatkavitz and Clydesdale, 1988).

Pepsin digestion. Thirty milliliters of a pepsin solution containing 59.1 mg pepsin/100 mL were added to 100 mL of DSIP. The mixture was incubated at 37°C for either 0,120 or 240 min at pH 2 (gastric pH). Ten milliliters of a 700 g/mL $FeCl_3$ solution (pH 2) were added to produce 50ppm $FeCl_3$ and the slurry incubated for 20 min at 37°C. The pH was increased to 5 with 1.0N and 0.1N NaOH to simulate intestinal pH. Adjustment to pH 5 inhibited the pepsin, as determined by the azocoll reagent (Amiot et al., 1981). A final incubation was conducted for 30 min at 37°C, after which the slurry was centrifuged at 2,235 X *g* for 15 min, and an analysis for soluble iron was performed on the supernatant.

Pepsin-pancreatin digestion. Following a 120 min pepsin digestion, the pH was increased to 5 and 30 mL of pancreatin solution (120.1 mg/100 mL) were added. The mixture was incubated at 37°C (pH 5) for 0.60 or 120 min, after which the volume was adjusted to 165 mL with prep tyrode buffer. After the addition of 10 mL of an $FeCl_3$ solution (875 mg/mL) at pH 2, which produced a concentration of 50 ppm $FeCl_3$, the pH was adjusted from 3 back to 5 with 1.0N and 0.1N NaOH. The final 30 min incubation, centrifugation and iron analyses were performed as previously described.

Digestion controls. Controls were conducted in which the DSIP fraction was replaced with an equal volume of distilled water (DDWE control). To evaluate any possible contributions of the enzymes to iron solubilization, controls were conducted in which distilled water replaced the DSIP fraction, an equal volume of 0.1N HCl replaced the pepsin solution and prep tyrode buffer replaced the pancreatin solution (DDW control). Controls without iron were evaluated to determine any contribution of endogenous iron to the soluble iron pool.

Gel filtration

The following experimental conditions were used for gel chromatography: (1) gel bed dimensions of 47 cm × 1.5 cm i.d.; (2) an eluant of prep tyrode buffer (pH 5); (3) a flow rate of 22 mL/hr using a peristaltic pump; (4) a sample volume of 3.4 mL; and (5) eluant volumes of 1.0 mL were collected using a linear fraction collector.

The chromatography partition coefficients (K_{av}) for the molecular weight standards and pooled protein fractions were calculated as follows:

$$K_{av} = \frac{Ve - Vo}{Vt - Vo}$$

where: Ve is the volume of solvent required to elute the solute from the bed; Vo is the elution volume of the substances completely excluded from the gel pores; Vt is the total bed volume.

A standard curve was drawn for Log MW vs K_{av} and used to determine the MW range of the pooled peptide fractions.

Digestion and fractionation of concentrated DSIP fraction for chromatography

One milliliter of a solution containing 17.2 mg pepsin/mL was added to 20 mL of the concentrated DSIP fraction. The pH was decreased to 2 with 6N HCl (gastric pH) and the solution incubated at 37°C for 120 min. The pH was increased to 5 with 1.0N and 0.1N NaOH. The digest was centrifuged at 27,000 x *g* for 30 min and decanted, and the supernatant was recentrifuged for another 10 min. This second centrifugation was necessary since the first did not remove all the particulate matter from the solution. The supernatant was frozen at −20°C in 4 mL aliquots until use.

After determination of the total soluble protein (A_{280}), 3.4 mL of the pepsin digest was applied to the column and the total soluble protein (A_{280}) measured in each 1.0 mL eluant collected. A chromatogram of A_{280} vs K_{av} was plotted, divided into different fractions, and individual 1.0 mL eluants were pooled according to these fractions. The same eluants from several column runs were pooled to obtain enough sample for the following studies.

Evaluation of pooled protein fractions

The total soluble protein (A_{280}) was measured and the quantity of free sulfhydryl groups was determined using the DTNB reagent (Ellman, 1959) in the pooled protein fractions.

A 30-min pancreatin digestion, as described previously, was performed at pH 5 after the enzyme solution and 3 mL of a 400 ppm $FeCl_3$ solution (pH 2) were added to 20 mL of each of the pooled protein fractions (pH 5). Following centrifugation, analyses for soluble iron, total soluble protein (A_{280}) and free sulfhydryl groups were performed. A control was conducted where each protein fraction was replaced with an equal volume of prep tyrode buffer.

Statistics

Analysis of variance test was used to evaluate the significance of treatments on the total soluble iron. Scheffe's test (Steel and Torrie, 1980) was used to assess the differences between individual means. The linear regression data was calculated for the change in the total soluble iron with time for the DSIP fraction (Steel and Torrie, 1980).

RESULTS & DISCUSSION

Effect of pepsin digestion time on the iron solubilizing properties of protein

The effects of pepsin digestion in a simulated gastrointestinal pH and ionic environment on iron solubility are shown in Table 1. Analysis of variance showed that the DSIP fraction solubilized significantly more iron at all digestion times ($p<0.01$) than a DDWE control. The DSIP fraction showed a significant linear relationship ($p<0.01$) with digestion time as well as a significant change in total soluble iron over time ($r^2=0.94$).

Analysis of variance showed that there was no significant difference ($p>0.05$) in the amount of iron solubilized during the pepsin digestion of a DDWE control when compared to a DDW control (Table 1). This provided evidence that pepsin did not bind any iron and, therefore, was not a factor in its solubilization. The small amount of soluble iron measured in the distilled water controls was probably due to soluble ferric hydroxides (Rabinowitch and Stockmayer, 1942; Spiro and Saltman, 1969).

Table 1—Effect of pepsin digestion time on the amount of iron solubilized at pH 5.0 from samples of dilute salt-insoluble protein containing 0.43 ppm endogenous iron and dilute salt-insoluble protein and distilled water controls containing 50 ppm exogenous $FeCl_3$

	Total soluble iron (ppm)[a,e]			
	Sample			
Digestion time (minutes)	DSIP[b] Exogenous Iron	Endogenous Iron	DDWE[c]	DDW[d]
0.00	0.92 ± 0.02	0.36 ± 0.03	0.47 ± 0.04	0.50 ± 0.03
20.0	1.26 ± 0.02	0.37 ± 0.04	0.46 ± 0.03	0.47 ± 0.04
140.0	2.20 ± 0.01	0.47 ± 0.05	0.47 ± 0.04	0.46 ± 0.03
260.0	2.56 ± 0.03	0.44 ± 0.06	0.44 ± 0.05	0.45 ± 0.03

[a] Values represent mean ± standard deviation.
[b] Dilute salt insoluble protein fraction.
[c] Distilled water control with enzyme.
[d] Distilled water control without enzyme.
[e] Total soluble iron is the micrograms soluble iron per 100mL-DSIP prepared from 10 g chicken.

The concentration of total soluble iron in the DSIP fraction containing 7.0 mg added iron (to produce a 50 ppm $FeCl_3$ solution) was significantly greater ($p<0.01$) at all digestion times than the control which contained only 0.06 mg endogenous iron, as determined in the 100 mL undigested DSIP (Table 1).

The change in the chemical profile of the soluble forms of iron with time at pH 5 for the DSIP fraction is shown in Fig. 1. The increase in soluble iron caused by this fraction was due to an increase in complexed iron. Only a small amount of total ionic iron, in the ferrous form, was measured at all four digestion times. Since the iron was measured in a complexed form, its absorption would be dependent not only on solubility but also on the stability of the iron chelate. The chelate must have a stability constant for iron greater than the iron hydroxides but the constant must be such that it allows the release of the iron to mucosal acceptors for absorption. Since the pepsin digestion products formed soluble complexes and prevented precipitation as the pH increased from 2 to 5 (gastric to intestinal conditions), they could explain, in part, the mechanism of the meat factor. Although the increase in total soluble iron during pepsin digestion was linear with time from 0 to 260 min, the intermediates of importance might require further digestion by pancreatin and, therefore, could be formed at any time during pepsin digestion. Since the absorption of dietary iron from a meat meal is maximal in the duodenum and proximal jejunum and occurs mainly in the first 2 postprandial hours as the meal empties from the stomach (Matseshe et al., 1980), the 120 min pepsin digestion was chosen to be followed by timed pancreatin digestion studies.

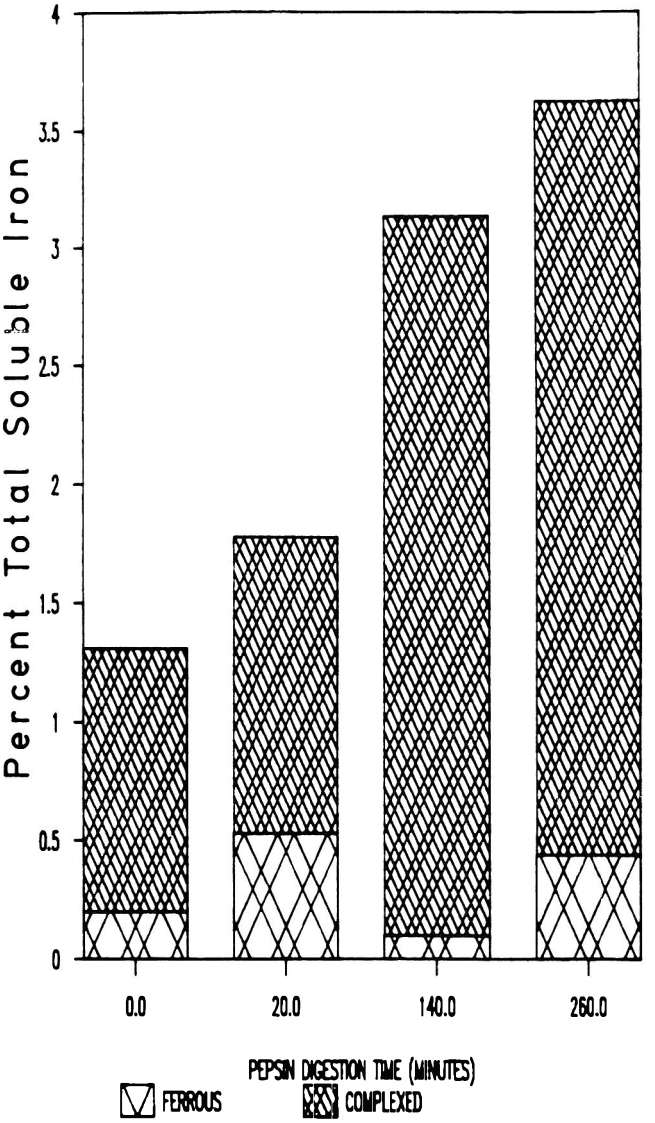

Fig. 1.—Effect of pepsin digestion time on the form and percent of total iron solubilized at pH 5.0 from samples of dilute salt insoluble protein fraction containing 50 ppm $FeCl_3$.

Effect of pancreatin digestion time on the iron solubilizing properties of protein

The results of the timed pepsin-pancreatin digestion studies on the iron solubilizing properties of the DSIP fraction and distilled water controls are shown in Table 2. Analysis of variance showed that the DSIP fraction solubilized significantly more iron at all digestion times ($p<0.01$) than the DDWE control. In addition, the amount of iron solubilized after 30 min pancreatin digestion with 50 ppm added $FeCl_3$ was significantly greater ($p<0.01$) than at 0, 90 or 150 min. The total soluble iron produced during the pancreatin digestion did not show a significant linear relationship and produced a low coefficient of determination ($r^2 = 0.11$). This indicated that increasing amounts of iron solubilizing pancreatin digestion products were not formed in a linear manner with time.

As seen with the pepsin digestion, there was no significant difference ($p<0.05$) in the amount of iron solubilized by a DDWE control versus a DDW control (Table 2). Therefore, the pancreatin, like pepsin, was not responsible for solubilizing the iron.

The change in the chemical profile of the soluble forms of iron with time at pH 5 for the DSIP fraction is shown in Fig. 2. The change in the total ionic iron, which in this case was all ferrous, with time (Fig. 2) parallels that of the total soluble iron as shown in Table 2. There was only a slight change in in the amount of iron measured as complexed (2.26%, 3.12%, 4.43% and 3.54%) at each of the four digestion times (0, 30,

Table 2—Effect of pancreatin digestion time on the amount of iron solubilized at pH 5.0 from pepsin digested samples of dilute salt insoluble protein and distilled water controls containing 50 ppm $FeCl_3$

	Total soluble iron (ppm)[a,e]		
	Sample		
Digestion time (minutes)	DSIP[b]	DDWE[c]	DDW[d]
0.0	2.36 ± 0.04*	0.63 ± 0.04	0.65 ± 0.04
30.0	13.71 ± 0.39*	0.60 ± 0.04	0.63 ± 0.04
90.0	11.51 ± 0.42*	0.58 ± 0.06	0.61 ± 0.02
150.0	8.93 ± 0.15*	0.56 ± 0.04	0.56 ± 0.04

[a] Values represent mean ± standard deviation.
[b] Dilute salt insoluble protein fraction.
[c] Distilled water control with enzyme.
[d] Distilled water control without enzyme.
[e] Total soluble iron is the micrograms soluble iron per 100 mL DSIP prepared from 10g chicken.
* Dilute salt-insoluble protein fraction was significantly different from the controls ($p < 0.01$)

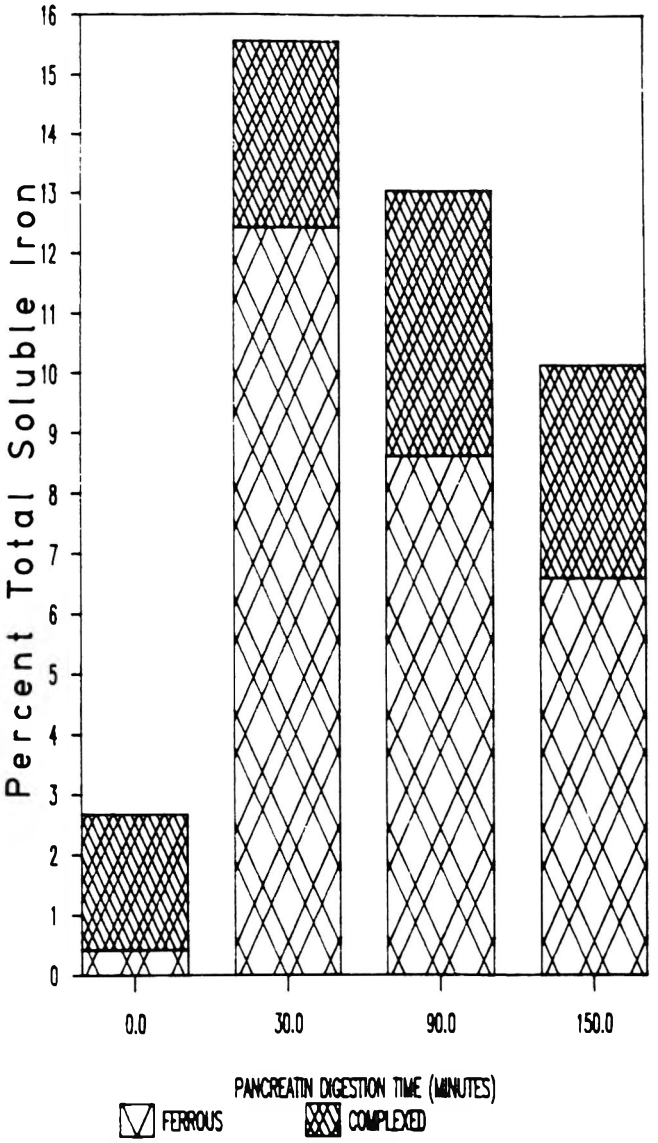

Fig. 2.—Effect of pancreatin digestion time on the form and percent of total iron solubilized at pH 5.0 from samples of dilute salt insoluble protein fraction containing 50 ppm $FeCl_3$.

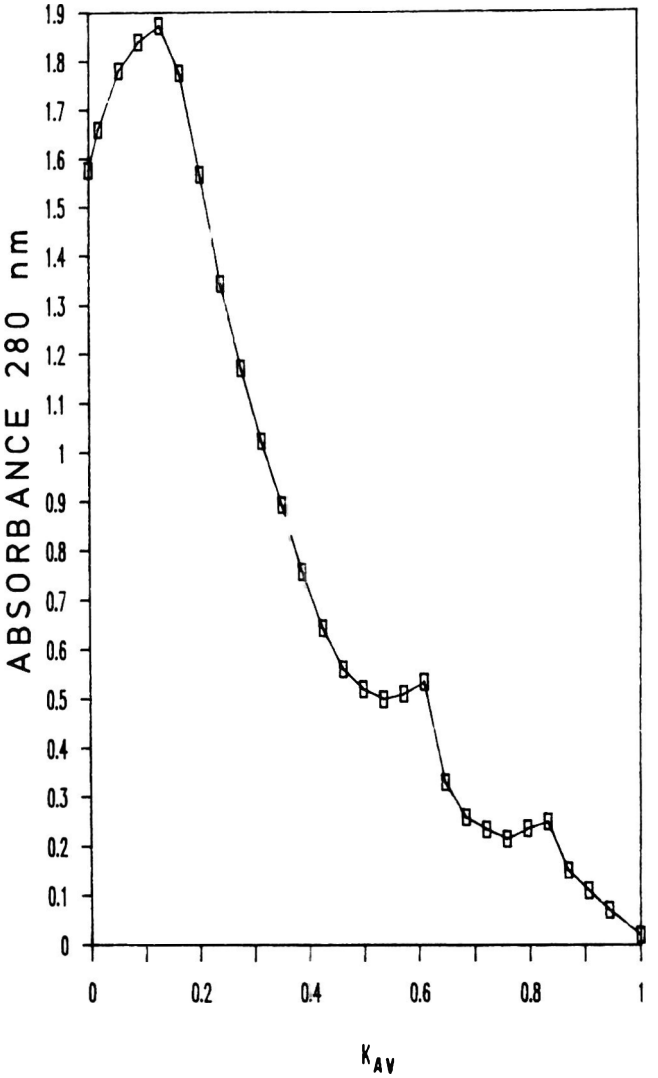

Fig. 3.—A chromatogram obtained by plotting A_{280} versus K_{AV} for the dilute salt insoluble protein fraction which was subjected to a 120 min pepsin digestion and then fractionated using Sephadex G-25 gel.

90 and 150 min). The iron bound to protein digestion products formed during the pepsin digestion could have been reductively released as ferrous iron by protein fragments formed during the pancreatin digestion or could have been removed from a weak iron complex by batho (Clydesdale and Nadeau, 1985). Since low molecular weight complexes of ferrous iron are absorbed *in vivo*, (Forth and Rummel, 1973; May et al., 1978), these complexes might contribute, in part, to the meat factor.

Soluble iron reached a maximum concentration after 30 min pancreatin digestion followed by a gradual decrease. This indicated that certain specific digestion intermediates might play an important role in solubilzing iron in vivo by chelation and/or reduction, thus enhancing subsequent absorption.

Effect of different molecular weight protein fractions on iron solubilization

Since the timed digestion studies showed that the maximal total soluble iron was present after a 120 min pepsin-30 min pancreatin digestion with the $FeCl_3$ added at the same time as the pancreatin, the concentrated DSIP fraction was subjected to a 120 min pepsin digestion and then separated on the basis of molecular weight. The 30 min pancreatin digestion, with $FeCl_3$, was performed on the eluted protein fractions. Adding iron to the eluted protein fractions after gel filtration prevented its adsorption on the chromatography column.

A chromatogram of A_{280} vs K_{AV} was plotted for the eluted protein pepsin digestion products (Fig. 3). The recovery from the column was greater than 90%. Based on mathematically calculated molecular weights from the elution standard curve, approximately 4% of the protein absorbing at 280 nm had a molecular weight above 6,200, 64% from 6,200–1,300 and 32% below 1,300. The MW range, where most of the soluble proteins were eluted (6,200–1,300), is represented by K_{AV} values 0.018 to 0.278 in Fig. 3.

The eluants from the chromatogram shown in Fig. 3 were divided into different fractions which are listed in Table 3. Possibly other proteins with molecular weights outside of the calculated ranges were in each fraction. Molecular weights determined by gel filtration do not account for coiling and folding of larger protein molecules (Reiland, 1971) or binding and subsequent release of peptides and/or amino acids. Following pancreatin digestion with 50 ppm added $FeCl_3$, only fractions 1, 2, 3 and the void volume solubilized significantly more iron ($p<0.05$) than a prep tyrode control (Table 4). Since the enhancing effect of animal protein on iron absorption has been shown to be dose-dependant (Layrisse et al., 1984), it

Table 3—Effect of different molecular weight protein fractions and a prep tyrode buffer control on the amount of iron solubilized from samples containing 50 ppm $FeCl_3$ and subjected to a 30 minute pancreatin digestion at pH 5.0[3w]

Elution volume (mL)	Peptide fraction	Molecular weight range[y]	Total soluble iron (ppm)[x]	Solubilization capacity[x,w]
27–29	Void volume	>6200	1.32 ± 0.05[x]	0.56 ± 0.02[y]
30–44	Fraction 1	6200–1300	8.46 ± 0.09[u]	3.06 ± 0.03[x]
30–38	Fraction 2	6200–2500	10.88 ± 0.12[y]	3.21 ± 0.03[w]
30–32	Fraction 2a	6200–5000	5.89 ± 0.05[w]	4.60 ± 0.04[t]
33–35	Fraction 2b	5000–3500	9.10 ± 0.02[t]	4.25 ± 0.01[u]
36–38	Fraction 2c	3500–2500	7.09 ± 0.05[v]	3.76 ± 0.03[v]
39–44	Fraction 3	2500–1300	0.75 ± 0.03[y]	0.35 ± 0.01[z]
45–55	Fraction 4	1300–400	0.69 ± 0.03[z,y]	
56–67	Fraction 5	400–100	0.50 ± 0.05[z]	
68–82	Fraction 6	<100	0.62 ± 0.05[z,y]	
–	Prep tyrode control	–	0.52 ± 0.03[z]	

[z] Solubilization capacities calculated for the protein fractions which solubilized significantly more iron than a prep tyrode control. Defined as micrograms iron solubilized per milligram of soluble protein. Soluble protein obtained by extrapolation from a standard curve of bovine serum albumin at A_{280}.

[y] Molecular weight was defined via elution standards on the gel filtration column.

[x] Values represent mean ± standard deviation.

[w] Total soluble iron is the micrograms soluble iron per 20mL of a dilute salt insoluble protein fraction prepared from 10g chicken.

[v] Means followed by different superscripts are significantly different ($p<0.05$).

was possible that there was an insufficient quantity of iron solubilizing peptides with a MW less than 1,300. Fraction 2, with an approximate MW range of 6,200–2,500 solubilized significantly more iron ($p<0.05$) than all the other peptide fractions. To further define this MW range, the protein fractions which were pooled to make up fraction 2 were divided into three aliquots based on their elution volume and referred to as fractions 2a, 2b and 2c (Table 3). When the protein in each of these aliquots was subjected to pancreatin digestion with $FeCl_3$, fraction 2b (MW range 5,000–3,500) solubilized significantly more iron ($p<0.05$) than fractions 2a and 2c but significantly less than the amount solubilized by the total fraction 2. Based on the results in Table 3, it appeared that the pepsin-pancreatin digestion products which solubilized the most iron were formed from pepsin digestion products which ranged in MW from 6,200–2,500.

In this study, the term "solubilization capacity" was introduced to allow more meaningful comparisons between fractions. It is defined as "the micrograms of iron solubilized per milligram of soluble protein". Its use as an in vitro index of potential enhancement of iron absorption is justified since the solubility of iron in the lumen of the gastrointestinal tract is the first prerequisite for its absorption (Clydesdale, 1983; Forth and Rummel, 1973). Table 3 lists the solubilization capacities calculated for the protein fractions which solubilized significantly more iron ($p<0.05$) than a prep tyrode control.

Fraction 2a showed the highest solubilization capacity ($p<0.05$) of all fractions as shown in Table 3. The solubilization capacities of fractions 2b and 2c were slightly lower with values of 4.25 and 3.76, respectively. Fractions 2a, 2b, and 2c had significantly greater ($p<0.05$) solubilization capacities individually than when combined as fraction 2. The solubilization capacities of fractions 1, 3, and the void volume were all significantly less than that of fraction 2.

When the total ionic iron was measured in fractions 2, 2a, 2b and 2c, approximately 95% of the soluble iron appeared to be in a complexed form and unavailable for reaction with batho. The other 5% iron was measured as ferrous. This could be due to stable iron complexes which did not release their iron to batho. For such bound iron to be bioavailable, it would probably have to be absorbed intact rather than give up its iron to a mucosal acceptor.

Determination of free sulfhydryl groups in different molecular weight protein fractions

Free sulfhydryl groups (-SH) were determined in the void volume and fractions 1, 2, and 3–6 after the following treatments: (1) before the pancreatin digestion; (2) after the pancreatin digestion with no added iron; (3) after the pancreatin digestion with 50 ppm added $FeCl_3$. Fractions 3–6 were pooled since none of these fractions solubilized a significant quantity of nonheme iron (Table 3). Thus, it was possible to evaluate the effect of pancreatin digestion (Treatments 1 and 2) and added iron (Treatments 2 and 3) on the formation of free sulfhydryl groups.

Within a given fraction, there was no significant difference ($p>0.05$) between treatments except for the void volume, where a significant difference was found between treatments 2 and 3 (Table 4). This indicates that in a given peptide fraction with a molecular weight less than 6200 (Fractions 1, 2, and 3–6), the free sulfhydryl groups formed did not bind or reduce the added iron in treatment 3.

Cysteine is an effective chelating agent for iron (Martinez-Torres et al., 1981). Jacobi et al. (1986) stated that cysteine increases the absorption of nonheme iron by forming a soluble complex through its thiol group (Taylor et al., 1985). However, in this study, the data indicated that the sulfyhdryl groups did not play a role in the increased amount of soluble iron noted in peptide fractions 1 and 2.

The presence of small peptides along with iron in the lumen of the gastrointestinal tract and the formation of iron-peptide chelates might play an important role in iron bioavailability. The formation of chelates in which the iron combines with electron donating ligands can solubilize the iron. This would prevent or delay hydrolysis and polymerization reactions which would occur at the pH of the duodenum and proximal jejunum, the primary site of iron absorption. The properties of the these peptides such as charge, conformation and amino acid sequence would help to explain their iron solubilizing ability.

This research contributes to a better understanding of the mechanism responsible for the potentiating effect of animal proteins on iron absorption, or the "meat factor." More complete identification of the peptides involved and a determination of their thermodynamic stability constants as well as the rate at which they transfer iron to mucosal acceptors are important physicochemical factors in iron bioavailability which should be investigated in future research. Elucidation of the

—Continued on page 1090

Table 4—Free sulfhydryl groups[a] determined in the pooled protein fractions

Protein fraction (MW range[c])	Treatment[b] (1)	(2)	(3)
	mmoles SH/L protein solution		
Void volume (>6250)	0.0102 ± 0.0007	0.0095 ± 0.0002	0.0042 ± 0.0003
Fraction 1 (6250–1300)	0.0237 ± 0.0022	0.0213 ± 0.0003	0.0209 ± 0.0005
Fraction 2 (6250–2550)	0.0238 ± 0.0024	0.0212 ± 0.0007	0.0208 ± 0.0004
Fractions 3–6 (2300–<100)	0.0057 ± 0.0020	0.0030 ± 0.0004	0.0018 ± 0.0005

[a] Determined using 5,5'-dithiobis-(2-nitrobenzoic acid).

[b] Values represent mean ± standard deviation. (1) before the pancreatin digest; (2) after the pancreatin digest with no added iron; and (3) after the pancreatin digest with 50 ppm added $FeCl_3$.

[c] Molecular weight was defined via elution standards on the gel filtration column.

Descriptive Profile Analysis of Cooked, Stored, and Reheated Chicken Patties

B. G. LYON

ABSTRACT

A trained panel evaluated off-flavor development in precooked, stored and reheated chicken patties. Factor analysis of intensity scores on 12 sensory attributes yielded three factors. Calculated Factor I scores increased with storage time and predominantly characterized cooked samples stored 2 and 3 days. Factors II and III characterized fresh cooked product and cooked product stored 0 or 1 day. Canonical Discriminant Analysis (CDA) revealed a spatial configuration that classified storage times according to attribute subsets corresponding to Factor scores. The second and third canonical variables classified samples stored 0 and 1 day differently from other stored samples and probably represented flavor characteristics which were important indicators of flavor change occurring within 24 hours of refrigerated storage.

INTRODUCTION

ALTHOUGH the general problem of warmed-over flavor (WOF) is recognized by the meat and poultry industries as a major problem in marketing new precooked, ready-to-heat and serve products, the specific mechanisms of WOF development are not clearly defined. The term, warmed-over flavor (WOF), was first used by Tims and Watts (1958) to describe the rapid onset of flavor deterioration in cooked meat during refrigerated storage for a short time. This flavor change correlated with 2-thiobarbituric acid (TBA) values, a measure of lipid oxidation.

It has been shown by analytical tests that there are changes in the lipid fractions of meats which are precooked, refrigerated for a short time and then reheated as well as in raw meats which are subjected to long-term frozen storage. The lipid changes that occur in stored meats are autoxidative processes that primarily involve the phospholipids during short-term refrigerated storage of cooked meats and that involve the triglyceride fractions during frozen storage of raw meats (Pearson and Gray, 1983).

Igene et al. (1985) and Johnson and Civille (1986) noted that sensory tests should be used with any analytical tests to study the problem of off-flavor development in precooked meats to validate the relationships of the analytical tests to the sensory perceptions. Sensory methods and descriptive terms used to define the flavor perceived in precooked, stored meats have varied (Poste et al., 1986). Some studies have included descriptive terms such as warmed-over, oxidized, rancid, stale, old and reheated to illustrate various flavor character notes present in precooked, stored and reheated meats. Most studies report sensory scores for intensity of warmed-over flavor or rancidity as a single attribute.

Poste et al. (1986) noted three phases of flavor changes discerned by a sensory panel during training to evaluate precooked stored pork. These were described as fresh-cooked, warmed-over (an intermediate stage, not yet rancid) and rancid (extremely oxidized flavor). Johnson and Civille (1986) proposed a list of terms and definitions appropriate for describing flavor changes in precooked, stored and reheated beef. This list also encompassed character notes describing fresh, intermediate and extreme off-flavor in beef. They noted that a common descriptive vocabulary would benefit studies to examine causes and prevention of WOF by providing an established protocol that would allow comparison and evaluation of data from different labs. A descriptive attribute profile for evaluating flavor changes in poultry resulting from precooking and reheating was reported by Lyon (1987). Relationships of that attribute profile to TBA values was reported by Lyon et al. (1988). The perception of sensory flavor change in the chicken products appeared to be melded in a complex descriptive profile of major character notes that changed during short-time storage.

The purpose of this study was to examine the sensory flavor change in precooked, stored, reheated chicken. Test products were 16 treatment combinations of chicken patties involving two formulations, two reheat methods and four storage times. Multivariate statistical programs (Powers, 1984) were used to explore a large volume of data to seek a perceptual model that might ultimately aid analytical chemical determinations and lead to a better understanding and control of WOF in poultry.

MATERIALS & METHODS

Test product

Products used were chicken patties (90g; 9.5 cm diam × 1.4 cm thick) made by two formulations, each containing equal proportions of ground white and dark meat. One formulation was made without added skin, and one formulation was made with 20% ground skin (Lyon et al., 1988). One set of raw patties (approximately 100) from each formulation was immediately packaged in plastic bags (five patties per bag, each pattie separated by waxed weighing sheets) and frozen at −34°C. These raw patties were used during testing as a freshly cooked control product. All remaining patties were cooked in a Despatch rotary reel oven set at 177°C to an internal temperature of 75°C to 80°C. Cooked patties were cooled quickly in a 2°C walk-in cooler. One set, designated as Cooked-0-day-stored sample, was packaged and frozen at −34°C within 1 hr. The remaining batches of patties were stored in trays covered with foil in a 2°C walk-in cooler for 1, 2 or 3 days. At the end of storage, patties were bagged and frozen at −34°C. All patties were made and precook treatments were done the same day. In preliminary work, the −34°C freezing treatment for a short time prior to evaluation had no significant effect on the products.

Reheat methods

Conventional and microwave ovens were used to reheat partially thawed patties. Reheating by both methods was done in combination ranges (GE-Model JHP97G) which was designed with the conventional household oven on the bottom and the microwave oven at the top. Patties were wrapped in foil (5 patties per treatment per foil pack) and heated 23–25 min in the conventional ovens set at 177°C. Patties for microwave reheating were placed in glass dishes, covered loosely with waxed paper and heated 2 to 2.5 min on each side at 70% power. All patties were prepared (cooked or reheated) just prior to panel evaluations and were served warm (55°).

Sensory evaluation

A ten-member trained panel, composed of employees of the center, participated in this study. The panel had previously participated in a 14-wk, (3 hr/wk) training program in which descriptive terms for

Author Lyon is with the USDA-ARS, Richard B. Russell Agricultural Research Center, P.O. Box 5677, Athens, GA 30613.

evaluation of chicken flavor changes due to storage time were developed (Lyon, 1987). That study detailed the use of panel input and statistical procedures into the final selection of twelve attribute terms to evaluate flavor change due to precooking, storing and reheating of chicken products. The twelve terms used in this study were chickeny, meaty, brothy, liver-organy, browned, burned, cardboard, warmed-over, rancid/painty, sweet, bitter, and metallic. Semi-structured 10-cm line scales for intensity were used which were anchored on the left side as 'weak' and on the right side as 'very strong'. Panelists indicated responses to intensity of the attributes by drawing a vertical line through the horizontal line scale (Stone et al., 1974). Marks were converted to scores by measuring the distance in mm from the left origin of the line scale. Range of the intensity scale values was 1 to 100.

Patties were grouped by formulation-reheating method, giving four groups of samples with four storage times in each group. There were 12 test sessions with one formulation-reheat group evaluated at each session. Groups to be evaluated were randomized among the twelve test sessions. Each of the samples in the four groups was evaluated three times by all panelists over the twelve sessions. A set of raw patties was cooked in a 177°C oven to an internal temperature of 75°C to 80°C at each test session to represent a freshly cooked control sample. The freshly cooked sample and the four reheated stored samples within a group were presented to panelists in random order at each test session. Panelists received two 2-cm diameter cores of each test sample, chosen randomly from the composited cores taken from 5 patties in each test sample set. Cores were placed in cups coded with 3-digit numbers. Cups were capped and placed in individual testing stations illuminated with 40-watt green lighting to mask color and appearance. Water and unsalted crackers were provided to the panelists for mouth-cleansing between samples.

Statistical analysis

Various statistical procedures were applied to the data using SAS statistical packages (SAS, 1985). Analysis of variance (ANOVA), general linear models (GLM) and multiple analysis of variance (MANOVA) were applied to evaluate panel performance, attribute appropriateness and effects due to formulation, reheat method and storage time effects. Among the sources of variation explaining differences in the sensory data, storage time was shown to be the primary contributor to differences detected in the samples (Lyon et al., 1988). Therefore, data were pooled over formulation-reheat method to focus on sensory changes due to storage time. Multivariate procedures included VARCLUS (VCA-variable cluster analysis), Canonical Discriminant Analysis (CDA) and Factor Analysis (FA).

RESULTS & DISCUSSION

ANOVA and MANOVA procedures were applied to panel data which included three replicated judgements per sample for each panelist. Probability selection criteria for retaining a panelist and an attribute were $P < 0.20$ and $P < 0.05$, respectively. All panelists were able to discriminate among the test samples for the attribute terms used for scaling. No attribute scales could be eliminated due to lack of sensitivity, although not all scales or panelists performed equally.

Mean scores for each attribute showed significant differences ($P < 0.01$) in storage times. These are visually displayed as sunray plots (also called spider webs) in Fig. 1 through 3, by plotting mean scores of each attribute for a particular sample on lines radiating from the center. For visual impact, the actual line scales have been shortened (representing about 60 mm instead of 100 mm). The figures illustrate that flavor changes occurred early in the cook-store regime. Samples that were cooked, cooled and frozen within 1 hr and then reheated (cooked, 0-day) showed a decrease in chickeny, meaty, brothy, liver/organy and sweet while all other attributes increased. In the 1-day stored samples, the chickeny, meaty, brothy, liver/organy and sweet scores were similar to the 0-day, but there was an increase in cardboard, warmed-over and rancid/painty. At 2-day storage (figure not shown), more changes occurred with a decrease in chickeny, meaty, brothy, liver/organy and sweet and a large increase in browned, burned, cardboard, warmed-over and rancid/painty. At 3-day storage (Fig. 3) the increases

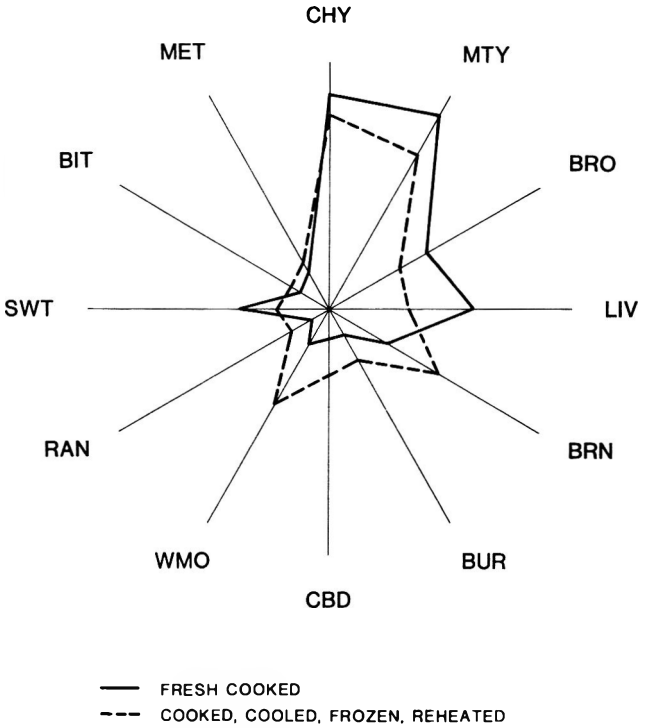

Fig. 1—Plots of mean scores on twelve descriptive attributes comparing freshly cooked product (—) with cooked, 0-day stored product (---). Abbreviated terms: CHY = Chickeny; MTY = Meaty; BRO = Brothy; LIV = Liver/Organy; BRN = Browned; BUR = Burned; CBD = Cardboard; WMO = Warmed-over; RAN = Rancid; SWT = Sweet; BIT = Bitter; MET = Metallic.

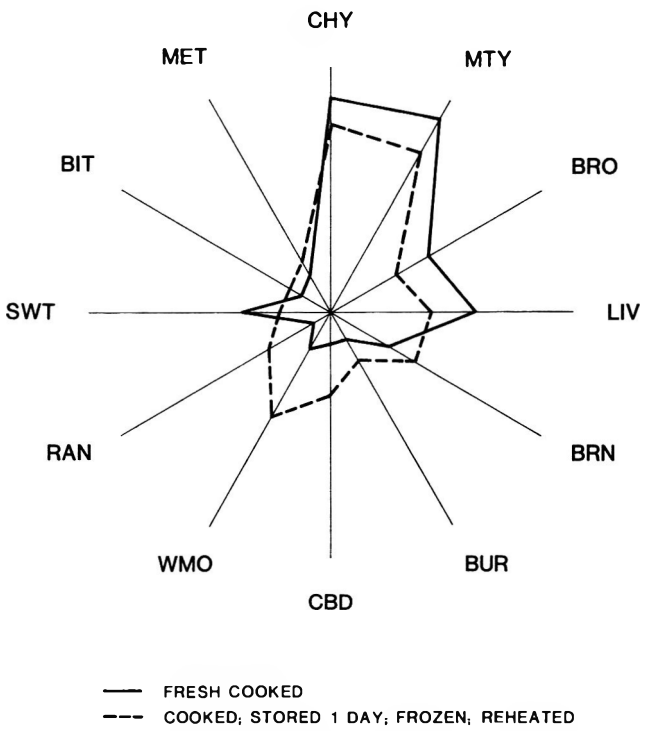

Fig. 2—Plots of mean scores on 12 descriptive attributes comparing freshly cooked product (—) with cooked, 1-day stored product (---). (See caption Fig. 1 for abbreviated terms).

in cardboard, warmed-over, rancid/painty, bitter and metallic were quite different in relation to the fresh-cooked sample.

Multivariate statistical programs enable data with multiple variables to be explored as a set rather than as individual pa-

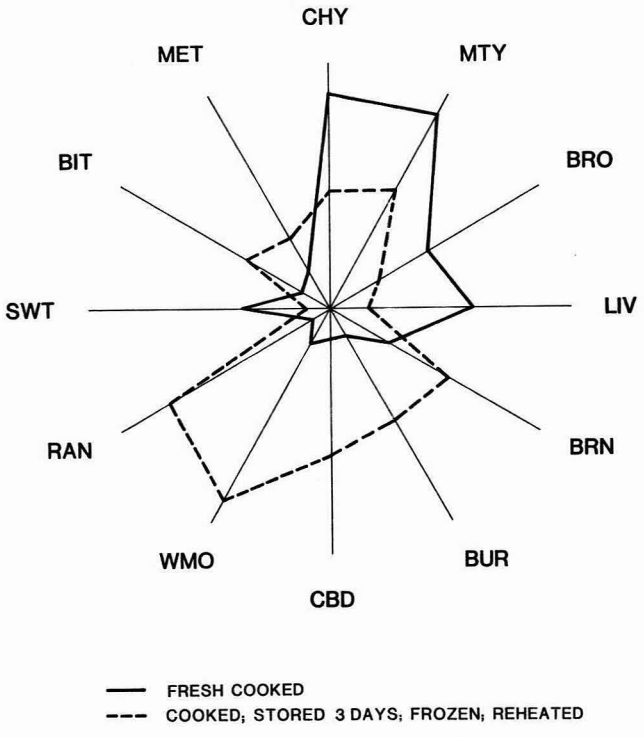

Fig. 3.—Plots of mean scores on 12 descriptive attributes comparing freshly cooked product (—) with cooked, 3-day stored product (---). (See caption Fig. 1 for abbreviated terms).

rameters (Powers, 1981). MVA programs all have a similar function in that they are used to seek simpler underlying associations of the relationships of the many parameters tested. Programs differ in the many mathematical functions applied to view these relationships (Bieber and Smith, 1986).

Relationships of descriptors to storage times of the samples were investigated by VARCLUS (VCA) procedure. The VCA procedure divides a set of numeric variables (terms) into nonoverlapping clusters. The terms associated with the cluster are a linear combination of variables (terms) with variance of the original variables maximized and explained by the cluster components. The procedure is similar to multiple group factor analysis except that a term appears in only one cluster component and makes no contribution to other components. When the freshly cooked samples were analyzed separately, there were three clusters extracted (Table 1). Four clusters were extracted when all precooked, stored and reheated samples were analyzed as a set or when only the two longest storage times (2 and 3 days storage) were used as a set. Three clusters were extracted when all samples were analyzed together.

Chickeny, brothy, and sweet were always grouped together regardless of the test data set analyzed. However, this cluster appeared as Cluster #3 in all analyses except for the analysis on the freshly cooked sample data set, in which these terms appeared as Cluster #2. Meaty and liver/organy were always grouped together. Metallic appeared with meaty and liver/organy in the analysis of freshly cooked only. In other data sets analyzed, metallic was included in different cluster numbers but usually with the terms cardboard, rancid and bitter.

Cluster #1, regardless of the data set analyzed, always included the term warmed-over. Cardboard, rancid/painty and bitter usually were included with warmed-over in Cluster #1, except for the analysis of the data set for the 2 and 3-day stored samples in which these terms were separated as an independent group. Browned and burned were always extracted together and usually appeared in the first cluster. In the analysis of stored, reheated samples, omitting freshly cooked samples, browned and burned formed a separate cluster. The reordering of groups of terms in the Varclus procedure, depending on the storage time of the samples in the data sets analyzed, indicated that different attributes assumed more importance at different storage times.

In Factor Analysis, linear combinations of terms are calculated to form new variables called Factors. Unlike the VARCLUS procedure, Factor Analysis does not exclude terms from the new factor variables but maximizes the contribution of high loading terms and minimizes those which contribute the least by rotation methods prior to the final solution. Three factors were extracted that explained 78% of the variation in the data (Table 2). Factor I had high loadings (correlations of terms to the Factor) for browned, burned, cardboard, warmed-over, rancid/painty and bitter. Factor II had high loadings for chickeny, brothy and sweet; and Factor III had high loadings for meaty, liver and metallic. These groupings of terms were very similar to the VCA, except that chickeny, brothy, sweet appeared in the second Factor (and the third cluster) and meaty, liver/organy appeared in the third Factor (and the second cluster).

Table 1—VARCLUS (Oblique Principal Component) clustering of attribute terms applied to sensory data by storage sets

		Descriptive terms and R^2 values associated with each cluster							
Storage set	Variation explained (%)	Cluster 1	R^2	Cluster 2	R^2	Cluster 3	R^2	Cluster 4	R^2
Freshly cooked	51	Browned	0.32	Chickeny	0.66	Meaty	0.57	–	
		Burned	0.45	Brothy	0.64	Liver/organy	0.69	–	
		Cardboard	0.70	Sweet	0.78	Metallic	0.51	–	
		Warmed-over	0.66						
		Rancid/painty	0.66						
		Bitter	0.43						
Stored 0, 1, 2, and 3 days	68	Cardboard	0.66	Meaty	0.73	Chickeny	0.70	Browned	0.72
		Warmed-over	0.63	Liver/organy	0.73	Brothy	0.73	Burned	0.72
		Rancid	0.73			Sweet	0.67		
		Bitter	0.63						
		Metallic	0.48						
Stored 2 and 3 days only	65	Browned	0.43	Meaty	0.77	Chickeny	0.57	Cardboard	0.56
		Burned	0.71	Liver/organy	0.77	Brothy	0.74	Rancid/painty	0.65
		Warmed-over	0.69			Sweet	0.65	Bitter	0.67
								Metallic	0.57
All samples	64	Browned	0.31	Meaty	0.75	Chickeny	0.71		
		Burned	0.57	Liver/organy	0.75	Brothy	0.76		
		Cardboard	0.66			Sweet	0.75		
		Warmed-over	0.72						
		Rancid/painty	0.72						
		Bitter	0.59						
		Metallic	0.38						

Table 2—Factor loadings (standard regression coefficients) of descriptive sensory attributes from SAS Factor Analysis

Terms[a]	Factor analysis Factor I[b]	Factor II	Factor III
CHY	−18	76[c]	−14
MTY	2	22	76[c]
BRO	− 8	80[c]	9
LIV	− 9	−14	88[c]
BRN	64[c]	12	12
BUR	84[c]	14	− 8
CBD	79[c]	− 1	−14
WMO	72[c]	−24	−19
RAN	78[c]	−10	−13
SWT	20	97[c]	3
BIT	75[c]	− 5	23
MET	56[c]	−12	50[c]

[a] Abbreviation for terms: CHY = Chickeny; MTY = Meaty; BRO = Brothy; LIV = Liver/Organy; BRN = Browned; BUR = Burned; CBD = Cardboard; WMO = Warmed-over; RAN = Rancid/Painty; SWT = Sweet; BIT = Bitter; MET = Metallic

[b] Values are multiplied by 100.

[c] Loadings >50.

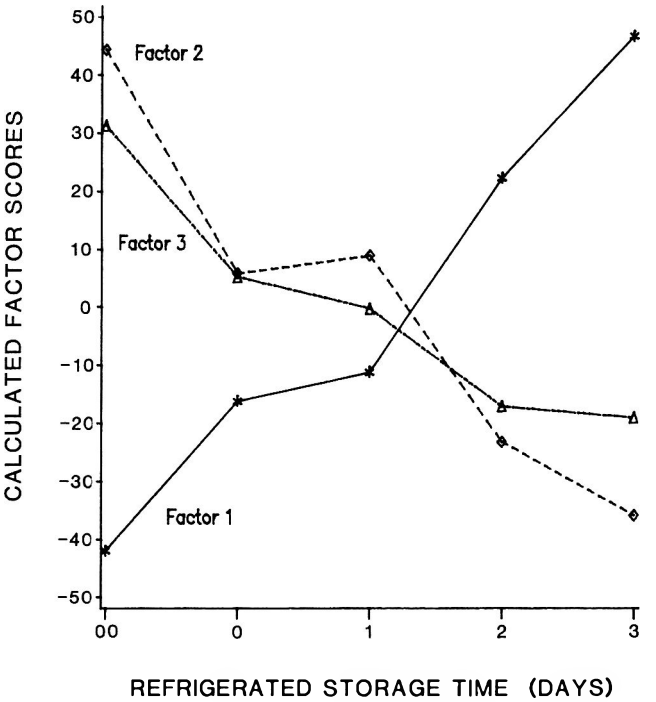

Fig. 4.—Calculated Factor Scores for each of the three factors extracted in Factor Analysis by refrigerated storage time (days). 00 = Freshly cooked product; 0 = cooked, 0-day storage; 1 = cooked 1-day storage; 2 = cooked, 2-day storage; and 3 = cooked, 3-day storage.

The output from factor analysis also gave scoring coefficients that were used to calculate factor scores from the standardized intensity scores of the attributes. Scores were calculated and plotted for storage time, designating the freshly cooked sample as 00-day (Fig. 4). Factor I (cardboard, rancid/painty) increased with storage time. Factor II and Factor III decreased with storage time. However, the rate of change was not linear. For the 00-day (fresh cooked) sample, the predominant Factors were II and III, defined by chickeny, brothy, sweet (Factor II) and meaty, liver/organy (Factor III). For storage of 2 and 3 day samples, the predominant factor was Factor I (cardboard, warmed-over, rancid/painty). Predominance of a specific factor was not as clear for the Cook-0 and 1-day samples. All three Factors appeared important in describing these samples, although Factor II (chickeny, brothy, sweet) scores were higher for these samples than scores for Factor I and Factor III. Factor II and Factor III almost intersected for the cook 1-day sample storage time.

Table 3—Canonical Discriminant Analysis (CDA) applied to descriptive terms used to characterize freshly cooked and precooked, stored, reheated chicken patties[a]

Sample[b]	Mahalanobis distances (above diagonal) and Probabilities of greater distances (below diagonal) A	B	C	D	E
A		14.25	21.06	25.56	30.70
B	0.024		11.59	14.15	19.12
C	0.006	0.049		12.64	14.87
D	0.003	0.024	0.036		6.96
E	0.001	0.008	0.020	0.239	

[a] Values above diagonal are distances between classes (product) and values below are probablities of the distance being greater. Based on three canonical variables derived from CDA.

[b] Samples are: A = Freshly cooked; B = Cooked, 0-day storage; C = Cooked, 1-day storage; D = Cooked, 2-day storage; E = Cooked, 3-day storage.

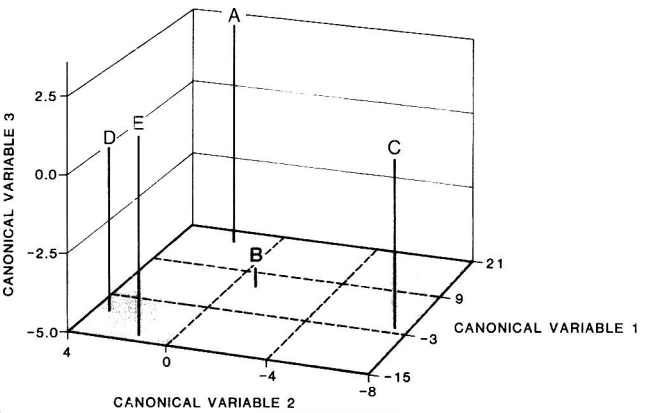

Fig. 5.—Spatial configuration of freshly cooked and cooked, stored, reheated chicken patties from Canonical Discriminant Analysis (CDA). (A = freshly cooked; B = cooked, 0-day storage; C = cooked, 1-day storage; D = cooked, 2-day storage; E = cooked, 3-day storage.)

Canonical Discriminant Analysis was performed to classify the samples based on the combined attribute relationships. This procedure first finds the linear combinations that best summarize differences in the samples and outputs these as single canonical variables. The samples are then classified along the canonical variables based on differences among the classes. Canonical Discriminant Analysis of the data resulted in three canonical variables. Table 3 shows the Mahalanobis distances and probabilities for obtaining greater distances among the storage time classes. Storage times of 2 and 3 days (Samples D and E) were not significantly different from each other, but were different from all other samples. Freshly cooked control (Sample A) and samples stored 0 and 1-day (Samples B and C) were significantly different from each other.

The spatial relationships are shown in Fig. 5 as a three dimensional plot of the samples classified on the three canonical variables. Canonical variable #1 explained most of the variation in the data. Sample positions plotted on Canonical variable #1 were ordered by storage time. Although samples B and C (cook 0 and 1-day) plotted close together on Canonical variable #1, they were different according to canonical variables #2 and #3. Sample C (cook 1-day) was different from all other samples on canonical variable #2, while sample B (cook 0-day) was extremely low on canonical variable #3. Interpretation of the meaning of canonical variables in terms of the attribute relationships was not usually possible. However, the spatial configuration of the samples helps to visualize differences noted in Fig. 4 of Factor score plots where scores for Factors II and III were almost the same for cook 0-day stored samples and all factors were fairly close but evenly separated for sample C (cook 1-day).

The cooked 0-day and 1-day samples may represent the in-

termediate stage noted by Poste et al. (1986). The terms warmed-over and rancid were not distinctly separated in the VARCLUS or Factor analyses. One reason may be that the 3-day storage was not long enough to accentuate the rancid character note. Storage treatments of pork used by Poste et al. (1986) went to 16 days.

The significance of the data in this study, viewed by several multivariate procedures, might be to emphasize the importance of focusing at storage times of 1-day or less to direct chemical/analytical studies to uncover causal factors in flavor change at very early times in refrigerated storage of precooked meat with subsequent reheating. By aiming at the determinations of compounds that relate to cardboard, rancid/painty at storage times beyond 1-day (24 hrs), researchers may be overlooking important contributions to flavor changes occurring at early stages of off-flavor development. Additionally, further studies that focus on analytically determined compounds that relate to Factors II and III (chickeny, meaty, brothy, liver/organy) might prove useful as these Factors, and the terms represented by them, are important descriptors of sensory change at 0 and 1-day (0 and 24 hr) storage. Maintenance of the flavor character notes represented by Factors II and III may be as important a goal in the problem of warmed-over flavor as is detection and prevention of off-flavor represented by Factor I.

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Mention of trade names does not imply endorsement by the author or by the U.S. Dept. of Agriculture to the exclusion of others not mentioned.

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mechanisim controlling the meat factor and iron uptake is extremely important in the search for more effective ways to improve iron nutrition.

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Preparation of Heat-induced Transparent Gels from Egg White by the Control of pH and Ionic Strength of the Medium

NAOFUMI KITABATAKE, ATSUSHI SHIMIZU, and ETSUSHIRO DOI

ABSTRACT

Egg white was dialyzed against distilled water or diluted with water, and the precipitates formed were removed by centrifugation. The supernatant gave a transparent gel after being heated at an acidic pH (2–4). At other regions of pH, except for the highly alkaline region, the gel or suspension was turbid upon heating. Insufficient centrifugation of the dialyzed egg white or the addition of NaCl to the supernatant after centrifugation resulted in a turbid gel on heating at even acidic pH. The removal of the slight precipitate formed at low ionic strength and the maintenance of low ionic strength during heating were both necessary for production of a transparent gel.

INTRODUCTION

THE TEXTURE and microstructure of heat-induced gels of food protein reflect the pattern of aggregation of the protein molecules, which is strongly affected by the period of heating, the temperature, the protein concentration and the conditions of the medium, such as pH and the ionic strength of the solvent (Foster and Rhees, 1952; Nakamura et al., 1978; Woodward and Cotterill, 1986). The interaction between each protein molecule denatured on heating is mainly governed by the surface net charge and the hydrophobic area exposed by heating of the protein molecules. The former usually gives rise to electrostatic repulsion and the latter to attractive forces between molecules. The balance of these interacting forces affects the state of products being heated (Schmidt, 1981; Gossett et al., 1984).

Ovalbumin, the main constituent of egg white, gives a transparent solution, transparent gel, turbid gel or turbid suspension, depending on the pH and ionic strength of the heating medium (Hegg et al., 1979; Egelandsdal, 1980; Hatta et al., 1986). A similar effect has been observed with other proteins (Clark and Lee-Tuffnell, 1986).

Egg white is not only a highly nutritious food, but also a food ingredient with various functional properties that make it useful as a food binder, gelling agent, thickener (Kinsella, 1982). The heating of egg white usually yields a turbid white gel. As food materials for transparent gels, agar or gelatin is usually used; however, these are less nutritious and heat-labile. If a transparent heat-induced egg white could be prepared by control of the heating and medium conditions, new uses of egg white as a novel and nutritious food ingredient, such as for cold desserts, meat binder, food for patients might be possible. The objective of this study was to examine various conditions in order to identify those needed to produce such a gel from egg white.

MATERIALS & METHODS

Materials

Egg white from which the chalaza was removed was stirred gently with a magnetic stirrer so as not to produce foam for 2 hr at room temperature, giving homogeneous egg white. After stirring, a coagulum floating on the surface of the egg white solution was removed by the spatula.

Authors Kitabatake, Shimizu, and Doi are with the Research Institute for Food Science, Kyoto Univ., Uji 611, Kyoto, Japan.

Measurements of turbidity and other gel properties

Turbidity of the heated sample was measured as follows. To 3.0 mL of sample in a special test tube (11.5 mm i.d. × 100 mm height) for turbidity measurement was added 0.15 mL 3M NaCl, the tube was heated for 1 hr at 80°C. After being cooled in tap water for 30 min, the test tube containing the sample was put directly in the cell holder of a Shimadzu (Kyoto) UV-240 spectrophotometer. The absorbance of the sample measured at 600 nm was used as the value of the turbidity.

When texture parameters were measured, 2.0 mL 3M NaCl was added to 40 mL sample in a screw-capped cylindrical vessel (40 × 75 mm), and heating and cooling were done as above. The textural properties of samples were measured at 20°C by use of a Rheoner RE-3305 (Yamaden Co., Ltd., Tokyo) with plunger 3.5 mm in diameter. The plate on which the sample in a cylindrical vessel was placed was set to move in both the upward and downward directions at 0.5 mm/sec. Gel was compressed with a 5 mm deformation. Gels were not fractured with this compression. When adhesiveness was to be measured, the chart speed was 240 mm/min, and the voltage input was 100 mV. Two consecutive bites were taken, and three measurements were made for each sample. Hardness, cohesiveness, adhesiveness and gumminess were measured and calculated by the method of Szczesniak (1963).

The water-holding capacity of the gels was measured as described by Eide et al. (1982) with some modifications. One to two grams gel was wrapped in a Miracloth (Calbiochem-Behring Corp. Behring Diagnostics, La Jolla, CA) and placed in a Centricone holder (Pharmacia, Stockholm) in a 50-mL centrifuge tube. The tube was centrifuged in a Hitachi superspeed centrifuge SCR 20B with an angle rotor (RPR 20-2) for 10 min at 20°C, at 1,000 rpm (125 × g) or 3,000 rpm (1,100 × g). The cloth-wrapped sample was weighed before and after centrifugation. The initial gel weight (G) and the weight (volume) of water released from the initial gel (W) were measured. The water-holding capacity was determined as (W) × 100/(G).

Measurement of electric conductivity

The electric conductivity of the solution was measured with a conductivity meter (CD-35M2, M & S Instruments, Inc, Tokyo) at 25°C.

RESULTS

Heating of egg white

The protein concentration of the egg white prepared in this study was 94.2 ± 1.7 mg/mL, and the preparation was clear with a slight yellow tinge. Its pH was about 9. The turbidity of egg white at various pH values, adjusted by the addition of HCl or NaOH, was measured without or with the addition of NaCl (150 mM) before and after being heated at 80°C for 1 hr (Fig. 1). Egg white before heating was not very turbid in the range of pH above 7. As the pH of the medium decreased from 7 to 4, the absorbance increased and then exceeded 2.0, indicating that the samples before or after the addition of NaCl were turbid in the region of pH 7 to 4. At around pH 2.5, the absorbance decreased and then increased again in the region of still lower pH.

When samples were heated with or without the addition of

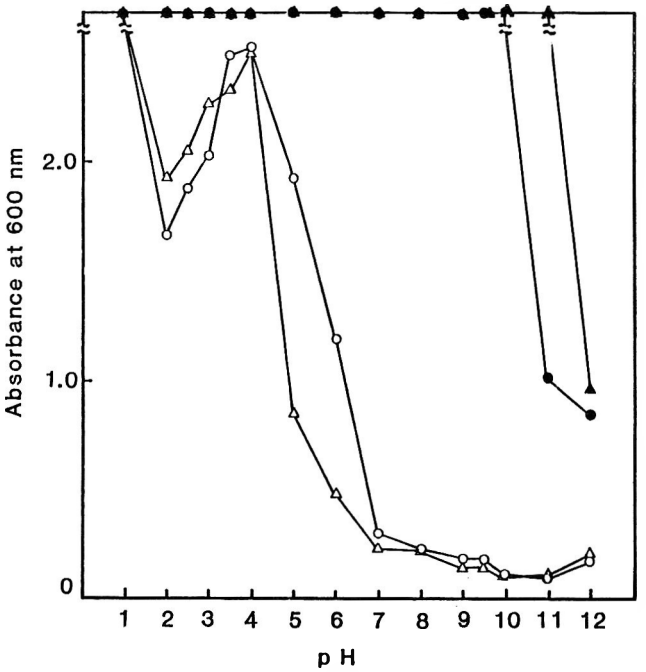

Fig. 1—Turbidity of egg white heated or not, with or without added NaCl. ○, Not heated, no added NaCl; △, not heated, with added NaCl (150 mM); ●, heated, without added NaCl; ▲, heated, with added NaCl (150 mM).

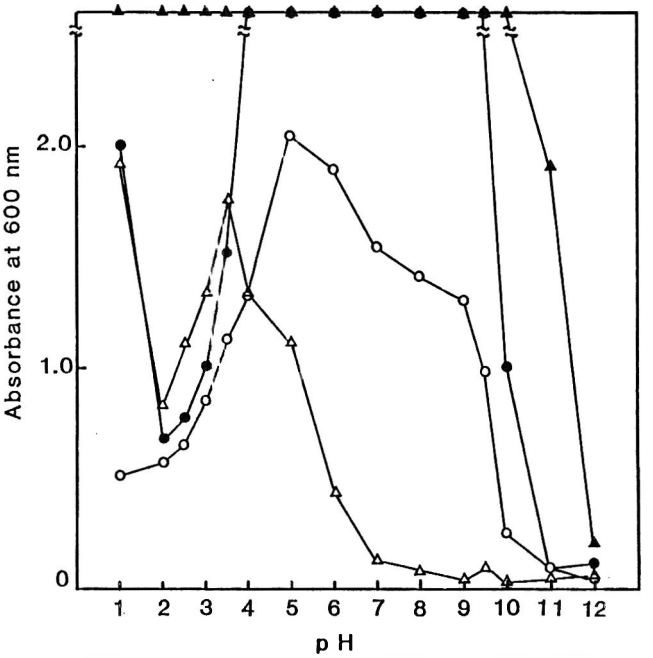

Fig. 2—Turbidity of egg white treated with dialysis and low-speed centrifugation. Egg white was dialyzed against water and the precipitate that formed was removed by centrifugation at 3,000 rpm. ○, Not heated, without added NaCl; △, not heated, with added NaCl (150 mM); ●, heated, without added NaCl; ▲, heated, with added NaCl (150 mM).

NaCl, they became a turbid gel except when heating was at pH 12 with NaCl or else at pH 11–12 without NaCl, which gave brownish transparent solutions. This color seemed to be produced by the Maillard reaction, and this type of transparent gel from egg white is well-known in Chinese cuisine for the preparation of *peetan*.

Effects of dialysis on turbidity and other gel properties

The heat-aggregation of ovalbumin and its turbidity is suppressed at low ionic strength (Hatta et al., 1986); however, even when no NaCl was added to egg white, the heated sample was turbid, as was that heated with NaCl in the experiment mentioned above. This might be because egg white contained enough salt in itself so that the addition of 150 mM NaCl did not affect the pH profile. Salt-free egg white was prepared by dialysis against distilled water exhausively. Electrical conductivity reached an almost constant value (about 100 μS). The precipitate that formed during dialysis was removed by centrifugation at 3,000 rpm (1,100 × *g*), and the supernatant was studied (Fig. 2) in experiments similar to those where results are shown in Fig. 1. The protein concentration of the supernatant (pH 9) was 72 mg/mL. The turbidity of the supernatant before being heated was greater than that of the original egg white in Fig. 1 at neutral and slightly basic pH regions. The turbidity peak was at pH 5. Added NaCl made the samples less turbid in neutral and slightly acidic pH regions; the turbidity peak was shifted to pH 4. The pH profile of the turbidity of the sample, heated with NaCl (150 mM), was similar to that shown in Fig. 1. Both with and without NaCl, the supernatant gelled on being heated at all regions of pH except 11 and 12.

The supernatant (about pH 9) obtained after dialysis and centrifugation at 15,000 rpm (28,000 × *g*) instead of 3,000 rpm was very clear. The protein concentration of this supernatant was 67 mg/mL. The turbidity of this clear solution was measured as described above, before and after being heated at various pH values both in the presence and the absence of NaCl (Fig. 3). Without NaCl and before being heated, the sample was turbid at pH 5.0 and 6.0, and transparent at other

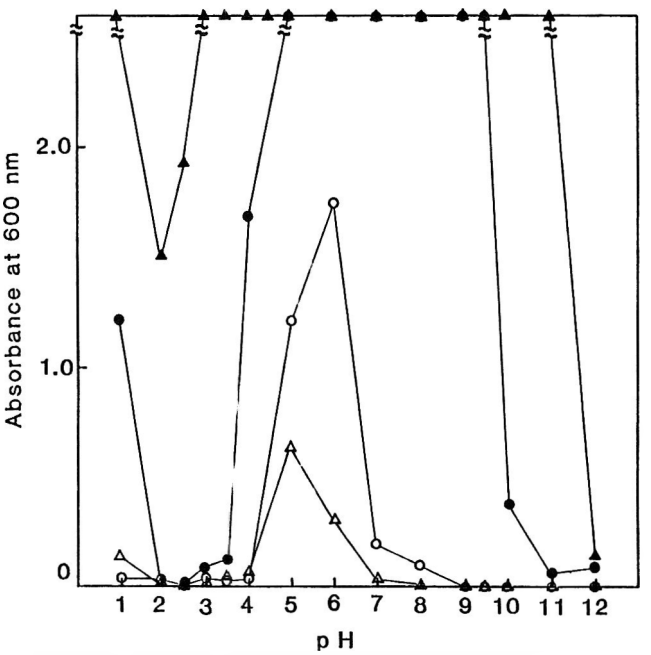

Fig. 3—Turbidity of egg white treated with dialysis and high-speed centrifugation. Egg white was dialysed against water and the precipitate that formed was removed by centrifugation at 15,000 rpm. Symbols are the same as in the legend to Fig. 2.

pH values. With the addition of NaCl (150 mM), the turbidity was reduced, and transparent or translucent solutions were obtained at all pH ranges. This may be an effect of salting-in. These samples were then heated as shown in Fig. 1. On being heated without NaCl, a transparent gel was formed at pH from 2.0 to 3.5 and also at 10.0 and above 10.0. On being heated with NaCl, the sample formed a translucent gel at acidic pH and a transparent one at pH 12.0, but at other pH values, turbid gels were obtained. Desalting and removal of the precipitate

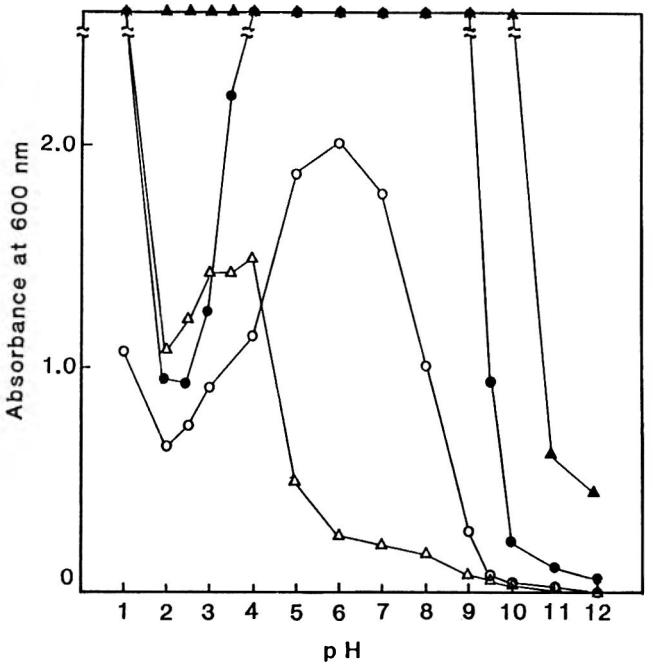

Fig. 4—Turbidity of egg white treated with twofold dilution and high-speed centrifugation. Egg white was diluted with an equal volume of water and the precipitate removed by centrifugation at 15,000 rpm. Symbols are the same as in the legend to Fig. 2.

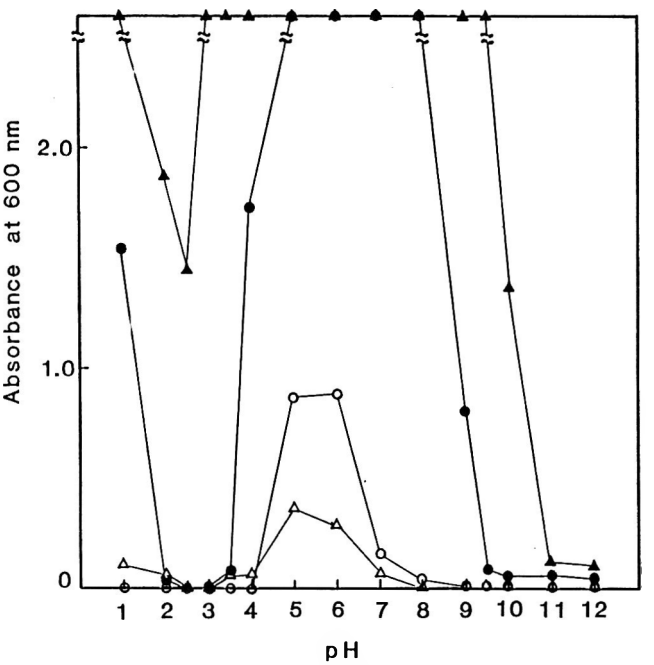

Fig. 5—Turbidity of egg white treated with fourfold dilution and high-speed centrifugation. Egg white was diluted with three volumes of water and the precipitate removed by centrifugation at 15,000 rpm. Symbols are the same as in the legend to Fig. 2.

that appeared from the egg white gave a transparent gel when heating was done at the region of acidic pH. Here, a transparent gel was prepared by the reduction of the ionic strength and heating at lower regions of pH. To reduce the ionic strength, dilution is a much simpler and faster technique than dialysis, so the dilution was used instead of dialysis to determine if similar results were obtained.

Effects of dilution on turbidity and gel properties

The supernatant obtained by centrifugation at 3,000 rpm after dilution with an equal volume of water (called the "twofold dilution method") was turbid or translucent in the range pH below 9 (Fig. 4) and transparent at and above pH 9. When NaCl was added to the supernatant and its pH was adjusted from 1 to 12, turbidity decreased in regions of neutral pH; samples were more turbid at pH 2.0 to 4.0 than without addition of NaCl. When the samples were heated, a turbid gel at pH 1.0, a translucent gel at pH 2.0–3.5, a turbid gel or suspension at pH 4.0–10.0 and a brown transparent solution at pH 12.0, were obtained by heating the samples without added NaCl. Heating with NaCl gave a turbid gel except at pH 10.0–12.0. By the twofold dilution method, a transparent sample was not obtained at acidic pH. Then egg white, diluted with three volumes of water (the "fourfold dilution method") was studied. By the fourfold dilution method, a clearer supernatant was obtained by centrifugation after dilution. This supernatant gave a slightly turbid suspension when adjusted to pH 5.0–7.0 (Fig. 5), which was much less turbid than with the twofold dilution method. By the addition of NaCl (150 mM), turbidity was reduced in the range of pH 4.0–7.0. When the supernatant was heated without added NaCl, turbid gels were formed at pH 1.0 and from 4.0 to 7.0. A turbid suspension was obtained at pH 8.0–9.0; however, there was a transparent solution at pH 2.0–3.0, a transparent gel at pH 3.5 and a transparent and yellow solution at pH 10.0–12.0. When the supernatant was heated with 150 mM NaCl at various pH, it became turbid except from pH 2.0 to 3.0 and pH 11.0 and 12.0. The gels were more transparent prepared by the fourfold dilution method than by the twofold dilution method; these gels were also much softer than those prepared by the twofold dilution method, because of the low protein concentration.

To prepare a heat-induced transparent, firm gel at acidic pH, both reduction of the salt concentration and maintenance of the protein concentration during heating were needed. Dialysis and high-speed centrifugation (15,000 rpm) treatment (called the "dialysis method") satisfied these requirements.

Effects of dialysis on other properties of gels

Textural parameters (hardness, adhesiveness and cohesiveness) of gels produced by the heating of a supernatant prepared by the dialysis method, were measured at various pH using a Rheometer in the textural mode. A peak of hardness was found at pH 3.5 (Fig. 6), at which turbidity was low, indicating that the gel was transparent. Transparent gels were obtained by heating at pH 2.0, 2.5, 3.0, and 12.0, but their hardness was low; the gels were soft. The peak of adhesiveness was found at pH 3.0, slightly acidic from the peak of hardness. The cohesiveness of the gel varied from sample to sample depending on the pH.

When heated with 150 mM NaCl at various pH, the samples gelled at every pH. Peaks of hardness were found around pH 4.0 and 12.0 (Fig. 7). Compared with heating without NaCl, the hardness of the sample at pH 4 was lower than that without NaCl; the peak of adhesiveness was at pH 4.0, a lower pH than for the peak of hardness. At pH 10.0, a large peak of adhesiveness was observed.

The water-holding capacity of gels prepared by heating of a solution dialyzed against water with or without the addition of NaCl was measured. The volume of water released from the gels prepared at various pH were plotted against pH (Fig. 8). Centrifugation at both 1,000 and 3,000 rpm was done. When centrifuged at 1,000 rpm, the gels prepared without NaCl had high water-holding capacity at pH 2.0–4.0, pH 7.0, and 8.0 (less water released), and low water-holding capacity at pH 5.0 and 6.0 (more water released). A part of the gel prepared above pH 9 passed through the Miracloth during centrifugation; values in these samples were not measured. The gels prepared with NaCl had lower water-holding capacity than

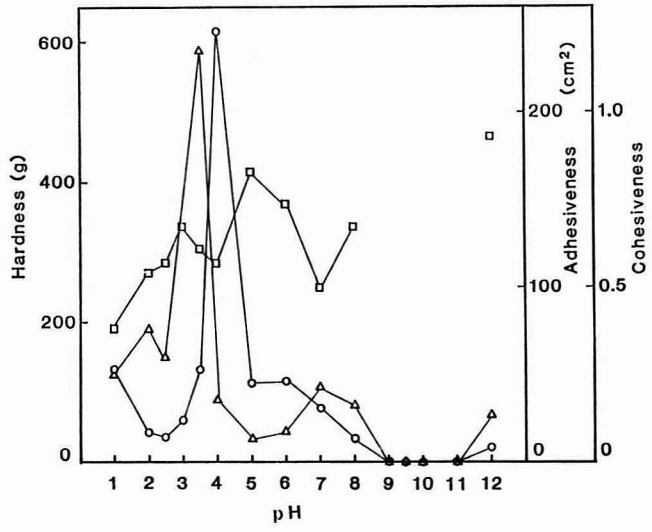

Fig. 6—Textural parameters of egg white treated with dialysis, high-speed centrifugation and heat. Egg white was dialyzed against water, centrifuged at 15,000 rpm to remove the precipitate and heated with no added NaCl. Hardness (○), adhesiveness (△), and cohesiveness (□) of these heat-treated egg whites were measured as described in Materials & Methods.

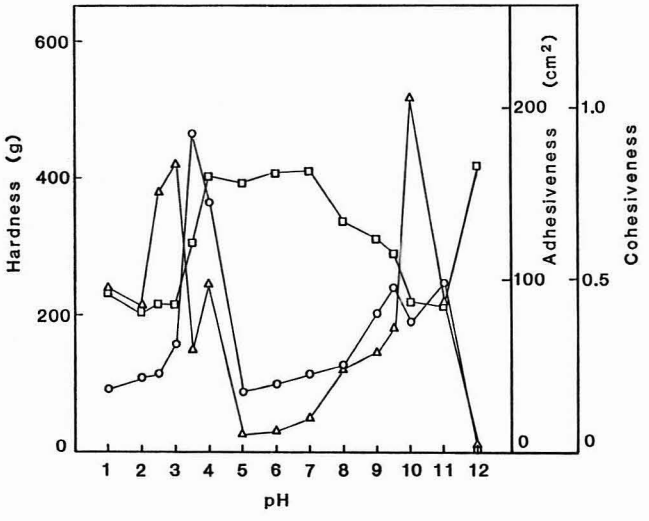

Fig. 7—Texture of egg white treated by dialysis, high-speed centrifugation and heat. Egg white was dialyzed against water, centrifuged at 15,000 rpm to remove the precipitate and heated with added NaCl (150 mM). Hardness (○), adhesiveness (△), and cohesiveness (□) of these heat-treated egg whites were measured as described in Materials & Methods.

those prepared without NaCl in all pH ranges measured; however, pH profile was similar to that of the gels prepared without NaCl. When centrifuged at 3,000 rpm, the gels released more water than when centrifuged at 1,000 rpm, but the pH profiles were similar, except more of the gel samples passed through the cloth by centrifugation at 1,000 rpm.

Effects of NaCl concentration on turbidity, textural properties, and water-holding capacity of the gels

Salt concentration during heating strongly affected the turbidity and hardness of the gels obtained, as described above. By changing the concentration of NaCl added to the supernatant after centrifugation at 15,000 rpm, the effects of NaCl concentration on turbidity and other properties of gels prepared at pH 2.5 were examined (Fig. 9). Below 75 mM, absorption at 600 nm did not exceed 0.4, and transparency was obtained,

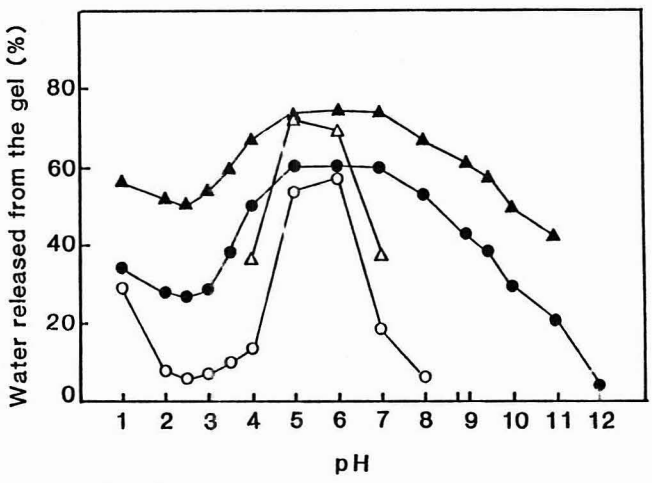

Fig. 8—Water-holding capacity of gels prepared from egg white. Egg white was dialyzed against water, centrifuged at 15,000 rpm to remove the precipitate and heated with or without NaCl. Gels obtained without NaCl were centrifuged at 1,000 rpm (○) and 3,000 rpm (△). Gels obtained with NaCl (150 mM) were centrifuged at 1,000 rpm (●) and 3,000 rpm (▲).

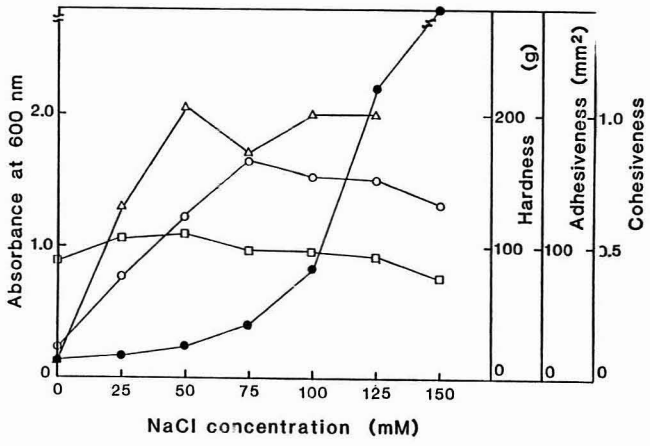

Fig. 9—Turbidity and textural parameters of egg whites heated at pH 2.5 with various concentrations of NaCl. Absorbance at 600 nm (●), hardness (○), adhesiveness (△), cohesiveness (□) of heat-treated egg whites were measured as described in Materials & Methods.

but above 100 mM, samples were turbid on heating. The hardness of the gel increased with the addition of NaCl. A hardness peak was found at 75 mM NaCl. A similar pattern was observed for adhesiveness. Cohesiveness was almost independent of the NaCl concentration, but the water-holding capacity of the gels was strongly influenced by NaCl. The amount of water released from the gel was plotted against the NaCl concentration (Fig. 10). The water-holding capacity increased up to about 1M NaCl; above this, it was constant. Thus, the egg white solution after dialysis and centrifugation at 15,000 rpm was a transparent solution when heated at pH 2.5 without NaCl, a transparent gel with 5–75 mM NaCl and a translucent or turbid gel when NaCl was above 75 mM. The results showed that maintenance of low ionic strength during heating at pH 2–4 and removal of insoluble materials formed at low ionic strength were both needed to obtain a transparent gel or solution from egg white on heating.

Reheating of the transparent gel and the effects of freeze-drying of the egg white

The gels prepared in this study, including transparent gels, were all "heat-set" gels that were not deformed by a second

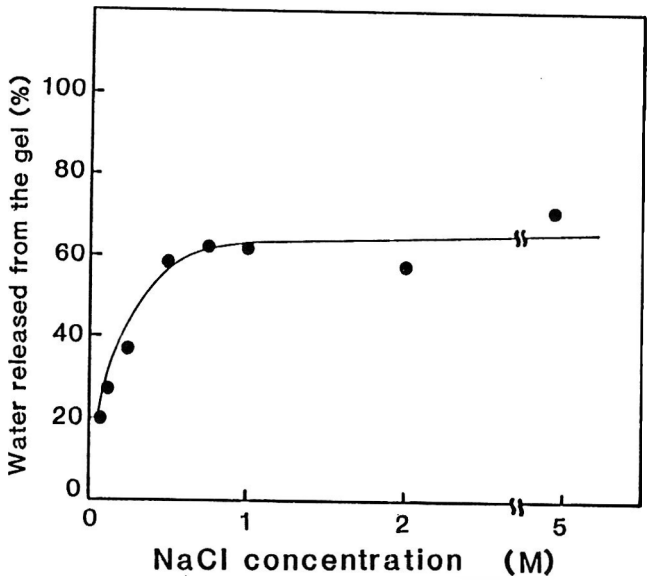

Fig. 10—Water-holding capacity of egg whites heated at pH 2.5 with various concentrations of NaCl.

heating. This property was different from results obtained with agar or gelatin gels (Morris, 1986; Ledward, 1986).

A supernatant obtained after dialysis and centrifugation at 15,000 rpm was freeze-dried. The powder obtained was rehydrated with distilled water and then heated. A similar transparent gel resulted. Freeze-drying did not lessen the ability to form a transparent gel.

DISCUSSION

THE pH PROFILE of the turbidity of heat-induced egg white gel was substantially the same as that of ovalbumin (Hatta et al., 1986). The turbidity of the heated ovalbumin sample changed drastically at pH 7.0–8.0, but the turbidity of egg white decreased at around 9 or 10 (Fig. 6; sample heated without NaCl). Two peaks of hardness were found in both pH profiles of ovalbumin (Hatta et al., 1986). With egg white, however, a peak in the higher pH region was absent (Fig. 6) or had shifted to a still higher pH (Fig. 7). These differences from ovalbumin probably arose because egg white contained many kinds of protein components. Some proteins with a high isoelectric point coagulated at regions of higher pH, which made the gel turbid or resulted in a rigid protein network.

The pH profile of the water-holding capacity was similar to that for turbidity. Turbid gels had a low water-holding capacity; that for transparent or translucent gels was higher. This showed that in the pH range where protein-protein interaction was strong, a coagulum of the protein was formed in the gel and water was easily excluded from the gel. Similar results have been reported for other proteins (Hermansson and Lucisano, 1982; Hermansson, 1986). At pH regions far from the isoelectric points of the main protein components of egg white, mutual repulsion of protein molecules increased and the binding areas on the denatured ovalbumin molecules were limited, so that clumps of aggregate (coagulum) were not formed, but linear aggregates were, which might form a protein network. This network should result in an increase in gel hardness.

The peak of adhesiveness was at a slightly lower pH than the hardness peak. This might be due to weakening of the protein-protein interaction at this pH by the increase in repulsion among the denatured ovalbumin molecules, so that a protein network was not formed, because the elongation of linear aggregates was restricted. However, a long, soluble linear aggregate might form; this would cause high viscosity. This aggregate kept its affinity to water, so the plunger was wet during the test. These effects resulted in high adhesiveness.

The alteration in the samples prepared at various pH was probably based on changes in the form of the molecular aggregates. This assumption is consistent with observations under the electron microscopy (Clark et al., 1981).

Whether the gel was clear or turbid depended on the protein-protein interactions in the medium. When this interaction was regulated by certain procedures, a clear gel or solution could be formed even if a turbid gel was normally obtained. For instance, some chemical modifications (Ma and Holm, 1982) or enzymatic modification (Kitabatake and Doi, 1985) give such results.

Heated egg white can be produced in forms other than a turbid gel. Nishikawa et al. (1984, 1985) prepared egg white that gave a transparent solution on being heated. In the method of Nishikawa et al. (1984), both the removal of the coagulum that occurred during foaming and autoclave treatment were necessary to obtain a transparent result. Perhaps, the protein component removed by dialysis in this study was the same as that removed by foaming in their study.

By polyacrylamide gel electrophoresis, the supernatant obtained by dialysis was found to contain conalbumin, ovomucoid, and other proteins in egg white as well as ovalbumin, and the removal of lysozyme and ovomacroglobulin by dialysis of egg white against water was observed (Kitabatake et al., 1988a). It seemed that ovomucin was also removed from egg white by various treatments applied in this study. Some ovomucin might be removed as a coagulum which formed during stirring of egg white and the other might be contained in the precipitate fraction occurred by dialysis or dilution. Hegg (1982) has reported that no transparent gel or opaque gels are obtained when purified conalbumin is heated; however, in this study conalbumin was also present in the transparent gel made with egg white at low pH when the ionic strength also was low. These differences might arise from differences in the heating conditions or the protein-protein interaction; that between ovalbumin and conalbumin might affect the formation of a coagulum.

This study showed that other proteins, not only ovalbumin, gave a transparent gel when heating and medium conditions, such as pH, ionic strength, and protein concentration, were controlled. Bovine serum albumin also gives a transparent gel (Clark and Lee-Tuffnell, 1986).

In the presence of NaCl, a clear gel was not obtained even at low pH, instead a turbid gel was formed. With ovalbumin (Kitabatake et al., 1987), first heating of dialyzed ovalbumin at lower pH gives a transparent gel in the presence of NaCl upon renewed heating (two-step heating method). The study on the preparation of such transparent gel from egg white in the presence of salt by two-step heating is shown in the following paper (Kitabatake et al., 1988b).

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Simultaneous Isolation of Avidin and Lysozyme from Egg Albumen

T.D. DURANCE and S. NAKAI

ABSTRACT

A single column cation exchange method was developed which allowed simultaneous recovery of lysozyme and avidin from undiluted egg white. A unique application-elution sequence was developed, involving accumulation of avidin on the column through several cycles of egg white application and lysozyme elution. Lysozyme was recovered with higher yields than reported for the isoelectric precipitation methods often used in the industry (86% vs 60–80%). Lysozyme peaks appeared homogeneous on SDS-PAGE. Avidin recovery was also as good or better than that of previously reported ion exchange methods (74%–80%). The purity of the avidin fraction (up to 40.9%) was superior to that of other reported primary avidin fractions.

INTRODUCTION

LYSOZYME AND AVIDIN are two proteins found in rather small quantities in egg white (3.5% dry basis and 0.05% dry basis, respectively). Each commands a significant commercial market and both can be removed from egg white without substantially altering its functional nutritional properties. In fact, the United States Food & Drug Administration (FDA) standard of identity for dried egg white has recently been altered to allow the sale of this product with the lysozyme and avidin reduced by ion exchange treatment (Anonymous, 1986).

Several methods for isolating and purifying lysozyme have appeared in the scientific literature. The classical method, involving direct precipitation from egg albumen at pH 9.5 by addition of 5% sodium chloride, is still used in industry (Alderton and Fevold, 1964). Recent modifications of this procedure have been briefly reviewed by Ahvenainen et al. (1980). Yields of 60–80% egg white lysozyme have been achieved with direct crystallization but a major disadvantage of these methods is that they require the addition of salts or other additives which contaminate and decrease the usefulness of the treated egg white.

Currently, the most efficient and probably the most widely used method for lysozyme isolation is a two-step ion exchange resin-isoelectric precipitation process. Egg albumen is mixed with a suitable ion exchange resin, commonly a modified carboxymethylcellulose, then filtered off. The resin is washed free of unadsorbed proteins, then placed in 5% sodium chloride which causes release of the lysozyme into solution. The lysozyme solution is then adjusted to pH 9.5, causing the lysozyme to precipitate. Lysozyme is redissolved, desalted, concentrated, and spray-dried.

Many affinity chromatography methods have been described for lysozyme isolation (Cherkasov and Kravchenko, 1967, 1969; Weaver et al., 1977; Imoto and Yagishita, 1973; Muzzarelli et al., 1978; Yoshimoto and Tsuru, 1974). Although good yields and purity have been achieved with these methods, they required specialized and expensive affinity supports which have not been widely used or proven in industry. Furthermore, dilution of the egg white was generally required.

Ahvenainen et al. (1980) reported the use of the commercially available cation exchange resin Duolite C-464 with good yield and purity of lysozyme. Their method, however, was most successful as a batch procedure and required a somewhat complex regeneration sequence between batches.

The earliest purification method for avidin involved alcohol precipitation from egg white (Eakin et al., 1941; Dhyse, 1954). Modifications of this approach have been widely used commercially but with complete destruction of the functionality of the remaining egg white. Melamed and Green (1963) achieved high purity with three successive purification steps on carboxymethylcellulose and Amberlite CG-50 ion-exchange resin, an effective but very time-consuming method. The primary product of their method contained 50% of initial avidin but purity was only approximately 1%. Yield and purity figures for other primary isolation procedures were 17% yield and 10-20% purity (Fraenkel-Conrat et al., 1952), 38% yield and 10% purity (Dhyse, 1954), 39% yield (Rhodes et al., 1958) and 80% yield with 24% purity (Green and Toms, 1970).

Two successful affinity methods for avidin have been described. Cuatrecasas and Wilchek (1968) bound avidin to biocytin-Sepharose columns and eluted with 6M guanidine-HCl at pH 1.5. Avidin was completely denatured by this treatment but could be renatured by dilution in 90% yield. Iminobiotin, which binds avidin at pH 11 but not at pH 4, was also employed as an affinity ligand for avidin. With this approach, yields of 95% and 99% avidin purity have been reported (Heney and Orr, 1981). Both affinity methods utilize expensive affinity supports with limited lifetimes. Also, affinity purified avidin may be only 75% as active as ion exchange purified avidin (Mock et al., 1985). Consequently, the affinity product has a lower value than ion exchange purified avidin. Furthermore, to date only ion exchange processes have been approved by the FDA for treatment of egg albumen. Therefore at present, economic factors strongly favor the ion exchange resin processes.

A convenient and practical column chromatography method for recovery of lysozyme with Duolite C-464 has recently been reported (Li-Chan et al., 1986). These authors indicated that a significant amount of avidin co-eluted with lysozyme. The main purpose of the study described here was to develop a procedure whereby these proteins could be resolved and isolated in separate fractions by a single cation exchange column.

Duolite C-464, a copolymer of methacrylic acid and divinyl benzene, has extensive aromatic ring structure and, therefore, might be expected to interact hydrophobically as well as electrostatically with proteins. A model of protein retention on ion exchangers has recently been proposed which allows estimation of the contributions of electrostatic and hydrophobic interactions to the binding of the protein at different salt concentrations (El Rassi and Horvath, 1986). This model, as well as the theoretical treatment of ion exchange resin retention put forward by Kopaciewicz et al. (1983), were employed to determine the relative importance of hydrophobic and electrostatic interactions between lysozyme and avidin and Duolite C-464.

MATERIALS & METHODS

Reagents

Duolite C-464 was supplied by the Diamond Shamrock Co. (Cleveland, OH). 2(4'-Hydroxyazobenzene)-benzoic acid (HABA), ion-exchange-purified avidin standards and 3X crystallized grade 1 lysozyme were obtained from Sigma Chemical Co. (St. Louis, MO).

Authors Durance and Nakai are with the Dept. of Food Science, Univ. of British Columbia, 248-2357 Main Mall, Vancouver, B.C., Canada V6T 2A2.

Egg white pretreatment

Egg whites were separated from eggs obtained from the University of British Columbia poultry farm or supplied frozen by Vanderpol's Eggs Ltd. (Surrey, B.C.). Before column application, egg albumen was passed through a Manton-Gaulin laboratory homogenizer (single stage, 6.9 MPa) to decrease viscosity.

Activity assays

Lysozyme activity was determined by the turbidimetric assay method (Anonymous, 1981) with modifications as reported by Li-Chan et al. (1986).

Avidin activity was measured by the HABA spectrophotometric method of Green (1965), with minor modifications. To 2 mL of sample solution, 1 mL of 0.2M sodium phosphate buffer, pH 7.2, was added, followed by 0.1 mL of 2 mM HABA. The absorbance of the avidin-HABA complex was read at 500 nm. Then 0.1 mL of 0.4 mM biotin solution was added to displace HABA and A_{500} was read again. The concentration of avidin was calculated as follows:

$$\text{Avidin (g/L)} = \frac{\text{Vol Assay} \times \text{MW avidin} \times \Delta A_{500}}{\text{Vol Sample} \times E \times 4} \quad (1)$$

where MW is the molecular weight of avidin (68,000), 4 is the number of biotin binding sites per avidin molecule and E is 34,000, the extinction coefficient of the avidin HABA complex.

Protein assays

Protein concentrations of samples were routinely determined from their absorbance at 280 nm. Protein content was calculated based on a $E^{1\%}$ values of 26.4 for lysozyme and 15.4 for avidin (Anonymous, 1981; Green, 1975).

Since lysozyme is the main contaminant of the avidin containing fractions, avidin purity can more accurately be estimated as follows, where $A_{s.a.}$ = avidin specific activity (mg/mL) and A_{280} = absorbance of fraction at 280 nm.

$$\text{Avidin purity (\%)} = \frac{A_{s.a.}}{A_{s.a.} + \frac{A_{280} - (A_{s.a.} \times 1.54)}{2.64}} \times 100\% \quad (2)$$

Electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed essentially according to Laemmli (1970), using a 10% acrylamide separation gel and 3% stacking gel containing 0.2% SDS. Samples containing 1–2 mg protein/mL were prepared in 0.01M sodium phosphate buffer (NaP) at pH 7.0, containing 2% SDS and 2% beta-mercaptoethanol; after heating at 100°C for 10 min, glycerol (to 10%) was added and 25 μL was applied to the sample slots. Electrophoresis was carried out at 100 volts for 4–5 hr. Gels were stained with Coomassie Brilliant Blue R-250 for 1.5 hr as described by Weber and Osborn (1969). Avidin and lysozyme were identified by comparison with commercial avidin and lysozyme standards.

Ion exchange isolation of lysozyme and avidin

Preliminary experiments and determination of optimum elution conditions for separation of lysozyme and avidin were carried out using a 7 mL (1.4 cm i.d.) column of Duolite C-464 resin. Subsequent laboratory experiments were performed with 25 mL (1.5 cm i.d.) columns or 170 mL (2.5 cm i.d.) columns of Duolite C-464. Homogenized egg white was applied to columns previously equilibrated with 0.1M NaP, at a flow rate such as to allow a contact time between the resin and egg white of 20–30 min. Immediately after egg white application, unadsorbed proteins were washed off the column with 5–10 column volumes of distilled water or starting buffer. Adsorbed proteins were eluted with buffer of increasing ionic strength, using either stepwise or gradient elution. Details of elution conditions are presented in the Results section.

A small pilot-scale experiment was performed with a 470 mL (5 cm × 18 cm) Duolite C-464 column. The process was partially automated with a Pharmacia C3 Process Controller (Pharmacia Canada Inc., Dorval, Que.) which controlled a series of electromagnetic valves and peristaltic pumps as diagrammed in Fig. 1.

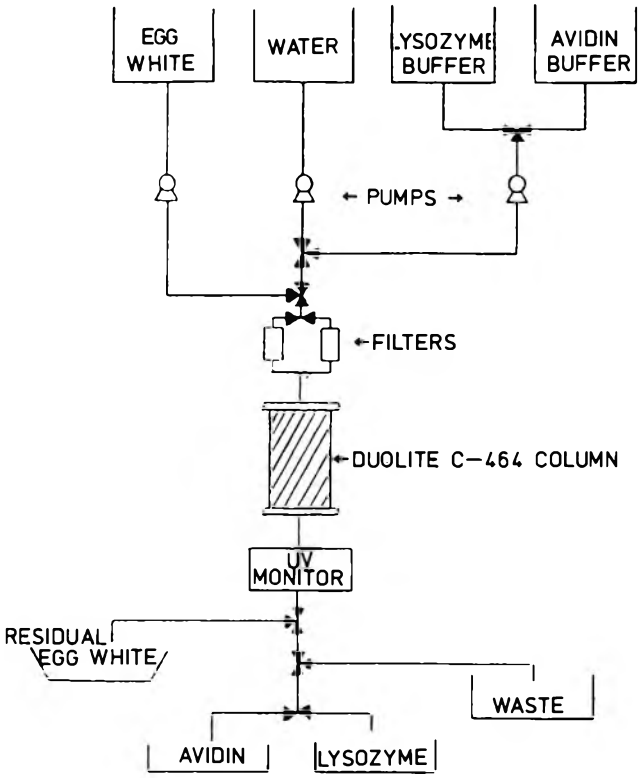

Fig. 1.—Diagram of a pilot plant apparatus for isolation of avidin and lysozyme from egg white by Duolite C-464 chromatography.

Simplex optimization

The mapping simplex optimization technique (Nakai et al., 1984; Nakai and Kaneko, 1985) was employed to increase the efficiency of the search for the best conditions for the separation of lysozyme and avidin from egg white. Three experimental factors known to have a bearing on the separation were selected for the optimization; (1) pH, (2) sodium chloride concentration of the limit buffer and (3) the shape of the elution gradient. Gradient shape was determined by the gradient power value P, such that $Y = X^P$, where Y was the volume ratio of the limit buffer and X was the corresponding elution volume fraction. Gradients had a total volume of 50 mL. Stepwise changes in the proportions of the starting buffer and the limit buffer (as defined by factors 2 and 3) were used to simplify the formation of the gradient. By varying the value of P, linear (P = 1), convex (P < 1) or concave (P > 1) gradients were possible. To simplify the optimization and decrease the number of experiments, only the characteristics of the avidin peak (i.e., avidin yield and avidin purity) were optimized. This approach was justified because a previous study (Li-Chan et al., 1986) had shown that lysozyme recovery was acceptable within the range of conditions used in this study.

The response value to be optimized was a function of both the purity of avidin in the avidin fraction and the yield of avidin as a percentage of the avidin applied in the egg white [i.e., response index = avidin purity (%) × avidin yield (%)].

Avidin binding capacity of Duolite C-464

A 2 mL column (3.1 cm × 0.5 cm) Duolite C-464 was equilibrated with 0.1M NaP, pH 7.9. A solution of 1.0 mg/mL avidin in NaP (50 mL) was applied at a flow rate of 0.17 mL/min and collected in 2 mL fractions. Eluant fractions were assayed for avidin by the HABA method.

Chromatography in high ammonium sulfate concentrations

A 1.5 cm × 18.0 column of Duolite C-464 was equilibrated with 0.025M NaP, pH 7.3 containing 0.15 molal to 2.25 molal ammonium sufate. Protein samples (5 mg avidin or lysozyme) were dissolved in 3 mL of the same buffer and applied to the column at a flow rate of 0.3 mL/min. Elution volume was taken as the point of maximum A_{280} for lysozyme or the point of maximum avidin activity for avidin. The

elution volume of the solvent front was determined with an injection of distilled water and detected as a decrease in eluant conductivity. The capacity factor, k, was calculated as:

$$k = (ev - ev_o)/ev_o \quad (3)$$

where ev = elution volume of a protein and ev_o = elution volume of the solvent front (i.e., water).

The values of log k and of m (m = molality of ammonium sufate) were fitted to the model of El Rassi and Horvath (1986) by multiple regression analysis performed with PC-Statistical Analytical System (SAS, 1985).

RESULTS

Simplex optimization of lysozyme-avidin separation

Li-Chan et al. (1986) found that avidin co-eluted with lysozyme when egg white was passed through a column of Duolite C-464 equilibrated at pH 8.0, washed with 0.1M NaP and isocratically eluted with 0.5M NaP. An example of this type of separation is given in Fig. 2. However, conditions for separation of avidin and lysozyme were not determined. In the present study, preliminary experiments in which these proteins were eluted with ionic strength gradients at different pH resulted in incomplete resolution and rather low yields. These results are shown in Table 1. In general, the responses were best in the pH range 7 to 8. The purity of the avidin fraction was a useful indication of the peak separation because avidin was less abundant than lysozyme. Therefore, unless the peaks were well separated, the avidin peak tended to be heavily contaminated by the much larger lysozyme peak.

Simplex optimization was used to assist in finding the best pH and salt gradient conditions. In an attempt to improve yield of avidin, water rather than NaP was used to wash the unadsorbed proteins from the column. The parameters and results of seven experiments are shown in Table 2. Although as much as 72% yield was obtained under these conditions, the avidin fraction was eluted with only fair resolution from the larger A_{280} peak, resulting in only 4.4% purity. Table 2 indicated that moderate improvement in the avidin response value was achieved by the optimization procedure. The purity of avidin was improved 5- to 33-fold as compared to untreated egg white.

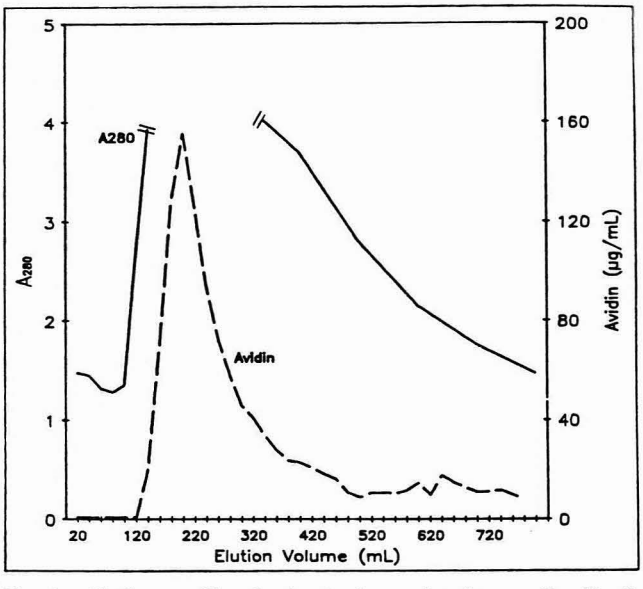

Fig. 2.—Elution profile of adsorbed proteins from a Duolite C-464 column (170 mL) by isocratic elution using 0.5M sodium phosphate buffer at pH 8.

Table 1—Preliminary separations of lysozyme and avidin from egg white on a 7 mL column of Duolite C-464

Elution conditions	Recovered proteins: Lysozyme yield (%)	Avidin yield (%)	Avidin purity (%)
NaP, pH 7.5, NaCl gradient	66	60	0.8
NaP, pH 8.0, NaP gradient	43	69	3.8
NP, pH 5.0, NaP gradient	67	84	1.3
pH 9.0, sodium carbonate gradient	61	59	3.3

Stepwise elution

The results of the optimization experiments indicated that manipulation of the pH and the shape of the simple 50 mL salt gradient was insufficient for satisfactory resolution of lysozyme and avidin. However, the optimization procedure did allow selection of the optimum pH for this buffer system as approximately pH 7.9. In fact, 7.9 is close to the upper buffering limit for NaP, so it was not possible to properly evaluate higher pH with this buffer. Since NaP was desirable for other reasons, such as economy and low toxicity, pH 7.9 was chosen for subsequent experiments.

Stepwise elution becomes practical when pH ceases to be a variable, since it becomes possible to accurately determine the salt concentrations that will elute lysozyme and avidin. This information was determined from linear gradient experiments and used to select elution buffers which would first elute all proteins adsorbed less strongly than avidin, followed by an avidin elution buffer. Table 3 shows the elution conditions and results of two experiments on the 7 mL column. Avidin activity was completely excluded from the first peak, and the purity of the pooled avidin fractions was higher than previously obtained. However, yield was poor at 44%. Nonetheless, the two-step approach was further investigated on a larger column.

Table 2—Factor levels for simplex optimization and resulting avidin recovery and purity for lysozyme-avidin separation from egg white by Duolite C-464 chromatography

	Factor Levels		Results		
pH	[NaCl] of limit buffer (M)	Power value[a]	Avidin purity[b]	Avidin yield (%)	Response index[c]
7.30	0.610	1.550	0.044	62.7	2.76
8.43	0.643	1.774	0.044	71.8	3.16
7.58	0.742	1.774	0.042	67.2	2.82
7.58	0.643	2.446	0.024	54.5	1.31
7.77	0.665	1.699	0.046	61.2	2.82
7.96	0.687	1.699	0.045	57.2	1.57
7.87	0.676	1.550	0.070	54.4	3.80

[a] Power value of gradient (P) such that $Y = X^P$, where Y is the NaCl concentration of the limit buffer and X is the corresponding elution volume fraction.
[b] Avidin purity in mg avidin/mg protein.
[c] Response index = avidin purity × avidin yield.

Table 3—Results of two-step elution for resolution of lysozyme and avidin fractions from egg white by Duolite C-464 chromatography on a 7 mL (1, 2) or a 170 mL (3) column

Elution conditions[b]	Avidin purity (mg avidin/mg protein)	Avidin yield (%)	Response index[a]
1(i) NaP, pH 8.45, 0.26M NaCl (ii) NaP, pH 8.45, 0.50M NaCl	0.117	44.0	5.15
2(i) NaP, pH 8.43, 0.26M NaCl (ii) NaP, pH 8.43, 0.43M NaCl	0.147	26.5	3.85
3(i) NaP, pH 7.90, 0.13M NaCl (ii) NaP, pH 7.90, 0.75M NaCl	0.088	59.3	5.22

[a] Response index = avidin purity × avidin yield.
[b] First step (i) for lysozyme elution and second step (ii) for avidin elution.

A 20-fold increase in column volume, with corresponding increase in flow rates and egg white application volume, were undertaken. Lysozyme and avidin elution conditions were altered slightly to improve separation and decrease tailing (Table 3; Fig. 3). The response value was slightly improved.

"Elution looping"

Although the response values of the two-step elution experiments were the best to date, further improvement was desired. The fraction designated the "avidin peak" in fact typically contained only 8.8% avidin. This suggested that because of the similarity in charge properties of avidin and lysozyme, combined with much the lower abundance of avidin, complete resolution of the protein peaks would be difficult by conventional elution conditions of ion-exchange chromatography. The large tailing peak composed mainly of lysozyme inevitably contaminated the small avidin-containing peak. A novel elution process was necessary for better resolution and improved purity of recovered avidin.

Apparently, an avidin peak of high purity was difficult to achieve as long as the ratio of avidin to other proteins (mainly lysozyme) on the column remained low. In an attempt to increase this ratio, a scheme was investigated in which avidin was allowed to accumulate on the resin through several cycles of egg white application and lysozyme elution with low ionic strength buffer. The term "elution looping" was coined to describe this process.

Two such multi-cycle experiments were performed on the 170 mL Duolite C-464 column, and the results are summarized in Table 4. Buffers and elution conditions for lysozyme and avidin were extrapolated from the gradient elution and two-step elution experiments. The A_{280} and avidin activity profiles from a five-cycle experiment are presented in Fig. 4. Both experiments resulted in improved resolution of avidin from other proteins. The five-cycle trial, with a response value of 10.37, was two-fold better than the previous best results. Both avidin purity and recovery improved.

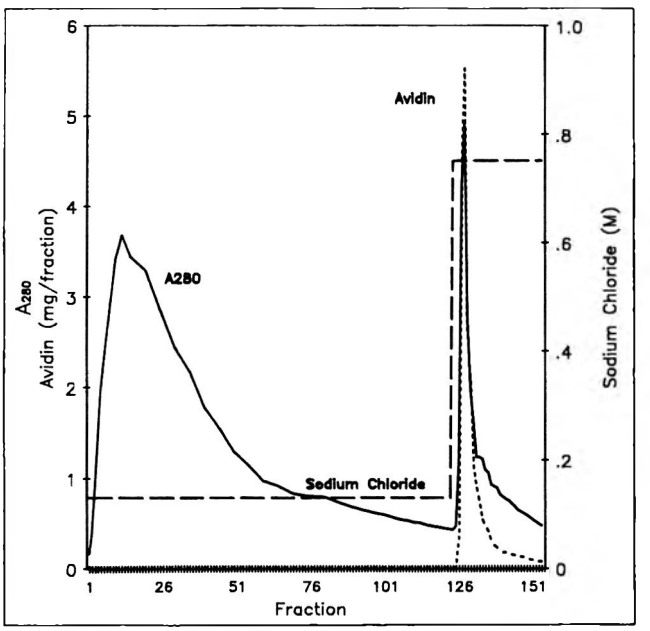

Fig. 3.—Elution profile of adsorbed egg white proteins from Duolite C-464 (170 mL resin) using a two-step elution process.

Table 4—Results of lysozyme-avidin separation from egg white by Duolite C-464 chromatography with multi-cycle elution looping

Elution conditions[b]	Avidin purity (mg avidin/ mg protein)	Avidin yield (%)	Response index[a]
3 cycles on 170 mL column	0.075	77.0	5.79
5 cycles on 170 mL column	0.129	80.4	10.37
8 cycles on 470 mL column	0.295	79.3	23.34
16 cycles on 25 mL column	0.409	74.0	30.27

[a] Response index = avidin purity (mg avidin/mg protein) × avidin recovery (%).

Small pilot-scale trial

The question of process scale-up is an important aspect of any procedure which has potential for industrial application. Although space and equipment restrictions precluded truly large-scale experiments in this study, an experiment was completed using a 470 mL (5.0 cm × 24 cm) resin column. A total of 14.2L of egg white was applied to the column in eight cycles over a 6-day period. Although the egg white was kept at 4°C except when actually on the column, the column and apparatus were at room temperature. The results are summarized in Table 4. SDS-PAGE profiles of representative fractions are presented in Fig. 5.

Capacity of Duolite C-464

The success of the five- and eight-cycle experiments raised a question as to the limits of elution looping; that is, how many cycles could be accommodated before avidin or lysozyme response would diminish. The lysozyme capacity of Duolite C-464 has been examined under similar conditions (Li-Chan et al., 1986). Approximately 19.3 mg lysozyme was applied per mL of Duolite C-464 before retention dropped below 90%. If the egg white applied per cycle per mL resin was 4 mL and lysozyme content was 3.5 mg/mL egg white, then approximately 35% of the resin ion exchange groups would be available for avidin. This is sufficient to adsorb 8.7 mg of avidin/mL resin or 40 to 50 cycles of egg white application, depending upon the exact concentration of avidin in the egg white. Although a 40-cycle trial was not undertaken, a 16-cycle experiment was performed on a small 25 mL column (Table 4 and Fig. 6) with substantial further improvement in avidin purity.

In practical terms, it is doubtful whether extension of the technique to the apparent limit of 40 to 50 cycles would result in proportional improvement in response. Purity of recovered avidin was plotted against the total amount of avidin loaded

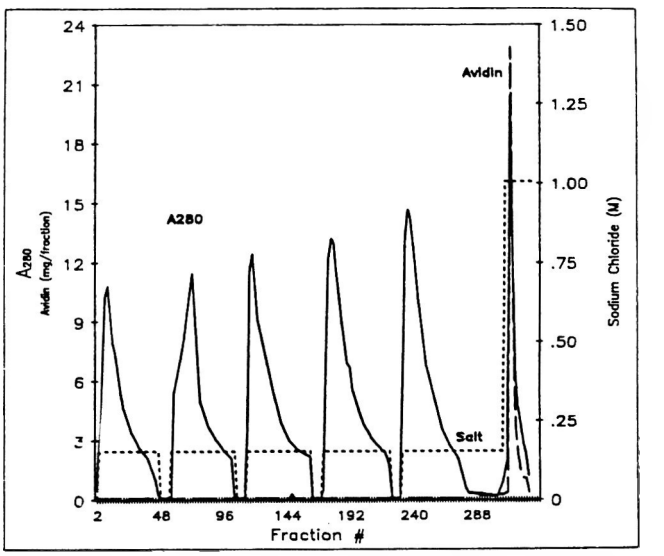

Fig. 4.—Profile of adsorbed egg white proteins eluted from a Duolite C-464 column (170 mL) by the "elution looping" process. Five cycles of egg white application and lysozyme elution were followed by a single elution of avidin.

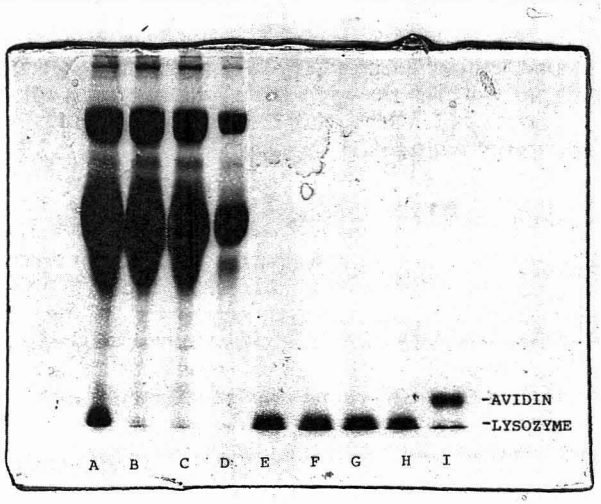

Fig. 5.—SDS-PAGE of proteins recovered from egg white with eight cycles of egg white application and lysozyme elution followed by a single avidin elution: (A) untreated egg white; (B) 1st cycle eluted egg white; (C) 4th cycle eluted egg white; (D) 8th cycle eluted egg white; (E) 1st cycle lysozyme; (F) 3rd cycle lysozyme; (G) 6th cycle lysozyme; (H) 8th cycle lysozyme; (I) avidin fraction.

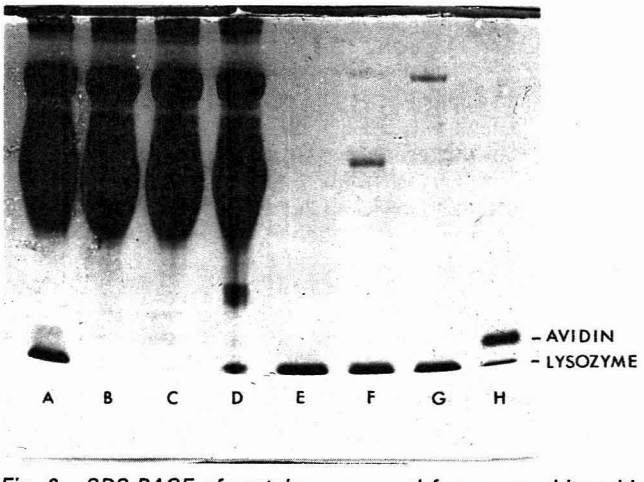

Fig. 6.—SDS-PAGE of proteins recovered from egg white with 16 cycles of egg white application and lysozyme elution followed by a single avidin elution: (A) untreated egg white; (B) 1st cycle eluted egg white; (C) 8th cycle eluted egg white; (D) 16th cycle eluted egg white; (E) 1st cycle lysozyme; (F) 8th cycle lysozyme; (G) 16th cycle lysozyme; (H) avidin fraction.

onto Duolite C-464 (Fig. 7). The avidin purity appeared to be approaching a maximum well before the estimated maximum resin loading of 8.7 mg/mL.

Protein-Duolite C-464 interaction

The net charge of a protein at a given pH has commonly been used to predict its behavior on ion-exchange resins (Kopaciewicz et al., 1983). The net charge of a protein is zero at its isoelectric point (pI), and increasingly positive as the pH falls or increasingly negative as the pH rises from the pI. According to the net charge approach, lysozyme (pI = 10.7) should bind more strongly than avidin (pI = 10.0) to a cation-exchange resin at pH 7.9. In fact, this has been shown to be true when the cation exchanger is carboxymethylcellulose (Durance and Nakai, 1988). However, the reverse situation was observed on Duolite C-464. Either the net charge concept was inadequate to explain the electrostatic binding of these proteins to Duolite

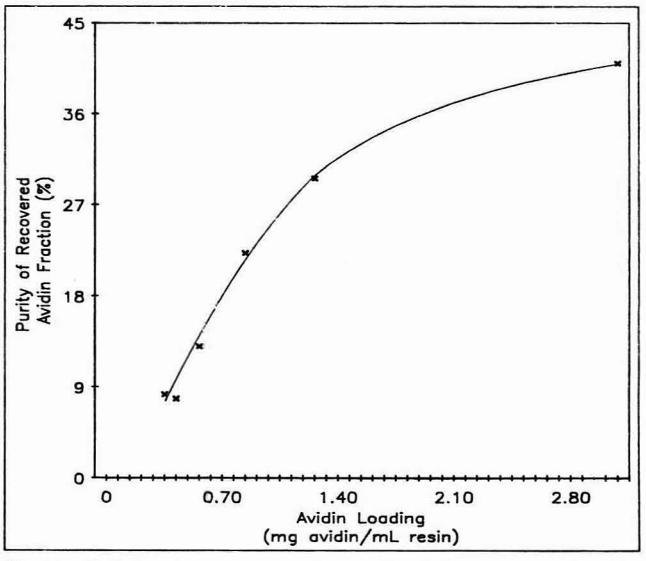

Fig. 7.—Relationship between the purity of the recovered avidin fractions and the concentration of accumulated avidin on the Duolite C-464 column.

C-464 or some other binding mechanism contributed to the retention on the column.

Duolite C-464, a co-polymer of methacrylic acid and divinyl benzene, has significant potential for hydrophobic interaction with proteins due to its aromatic ring content. This raises the question of whether hydrophobic interaction might favor the retention of avidin on the column and account for the unusual elution pattern.

A recently proposed model of protein interaction on ion exchanger allows estimation of electrostatic and hydrophobic binding parameters by examining the effect of neutral salt concentration in the eluant on protein retention (El Rassi and Horvath, 1986; Horvath et al., 1985). Although this model was developed for use with high-performance ion-exchange chromatography data where considerably greater precision was possible, it appeared applicable to open column data as well, albeit with greater experimental error. Capacity factors (k) were determined for avidin and lysozyme at seven different ammonium sulfate concentrations in 25 mM sodium phosphate buffer, pH 7.3. Although pH 7.9 would have been more in keeping with the experimental conditions employed previously, 25 mM sodium phosphate had insufficient buffering capacity at the higher pH. A higher phosphate concentration might have contributed unduly to the total salt concentration and obscured the effect of ammonium sulfate concentration. The data were fitted to the following equation:

$$\log k = A + B \log m + C \cdot m \qquad (4)$$

where k is the capacity factor, m is the molality of ammonium sulfate, and A, B, and C are parameters for a given solute, solvent, resin, pH and salt combination (Fig. 8). The values of these constants together with R^2, the coefficient of determination, are presented in Table 5. Parameters B and C are of particular interest. B, the limiting slope at sufficiently low salt concentration, is a measure of solute-resin interaction in conditions which maximize electrostatic interaction and minimize hydrophobic interaction. C is the corresponding parameter in charge quenching, high salt conditions, in which electrostatic interactions are insignificant and hydrophobicity governs protein-resin interactions.

In the case of avidin and lysozyme, the absolute values of both B and C were greater for avidin, indicating a greater potential for both electrostatic and hydrophobic interaction with Duolite C-464 at appropriate salt concentration. The manner in which these observations and data were to be applied to the

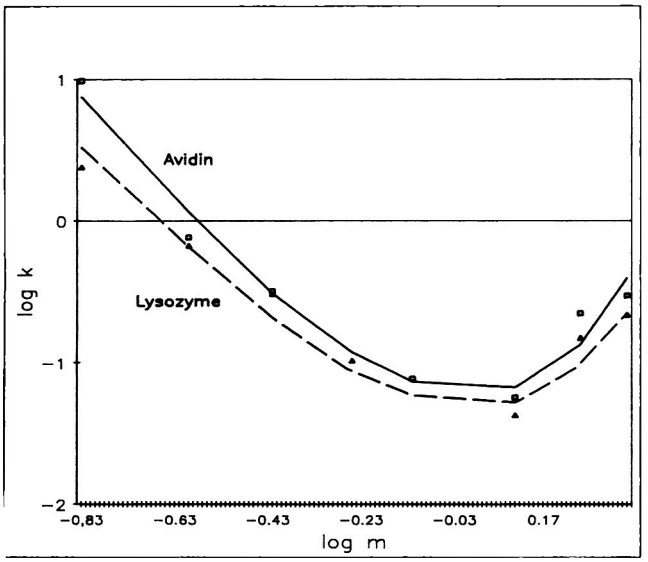

Fig. 8.—Plot of logarithmic capacity factors of lysozyme (▲) and avidin (□) against the logarithm of ammonium sulfate molality (log m). Elution was isocratic with 25mM NaP buffer, pH 7.3, at different ammonium sulfate concentrations.

Table 5—Electrostatic (B) and hydrophobic (C) interaction parameters for retention of avidin and lysozyme on Duolite C-464. Intercept (A) and R^2 of multiple regression are also included

	A	B	C	R^2
Avidin	−3.16	−4.53	1.93	0.9653
Lysozyme	−2.95	−3.90	1.63	0.9026

Table 6—Retention volumes of lysozyme and avidin absorbed from egg white onto Duolite C-464 and eluted with linear gradients of four different salts

		Avidin		Lysozyme	
Eluting[a] salt	σ[b]	Eluant volume (mL)	Salt (M) at elution	Eluant volume (mL)	Salt (M) at elution
NaCl	1.64	100	0.40	60	0.24
NH_4Cl	1.39	71	0.28	44	0.17
$(NH_4)_2SO_4$	2.16	35	0.32	25	0.22
Na_2HPO_4	2.02	10	0.04	15	0.06

[a] Columns were equilibrated with 0.1M NaP, pH 7.5 and all gradients were in the same buffer.

[b] Molal surface tension increments of eluting salts.

adsorption of lysozyme and avidin from egg white was not immediately apparent. The differences in pH (7.9 vs 7.3) may alter the interaction slightly but experiments in this laboratory have demonstrated that the general elution pattern with phosphate buffers and sodium chloride gradients was similar between pH 7 and pH 9 (data not shown). In retrospect, the determination of B and C in sodium chloride solutions rather than ammonium sulfate would have simplified interpretation. However, preliminary experiments showed that the elution order of lysozyme and avidin was the same in gradients of these two salts (Table 6). The fact that avidin was adsorbed more strongly at high ammonium sulfate molality seemed to suggest that hydrophobic forces might help to explain the elution order. On the other hand, contradictory evidence also existed. When avidin and lysozyme were chromatographed on aliphatic hydrophobic interaction chromatography (HIC) or Phenyl-Sepharose columns, lysozyme was found to bind more strongly than avidin (Durance and Nakai, 1988). This does not necessarily conflict with the results obtained with Duolite C-464, since the accessibility and density of hydrophobic regions in both the resin and the protein are known to affect the capacity and selectivity of HIC (Hofstee and Otillio, 1978). However, it does seem that there is not a clear-cut relationship between protein HIC binding potential and retention on Duolite C-464.

Also, although retention of avidin and lysozyme at high ammonium sulfate molality was clearly demonstrated, under the conditions used for isolation from egg white, both proteins were bound at low salt concentrations and ultimately eluted with increasing salt. Since increasing salt concentration would tend to strengthen hydrophobic bonds and only weaken electrostatic bonds, electrostatic interactions must play a dominant role. The interaction parameters as determined here confirmed that the electrostatic interaction of avidin with Duolite C-464 was also greater than that of lysozyme.

A final indication of the dominant role of electrostatic forces in this separation was obtained from the effect of different neutral salts on the elution pattern. In preliminary experiments, gradients of different salts (ammonium sulfate, sodium phosphate, ammonium chloride and sodium chloride) were used to elute lysozyme and avidin from Duolite C-464. Elution volumes with each salt are presented in Table 6. According to the solvophobic theory of hydrophobic interaction chromatography, the strength of salt-mediated hydrophobic bonds is directly proportional to the molal surface tension increment (σ) of the salt (Melander and Horvath, 1977). In this case, a comparable trend was not observed. If, on the other hand, one assumed that the relative retention of avidin and lysozyme on Duolite C-464 was primarily an electrostatic event, but one which was not completely defined by the net charge of the proteins, the observed phenomena were consistent with previously reported results.

Kopaciewicz et al. (1983) examined the influence of various salts on protein retention by strong sulfonic acid cation exchangers. They found that the nature of both the cation and the anion in the salt gradient affected protein retention times and that different proteins responded differently to different salts. In the case of lysozyme, the retention time was greater in sodium chloride gradients than in the sodium phosphate as observed here. The observation that the net charge does not adequately predict the ion-exchange behavior of proteins even in the absence of significant hydrophobic binding has been reported as well (Kopaciewicz et al., 1983). The phenomena have been ascribed to charge asymmetry on the protein molecule. Even at its pI, where net charge is zero, proteins have many charged groups on their surfaces. If the charge distribution is not uniform, this will lead to an uneven electrostatic potential depending on the orientation of the molecule with respect to the charged groups on the ion-exchange resin. Thus, proteins may be oriented on the ion exchanger in a non-random fashion, leading to deviation from the ideal relationship between net charge and protein binding (Gorbanov et al., 1986).

CONCLUSIONS

A SINGLE COLUMN cation exchange method which allowed simultaneous recovery of lysozyme and avidin as separate peaks from undiluted egg white was developed. Lysozyme was recovered with high purity and at higher yields than reported for the isoelectric precipitation methods often used in the industry (86% vs 60–80%). Avidin recovery was also as good as or better than previously reported ion exchange methods (74–80% vs 60–80%). The purity of the avidin fraction (up to 40.9%) was higher than previously reported preparations, although lower than is desirable for some applications.

Avidin was shown to have a greater potential for both electrostatic and hydrophobic interactions with Duolite C-464 than lysozyme. Under the chromatographic conditions of this separations method, however, electrostatic interactions were dominant. Finally, the "elution looping" method described and used in this study might prove valuable in other protein purifications where a trace protein must be separated from a more abundant protein of similar charge.

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Functional Properties of Heat-Treated Egg Yolk Low Density Lipoprotein

TOMOMI TSUTSUI

ABSTRACT

Egg yolk low density lipoprotein (LDL) was heated at various temperatures from 55°C to 100°C for 5 min and transmission, extractability of lipid, emulsifying property and foaming property of each solution were examined. Transmission of LDL solution decreased for treatments above 60°C and was almost 0 at 75°C. Above 70°C, lipid extractability gradually decreased with increase of treatment temperature. Emulsifying property and apparent viscosity were significantly correlated with the extractability of lipid.

INTRODUCTION

EGG YOLK is an excellent emulsifyer. A main component of egg yolk is the low density lipoprotein (LDL). Its emulsifying capacity is higher than that of high density lipoprotein (HDL), and the diameter of the LDL emulsion is smaller than that of the HDL emulsion (Oshida, 1976). However, lipoproteins are usually denatured more readily than other proteins (Lea, 1957), for example, above 60°C, the lipoprotein is denatured irreversibly (Franzen et al., 1969). Parkinson (1970) observed denaturation of lipoprotein complex from heat-treated whole egg. Emulsifying properties of egg yolk were not damaged by moderate heat treatments (Miller and Winter, 1951; Palmer et al., 1969; McCready et al., 1971; Varadarajuru and Cunningham, 1972; Cotterill et al., 1974), but there are no reports concerning the functional properties of heat-treated LDL. The purpose of present study was to provide information on the chemical and functional properties of heat-treated LDL.

MATERIALS & METHODS

Preparation of low density lipoprotein (LDL)

Day old eggs were obtained from one strain of leghorns (hy-Line). Egg yolk was separated from egg white, pooled and diluted with an equal weight of 0.16 M NaCl. This solution was mixed for 30 min at 4°C, then centrifuged at 45,000 G for 30 min under refrigeration. The plasma (upper layer) was collected, adjusted to 1M NaCl concentration and centrifuged at 90,000x *g* for 16 hr under refrigeration. The floating LDL was collected, dissolved in 1M NaCl solution and centrifuged using the same conditions. The floating LDL was collected and dialyzed against 0.05M Tris HCl buffer, pH 8.0.

Heat treatment

A four percent LDL solution (5 mL) was heated at various temperatures from 55°C to 100°C for 5 min; then each solution was cooled in ice water.

Analyses

The absorbance of the LDL solutions at 650 nm was measured using a model 100-60 Hitachi Seisakusho spectrophotometer to obtain turbidity. The apparent viscosity was determined with a model EDL, tokyo keiki viscometer at 25°C and shear rate and shear stress were calculated using revolutions per minute (rpm) and a radius of a rotor of the viscometer.

Author Tsutsui is with the Seitoku Junior College of Nutrition, Nishishinkoiwa 1, Katsushika-ku, Tokyo, 124, Japan.

Preparation of antisera

Antisera against LDL were prepared from rabbits as follows: the antigen emulsion with Freunds incomplete adjuvant was injected three times into the ear at 1-wk intervals, followed by one intravascular injection as a booster.

Agar gel precipitation reaction

Agar gel precipitation reaction was conducted according to the method of Ouchterlony (1963). An agar gel (1.5% dissolved in a saline solution) was used. Antiserum to LDL was added into the center well, LDL solution (native or heat-treated LDL solution) was added into the other wells and then incubated for 18 hr at 37°C.

Extraction of lipid

Five milliliters of ethyl ether were added to 5 mL of a 4% LDL solution (native or heat-treated LDL solution), mixed well and left for 24 hr. Then the other layer (upper layer) was collected. The lower layer was washed twice using 5 mL ether. The ether layers were pooled, dried by heating and the amount of lipid was determined by weight.

Emulsifying tests

The emulsifying activity index (EAI) of Pearce and Kinsella (1978) was used with minor modifications. Three milliliter of 0.5% LDL solutions and 1 mL corn oil were blended in a Nihon Seiki Ace Homogenizer AM-11 at 12,000 rpm for 1 min. After 1 min standing, an aliquot of the emulsion was taken from the bottom of the vessel and diluted 200 times with 0.05M Tris HCl buffer, pH 8.0 containing 0.1% sodium dodecyl sulfate. The absorbance at 500 nm was measured in a Hitachi 100-60 spectorphotometer. Emulsifying capacity (EC) was determined by an oil titration method. Emulsions were prepared in a glass tube equipped with electrodes, attached to a Nihon Seiki Ace Homogenizer (AM-11). Two milliliters of 0.5% protein

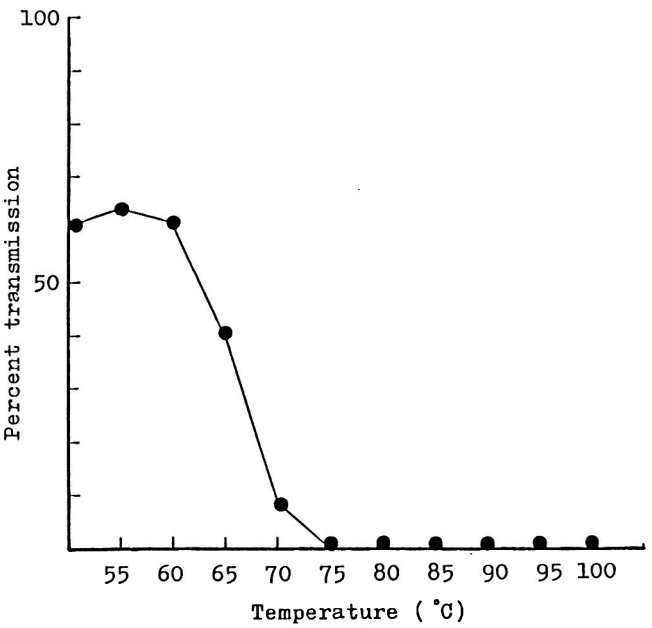

Fig. 1—Turbidity of LDL as a function of heating temperature.

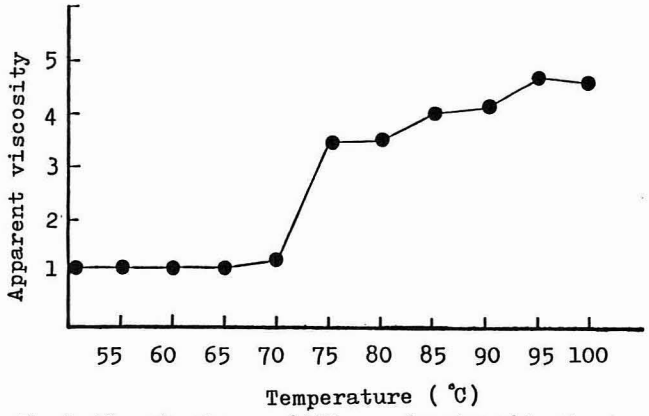

Fig. 2—Viscosity change of LDL as a function of heating temperature.

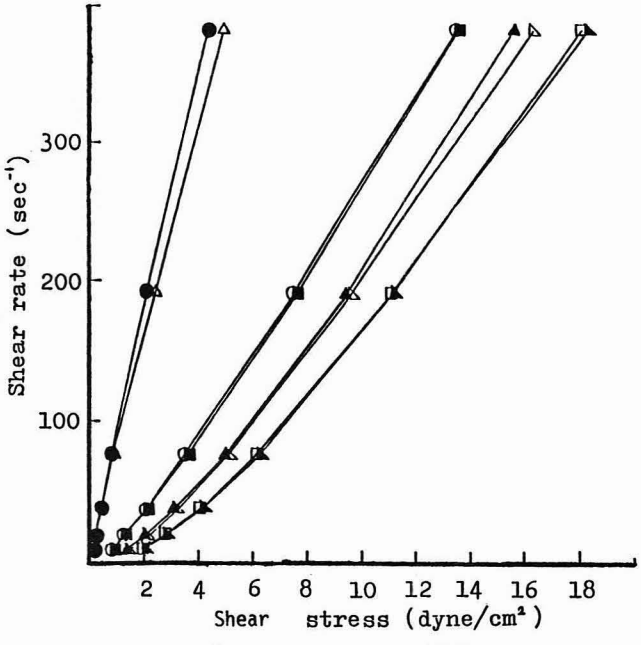

Fig. 3—Shear rate-shear stress curves of LDL as a function of heating temperature. (●, native ~ 65°C; △, 70°C; ○, 75°C; ■, 80°C; ▲, 85°C; ◺, 90°C; □, 95°C; ◣, 100°C).

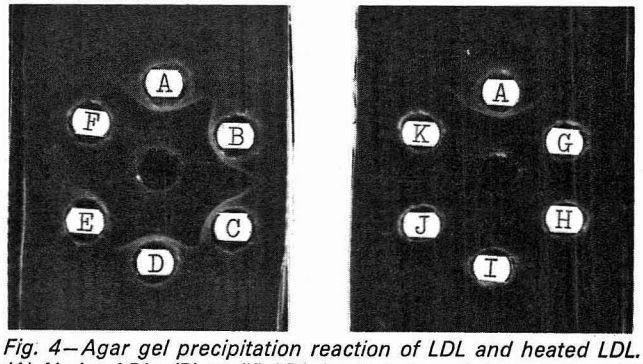

Fig. 4—Agar gel precipitation reaction of LDL and heated LDL. (A) Native LDL; (B) to (K) LDL heated at each temperature (B) −55°C; (C) −60°C; (D) −65°C; (E) −70°C; (F) −75°C; (G) −80°C; (H) −85°C; (I) −90°C; (J) −95°C; (K) −100°C.

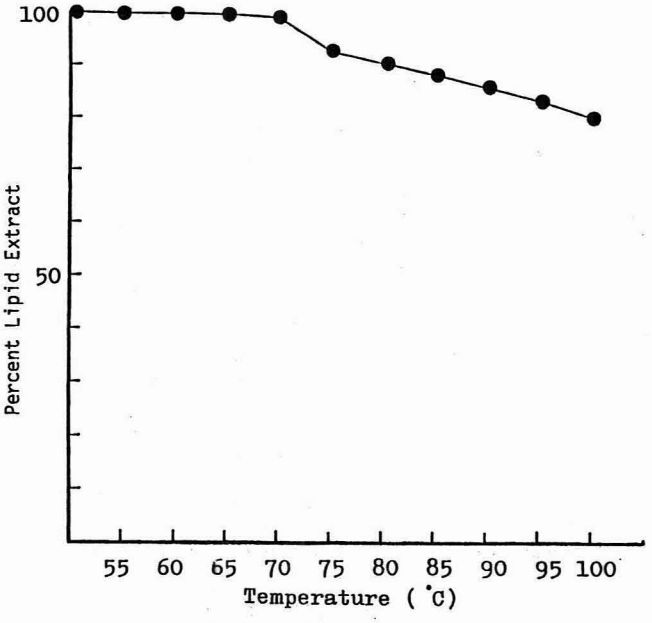

Fig. 5—Percent lipid extracted as a function of heating temperature.

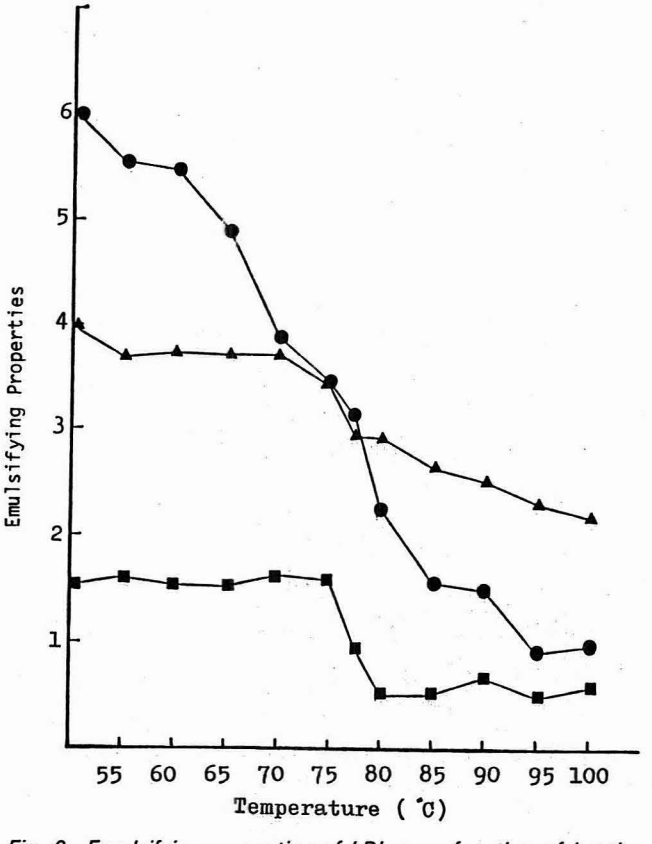

Fig. 6—Emulsifying properties of LDL as a function of heating temperature. [●−, Emulsifying activity index ×10⁻¹ (m²/gl); ▲−, emulsifying capacity (mL); ■−, emulsifying stability index (min)].

solution were titrated with corn oil at a flow rate of 0.6 mL/min mixing at 10,000 rpm. Oil addition was terminated when phase inversion was indicated by a sudden increase in electrical resistance on the volt-ohm meter. The weight of oil added was calculated by the difference in weight of the glass tube before and after oil titration; the weight was converted to volume (specific gravity = 0.926). Emulsion stability was determined by the method of Peace and Kinsella (1978). To prepare an emulsion, 1 mL corn oil and 3 mL 0.5% LDL solution in 0.05M Tris HCl buffer, pH 8.0, were homogenized in a Nihon Seiki Ace Homogenizer AM-11 at 12,000 rpm for 1 min at 20°C. A one-tenth milliliter portion of the emulsion was taken from the bottom of the container at 1 min intervals immediately after homogenization and diluted with 5 mL of 0.1% SDS solution. The turbidity of the diluted emulsion was then monitored at 500 nm with time.

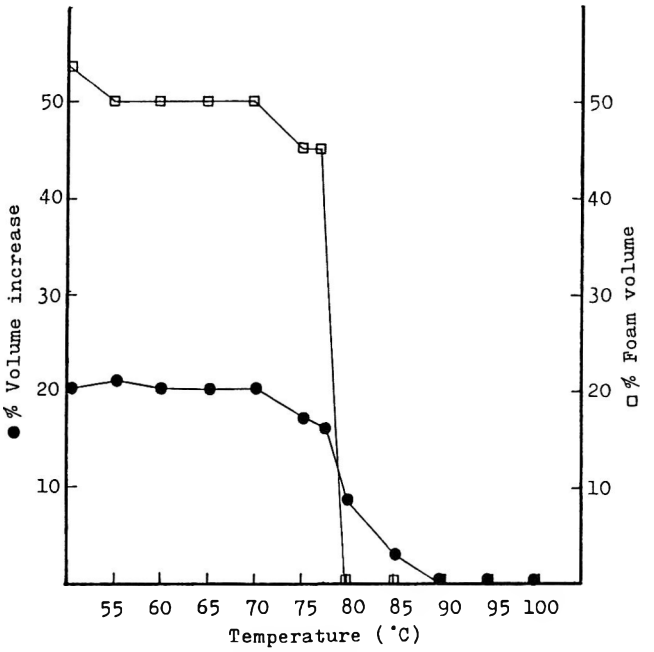

Fig. 7—Foaming properties of LDL as a function of heating temperature.

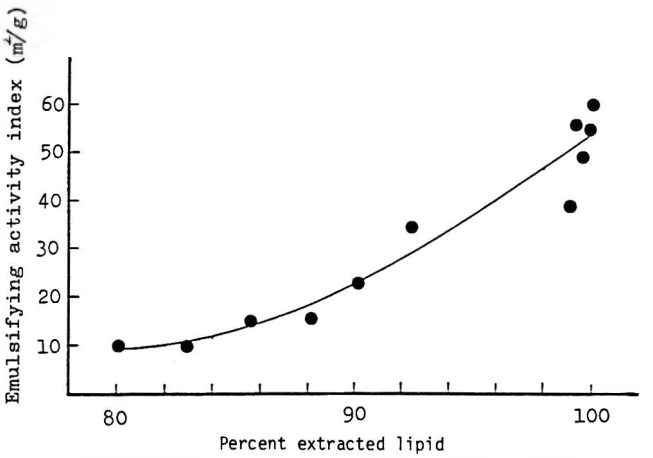

Fig. 8—Relationship between emulsifying activity index and percent extracted lipid.

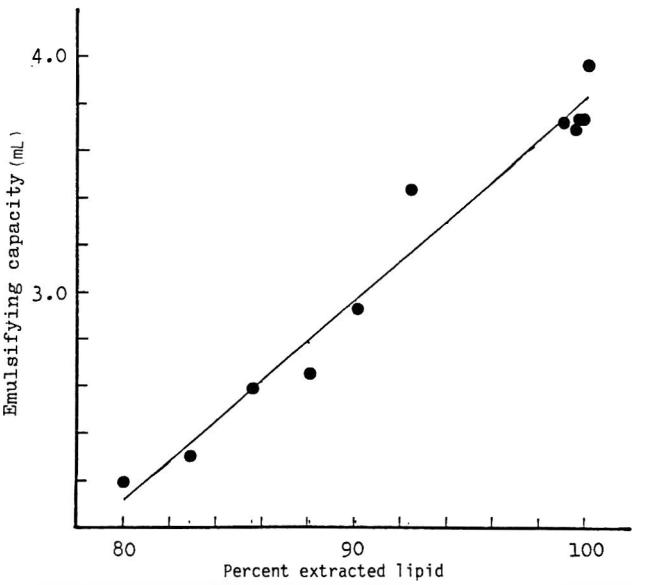

Fig. 9—Relationship between emulsifying capacity and percent extracted lipid.

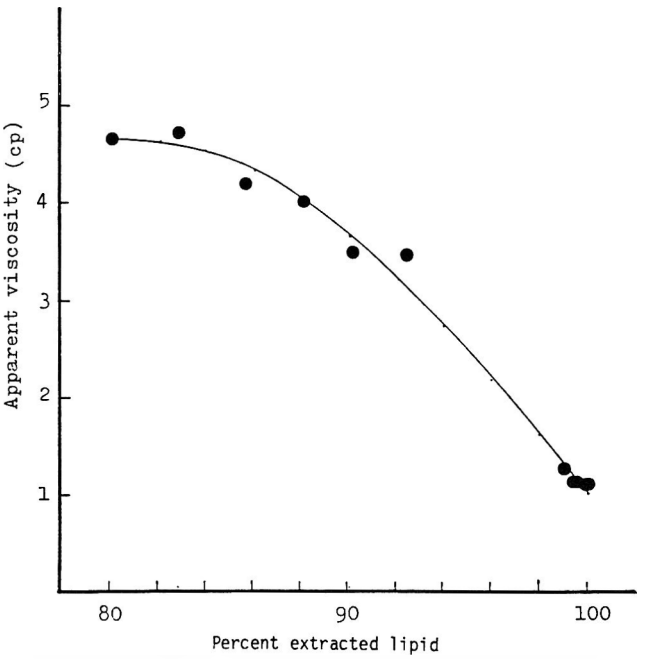

Fig. 10—Relationship between apparent viscosity and percent extracted lipid.

Foaming test

Foaming capacity and foam stability were determined by the method of Tasneem and Subramanian (1986). Fifteen milliliters of 1% LDL solution (native and heat-treated LDL) were blended for 5 min in a Nihon Seiki Ace Homogenizer (AM-11) and immediately transferred to a 50-mL measuring cylinder. The total (liquid and foam) and the liquid volumes were recorded at 0.5 and 30 min after whipping; foam stability was expressed as percent foam volume after 30 min.

RESULTS & DISCUSSION

BANASZAK and McDonald (1962) reported that heat-treated LDL (50°C for 1 hr) maintained its original characteristics. Transmission of heat-treated LDL solutions (60°C for 5 min) was almost the same as that of native LDL but contained a small amount of aggregated product with eluted in the void volume upon gel filtration (Tsutsui and Obara, 1980). At treatments above 65°C, the transmission of LDL suddently decreased with an increase in temperature, and the transmission was 0 at 70°C (Fig. 1). The apparent viscosity curve for LDL also indicates a sudden increase at treatments above 70°C (Fig. 2). Below 70°C, LDL solutions followed Newtonian flow behavior (Fig. 3). Throughout a broad shear rate range, heat-treated LDL (below 70°C) indicated almost the same apparent viscosity. But above 70°C, the LDL solutions followed non-Newtonian flow behavior and the apparent viscosity decreased with an increase of shear rate. These data indicated that the aggregation product of LDL was destroyed by the increase of shear rate.

Agar gel precipitation reaction of LDL and heat-treated LDL displayed the precipitation reaction below 70°C, but above 75°C, heat-treated LDL did not give the precipitation reaction (Fig. 4). Perhaps, the LDL heated above 75°C could not enter into the agar gel due to aggregation.

The amount of extracted lipid from heat-treated LDL decreased above 75°C. The extracted lipid at 75°C ws 92.4% of that of native LDL, then it decreased with an increase of the treatment temperature (Fig. 5). The aggregation product of LDL may contain lipid in the interior of the structure, and the

protein portion may play an important role in aggregation between LDL particles.

There is increasing evidence that the emulsifying properties of egg yolk are not damaged by moderate heat treatment (Miller and Winter, 1951; Palmer et al., 1969; McCready et al., 1971; Varadarajuru and Cunningham, 1972; Cotterill et al., 1974). Cotterill et al. (1976) reported that pasteurized egg yolk containing 10% sodium chloride had the same emulsifying capacity as that of native egg yolk. Emulsifying activity of heat-treated LDL gradually decreased with an increase of the treatment temperature (Fig. 6), but emulsifying capacity and emulsifying stability of heat-treated LDL decreased only above 75°C. These results were in good agreement with the morphological change of LDL. Graham and Kamat (1973) made madeira cake using egg yolk and reported that the plasma of egg yolk played an important role as a foaming agent. A main component of plasma is LDL. Native LDL displayed weak-foaming properties, but the foaming capacity and foam stability decreased greatly above 77.5°C (Fig. 7).

We suggest a relationship between morphological change of LDL and the functional properties of LDL. Based on the above results, the percent extracted lipid (PEL) was selected as an index of morphological change of LDL, because PEL underwent a large decrease above 75°C. Figure 8 indicates the relationship between PEL and emulsifying activity index. Linear regression analysis after linearization showed that PEl was significantly correlated with the regression equation:

$$Y_1 = 1112.967 - \frac{17646.851}{X} + \frac{7052629.608}{X}$$

$$(r = 0.967,\ n = 11\ p<0.001)$$

where Y_1 = Emulsifying Activity Index and X = PEL. The relationship between PEL and emulsifying capacity is shown in Fig. 9. PEL also significantly correlated with emulsifying capacity with the regression equation:

$$Y_2 = 19.197 - \frac{2222.688}{X} + \frac{68622.671}{X^2}$$

$$(r = 0.984,\ n = 11,\ p<0.001)$$

where Y_2 = Emulsifying Capacity and X = PEL. The relationship between PEL and the apparent viscosity according to the regression equation:

$$Y_3 = -96.348 + \frac{16458.962}{X} - \frac{667142.758}{X^2}$$

$$(r = 0.996,\ n = 11,\ p<0.001)$$

where Y_3 = Apparent Viscosity and X = PEL.

In conclusion, it was confirmed that functional properties and the apparent viscosity of heat-treated LDL were significantly correlated with the percent extracted lipid (Fig. 10) and that a morphological change of LDL, induced by heating, affected the functional properties of LDL.

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Emulsifying Properties of Food Proteins: Bovine Micellar Casein

ZAHURUL HAQUE, JACEK LEMAN, and JOHN E. KINSELLA

ABSTRACT

The capacity of micellar casein isolated from bovine milk to stabilize oil in water (o:w) emulsions was studied. Emulsion surface area per unit mass of protein, i.e., emulsifying activity (EA), was highest at a protein concentration of 1% and o:w ratio of 1:9. The surface concentration of micellar casein was related to the o:w ratio being highest at an o:w of 4:6. Interfacial surface area decreased at pH 6.7 reflecting micellar stability and improved with increasing pH apparently because of destabilization of the casein micelle. Emulsion stability was high when the EA was high. The apparent viscosity of the emulsions was inversely related to EA.

INTRODUCTION

PROTEINS with differing sizes, flexibility, charge, hydrophobicity, and association tendencies may display different emulsifying properties (Haque and Kito, 1984; Kinsella, 1984; Halling, 1981). Milk caseins are flexible, amphipathic and possess good surface activities (Kinsella and Whitehead, 1987). The average hydrophobicities of α_s-, β- and κ-caseins are 4.9, 5.6, and 5.4 KJ mol^{-1}, respectively (Bigelow, 1967). This imparts a strong tendency for noncovalent self-association that contributes to the formation of the casein micelle which is the dominant protein constituent (78–85%) of milk.

The relative efficiency of caseins in the micellar state compared to the nonmicellar casein in emulsion stabilization, is not well characterized although it is known that casein micelles and sodium caseinate have different adsorption properties (Morr, 1981). Casein micelles remain intact during homogenization (Walstra and Jenness, 1984), can withstand high temperatures (Schmidt, 1982) and are easily redispersed after the drying of milk (Dickenson and Stainsby, 1982). Their relative capacities in stabilizing emulsions is of practical interest for the use of milk powders.

This study was conducted to determine the emulsifying properties of micellar casein using a laboratory homogenizer under conditions that allowed control of all variables including energy input.

MATERIALS & METHODS

RAW SKIMMED MILK (Holstein) was obtained from the Cornell Dairy. Peanut oil was commercial edible grade (921 kg/m^3). α-casein and Coomassie Brilliant Blue R-250 were from Sigma Chemical Co. (St. Louis, MO). All other reagents were analytical grade.

Preparation of Micellar Casein

The raw skimmed milk was further delipidated by centrifugation at 4068× *g* (5000 rpm) (GSA rotor) in a Sorvall RC-5 superspeed refrigerated centrifuge for 20 min at 20°C. The mat of milk lipids (top layer) that was formed was carefully removed using a spatula and the centrifuged milk was then filtered (Whatman #1) to remove all traces of precipitate and separated fats. The filtrate was centrifuged at 45,440 × *g* (19500 rpm) for 20 min at 20°C in the same centrifuge using a SS-34 rotor. The supernatant thus obtained was discarded and the precipitate, comprising the casein micelles, was redispersed in Jenness-Koops buffer (Jenness and Koops, 1962) at pH 6.7 and again centrifuged under the same conditions. The resulting precipitate was analyzed for the protein content, used in the emulsification experiments or freeze dried and stored over desiccant at −10°C. The freeze dried micellar casein dispersed in Jenness-Koops buffer yielded data that were identical to the freshly prepared micellar casein. The protein concentration was determined by the modified Lowry method of Wang and Smith (1975). α-Casein was used as the reference protein to determine the protein content.

Emulsification activity

Emulsions were prepared under constant conditions including energy input using a recirculating valve homogenizer (Tornberg and Lundh, 1978; Haque and Kinsella, 1988a). The inlet pressure was held constant at $414 \cdot 10^3$ Pa, the mean stroke volume was 4.2 mL, load volume was 20 mL and all experiments were conducted at (25°C). Distilled water was used to calibrate the valve homogenizer. The valve load was adjusted to produce a valve head pressure of 3.44×10^6 Pa for water which gave a mean energy input per unit volume of ca. 3.8 J 10^6 m^{-3} for the emulsions. The energy input per unit volume (E) was calculated on the basis of the valve head pressure (ΔP_{max}) and the time of valve lift (T_v) as described (Haque and Kinsella, 1988a).

The freeze-dried micellar casein was dispersed in the Jenness-Koops buffer (Jenness and Koops, 1962) that simulates the salt and mineral composition of the milk plasma. The pH was carefully adjusted to the desired level (6–9). Aliquots of the casein dispersion were weighed and mixed with an appropriate amount of oil in a Janke-Kunkel TP18-10 blender (5 sec) and the mixture was then immediately transferred to the hopper of the valve homogenizer and emulsified as described. The O:W ratio is referred to in the text as 1,2,3 and 4 and represents 10, 20, 30, and 40% oil, respectively. After 20, 40, 60, 120, 180, 240, 360, and 480 strokes (corresponding to energy inputs of 76, 152, 228, 456, 684, 912, 1368, and 1824 10^6 J m^{-3}, respectively), duplicate samples of the emulsion were pipetted, diluted immediately in 0.5% SDS in 10 mM imidazole buffer, pH 6.7 and the turbidity measured at 600 nm to calculate the total surface area of the emulsion i.e. emulsifying activity (Pearce and Kinsella, 1978).

Creaming stability

The creaming stability of the emulsion was determined because it is an important event in emulsion instability (Tornberg, 1978). There is a difference in density between the dispersed phase and the continuous phase. To determine the creaming stability of the emulsions, a gravitational field was applied to the emulsion by centrifuging the freshly prepared emulsion in 5 mL disposable glass tubes using an IEC desk-top clinical centrifuge at 769 × *g* (25°C). After centrifugation times of 2, 4, 8, and 16 min, emulsion samples (10 μL) were pipetted from the bottom of the centrifugation tube and immediately diluted in the SDS solution to determine surface area per unit mass of protein as described (Pearce and Kinsella, 1978).

Surface concentration of micellar casein (Γ)

The Γ of micellar casein in oil-in-water emulsions under different conditions was determined. Emulsions were centrifuged at 769 × *g* (25°C) for 50 min. The resulting mat (top layer), comprised of emulsified oil droplets plus the proteins adsorbed in the interfacial film, was carefully removed. The centrifugation step was repeated thrice and the protein content of the final aqueous supernatant was determined. The difference between the initial protein concentration (C_p)

Authors Leman, and Kinsella are with the Inst. of Food Science, Cornell Univ., Ithaca, NY. 14853. Author Haque is now with the Dairy Science Dept., Mississippi State Univ., Drawer DD, Mississippi State, MS 39762.

of the dispersion and the concentration in the final aqueous supernatant corresponded to the protein adsorbed to the oil at the interface (C_i). This value was used instead of the original concentration of protein to estimate interfacial protein concentration (Haque and Kinsella, 1988b).

Apparent viscosity

To determine if viscosity was related to emulsion activity and stability, the apparent viscosity of the casein stabilized emulsion was measured directly during homogenization by monitoring pressure at the valve head (Haque and Kinsella, 1988a). The maximum valve head pressure drop (ΔP_{max}) was measured using a pressure transducer coupled to a laboratory computer through a suitable analog/digital converter. Data were acquisitioned at the rate of 20/sec and third degree polynomial fits (least square) of the data points were recorded for each of the conditions studied for comparison with the emulsion activity data.

RESULTS & DISCUSSION

THE EMULSION SURFACE area formed per unit mass of micellar casein under constant condition of pH, temperature, and protein concentration increased with energy input (Fig. 1). The optimum surface area formed at a 1% protein concentration and o:w ratio of 4 (pH 6.7) for micellar casein was <100 m^2/g protein compared to >450 m^2/g for 0.5% acid precipitated casein under the similar conditions (Haque and Kinsella, 1988c).

Energy input had a direct effect on the formation of new surface. At all the concentrations of protein (1 to 3% weight/volume of oil-water mixture), surface area per unit mass of protein decreased as the o:w ratio was increased, e.g., the optimum surface area at a constant protein concentration of 1%, pH 6.7, was 245 m^2/g at an o:w ratio of 1 compared to 90 m^2/g at an o:w ratio of 4 (Fig. 1). This may reflect a higher surface concentration of protein (Γ) at the higher o:w ratio (4) compared to lower o:w ratio (<4 of oil) (Fig. 2), e.g. 12 mg protein per m^2 surface area at o:w = 4 compared to 6 mg at o:w = 1. This observation was similar to the observations with BSA where the Γ was related to the o:w ratio (Haque and Kinsella, 1988b).

In an emulsion, proteins in the bulk solution migrate to, and concentrate at the interface depending on the amphipathicity of the protein (Graham and Phillips, 1979a; Haque and Kito, 1984). The surface concentration of the protein at the interface may reflect its affinity for and retention at the interface. The decreased EA of micellar casein compared to nonmicellar casein may be because the latter can spread more easily at the interface (Morr, 1982) whereas the casein micelles are large (mean diameter ~120 nm) and unable to cover a comparable surface area. The large velocity gradient at the valve-head of the homogenizer (Haque and Kinsella, 1988a) and viscous forces may disrupt the casein micelles to some extent resulting in an increase in the total number of colloidal particles available for interaction. However, the number of micellar casein particles may have limited coverage of the oil surface area even at the highest protein concentration (3%) used in this study.

There was a marked decrease in the EA of casein micelles at around pH 6.7 at all the o:w ratios and protein concentrations studied. When the energy input was fixed at 680 J 10^6 m^{-3} at protein concentration of 1%, the emulsion surface area was 140 m^2/g protein at pH 6.7 compared to the maximum of 270 m^2/g at pH 8 (Fig. 3A). The lower surface area obtained around pH 6.7 may reflect increased stability of the casein micelle at that pH because dissociation of the micelle to smaller components should increase protein available for film formation at the o:w interface.

The apparent viscosity of the emulsions, determined from the pressure at the valve head of the homogenizer (Haque and Kinsella, 1988a), was inversely related to the surface area data, i.e., when the surface area was high (small droplets), the viscosity was low and *vice versa* and this became more pronounced as the o:w ratio was increased (>2) (Fig. 3B). Increases in the emulsion viscosity may reflect the unfolding of the interfacial proteins. It is known that the intrinsic viscosity is highly sensitive to molecular shape (Cantor and Shimmel, 1980).

A possible relationship between the apparent viscosity and the emulsion activity of oil-in-water emulsions has been reported (Haque and Kinsella, 1988a). Cross-bridging between adsorbed micelles may increase emulsion viscosity of micellar casein emulsions (Darling, 1982). For polyelectrolyte stabilized emulsion, van Vliet et al. (1978) have shown that the concentration of an elastically effective polyelectrolyte chain between two emulsion globules is related to the equilibrium separation $\bar{o}$ by:

$$V = c\, n_b / A^* \bar{o} \qquad (1)$$

where A^* is the effective contact area between globules and n_b is the number of cross-links between the two globules. The factor c is the number of elastically effective chains per cross-link. The observed changes in the viscosity of the micellar casein emulsion could arise from the changes in the effective contact area and/or number of cross-links between the emulsion globules. Viscosity and the degree of cluster formation are also influenced by disperse phase fraction (ø) (Dickinson and Stainby, 1982).

The effect of protein concentration (1–3%) on the EA of micellar casein was observed at various pH steps; the o:w ratio was fixed at 1 (i.e. 10%), a point at which the highest EA observed (Fig. 1). The EA increased at pHs >8 (Fig. 4A) and this may be because the electrokinetic potential of the casein

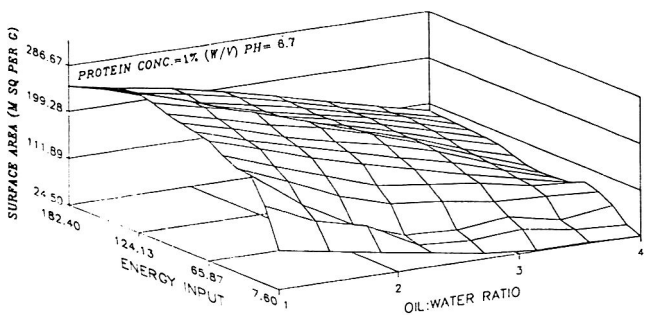

Fig. 1—Effects of energy input and oil:water ratio on the interfacial surface area of micellar casein emulsions. Protein concentration was 1% (w/v) at pH 6.7. Surface area is in m^2 per gram of protein used, energy input is in 10^7 J m^{-3}, and o:w ratios 1, 2, 3 and, 4 represent 10, 20, 30, and 40% oil, respectively.

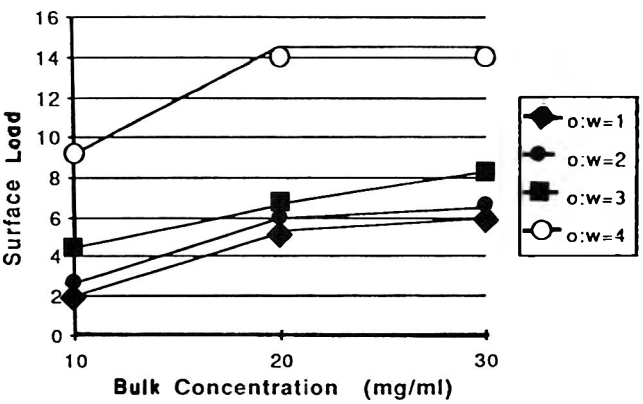

Fig. 2—Effect of oil:water ratio and bulk protein concentration on the surface load (surface) of micellar casein at the emulsion interface. Surface is in mg/m^2. Energy input was 680 10^6 J m^{-3} and the pH was 6.7.

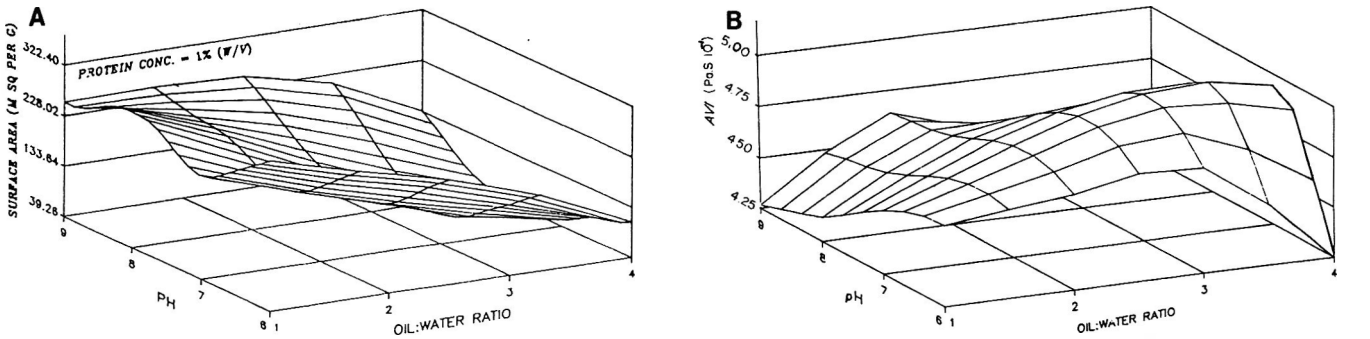

Fig. 3—Effect of pH and oil:water ratio on micellar casein emulsions. Energy input was fixed at 680 10^6 J m^{-3}. All other conditions were as in Fig. 1. (A) The interfacial surface area; (B) The apparent viscosity index (AVI) in Pa·s.

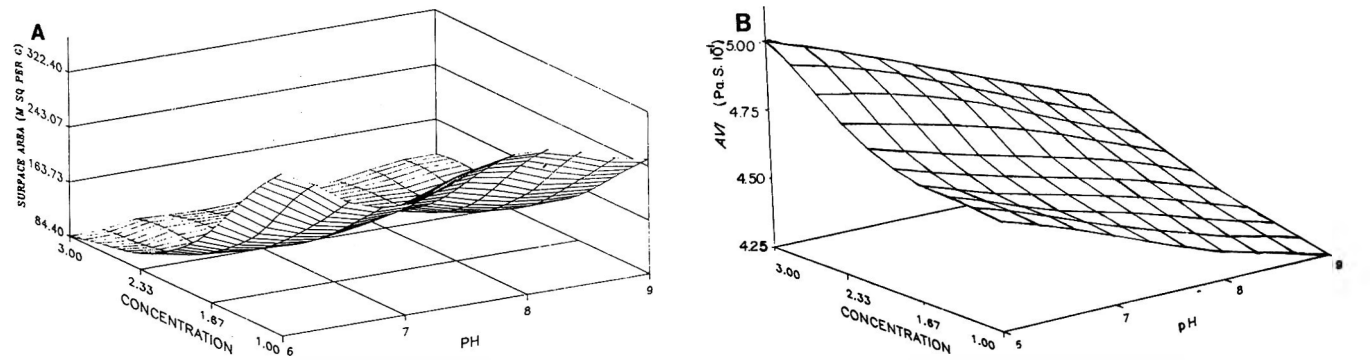

Fig. 4—Effect of protein concentration and pH on micellar casein emulsions. Energy input was fixed at 680 10^6 J m^{-3}. Protein concentration is in percent (w/v). All other conditions were as in Fig. 3. (A) The interfacial surface area; (B) On the apparent viscosity (Pa·S) in Pa·s.

micelle increases with pH (McMahon and Brown, 1984) resulting in dissociation of the micelles.

The apparent viscosity was inversely related to the EA and was low in the regions that corresponded to optimum surface area (Fig. 4B). The observed viscosity as a function of pH cannot be explained in terms of the viscosity of the micellar casein solution. There is little difference in the viscosity of casein dispersions in the near neutral and alkaline region (pH 6-9) (Korolczuk, 1982).

Creaming stability

The creaming stability of the micellar casein emulsion was tested at the different pHs and o:w ratios. The stability improved under conditions that gave good EA (Fig. 5); the profile of creaming stability was comparable to EA under identical conditions (Fig. 3A). Stability was high at pH 6 and o:w ratio of <2, decreased around pH 6.7 and then increased with pH >7. The emulsion stability as a function of protein concentration and o:w ratio was again optimum under conditions that gave high EA (Fig. 6).

The creaming (or settling) speed of a single oil globule is described by the equation:

$$V = 2a^2 | \Delta\rho | g/9\eta \qquad (2)$$

where $\Delta\rho = \rho - \rho_o$, a is the radius of the dispersed phase globules, η is the shear viscosity of the liquid in a centrifugal field, and g is the local acceleration of free fall. Because we used a centrifuge, g is replaced by w^2R where w is the angular velocity and R is the distance from the center of the rotor. Creaming is reduced by small disperse phase globules. The

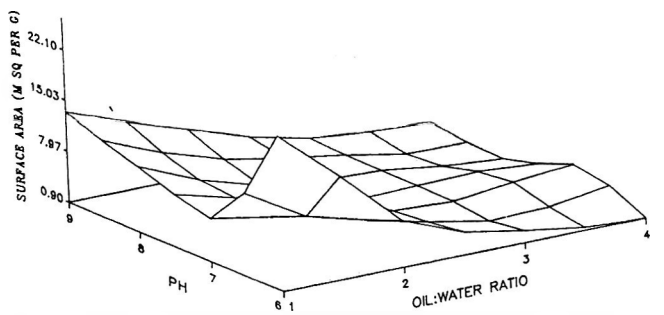

Fig. 5—Effect of pH and oil:water (o:w) ratio on the creaming stability of micellar casein emulsions. The centrifugation time (769 × g, 25°C) for determining the creaming stability was 4 min. For making the test emulsions, the protein concentration was 1% (w/v), energy input was 1824 10^6 J m^{-3} and the pH was 6.7. The surface area and o:w ratio are explained in Fig. 1.

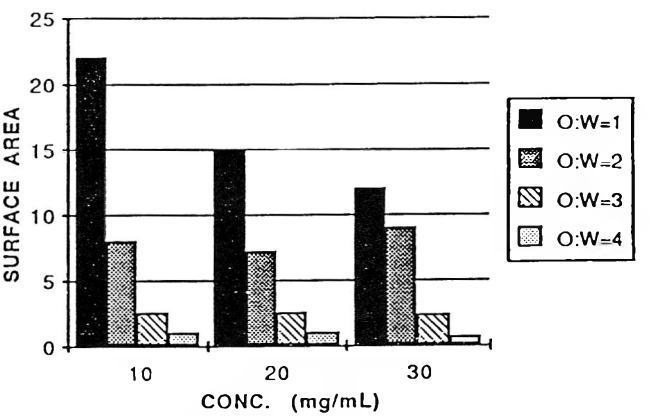

Fig. 6—Effect of protein concentration and o:w ratio on the creaming stability of micellar casein emulsions. All conditions are as in Fig. 5.

improved stability of micellar casein emulsions at the lower concentration range is explained by the fact that the smallest globules were obtained at this region.

Conditions conducive to emulsion stability also yielded the least viscous emulsions. Polymer bridging (Vincent, 1974), i.e., sharing of the same interfacial aggregate by more than two oil globules (Eq. 1), could contribute to emulsion viscosity which is expected to improve creaming stability. However, since polymer bridging causes the attachment of more than one oil globule to the same interfacial protein aggregate forming a floccule, the rate of creaming of a single oil globule under an applied gravitational field would be affected by the size of the floccule and not by the individual emulsion globules that constitute the floccule. Therefore increased apparent viscosity was not always associated with increased stability.

The principal constituent that contributes to polymer bridging in dairy emulsions is micellar casein (Ogden, 1973). Darling (1982) noted that polymer bridging causes an increase in emulsion viscosity and is responsible for flocculation during homogenization of dairy emulsions.

The lowest EA of micellar casein occured at pH 6.7, and the EA increased with increasing pH. The apparent viscosity of the emulsion, which was inversely related to the EA and to the creaming stability of the emulsion, may be useful for monitoring emulsion properties during homogenization.

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Estimation of Calcium Status in Selected Food Systems

LESLEY A. KEANE, NORMAN N. POTTER, and JOHN W. SHERBON

ABSTRACT

Ionic and total dialyzable calcium in nonfat dry milk, soy protein isolate, and a 50:50 mixture of the two were estimated in dialysates before and after *in vitro* digestion by HCl/pepsin and pancreatin/bile. Heat pasteurization did not affect % ionic or % total dialyzable calcium, before or after digestion, for the three food systems. Digestion increased % ionic dialyzable calcium for non-fat dry milk and the mixture, and increased % total dialyzable calcium for all three food systems. After digestion, % total dialyzable calcium did not differ between the milk and the mixture. Soy protein did not bind substantial amounts of calcium from the milk.

INTRODUCTION

HIGH INCIDENCE of osteoporosis among postmenopausal women has resulted in growing interest in calcium intakes and status. Since two-thirds of U.S. women ages 18–30 fail to consume the recommended daily allowance for calcium (Stark, 1984), foods of high calcium availability become especially important for these individuals.

Several authors have reported solubility of minerals in foods subjected to peptic-pancreatic digestion to be indicative of their bioavailability (Narasinga Rao and Prabhavathi, 1978; Miller et al., 1981; Wien and Schwartz, 1983). However, calcium solubility in peptic-pancreatic digests of food is not consistently related to *in vivo* calcium bioavailability (Zemel, 1984). Zemel suggested that formation of stable, soluble calcium complexes that are not readily absorbed may be responsible for the discrepancy.

Dairy foods are generally regarded as excellent sources of calcium whereas soy foods are viewed as poor sources because of their fiber and phytate constituents which may bind substantial quantities of mineral (Reinhold et al., 1975; Camire and Clydesdale, 1981; Grynspan and Cheryan, 1983). However, the ability of soy protein, itself, to bind mineral has not been thoroughly investigated.

The purpose of the present study was to estimate calcium status before and after digestion in three food systems, reconstituted nonfat dry milk, soy protein isolate, and a 50:50 mixture of the two.

MATERIALS & METHODS

Food systems

Nonfat dry milk (O-At-Ka Milk Products) and soy protein isolate (Ralston Purina Co.) were reconstituted with deionized, distilled water to a protein concentration of 3.6%. Sucrose was added to the soy protein isolate to the level of lactose found in the non-fat dry milk (59.5%). A 50:50 mixture of the reconstituted non-fat dry milk and reconstituted soy protein isolate plus sucrose also was prepared. These food systems were held for 72 hr at 10°C prior to use to allow for calcium equilibration. Aliquots of these systems then were heat pasteurized at 63°C for 30 min.

Digestion and dialysis

An adaptation of the *in vitro* method for iron availability of Miller et al. (1981) was used. For the after-digestion samples, 62 mL aliquots of each food system were acidified to pH 2, in duplicate, and incubated with 3 mL pepsin (I.C.N. Biochemicals, 5 × N.F., 0.024g pepsin/1.2g protein suspended in 0.1 N HCl) at 37°C, with shaking, for 2 hr. To one of each duplicate, 15 mL of a suspension of pancreatin (I.C.N. Biochemicals, 1 × N.F., 0.04g pancreatin/1.2g protein) and bile (Sigma Chemical Co., 0.0625g bile/1.2g protein) in 0.1 M $NaHCO_3$ was added. These aliquots were then titrated to pH 7.5 with 1 N KOH to determine the normality of sodium bicarbonate solution to be subsequently used in dialysis bags to neutralize the remaining pepsin digests. Twenty mL dialysis bags (Spectrapor 1 dialysis tubing, 6,000-8,000 dalton MW cut-off, Fisher catalog #08-670C), filled with sodium bicarbonate solution, were then immersed in the remaining pepsin digests and incubated for 30 min at 37°C, with shaking. Pancreatin/bile (15 mL) was added to the pepsin digests and digests plus dialysis bags were incubated at 37°C with shaking for an additional 2 hr. During dialysis, the bicarbonate in the dialysis bag passed through the membrane into the food sample and calcium released from the food passed through the membrane into the dialysis bag. For the before-digestion samples, aliquots of 80 mL of the food systems were dialyzed against 20 mL dialysis bags, filled with deionized, distilled water, for 2.5 hr at 37°C with shaking.

Calcium analyses

Five mL aliquots of food systems were dry-ashed at 450°C for 24 hr in a muffle oven. The ashed samples were then dissolved in 1 mL concentrated nitric acid, heated at 80°C until dry, and redissolved in 1 mL concentrated hydrochloric acid and 2 mL deionized, distilled water. Samples were diluted with 0.1 N HCl and total calcium was determined by atomic absorption spectrometry using an air-acetylene flame (Perkin Elmer Spectrophotometer Model # 360). Lanthanum at a concentration of 500 ppm was added to all samples and standards to avoid formation of calcium phosphate complexes.

Aliquots of 5 mL of the dialysates were ashed and total calcium concentration was determined by atomic absorption spectrometry. Aliquots of 10 mL of the dialysates were analyzed for ionic calcium concentration using the calcium ion selective electrode (Orion Research, Inc.). The ionic strength of samples and standards was adjusted above 0.08M with 4M KCl so that the calcium activity coefficient was constant and the calcium activity was directly proportional to concentration (Orion Research, Inc., 1986). Ionic calcium concentration in samples was determined from the ionic calcium standard curve of m volts vs. ionic calcium concentration.

Assuming calcium equilibrium, % ionic dialyzable calcium was defined as the amount of ionic calcium that was dialyzable (ionic calcium concentration in dialysate × total volume of digest plus dialysate), expressed as a percentage of total calcium in the food. Percent total dialyzable calcium was defined as the amount of total calcium that was dialyzable (total calcium concentration in dialysate × total volume of digest plus dialysate), expressed a percentage of total calcium in the food.

Statistical analyses

Experiments were repeated four times on four different days. An analysis of variance of % ionic and % total dialyzable calcium with the variables of pasteurization, digestion, and protein source, and blocking by day was run on the statistical analysis system, SAS, using the PROC GLM option (SAS, 1985). The effects of heat pasteurization, digestion, and protein source on % ionic and % total dialyzable calcium were determined using linear contrasts of the means (Snedecor and Cochran, 1980).

RESULTS & DISCUSSION

TOTAL CALCIUM LEVELS in the nonfat dry milk, soy protein isolate, and the mixture were 121.4, 5.6, and 64.0 mg/100g respectively. These values agree well with values re-

Author Keane is with M&M/Mars, Inc., Hackettstown, NJ 07840. Authors Potter and Sherbon are with the Dept. of Food Science, Cornell Univ., Ithaca, NY 14853.

Table 1—Mean % ionic dialyzable calcium in nonfat dry milk (NFDM), soy protein isolate (SPI), and a mixture of the two (MIX)

Sample	Treatment[b]	Mean % ionic dialyzable calcium[a] Before digestion	After digestion	S.E. Mean	pr > |t|[c]
NFDM	U	10.6	18.0	2.7	0.01*
NFDM	P	8.0	16.6		
SPI	U	17.7	15.5	4.1	0.29
SPI	P	11.0	22.9		
MIX	U	13.4	31.2	2.8	0.0001*
MIX	P	12.0	38.8		

[a] Means are averages of 4 trials.
[b] U = unheated, P = heat pasteurized.
[c] Results of t test on comparison of before vs. after digestion, combining unheated and heat pasteurized treatments, expressed as probability of exceeding the computed t value (pr > |t|).
* Means are significantly different before vs after digestion at p < 0.05.

Table 2—Mean % total dialyzable calcium in nonfat dry milk (NFDM), soy protein isolate (SPI), and a mixture of the two (MIX)

Sample	Treatment[b]	Mean % total dialyzable calcium[a] Before digestion	After digestion	S.E. mean	pr > |t|[c]
NFDM	U	28.5	50.2	2.7	0.0001*
NFDM	P	28.6	53.1		
SPI	U	19.8	67.0	12.8	0.002*
SPI	P	14.1	66.8		
MIX	U	27.4	59.4	2.4	0.0001*
MIX	P	26.9	62.8		

[a] Means are averages of 4 trials.
[b] U = unheated, P = heat pasteurized.
[c] Results of t test on comparison of before vs. after digestion, combining unheated and heat pasteurized treatments, expressed as probability of exceeding the computer t value (p > |t|).
* Means are significantly different before vs. after digestion at p < 0.05.

ported in the literature (Hargrove and Alford, 1974; Stark, 1984; Kim and Zemel, 1986; Ralston Purina, 1986).

Values for % ionic dialyzable calcium and % total dialyzable calcium are given for the three food systems (Tables 1 and 2). In milk, approximately 30% of the calcium is in the serum phase (Walstra and Jenness, 1984), which is approximately the same amount estimated (Table 2) to be dialyzable in non-fat dry milk before digestion. Kim and Zemel (1986) also estimated potential bioavailability in foodstuffs. They reported 30.4% soluble ionic calcium and 60.8% total soluble calcium as available in pepsin/pancreatin digested skim milk. The lower values of % ionic and total dialyzable calcium after digestion in Tables 1 and 2 may be more accurate for estimates of available calcium since Zemel (1984) reported that soluble calcium from digested foods overestimated bioavailable calcium compared to *in vivo* estimates. In the present study, % total dialyzable calcium was generally greater that % ionic dialyzable calcium. This indicates that more than just ionic calcium was dialyzable. The digestion treatment released significant amounts of protein bound calcium in all of the food systems since both ionic and total dialyzable calcium increased.

There were no significant differences in % ionic dialyzable calcium or % total dialyzable calcium in any of the systems due to heat pasteurization ($p > 0.05$). Hence, in determining the effect of the digestion treatment, the unheated and heat pasteurized treatments were combined. The % ionic dialyzable and % total dialyzable calcium averaged significantly higher ($p < 0.05$) after digestion for non-fat dry milk and the mixture (Tables 1 and 2). Only % total dialyzable calcium averaged higher after digestion for the soy protein isolate ($p < 0.05$). The failure to see a difference in % ionic dialyzable calcium might be caused by the ionic calcium concentration in the soy protein isolate sample nearing the detection limits of the electrode (10^{-5}M).

In comparing the nonfat dry milk (NFDM), soy protein isolate (SPI), and the mixture (MIX), with unheated and heat pasteurized treatments combined, it can be seen that before digestion, % ionic dialyzable calcium was in the order, SPI > MIX > NFDM. The only significant difference was for SPI > NFDM ($p < 0.05$). In milk, calcium may be present in the form of micellar calcium phosphate. Soy protein isolate does not have this form of calcium so there may be more calcium in the ionic state. This is further indicated since % ionic dialyzable calcium and % total dialyzable calcium for SPI before digestion were approximately the same (Tables 1 and 2). The calcium in SPI that was dialyzable may have been essentially all ionic calcium. Also, the addition of sucrose to aqueous solutions has been found to increase calcium activity, hence concentration (Geerts et al., 1983). Sucrose was added to the soy protein isolate and this may have accounted for the greater % ionic dialyzable calcium observed.

Further, before digestion, % total dialyzable calcium was in the order, NFDM > MIX > SPI, with significant differences for both NFDM and MIX being greater than SPI ($p < 0.05$). Again, only ionic calcium may have dialyzed in the case of SPI, whereas both ionic and collidal forms of calcium from milk were able to dialyze. In NFDM and MIX, the % total dialyzable calcium was greater than the % ionic dialyzable calcium (Tables 1 and 2), indicating that other forms of dialyzable calcium were present.

After digestion, % ionic dialyzable calcium was in the order, MIX > SPI > NFDM, with significant differences for MIX being greater than both NFDM and SPI ($p < 0.05$). The soy protein and the milk protein alone had far less % ionic dialyzable calcium than when the two protein sources were combined. The reason for this is not certain. A possible explanation may be that in the NFDM and SPI, there was binding of calcium to peptides. But, in the MIX, there was interaction of the peptides from the two protein sources, allowing more ionic calcium to remain free.

The binding of calcium to peptides did not inhibit dialysis of calcium, as evidenced by all three food systems not differing significantly in % total dialyzable calcium after digestion. Rather, it appears to have altered the ionic-nonionic calcium equilibrium. From these data, it also may be concluded that soy protein does not bind substantial amounts of calcium from milk.

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Direct Measurement of the Attractive Force Between Individual Cooked Rice Grains of Sticky and Flaky Cultivars

SEUNG-JU LEE and M. PELEG

ABSTRACT

The attractive force between juxtapositioned cooked rice grains of five sticky and flaky cultivars was determined using a surface tensiometer. The attractive force between the sticky grains was in the order of 800–1700 dynes and between the flaky ones, 200–1000 dynes. Increase of the water-to-rice ratio during cooking, in the range 0.75:1 to 1.9:1, resulted in an increase of the attractive forces. Determination of the amount of solids extracted during cooking in excess water indicated that stickiness was most probably associated with extractability.

INTRODUCTION

THE STICKINESS of cooked rice is considered one of its most important textural characteristics. For most westerners, stickiness is an undesirable property of rice. Consequently, the varieties consumed in the west are mostly of the flaky or nonsticky type. Plant geneticists who work on the improvement of rice varieties have great concern for this attribute and considerable effort is invested by rice processors to keep their products from becoming sticky. For most Orientals the situation is somewhat reversed, and primarily because of sensory perference, a certain degree of stickiness is not only acceptable but is, in fact, highly regarded (Tanaka, 1986).

The terms "stickiness" or "flakiness" in the context of cooked rice are intuitively clear. It is a different matter to determine the stickiness of rice objectively and to characterize its intensity in quantitative terms. The food literature has few reports on the application of instrumental methods for stickiness determination. They are usually based on the force required to pull a device imbedded in the cooked rice (Kurasawa et al, 1962; Bhattacharya et al., 1976) or pressed upon it (Mossman et al, 1983; Fellers et al., 1983). The latter was also applied to compressed individual cooked grains and the force was treated as "adhesiveness" according to the "Texture Profile Analysis" terminology (Tsuji, 1981). Other methods were based on sieving (Bhattacharya et al., 1976) or relating stickiness to other mechanical parameters (Blakeney, 1979; Mohandoss and Pillaiyer, 1981). The difficulty with most of these methods is that their geometry is arbitrary and ill-defined and, therefore, their results depend, to a large extent, on the particular features of the mechanical device used and the size of the rice sample. Furthermore, it has long been recognized that the stickiness of cooked rice depends not only on the rice cultivar, quality (especially with respect to size of the broken grains fraction) and age but also on the cooking conditions. The latter primarily refers to the water to rice ratio, the cooking time and the temperature (Batcher et al, 1963; Gariboldi, 1973; Fellers et al., 1983). The biochemical causes of stickiness and their relation to the rice amylose contents have been thoroughly investigated (Juliano, 1985). The purpose of this communication was to demonstrate that the attractive forces between individual cooked rice grains were not only of measurable magnitude but could also be used as a physical criterion to distinguish between sticky and flaky rice cultivars.

Authors Lee and Peleg are with the Dept. of Food Engineering, Univ. of Massachusetts, Amherst, MA 01003.

MATERIALS & METHODS

RICE of the Lemont, Rexmont, Toro-2, Mars and Rico-1 cultivars was provided through the courtesy of USDA-ARS Regional Rice Quality Laboratory at Beaumont, TX and the Rice Experiment Station of the Louisiana State University at Crowley, LA. The major physical characteristics of these samples are summarized in Table 1.

Samples of the rice were cooked with different water ratios following the procedure of Mossman et al. (1983). Eight grams rice were placed in a 30 mL beaker to which a weighed amount of distilled water—6, 9, 12, or 15g—was added. The beaker was placed on a screen above boiling water in a covered pan 25 cm diameter and 15 cm deep. After steaming for 20 min, the heat was turned off, and the sample was held in the pan for an additional 10 min. The beaker was then removed, sealed with a vinyl wrap and placed upside down to cool for 40 min at room temperature of about 25°C before testing. The cooked rice was then taken from the beaker and divided into four sections starting from the top. Grains for the test were taken only from the center of the second and third sections.

Attractive force measurements

Individual grains were mounted one on top of the other in a parallel portion in a special device shown schematically in Fig. 1. They were slightly pressed together by a finger at an average force of about 15g as determined by performing the pressing over an analytical balance.

Table 1—Physical characteristics of the rice grains used in the reported study

Cultivar	Grain shape	Dimensions (mm)	Stickiness classification	Moisture content (%)	Broken grains (%)	Source
Lemont	long	6.7×2.0	nonsticky (flaky)	12.1	3.0	Louisiana
Rexmont	long	6.8×1.9	nonsticky (flaky)	12.1	1.1	Texas
Toro-2	long	7.4×2.0	sticky	11.7	3.4	Louisiana
Mars	medium	5.2×2,3	sticky	11.1	4.2	Louisiana
Rico-1	medium	5.3×2.4	sticky	12.4	4.4	Texas

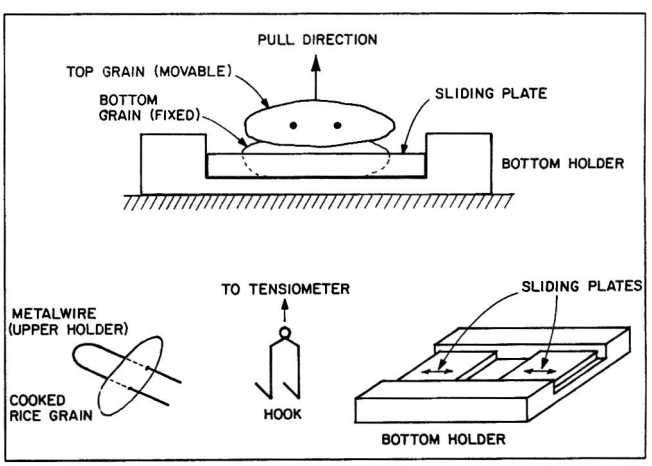

Fig. 1—Schematic presentation of the device used to hold and pull apart two cooked rice grains.

Table 2—Comparison of the attractive forces between cooked rice grains cooked at different water-to-rice ratios[a]

Water to rice ratio:	0.75:1		1.13:1		1.5:1		1.9:1	
Cultivar	Mean force (dyne)	Coef. of variance (%)	Mean force (dyne)	Coef. of variance (%)	Mean force (dyne)	Coef. of variance (%)	Mean force (dyne)	Ccef. of variance (%)
Lemont	490[c]	46	980[b]	20	910[c]	29	880[c]	38
Rexmont	200[d]	71	200[c]	78	250[d]	50	370[d]	50
Toro-2	1200[e]	32	1530[e]	17	1390[e]	23	1710[e]	34
Mars	910[b]	26	1180[b]	24	980[c,b]	24	1330[b]	15
Rico-1	840[b]	31	1170[b,e]	49	1270[b,e]	35	1300[b]	10

[a] Data from at least eight replicates of grains oriented at a parallel position.
[b-e] Different letters at the same column indicate significant difference at a probability level of $P \leq 0.05$.

The whole device was then placed under the arm of a Rosano Surface Tensiometer (Biolar Corp., North Graftan, MA) as shown in Fig. 2.

The tensiometer was activated to pull the grains apart and the force to do so (F) was recorded. The instrument was then balanced with the top grain hanging in the air and the corresponding weight, W (of the suspended grain, metal wire, hook and connecting wire) determined. The net attractive force, T, between the grains is given by:

$$T = F - W \quad (1)$$

and is reported in dynes. All the measurements were made in at least 8 replicates where each test was performed on a fresh pair of grains. All the experiments were repeated, as described above, with samples of newly cooked rice.

Solid extraction and the surface tension of the cooking water

Rice samples were cooked in excess water (10:1 ratio) to compare the extraction of solids from the grain. The solids of the water were determined by air drying at 105°C, and the surface tension of the cooking water at 25°C was determined using the Rosano tensiometer operated in its standard mode.

Statistical analysis

The significance of the differences between cultivar was tested by the Student-Newman-Keul's procedure at a signficance level of $p \leq 0.05$ (Ott, 1984).

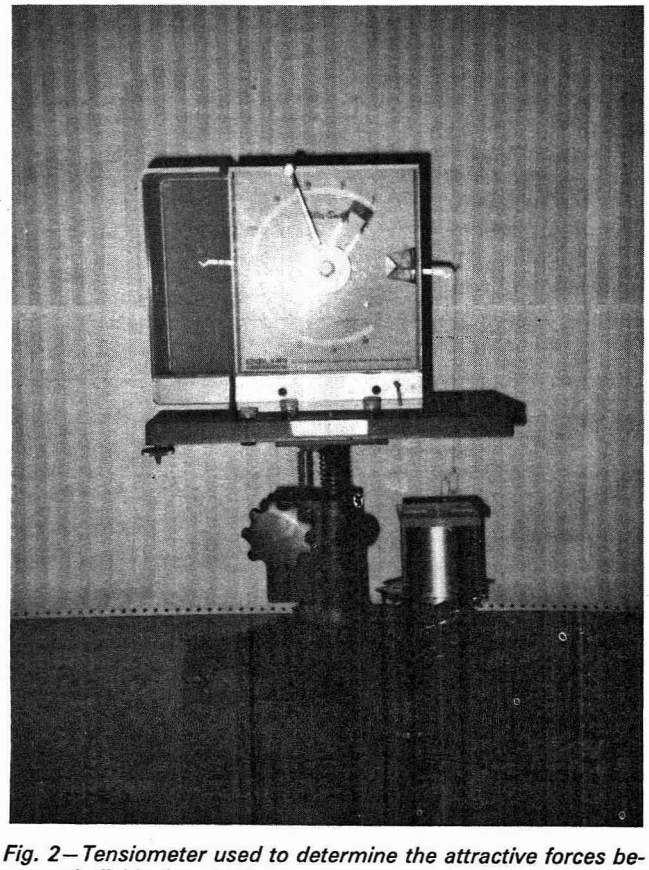

Fig. 2—Tensiometer used to determine the attractive forces between individual cooked rice grains.

RESULTS & DISCUSSION

THE INTERGRAIN attractive forces of the five rice cultivars are listed in Table 2. The table clearly shows that the varieties known to be "flaky", namely Lemont and Rexmont, had attractive forces on the order of 200–1000 dynes, depending on the water/rice ratio. Under the same cooking conditions, the attractive forces between grains of the sticky cultivars, namely Toro-2, Mars and Rico-1, were in the range of 800–1700 dynes. The measurement scatter was fairly high and found to be in the range of 10–80% when expressed in terms of the coefficient of variance, i.e., the standard deviation divided by the mean. The variation was largely due to the test's imperfect geometry. This was evident from the fact that tests performed with the grains in cross instead of parallel position had a much larger variation that did not allow for meaningful interpretation of the data. It appeared that other factors, such as slight differences in the cooling time or variations in the pressure applied to the grains, were of minor signficance. This is for the reason

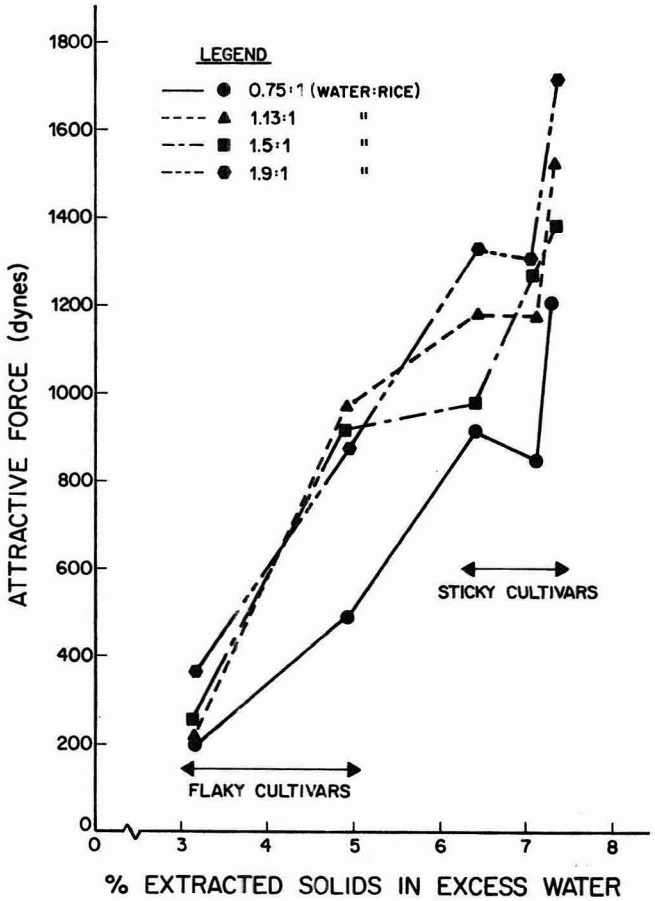

Fig. 3—Relation between the amount of extracted solids in excess water, and the attractive force between cooked rice grains as measured in rice cooked with various water to rice ratios. (For extraction conditions see Table 3).

Table 3—Surface tension of the cooking water when water was added in excess[a]

Variety	Total solid loss to water (%)	Surface tension at 25°C (dyne cm^{-1})
Lemont	4.9[c]	43
Rexmont	3.2[d]	38
Toro-2	7.3[e]	36
Mars	6.4[b]	36
Rico-1	7.1[e]	35
Water	—	71(measured) 72(theoretical)

[a] Water to rice ratio was 10:1 and the cooking was done at 77°C for 40 minutes. (The figures are means of three replicates).
[b-e] Different letters indicate a significant difference at a probability of $p \leq 0.05$.

that a very similar scatter pattern was observed when the whole set of tests was repeated with freshly cooked samples. Again, and not unexpectedly, the variation, in relative terms, was higher in the flaky cultivars, i,e., up to 80%, than in the sticky cultivars where it was generally in the range of only 20–30%. When the absolute magnitude of the forces was taken into account, it appeared that the test accuracy was in the order of 200–300 dynes (at least two orders of magnitude higher than the tensiometer accuracy). Since the differences between the sticky and flaky varieties were by far larger, the procedure, despite its crudeness, enabled the classification of rice stickiness by the attractive forces between its cooked grains.

With the aid of statistical analysis, it was also possible to detect lesser differences. Thus, it appeared that the samples tested in this study could be ordered according to their stickiness. The result was in a decreasing order of stickiness, Toro-2, Mars and Rico-1, Lemont and the most flaky Rexmont.

As can be seen in Table 2, the same order was generally maintained at all the tested water/rice ratios. This was confirmed with the results of the second set of data where the same order of stickiness was also observed.

As previously mentioned, rice samples were cooked with excessive amounts of water to test the hypothesis that there might be gross differences in the properties of the extract obtained from the sticky and flaky cultivars. The results are summarized in Table 3. In general, more solid was extracted from the sticky grains compared with the flaky ones, indicating that extractability and stickiness may be related as shown in Fig. 3. It ought to be mentioned though that the extraction data were obtained under arbitrary conditions and, therefore, need not, on theoretical grounds, be indicative of the amount and composition of the solids that are extracted under common cooking conditions. The amount of extracted material can also be regulated, at least to some extent, by the amount of broken grains (Table 1), and part of the observed stickiness was most probably due to this factor. However, repeating the test with rice samples from which all the broken grains were removed prior to cooking yielded very similar results to those shown in Table 2, suggesting that the broken grains contribution to the measured stickiness was not a decisive factor, at least under the conditions reported in this work. No pattern was observed in the analysis of the surface tension (Table 3), suggesting that surface tension of a liquid layer, if it existed at all, was not a major contribution to rice stickiness.

CONCLUSIONS

DIFFERENCES between sticky and nonsticky varieties could be quantified in terms of the attractive forces between individual grains, despite the large observed variation scatter that primarily stemed from the non-uniform features of the contact area between the grains and to a lesser extent from other factors including the non-uniform pressure that was applied to the grains. From a technical point-of-view, the method was very simple to perform and did not require sophisticated or expensive equipment nor special training for the operator. The measurements could be repeated successively on a number of grain pairs, thus achieving a sufficient number of replicates for a required statistical significance. In this way, at least in principle, the effect of water to rice ratio on stickiness could also be quantified in objective terms. It is also possible that the method could be applied to other factors that are known to affect rice stickiness such as percent broken grains, amylose content, age and storage conditions.

The method would be greatly improved if a mathematical procedure could be developed to account for the differences in contact area and geometry among the grains of different cultivars. Despite its crudeness, however, and in comparison with other published methods, the method is still the most convenient way to minimize the role of arbitrary factors in stickiness evaluation, especially when the latter involve the sample size and the overall test geometry. Because, the described method provides information that is directly related to the intensity of the grains surface adhesiveness, it can be considered as a truly objective method for stickiness assessment in clearly defined physical terms.

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Psychophysical Relationships Between Perceived Sweetness and Color in Lemon- and Lime-Flavored Drinks

H.A. ROTH, L.J. RADLE, S.R. GIFFORD, and F.M. CLYDESDALE

ABSTRACT

The effect of color, as measured on the Gardner XL-23 colorimeter, on the sweetness perception of a series of lime- and lemon-colored and flavored beverages was quantified by taste panelists using magnitude estimation. The regression lines for each colored series in the lemon drinks were found to differ significantly ($p<0.05$) indicating that color had a significant effect on sweetness and the inequality of the slopes of the lines obtained when sweetness was plotted versus log color further confirmed this effect. A sucrose level of 4.4% maximized the effect of color on sweetness perception in both the lime ($p<0.01$) and lemon ($p<0.05$) drinks.

INTRODUCTION

COLOR is a major contributing factor in the initial acceptance or rejection of a food product. Early in cognitive development, one learns to characterize foods by associating them with specific colors. The understanding of the complex relationships between color and the perceived taste of food has been the goal of many researchers (Anthoney et al., 1984; Cardello et al., 1979; Clydesdale, 1984; Dubose et al., 1980; Gifford and Clydesdale, 1986; Johnson et al., 1982, 1983).

The interrelationships between sweetness perception and color are important criteria in the product development process; hence, considerable research has been directed to this area. Early work by Pangborn and Hansen (1960) did not reveal any color-sweetness interactions when unflavored solutions of distilled water, food coloring and sucrose were evaluated. Pangborn and Hansen's (1963) findings with pear nectar drinks, however, showed that green-colored samples were often equated with lower sweetness values. This same relationship of green color with sweetness was encountered in Maga's (1974) threshold studies. Maga (1974) found that the color green statistically increased taste threshold sensitivity while yellow decreased taste sensitivity. This effect was explained by the hypothesis that a green color is associated with a sour or unripened fruit.

DuBose et al. (1980) and Tuorila-Ollikainen et al. (1984) both found that flavor attributes significantly influenced overall acceptability whereas color had little significance. However, they did not provide a specification of the flavor identity of the sample to the panelists. Earlier studies by Tuorila-Ollikainen (1982), in which the flavors were specified, showed that color changes did indeed influence acceptability. Although color was not found to be the major contributing factor in product acceptability, it is well documented that color is a necessary cue for flavor identification. Martens et al. (1983) found that even a slight change from an accustomed hue may have significant effects on preference ratings. Kostyla (1978) found the addition of yellow color to cherry- and strawberry-flavored drinks decreased flavor perception by 3-4% and sweetness by 2%. The addition of blue color reduced tartness and fruit flavor by 20%, and the addition of red color increased sweetness by 5-10%, although the sucrose concentration was unchanged. Further work by Johnson et al. (1982, 1983) found that in red, cherry- and strawberry-flavored drinks sweetness perception increased with increasing color over a narrow range of sucrose concentrations. At higher sucrose and color concentrations, perceived sweetness was found to be influenced by pleasantness effects and color acceptability.

The present research was undertaken to investigate the psychological relationships between perceived sweetness, sucrose concentration, and color intensity in lemon- and lime-flavored drinks. At constant levels of lime flavor, sucrose concentrations and lime color intensity were varied. An identical protocol was followed for the lemon flavor and color using untrained panelists and the technique of magnitude estimation to characterize all relationships.

Authors Roth, Gifford, and Clydesdale are with the Dept. of Food Science & Nutrition, Massachusetts Agricultural Experiment Station, Amherst, MA 01003. Author Radle is with Nabisco Brands, Inc., 2111 Rte 208, Fairlawn, NJ 07410.

MATERIALS & METHODS

TASTE PANELS were organized from ten, untrained students in the Dept. of Food Science & Nutrition at the Univ. of Massachusetts. These volunteer panelists ranged in age from 20 to 25 yr. All panelists were screened for normal color vision using the Ishihara (1977) test for color blindness. Correct identification of all eleven test plates was required of each participant. To familiarize the panelists with magnitude estimation, a short training session was employed, using a series of lines and shapes as described by Moskowitz (1977).

The panels were conducted in controlled-atmosphere booths, illuminated by 100-watt light bulbs. The samples were formulated using freshly made, double-distilled water, prepared using a Corning model AG-3, 220 volt, 2300 watt Still and Bantom Demineralizer model BO-5 (Barnstead Still and Sterilizer Corp; Boston, MA). The colors were mixed with the double-distilled-deionized water at the start of each six day testing period. Sucrose and flavors were added to the appropriate colored solutions 24 hr prior to the test session and stored in covered glass flasks at room temperature (24 ± 1°C) and served in clean, clear, tasteless and odorless 100 mL glasses. During each testing series, the panelists would taste once in the morning and again in the afternoon so that two observations per sample, per panelist, per day were obtained. All samples were assigned a three-digit number and were presented to the panelists in a randomized order so that each panelist was tasting the samples in a different order. The reference was always placed on the far left side of the tray.

Sample formulation

Prior to the formulation of five green and five yellow colors triangle tests were conducted to determine if the food colors contributed any taste or flavor attributes to the double-distilled-deionized water. The highest and lowest concentrations of colorants used in the magnitude estimation were evaluated by 25 panelists against the double-distilled-deionized water. The colors of the solutions were masked by serving them in red-colored glasses under red lights. The results indicated that the food colors did not have any significant effect on the taste of the samples. In formulating the green colors, increasing volumes of 0.0005% Green 3 (Warner-Jenkinson Co. No. 6503, St. Louis, MO) were added to decreasing volumes of 0.005% Yellow 5 (Warner-Jenkinson Co. No. 8805). In formulating the yellow colors, increasing volumes of 0.01% Yellow 5 (Warner-Jenkinson Co. No. 8805) were added to double-distilled-deionized water. Natural lime flavor at 0.03% (WJ Flavors St. Louis, MO) and natural lemon flavor at 0.04% (WJ Flavors) were added to the respective drinks at a constant level throughout the study. The middle color level for both the lemon and lime drinks was chosen as the reference to evaluate psychological responses in the lighter and darker solutions.

The tristimulus values for the samples were measured directly, using the transmission mode of the Gardner XL-23 colorimeter (Gardner Laboratory Inc. Bethesda, MD), and color function $\cot^{-1}$ (a/b) was calculated.

Both the lemon and lime drinks at each color level were sweetened with the following concentrations of sucrose: 3.2, 3.6, 4.0, 4.4, and 4.8% (w/v). Five different color series (C_1 to C_5) were thus produced, each of which had the same succession of sucrose concentrations. This allowed each concentration of sucrose (for a total of five) to be evaluated for sweetness perception at five different color levels. Any differences in sweetness perception due to color would then be reflected by differences in the slopes of the lines representing each color series when log sweetness is plotted versus log % sugar.

A randomized 6-day schedule was set up so that at the end of the testing period all five sweetness levels had been evaluated with all five colors. The reference was sweetened with 4.0% sucrose which fell in the middle of the sucrose range.

Magnitude estimation

The technique of magnitude estimation, using a no-modulus approach, was utilized in both the lime and lemon studies (Stevens, 1957). The panelists used a sip and spit method in evaluating the sweetness of the samples. The panelists were asked to first sip and spit out a small portion of the double-distilled-deionized water provided. The panelists were then asked to sip and spit out a small portion of the reference and assign it any positive number based on its sweetness. Next, the panelists were asked to rinse with double-distilled-deionized water and sip and spit out the first sample and assign it a number reflecting its ratio of sweetness as compared to the reference. If the sample tested were found to be twice as sweet as the reference, it was assigned a number twice as large. The panelists were asked not to retaste the samples after a number had been assigned, although they were permitted to retaste the reference at any time throughout the testing session. All the magnitude estimates were subjected to the normalization procedure of Cardello (1977) as modified by Kostyla (1978). The procedure is summarized as follows: (a) calculation of the prenormalized geometric mean from all of the magnitude estimates ascribed to the same sweetness level in each of the five colors; (b) calculation of the pre-normalized grand geometric mean from the prenormalized mean calculated in (a); (c) multiplication of each judge's magnitude estimate by the ratio of the grand geometric mean: sample geometric mean; (d) calculation of the geometric average of the values obtained in (c); (e) conversion of the normalized average score to logarithmic values. A computer program developed by Kostyla (1978) was used to normalize the magnitude estimation data.

Magnitude estimation Test 1. Effect of color on sweetness evaluation in the lime and lemon beverages

This test was designed to investigate the effect of color on sweetness perception. A series of different colored solutions (1–5) were evaluated across the same range of sucrose concentrations. The panelists were asked to compare the sweetness levels of the samples to that of the reference. The test evaluated the potential of utilizing color to create the same sweetness perception as a lower concentration of sucrose.

Magnitude estimation Test 2. Evaluation of color intensity of the lime and lemon drinks

This test was designed to confirm that the panelists were able to perceive color intensity differences among the samples. The panelists were asked to visually assess the color intensity of each sample compared to the reference. This test was performed on the first day of testing of both the lime and lemon drinks, since all five colors were represented on this day.

Statistical analysis

Log-perceived sweetness was plotted versus both log % sugar and the color function, $\cot^{-1}$ (a/b). The slope, Y-intercept, coefficient of determination, r^2, and all one-way and two-way analysis of variance were calculated using the HP-41C/CV Alphanumeric Full Performance Programmable Calculator, with the HP-41C Statistics Package application module insert. The equality of regression lines across groups were tested using the BMDPIR (Health Sciences Computing Facility, Univ. of California, Los Angeles) biomedical computer program.

RESULTS & DISCUSSION

Magnitude estimation Test 1

The normalized magnitude estimates obtained from the evaluation of sweetness in the five colored solutions were plotted as log-sweetness versus log percent sugar (Fig. 1 and 2). The results of the regression analysis for these lines are given in Table 1. The coefficients of determination for the lime and lemon groups with the same color ranged from 0.82–0.99, thus showing a power function (exponential) relationship between the psychological perception of sweetness and sucrose concentration within a given colored system. The values for the exponent, n, (Table 1) for both the lime and lemon drinks were in agreement with Steven's (1957) power law. This law describes the relationship between physical intensity and the estimate a subject designates to it. Perceived sweetness has been found to fall into the group of linear functions which are governed by slopes greater than 1.0 in the log-log scale. They are said to characterize expansive continua which are an accelerating function of the physical ratios that produce it (Moskowitz, 1974). Stevens (1960) has reported exponents from 1.30 to 1.50 describing the perception of sweetness, both in water and in food products. Johnson et al. (1982, 1983) reported exponents ranging from 2.04–2.75 in sweetened, strawberry-colored and -flavored solutions, and 1.82–2.15 in cherry-colored, sweetened and flavored solutions. The value of the exponents in the present studies ranged from 1.77 to 2.61 for the lime-colored, sweetened and flavored solutions and from 1.81 to 3.19 for the sweetened lemon-colored and flavored solutions. The wide range of exponents may be explained by the diverse color intensities used. The rate of increase in sweetness perception across the same range of sucrose concentrations was directly correlated with color. The yellow, lemon colors resulted in a highly accelerated function, whereas the green, lime colors produced smaller exponents similar to the red cherry and strawberry drinks.

When the regression lines (Fig. 1 and 2) across groups were

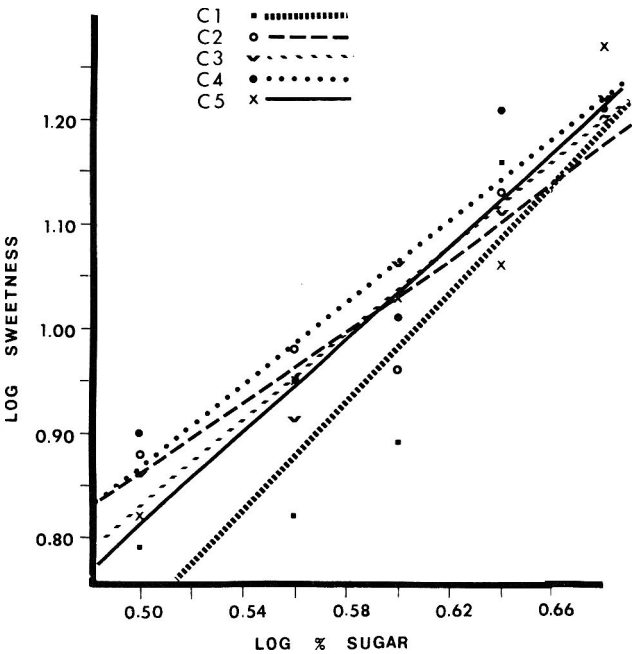

Fig. 1—Perceived sweetness (log sweetness) of five samples colored to simulate lime colors plotted versus log sugar concentration (3.2–4.8% sucrose). Each data point is based on the log geometric mean of the magnitude estimates for each sample. Each color level contained increasing amounts of 0.0005% Green 3: C1 = 10%, C2 = 25%, C3 = 30%, C4 = 50%, C5 = 90%, and decreasing amounts of 0.005% Yellow-5: C1 = 90%, C2 = 75%, C3 = 70%, C4 = 50%, C5 = 10%.

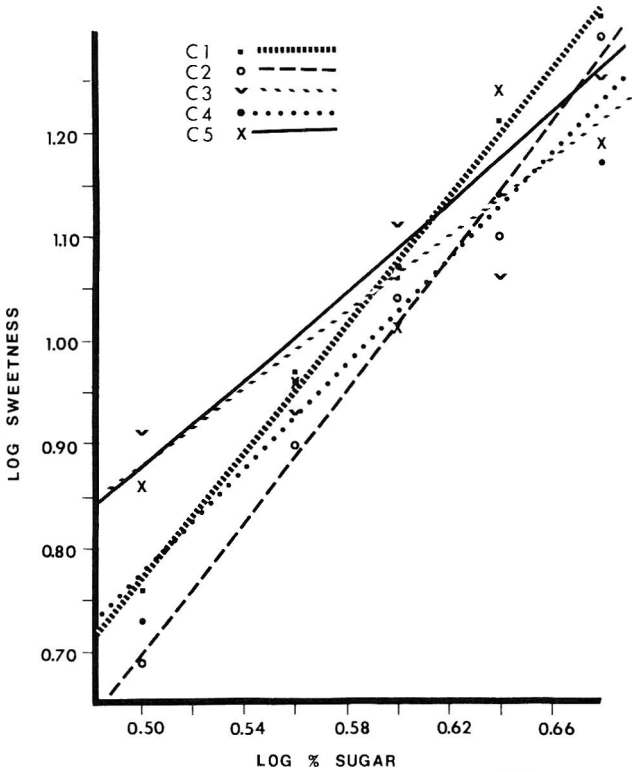

Fig. 2—Perceived sweetness (log sweetness) of five samples colored to simulate lemon colors plotted versus log sugar concentration (3.2–4.8% sucrose). Each data point is based on the log geometric mean of the magnitude estimates for each sample. Each color level contained decreasing amounts of 0.01% Yellow 5: C1 = 75%, C2 = 25%, C3 = 10%, C4 = 1%, C5 = 0.1%.

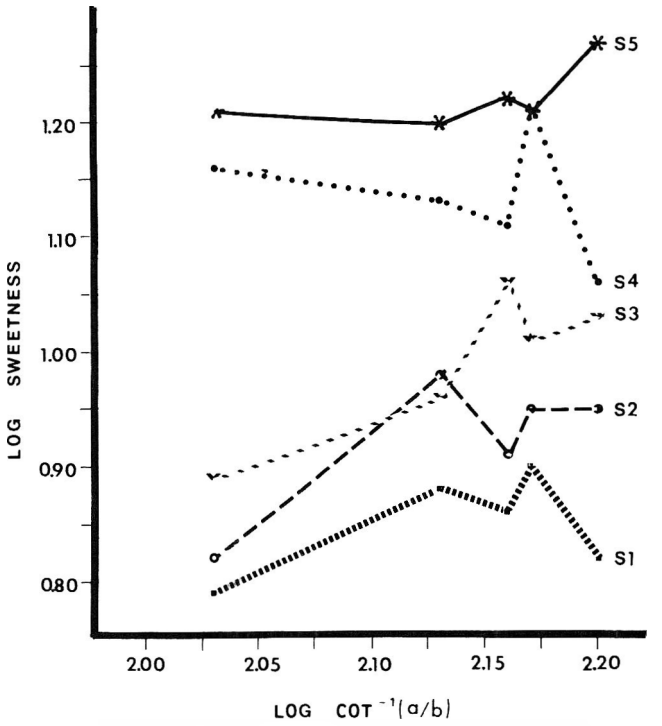

Fig. 3—Perceived sweetness (log sweetness) in five different concentrations of sucrose versus log cot⁻¹ (a/b). Each lime color contained increasing amounts of 0.0005% Green 3: C1 = 10%, C2 = 25%, C3 = 30%, C4 = 50%, and decreasing amounts of 0.005% Yellow 5: C1 = 90%, C2 = 75%, C4 = 50%, C5 = 10%. Each data point is based on the log geometric mean of the magnitude estimates for each sample. Sucrose levels: S1 = 3.2%, S2 = 3.6%, S3 = 4.0%, S4 = 4.4%, S5 = 4.8%.

Table 1—Regression analysis of perceived sweetness versus sucrose concentration

Physical parameter[a]	Y-Intercept (log)	Slope n (log)	Coefficient of determination r^2
	Lime[b]		
Color 1	−0.58	2.61	0.86
Color 2	−0.03	1.77	0.89
Color 3	−0.20	2.06	0.96
Color 4	−0.11	1.96	0.88
Color 5	−0.33	2.28	0.93
	Lemon[c]		
Color 1	−0.76	3.06	0.99
Color 2	−0.90	3.19	0.99
Color 3	−0.02	1.81	0.82
Color 4	−0.45	2.46	0.93
Color 5	−0.20	2.15	0.88

[a] Lime colors: Color 1-10% 0.0005% Green 3, 90% 0.005% Yellow 5. Color 2-25% 0.0005% Green 3, 75% 0.005% Yellow 5. Color 3–30% 0.0005% Green 3, 70% 0.005% Yellow 5. Color 4–50% 0.0005% Green 3, 50% 0.005% Yellow 5. Color 5–90% 0.0005% Green 3, 10% 0.005% Yellow 5.

[b] Lemon colors: Color 1–75% 0.01% Yellow 5, Color 2–25% 0.01 Yellow 5. Color 3–10% 0.01% Yellow 5. Color 4–1% 0.01% Yellow 5. Color 5–0.1% 0.01% Yellow 5.

[c] Sucrose concentrations for the lime and lemon experiments: 3.2%, 3.6%, 4.0%, 4.4%, and 4.8% (w/v).

tested for equality, those representing the lemon drinks were found to differ significantly ($p<0.05$) while the regression lines for the lime drinks did not. Upon examination of Fig. 1 and 2, representing the lemon and lime drinks, it can be noted that there are various points of intersection. If color had no effect on sweetness, all the lines for the lemon and lime drinks would have been parallel with no crossover points. Johnson et al. (1982, 1983) observed a similar crossover effect in strawberry- and cherry-colored and flavored drinks.

The normalized magnitude estimates obtained from the evaluation of sweetness in the five colored solutions were plotted (Fig. 3 and 4) as log sweetness versus log cot^{-1} (a/b). Cot^{-1} (a/b) is the geometric relationship derived from a plot of the Hunter a,b color values at a constant L value, which represents a function of color (Francis and Clydesdale, 1975). The results of linear regressions performed on the points may be seen in Table 2. At the highest sweetness concentration (sweetness 5), a linear relationship was observed in the lime beverages. However, linearity decreased as the sugar concentration decreased, indicating some confusion in sweetness perception. This means that color may have a greater effect on sweetness perception as sucrose concentration decreases. Regression analysis of the lemon beverages showed the same trend with the highest coefficient of determination at the highest sweetness level being 0.37. However, an r^2 of 0.37 did not account for enough variability in the data to conclude that a linear relationship existed. If color had no effect, all the lines would be similar, parallel, and show a linear relationship. Therefore, it must be concluded that color influenced the perception of sweetness at the sucrose concentrations evaluated in this study.

Analysis of variance conducted for both experiments indicated that the panelists were able to note significant differences ($p<0.01$) among sucrose concentrations. A two-way analysis of variance between perceived sweetness and color showed no significant interaction in the lime and lemon drinks. Looking at each sugar level separately, utilizing a one-way analysis of variance, a highly significant interaction ($p<0.01$) between perceived sweetness and color was observed in the lime drinks at a sucrose concentration of 4.4% (Table 3). Significance was not found at the other sucrose levels. A large decrease was observed between color 4 and color 5 which may be attributed to pleasantness effects seen at the highest color levels as found in studies by Johnson et al. (1982, 1983). A significant interaction ($p<0.05$) between perceived sweetness and color was also observed in the lemon drinks at a sucrose concentration of 4.4% (Table 3). There were no pleasantness effects ob-

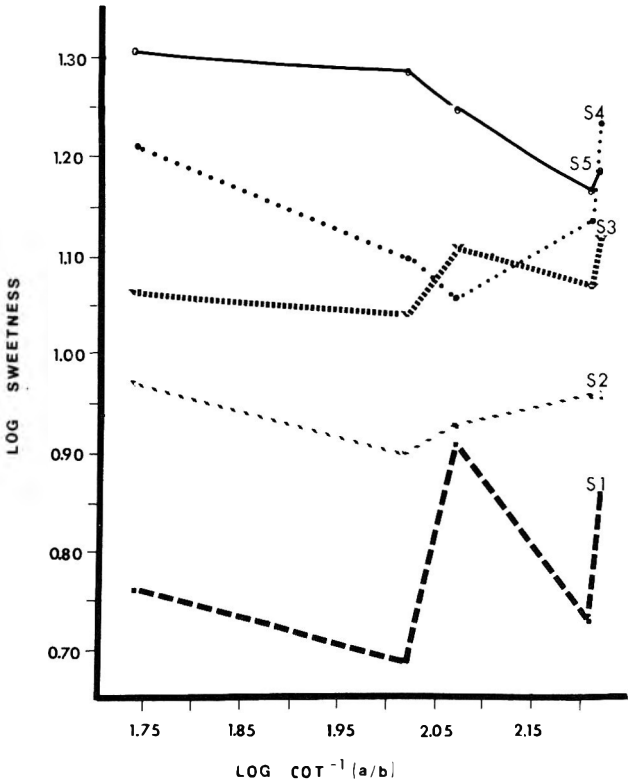

Fig. 4—Perceived sweetness (log sweetness) in five different concentrations of sucrose versus log cot^{-1} (a/b). Each lemon color contained decreasing amounts of 0.01% Yellow 5: C1 = 75%, C2 = 25%, C3 = 10%, C4 = 1%, C5 = 0.1%. Each data point is based on the log geometric mean of the magnitude estimates for each sample. Sucrose levels: S1 = 3.2%, S2 = 3.6%, S3 = 4.0%, S4 = 4.4%, S5 = 4.8%.

Table 2—Analysis of variance of perceived sweetness versus sucrose concentration

Sucrose conc	Color 1	Color 2	Color 3	Color 4	Color 5	F-Value
			Lime[a]			
3.2	0.79	0.88	0.86	0.90	0.82	1.01 N.S.
3.6	0.82	0.98	0.91	0.95	0.95	1.38 N.S.
4.0	0.89	0.96	1.06	1.01	1.03	1.59 N.S.
4.4	1.16	1.10	1.11	1.21	1.06	3.97 **
4.8	1.21	1.20	1.22	1.21	1.27	1.71 N.S.
			Lemon[b]			
3.2	0.76	0.69	0.91	0.73	0.86	1.92 N.S.
3.6	0.97	0.90	0.93	0.96	0.96	0.33 N.S.
4.0	1.06	1.04	1.11	1.07	1.12	0.50 N.S.
4.4	1.21	1.13	1.06	1.14	1.24	2.68 *
4.8	1.31	1.29	1.25	1.17	1.19	1.58 N.S.

[a] Lime colors, 1–5, are the same as Table 1.
[b] Lemon colors, 1–5, are the same as Table 1.

served in this case as an increase in yellow color corresponded to an increase in perceived sweetness. It might be concluded that a 4.4% sucrose level was the optimum at which the effects of color on sweetness perception might be observed in lime and lemon drinks under the conditions of this study.

Magnitude estimation Test 2

Evaluation of the intensities of the green and yellow colors in the samples showed that the panelists were able to perceive color differences ($p<0.01$). In both experiments power functions were obtained with the absolute value of the exponent exceeding one. This indicated that a linear change in the color function cot^{-1} (a/b) correlated with an increase in perceived color intensity.

Table 3—Regression analysis of perceived sweetness versus cot^{-1} (a/b)

Physical parameter[a]	Y-Intercept (log)	Slope n (log)	Coefficient of determination r^2
		Lime[b]	
Color 1	0.81	−0.02	6.59×10^{-4}
Color 2	1.92	−0.42	0.24
Color 3	2.25	−0.59	0.34
Color 4	2.97	−0.84	0.72
Color 5	2.05	−0.39	0.82
		Lemon[c]	
Color 1	1.42	−0.28	0.04
Color 2	0.44	0.22	0.26
Color 3	1.50	−0.19	0.13
Color 4	0.19	0.43	0.14
Color 5	−0.03	0.57	0.37

[a] Sucrose concentrations, 1–5, for both lime and lemon Drinks are the same as listed in Table 1.
[b] Lime colors,1–5, are the same as in Table 1.
[c] Lemon colors, 1–5, are the same as in Table 1.

SUMMARY & CONCLUSIONS

THE PSYCHOPHYSICAL RELATIONSHIP between sweetness perception and sucrose concentration supported Steven's power law, producing a power function (exponential) relationship. The ranges observed for the exponent, n, for the lime and lemon drinks were higher than those proposed by Steven's, thus representing a more accelerating function. A significant difference ($p<0.05$) was found when comparing the regression lines produced when log perceived sweetness was plotted versus log sucrose concentration in the lemon-colored drinks. The regression lines for both the lemon and lime beverages showed various points of crossover, thus illustrating the confusion in sweetness perception brought about by changing colors.

A sucrose concentration of 4.4% was found to be optimum for the production of a highly significant ($p<0.01$) effect of color on sweetness perception in the lime drinks and a significant relationship ($p<0.05$) in the lemon drinks. When log-perceived sweetness was graphically evaluated against log cot^{-1} (a/b) at each sweetness level, lines of unequal slopes were produced for both the lime and lemon drinks, thus further supporting an effect of color on the perception of sweetness. The psychophysical relationship between sweetness and color is complex and related to the concentration of sucrose, color intensity, and pleasantness effects. The success in comparing this model system to a complex multivariable commercial situation has yet to be investigated.

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Radiation Decontamination of Tea Herbs

BRANKA KATUŠIN-RAŽEM, STJEPAN MATIĆ, DUŠAN RAŽEM, and VLADO MIHOKOVIĆ

ABSTRACT

The incidence and microbial load of several tea herbs on the retail market are high and decrease approximately in the order: chamomile flowers, mint leaves, linden flowers, and dog-rose hips. Irradiation is proposed as an adequate decontamination method and the radiosensitivity of contaminating microflora is measured. Combining the knowledge of the initial contamination and the radioresistance of the contaminants, the dose requirement for efficient decontamination can be calculated. The dose satisfying commercial requirements does not exceed 10 kGy. Chemical changes in pharmacologically active components and those essential for the appearance and aroma of tea herbs were investigated. No changes of the yield and composition of ethereal oils, color pigments, polyphenols, and carotenoids were found at doses up to 10 kGy.

INTRODUCTION

DRY PLANT MATERIALS often harbor a variety of microorganisms as a consequence of growing and harvesting conditions and post-harvest handling. A survey of the microbiological quality of these materials on the retail market shows the presence of both a wide variety and a large number of microorganisms (Katušin-Ražem et al., 1983). A number of microbial spores can survive room temperature extraction by alcohol as well as the industrial practice of room temperature extraction enhanced by ultrasound (Katušin-Ražem et al., 1985). A fraction of thermophilic microflora can survive hot water treatment in preparing infusions (Bernard, 1983; Saint Lèbe et al., 1985).

Microbial contamination of dry products of vegetable origin, and its persistence, jeopardizes subsequent use of these materials for food, pharmaceutical, and cosmetic applications. Applicable decontamination methods are therefore of considerable concern from economic, public health, and environmental aspects. The decontamination method which uses irradiation by gamma rays or fast electrons (Josephson and Peterson, 1982, 1983; Anon, 1982) has recently been receiving growing attention. The practice of radiation decontamination of spices, condiments and dry food ingredients has already been well developed (Farkas, in press). Relatively less attention has been paid to those natural plant products which are suitable for the preparation of infusions (herbal teas), as well as food ingredients and flavoring aids (Farkas, 1985).

In our previous papers we demonstrated the feasibility of radiation decontamination of dry chamomile flowers (Katušin-Ražem et al., 1983; 1985) and chamomile extracts (Katušin-Ražem et al., 1985). The purpose of the present work is to provide a general understanding of the problem of microbial contamination of dry herbs, and to assess the potentials of radiation as a decontamination method.

MATERIALS & METHODS

The incidence of microbial contamination of chamomile flowers, mint leaves, linden flowers, dog-rose hips and sage leaves was investigated in suspected samples taken from the market over the past several years. The survival of microorganisms in infusions was studied in both indigenously and artificially contaminated herbs. Radiation survival of microorganisms was studied in artificially contaminated herbs; earlier data on indigenously contaminated chamomile flowers are shown for comparison. All materials were acquired shortly after harvesting and drying. They were of the usual commercial quality available, and were marketed by various retailers.

As there was difficulty in finding indigenously contaminated material, other than chamomile, which was contaminated at a level conveniently high for radiation resistance studies at the time of this work, artificially contaminated samples were prepared as follows: an isolate of the microflora characteristic for the given plant material was raised on nutrient agar. It was then suspended in physiological solution and sprayed back onto the respective plant materials, which were then left undisturbed for several (4–5) days before further analysis.

The infusions were prepared by pouring 200 mL boiling water over a specified quantity of plant material in a cup, according to the manufacturer's instructions. Samples for microbiological analyses were taken after 10 min.

Ten-gram samples of dry plant materials were irradiated in sealed polyethylene pouches with ^{60}Co gamma rays at a dose rate of 50 Gy/min. Samples were homogenized after irradiation and portions were taken for analyses.

Microbiological analyses

Microbiological screening of dry plant materials and the monitoring of the survival of microorganisms after heat and radiation treatments were performed according to the existing regulations (Regulation, 1980). Colony-forming units (CFU), germinating mold spores, *Salmonellae*, sulfite-reducing *Clostridia*, coagulase-positive *Staphylococcus aureus* and *Proteus spp.* were determined by inoculation of proper nutrient media and incubation for a specified time at a specified temperature, and preceded, if necessary, by appropriate heat treatment, as follows: CFU: on nutrient agar incubated at 32°C for 72 hr; sporogenic bacteria: same as above after a 5 min boiling water dip; germinating mold spores: on Sabouraud agar incubated at 32°C for 24 hr followed by 48 hr at 20°C; *Salmonellae*: on selenite broth incubated at 37°C for 24 hr, followed by seeding SS and Wilson-Blair bismuth sulfide agar and subsequent incubating at 37°C for 24 to 48 hr; coagulase-positive *Staphylococcus aureus*: on salt broth incubated at 37°C for 24 hr, followed by seeding Baird-Parker ETGP agar for subsequent dilutions and incubation at 37°C for a further 24 to 48 hr; sulfite-reducing *Clostridia:* after a 10 min dip in an 80°C bath the culture was incubated in sulfite agar for 3 to 5 days at 37°C.

Where necessary, the results were further confirmed by observing reactions to specific substrates. Enterobacteria were determined by direct inoculation on violet red bile agar (Mossel, 1957); coliform bacteria: on brilliant green broth incubated at 37°C for 24 to 48 h; *E. Coli*: by seeding positive tubes of brilliant green broth on Endo agar incubated at 37°C for 24 hr.

Chemical analyses

Chemical composition was expressed per 100g dry matter. Drying was performed according to the Yugoslav Pharmacopeia by heating plant materials at 104°C for 2 hr and an additional period of 40 min (Pharm. Yug. III, 1972).

Ethereal oils of mint and sage leaves were obtained by steam distillation for 2.5 hr in a Clevenger apparatus, according to the Yugoslav Pharmacopeia (Pharm. Yug. III, 1972). Ethereal oils were analyzed by gas chromatography using a Varian Aerograph 2880 model with a flame ionization detector.

Gas chromatography of mint oil was carried out with a 180 cm × 3 mm o.d. stainless steel column packed with 10% Carbowax 20 M on Chromosorb AW 100/120. The column was operated isothermally

Authors Katušin-Ražem and Ražem are with the "Ruder Bošković" Institute, 41000 Zagreb, Yugoslavia, Authors Matić and Mihoković are with the Public Health Institute of Croatia, 41000 Zagreb, Yugoslavia.

at 130°C at a carrier gas flow of 14 mL nitrogen per min. A pentane solution of ethereal oil was used, using 2-octanol as an internal standard. Positive identification of the chromatographic peaks was performed by comparison with the standards menthone, isomenthone and menthol, and with literature data (Masada, 1976). The response of the FID detector to these compounds relative to the internal standard was between 0.88 and 1.05 g/g standard. A hexane solution of ethereal oil of sage was chromatographed on the same column, temperature programmed from 70°C isothermal for 10 min to 200°C, at a carrier gas flow rate of 25 mL nitrogen per min. Mesitylene was used as an internal standard. The identification of the chromatographic peaks was performed by comparison with the standards α-pinene, limonene, camphor and borneol, and with literature data (Masada, 1976). The response of the FID detector to these compounds relative to the internal standard was between 0.91 and 1.07 g/g standard.

Chlorophyll *a* and chlorophyll *b*, pheophytin *a* and pheophytin *b*, as well as total chlorophyll and total pheophytin were determined spectrophotometrically in 80% aqueous acetone extracts of mint and sage leaves (Vernon, 1960). The percent retention of chlorophylls was also determined as a function of dose.

Total polyphenols were determined in a 30% aqueous ethanolic extract of linden flowers by spectrophotometry of the blue complex formed with the Folin reagent. Nonprecipitated polyphenols were determined in the same way in a supernatant liquid after precipitation of tannins with milk serum (Gstirner and Korf, 1966). The method was calibrated with pyrogallol whereby $\epsilon' = 140$ mL (mg cm)$^{-1}$ was obtained at the maximum absorption wavelength of 770 nm.

Total flavonoids were determined by spectrophotometry of the yellow complex with aluminium chloride, which is formed in an ethyl acetate solution after hydrolysis of linden flowers with HCl in an acetone solution (Hörhammer et al., 1961). The method was calibrated with rutine whereby $\epsilon' = 21.8$ mL (mg cm)$^{-1}$ was obtained at the maximum absorption wavelength of 415 nm.

Vitamin C was determined spectrophotometrically as oxazone with 2,4-dinitrophenyl hydrazine after oxidation with bromine of a metaphosphoric acid extract of ground dog-rose hips (Roe and Oesterling, 1944). The method was calibrated with ascorbic acid whereby $\epsilon' = 34.5$ mL (mg cm)$^{-1}$ was obtained at the maximum absorption wavelength of 540 nm.

Carotenoid pigments were extracted from dog-rose hips by a mixture of petroleum ether and acetone (1:1). Biologically active α-, β- and γ-carotene were separated from other carotenoid pigments on an alumina column with 2% acetone in petroleum ether and were determined by spectrophotometry (Goodwin, 1955). They were expressed as β - carotene taking $\epsilon' = 250$ mL (mg cm)$^{-1}$ at the maximum absorption wavelength of 448 nm.

RESULTS

THE RESULTS of the microbiological survey of chamomile flowers, mint leaves, linden flowers, and dog-rose hips are shown in Fig. 1 to 4. Chamomile flowers have already been recognized as the most heavily contaminated material (Katušin-Ražem et al., 1983), while the contamination of mint leaves, linden flowers, and dog-rose hips falls in that order. The presence of germinating mold spores was approximately equally distributed in chamomile flowers, linden flowers, and dog-rose

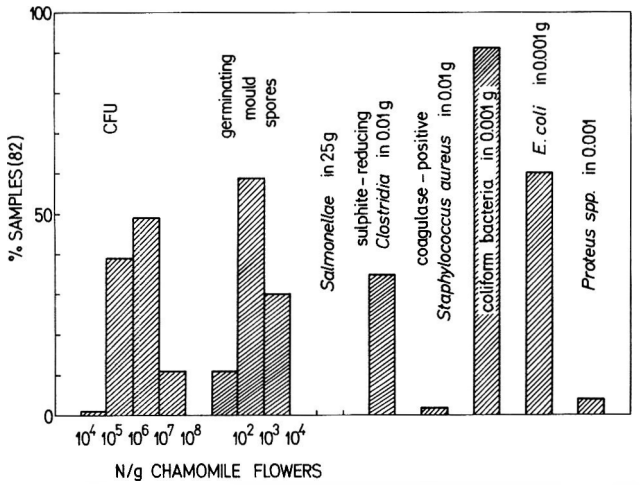

Fig. 1—Distribution of microbiological contamination of chamomile flowers.

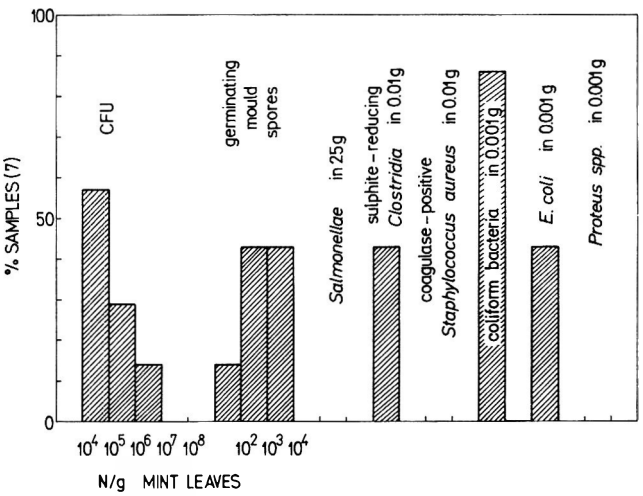

Fig. 2—Distribution of microbiological contamination of mint leaves.

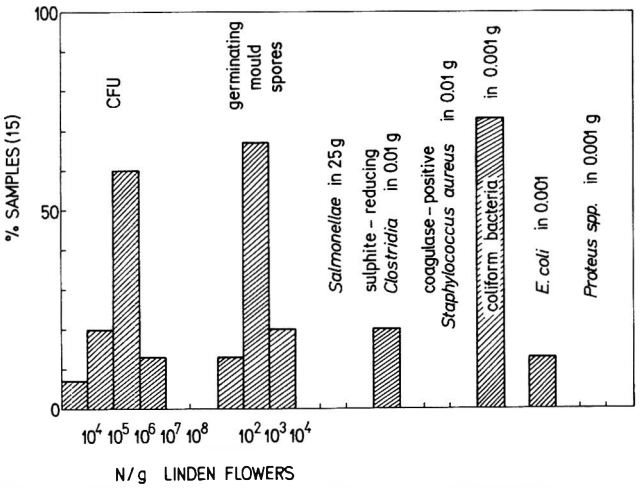

Fig. 3—Distribution of microbiological contamination of linden flowers.

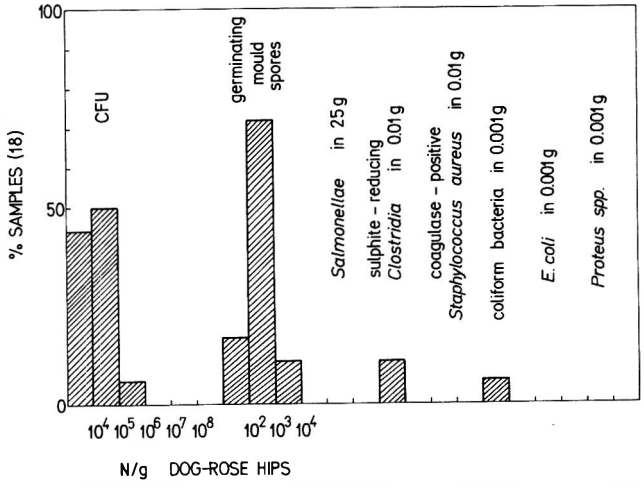

Fig. 4—Distribution of microbiological contamination of dog-rose hips.

hips, while mint leaves appearred susceptible to a somewhat higher contamination by molds. None of the samples contained *Salmonella* in 25g and, except for 2% of chamomile flowers, none contained coagulase-positive *Staphylococcus aureus* and *Proteus* spp. The incidence of sulfite-reducing *Clostridia* decreased from mint leaves, chamomile flowers, and linden flowers to dog-rose hips. The incidence of coliform bacteria and *E. coli* similarly decreased from chamomile flowers, mint leaves and linden flowers, to complete absence in dog-rose hips.

The determination of the number of microorganisms which survived hot-water treatment in the preparation of infusions was performed with both indigenously and artificially contaminated tea herbs, with the exception of sage leaves, which were only artificially contaminated (Table 1).

There was only a minor difference in the fraction of the total microflora surviving hot-water treatment of indigenously and artificially contaminated chamomile and linden flowers, about 2% in both cases. However, the population indigenous to mint leaves and dog-rose hips seemed to be much more resistant to heat than the artificially raised population (about 80% survival of the former, as compared to about 2% survival of the latter). Microbes in artificially contaminated sage leaves had an intermediate survival rate of about 25%. Total counts of indigenous microflora in mint leaves and dog-rose hips were about two orders of magnitude lower than those usually found in chamomile and linden flowers (Fig. 1 to 4). This must be due to the different physical, chemical, and biological characteristics of these materials which make them selective habitats for various populations of microorganisms.

Sulfite-reducing *Clostridia* were enumerated by the highest negative dilution. From these data, the maximum probable number, MPN, was calculated and used for the calculation of the survival rate. This procedure does not affect the survival rate which is a relative number. Sulfite-reducing *Clostridia* were found less frequently in linden flowers and dog-rose hips than in chamomile flowers and mint leaves (Fig. 1 to 4). When present in indigenously contaminated samples of linden flowers and dog-rose hips, however, the probability of their subsequent identification in infusions is relatively high and comparable to the more frequently and more heavily contaminated chamomile flowers and mint leaves. Moreover, 4% survival in dog-rose hips was the highest survival rate of sulfite-reducing *Clostridia* recorded in this work. Germinating mold spores and coliform bacteria did not survive hot-water treatment of any tea herb, neither in indigenously nor in artificially contaminated samples.

Radiation survival curves in mint leaves and dog-rose hips are shown in Fig. 5 and 6 as typical examples. All data were obtained with artificially contaminated plant materials except for chamomile flowers, for which a sample with high indigenous contamination was available. All dose survival curves were approximated by straight lines in order to obtain a parameter for intercomparison: decimal reduction dose D_{10}. The values of D_{10} are tabulated in Table 2.

Maximum probable number of sulfite-reducing *Clostridia* was used for the construction of the dose survival curves (Fig. 7). A possible effect of this procedure on the dose survival curves would be their shift upwards within one order of magnitude, but this should not affect their slopes. From these slopes, D_{10} values of sulfite-reducing *Clostridia* were calculated in various tea herbs (Table 2).

Generally, the action of radiation on the microflora of tea herbs was not very selective. With the exception of sulfite-reducing *Clostridia*, the slopes of the dose survival curves for the same species of microorganisms were similar in all tea herbs investigated. The absence of breaks on dose survival curves indicates that the population, externally grown on a nutrient substrate and subsequently inoculated onto the same substrate, was radiobiologically the same population as the one already thriving on that substrate. They behaved as a homogeneous population with a common value of D_{10}. The values of D_{10} found in various plant materials, as well as in indige-

Table 1—Microorganisms surviving in infusions prepared from indigenously and artificially contaminated tea herbs

Tea herb	g/200 mL	Species of microorganism	Indigenous contamination: Herb	Indigenous contamination: Infusion[b]	Survival (%)	Artificial contamination: Survival (%)
Chamomile flowers	1	Total count, CFU/g[a]	6,200,000 ± 920,000	120,00 ± 28,000	1.9	2.6
		Sulfite-reducing *Clostridia*, MPN/g	70	0.7	1	0.7
Mint leaves	1.5	Total count, CFU/g[a]	39,000 ± 6,000	31,000 ± 7,000	79	2.6
		Sulfite-reducing *Clostridia*, MPN/g	4.3	0.01	0.2	1
Linden flowers	2.3	Total count, CFU/g[a]	1,900,000 ± 400,000	32,000 ± 8,000	1.7	0.7
		Sulfite-reducing *Clostridia*, MPN/g	7	0.04	0.6	1
Dog-rose hips	3	Total count, CFU/g[a]	37,000 ± 11,000	30,000 ± 20,000	81	1.9
		Sulfite-reducing *Clostridia*, MPN/g	10	0.4	4	0.3
Sage leaves	2	Total count, CFU/g[a]				25
		Sulfite-reducing *Clostridia*				0.1

[a] Three replicates of same sample ± S.D.
[b] Calculated from CFU or MPN per mL infusion and g/200 mL.

Table 2—Decimal reduction dose in dry plant materials D_{10} (kGy)

Species or groups of microorganisms	Indigenous contamination: Chamomile flowers	Artificial contamination: Chamomile flowers	Mint leaves	Linden flowers	Dog-rose hips	Sage leaves
Total count	1.70	1.43	1.14	1.37	1.27	1.64
Sporogenic bacteria	2.03	1.52	1.89	1.92	1.15	2.17
Germinating mould spores	1.08	1.35	1.30	0.79	0.63	
Enterobacteria	0.90	0.88	0.40	1.16	0.76	0.65
E. coli		0.81	0.41	1.37	0.59	
Sulfite-reducing Clostridia	3.46	0.50	1.00	0.96	1.26	1.64

nously and artificially contaminated chamomile flowers, were similar for the same species of microorganisms, again with the exception of sulfite-reducing *Clostridia*. From typical initial contamination levels given in Fig. 1 to 4, and the values of decimal reduction dose D_{10} given in Table 2, it follows that commercial requirements for dry tea herbs can be met by irradiation not exceeding 10 kGy.

Based on the stand of the Joint FAO/IAEA/WHO Expert Committee on Food Irradiation (JECFI, 1977), that the chemiclearance approach provides an adequate basis for ascertaining the wholesomeness of irradiated foods, chemical analyses of the essential components of dry plant materials were made as function of dose.

The chemical composition of ethereal oil of chamomile as a function of dose has already been reported (Katušin-Ražem et al., 1985). In agreement with these results, the composition of ethereal oils of mint and sage did not change with a radiation dose up to 10 kGy (Tables 3 and 4, respectively). Sage was intentionally over-irradiated with 110 kGy, whereby the concentrations of the components of ethereal oil were reduced to about 70% of the original concentrations. Camphene and α-pinene appear more sensitive to irradiation than other components. No new peaks could be detected in the chromatograms.

The characteristic green color of mint and sage leaves is due to the presence of chlorophylls and pheophytins. Their concentration did not change with irradiation up to 15 kGy (Tables 5 and 6, respectively), and not even at 110 kGy in over-irradiated sage. Radiation-induced damage to the leaf tissue must be negligible, because the percentage of chlorophylls extracted into 80% aqueous acetone does not change even at 110 kGy.

Total polyphenols in linden flowers did not change with a dose of up to 10 kGy. However, a redistribution between nonprecipitated and precipitated polyphenols occurred with irradiation at 5 kGy (Table 7): the concentration of precipitated polyphenols decreased while that of nonprecipitated increased.

Total flavonoids in linden flowers (Table 7) and carotenoids in dog-rose hips (Table 8) also did not change with dose. The only marked change with irradiation is the content of vitamin C in dog-rose hips, which was reduced to 50% of the original concentration by 10 kGy dose (Table 8).

DISCUSSION

LARGE VARIATIONS in microbiological quality of herbs and spices have been reported (Julseth and Deibel, 1974; Schwab et al., 1982; Baxter and Holzapfel, 1982). The microbiological status of dry plant materials is the result of growing conditions, harvesting procedures, and post-harvest handling, as well as the selective actions characteristic of a given substrate. Although the physical characteristics of the surfaces are likely to play a role in colonization by microorganisms (Kedzia and Holderna, 1984), chemical composition is dominant in determining bactericidal (Baxter and Holzapfel, 1982), antifungal (Guèrin and Reveillère, 1984, 1985) and anti-yeast (Conner and Beuchat, 1984) activities.

Although mint extracts have shown a strong antifungal activity (Guèrin and Reveillère, 1985), mold spores were found in all mint samples (Fig. 3). Mild antifungal activity was found in sage and chamomile extracts (Guèrin and Reveillère, 1985), as well as in linden and dog-rose extracts (Guèrin and Reveillère, 1984; Chesne et al., 1984). Sage extracts had no anti-

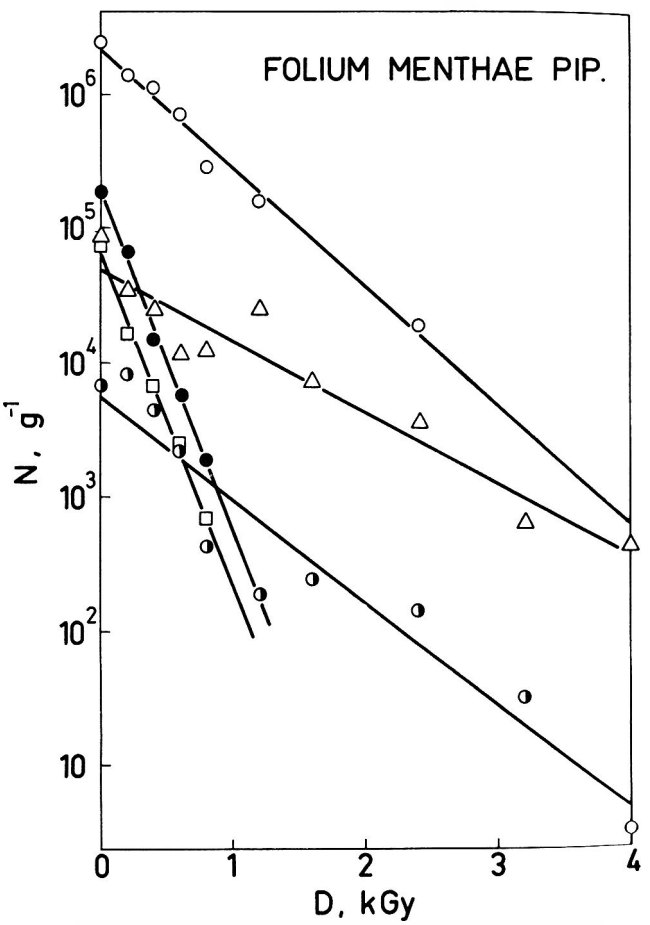

Fig. 5—Dose survival curves in artificially contaminated mint leaves. Each point is the average of three replicates of same sample. ○, colony-forming units (CFU); △, spore-forming aerobic bacteria; ◐, germinating molds spores; ●, enterobacteria; □, E. coli.

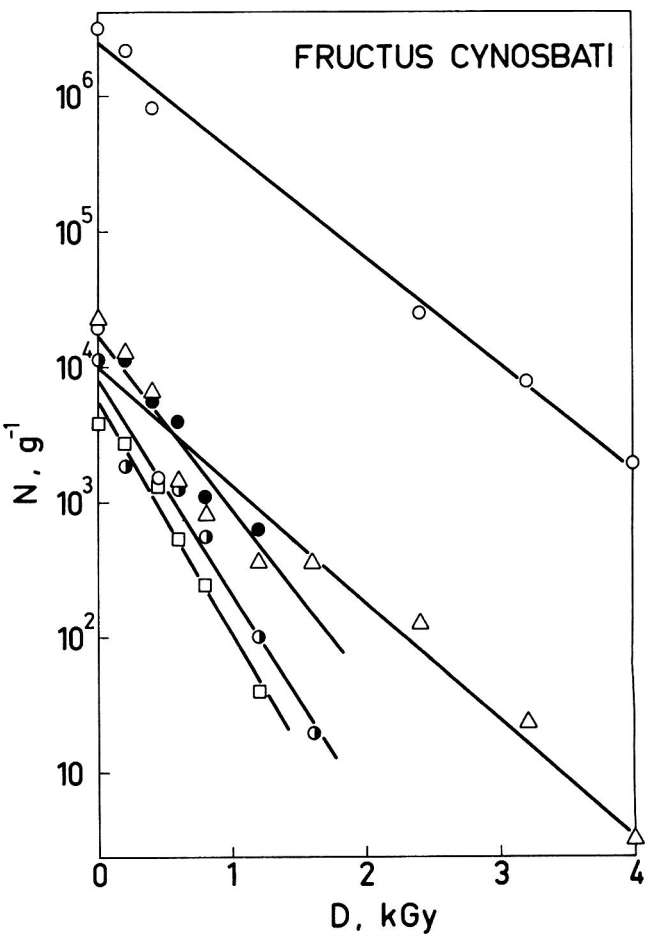

Fig. 6—Dose survival curves in artificially contaminated dog-rose hips. Each point is the average of three replicates of same sample. Symbols same as in Fig. 5.

yeast activity (Conner and Beuchat, 1984). Enterobacteriaceae persist in stored chamomile for over a year's period (Grabowska and Kedzia, 1982).

None of the five tea herbs which are dealt with in the present work seems to possess an inherent antimicrobial activity. High levels of contamination of sage (Lerke and Farber, 1960), chamomile (Grabowska and Kedzia, 1982; Bernard, 1983) and mint (Lutomski and Ježowa, 1973; Bernard, 1983) were also reported in the U.S., Poland, and France, respectively. An illustration of poor handling is the presence of insect fragments, rodent hair, feather barbules, mites, thrips, and aphids in spices and herbs (Gecan et al., 1986). Contamination of animal origin indicates an increased probability of microbial contamination as well. The absence of antimicrobial activity was proved for chamomile oil (Morris et al., 1979).

The absence of *Salmonella* and coagulase positive *Staphylococci* in our samples is in agreement with the findings of other authors (Baxter and Holzapfel, 1982). The higher incidence of *Enterobacteriaceae* and *Clostridia*, however, indicates higher probability of faecal contamination of our samples. Some authors maintain that an infinite number of soil bacteria and fungi may be permitted in plant materials intended for the preparation of tinctures, juices and alcoholic extracts, as well as for the preparation of infusions and decoctions (Kedzia and Holderna, 1984). However, there is sufficient evidence, both in literature (Baxter and Holzapfel, 1982; Bernard, 1983) and in our own previous (Katušin-Ražem et al., 1985) and the present work which shows that these procedures are survived by a number of sporeforming and thermoresistant bacteria.

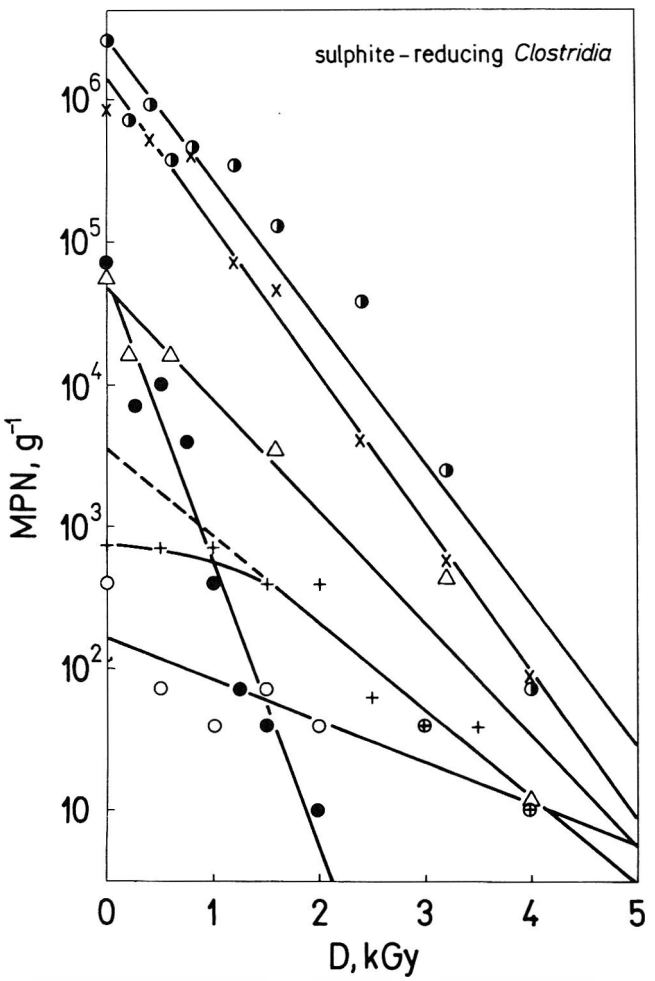

Fig. 7—Dose survival curves of sulfite-reducing Clostridia *in various tea herbs, as obtained from the maximum probable number, MPB. ◐, artificially contaminated mint leaves, three dilutions, six repetitions each; X, artificially contaminated linden flowers, three dilutions, six repetitions each; △, artificially contaminated dog-rose hips, three dilutions, six repetitions each; +, artificially contaminated sage leaves, three dilutions, three repetitions each; ●, artificially contaminated chamomile flowers, two dilutions, three repetitions each; ○, indigenously contaminated chamomile flowers, two dilutions, three repetitions each.*

Fumigation with ethylene oxide was extensively used for decontamination until recently. Since the recognition of its harmful effects in foods and the subsequent banning of the practice in many countries, further charges are not really necessary. Only a piece of evidence relevant for tea herbs shall be mentioned: ethylene oxide is readily absorbed in mint, and its dilution with other gases does not reduce the maximum attainable concentration (Chaigneau, 1983 b). Plant materials still contain 2-chloro ethanol two years after the treatment (Chaigneau, 1983 a), and it has been known for a long time that it cannot be removed by boiling (Wesley et al., 1965). These considerations further substantiate the need to use irradiation for the decontamination of dry plant materials as the only alternative "cold" process. This has been already realized and the process has been legally cleared in many countries (Anon., 1987).

The survival rate with hot-water treatment cannot be easily generalized because of many exceptions. With the exception of sage, about 2% of inoculated microflora survive hot-water treatment. The same is true for indigenously contaminated chamomile and linden flowers which typically support large total counts. Mint leaves and dog-rose hips, on the other hand, support much smaller indigenous populations with apparently inherent thermal resistivity, which, however, seems to be lost by cultivation on nutrient agar. An anomalously high survival rate was repeatedly found in artificially contaminated sage.

The radiosensitivity of total aerobic microflora in tea herbs is similar to the radiosensitivity of the total aerobic microflora found in other dry materials of vegetable origin, such as spices (Grecz et al., 1986; Weber, 1983). As expected, sporogenic bacteria are generally more resistant and mold spores are generally less resistant, while *Enterobacteriaceae* are generally the least resistant to irradiation. Although a very efficient microbicidal agent, radiation is not expected at the same time to be very selective; the use of artificially contaminated samples for radiation sensitivity studies appears justifiable.

The radiosensitivity data (D_{10} values) for sulfite-reducing *Clostridia* in indigenously and artificially contaminated chamomile flowers were not obtained in the same sample of chamomile flowers. (Only about 1/3 samples contain sulfite-reducing *Clostridia* in 0.01g, Fig. 1). The sevenfold difference between them may reflect the variability of strains and the concomitant variability of radiation resistances to be expected in natural samples. Although this problem deserves more attention, it is not imminent in tea herbs due to the relatively low incidence and generally low initial contamination levels. Thus it is usually handled within the 10 kGy processing dose.

In comparison with data in the literature (Katsiotis et al., 1985), the yield and quality of the essential oil of mint is influenced more by the degree of comminution of the plant material than by irradiation. Similarly, seasonal variations (Pi-

Table 3—Chemical composition of ethereal oil of mint (mg per 100g dry matter)[a]

Component	Dose, kGy		
	0	5	10
β-Pinene	5.7 ± 0.6	6.6 ± 0.3	6.4 ± 0.5
1.8-Cineol	19.3 ± 2.4	20.1 ± 1.8	20.3 ± 1.3
Menthone	61.1 ± 10.1	61.1 ± 13.1	65.0 ± 9.8
Isomenthone	9.6 ± 1.3	10.6 ± 2.5	9.9 ± 1.3
Menthyl acetate	15.2 ± 2.2	14.2 ± 1.1	16.0 ± 1.8
Neomenthol	12.3 ± 2.1	13.9 ± 3.0	14.5 ± 2.8
Neoisomenthol	10.3 ± 1.3	11.1 ± 2.4	12.8 ± 1.2
Menthol	164.0 ± 22.3	144.2 ± 12.3	166.1 ± 13.4
Piperitone	8.6 ± 0.4	8.2 ± 1.1	11.0 ± 0.8

[a] Four replicates of same sample (four injections each) ± S.D.

Table 4—Chemical composition of ethereal oil of sage (mg per 100g dry matter)[a]

Component	Dose, kGy 0	5	10	110
α-Pinene	6.3 ± 0.2	5.7 ± 0.3	5.2 ± 0.8	3.5 ± 0.3
Camphene	10.0 ± 0.6	8.7 ± 1.0	7.0 ± 1.1	4.3 ± 0.9
β-Pinene	3.6 ± 0.4	3.0 ± 0.4	2.9 ± 0.5	3.0 ± 0.4
Myrcene	2.5 ± 0.1	2.6 ± 0.1	2.5 ± 0.2	1.7 ± 0.1
Limonene	6.1 ± 0.4	5.7 ± 0.5	5.1 ± 0.6	4.1 ± 0.1
1.8-Cineol	40.1 ± 1.6	37.0 ± 2.2	35.2 ± 4.7	27.3 ± 1.9
Thujone	195.9 ± 8.1	188.3 ± 10.2	190.8 ± 12.9	150.8 ± 4.6
Campho-	162.4 ± 6.8	156.6 ± 14.0	152.0 ± 12.0	125.1 ± 6.5
Bornyl acetate	60.0 ± 2.8	53.2 ± 5.4	51.6 ± 2.4	41.9 ± 2.1
Borneol	27.3 ± 1.3	26.7 ± 3.2	28.2 ± 4.1	22.4 ± 1.3

[a] Four replicates of same sample (four injections each) ± S.D.

Table 5—Chlorophylls and pheophytins in the mint leaves (mg per 100g dry matter)[a]

	Dose, kGy 0	5	10	15
Chlorophyll *a*	362.7 ± 17.6	355.8 ± 8.8	348.2 ± 5.9	341.7 ± 7.0
Chlorophyll *b*	252.3 ± 12.4	258.1 ± 6.5	262.8 ± 4.7	248.0 ± 5.2
Total chlorophyll	608.9 ± 29.6	606.9 ± 15.0	601.4 ± 10.4	581.3 ± 12.1
Pheophytin *a*	329.2 ± 13.5	320.9 ± 10.0	320.9 ± 6.3	313.9 ± 9.2
Pheophytin *b*	187.1 ± 7.6	185.7 ± 5.7	185.7 ± 3.7	181.8 ± 5.2
Total pheophytin	516.2 ± 21.4	506.6 ± 15.7	506.6 ± 10.0	495.7 ± 14.4
% Chlorophyll *a*	88.0 ± 3.5	87.2 ± 1.8	88.1 ± 5.3	89.9 ± 2.6
% Chlorophyll *b*	63.5 ± 2.4	60.4 ± 1.3	60.3 ± 3.7	62.5 ± 1.7
% Total chlorophyll	78.7 ± 2.9	76.8 ± 1.5	77.4 ± 4.4	79.5 ± 2.4

[a] Six replicates of same sample ± S.D.

Table 6—Chlorophylls and pheophytins in sage leaves (mg per 100g dry matter)[a]

	Dose, kGy 0	5	10	15	110
Chlorophyll *a*	122.2 ± 6.1	116.7 ± 2.7	121.1 ± 2.6	110.5 ± 2.4	123.8 ± 3.1
Chlorophyll *b*	78.4 ± 6.4	75.8 ± 4.7	73.1 ± 12.0	66.9 ± 4.5	89.1 ± 6.0
Total chlorophyll	200.9 ± 6.6	193.0 ± 4.2	194.7 ± 11.3	178.3 ± 7.7	212.7 ± 5.6
Pheophytin *a*	91.6 ± 1.9	90.8 ± 0.9	91.3 ± 2.1	82.0 ± 0.4	92.9 ± 0.4
Pheophytin *b*	48.7 ± 2.7	48.9 ± 2.2	48.5 ± 3.3	43.8 ± 0.9	51.9 ± 1.1
Total pheophytin	140.3 ± 2.1	139.8 ± 1.8	139.8 ± 2.5	125.7 ± 0.8	144.8 ± 0.9
% Chlorophyll *a*	66.3 ± 3.2	70.1 ± 1.6	66.8 ± 4.3	65.4 ± 1.4	66.4 ± 1.6
% Chlorophyll *b*	50.2 ± 3.9	53.2 ± 3.1	54.8 ± 8.9	53.2 ± 3.5	47.2 ± 3.1
% Total chlorophyll	59.5 ± 1.9	63.3 ± 1.3	62.1 ± 3.6	60.5 ± 2.6	58.4 ± 1.5

[a] Three replicates of same sample ± S.D.

Table 7—Polyphenols expressed as pyrogallol and total flavonoids expressed as rutine in linden flowes (mg per 100g dry matter)[a]

	Dose, kGy 0	5	10
Total polyphenols	3072 ± 71	2761 ± 80	2910 ± 73
Nonprecipitated polyphenols	1051 ± 63	1140 ± 61	1155 ± 65
Precipitated polyphenols	2021 ± 96	1683 ± 135	1755 ± 94
Total flavonoids	681 ± 16	697 ± 79	665 ± 32

[a] Five replicates of same sample ± S.D.

Table 8—Carotenoids expressed as β-carotene and vitamin C in dog-rose hips (mg per 100g dry matter)[a]

Dose, kGy	Carotenoids	Vitamin C
0	3.62 ± 0.13	474 ± 24
5	3.49 ± 0.12	407 ± 21
10	3.58 ± 0.14	238 ± 10
15	3.60 ± 0.13	

[a] Seven replicates of same sample ± S.D.

tarević et al., 1984) and variations due to different localities (Kuštrak et al., 1984; Raić et al., 1985) are responsible for a much larger variability of the yield and quality of the essential oil of sage than irradiation, even including the overdose of 110 kGy.

The stability of chlorophylls in irradiated dry mint and sage leaves is greater than in irradiated parsley (Josimović, 1983); while the chlorophyll content of parsley was reduced to 94% at 50 kGy, the dose of 110 kGy was unable to produce any measurable change in chlorophylls and pheophytins, and their extractability. Generally, the stability of these pigments in dry materials is much higher than in fresh vegetables (Diehl, 1982).

Polyphenols are known to be antioxidants and their radiation-induced degradation would not be unexpected under aerobic conditions of irradiation. The failure to observe any changes of total polyphenols can be explained by the absence of oxidative changes in irradiated dry materials, or the inability of polyphenols to take part in them due to the absence of water in the medium. A slight increase of non-precipitated polyphenols is probably due to radiation-induced fragmentation of larger tannins.

The amount of total flavonoids in linden flowers is smaller than in chamomile flowers, but their resistance to radiation-induced changes when irradiated in dry plant material is similar: up to 10 kGy no loss could be detected within the experimental error. Moreover, total flavonoid content of liquid extract was stable at a dose of up to 15 kGy and 6 months' storage (Katušin-Ražem et al., 1985).

The loss of total carotenoids in various irradiated foods varies (Diehl, 1983; Tobback, 1977). High stability of carotenoids found in dog-rose hips parallels that found in other vegetable materials with low moisture content, such as ground paprika and corn.

The only component of dry tea herbs that undergoes any radiation-induced change found in this work is vitamin C in dog-rose hips. Ascorbic acid is easily destroyed in foods by oxidation during ripening, drying or irradiation (Tobback, 1977).

Ripening and drying, however, appear more detrimental to vitamin C in dog-rose hips than irradiation (Mihelić and Vajić, 1974).

CONCLUSIONS

THE INCIDENCE and microbial load of tea herbs on the retail market decreases approximately in the order: chamomile flowers, mint leaves, linden flowers, dog-rose hips. *Salmonellae*, coagulase-positive *Staphylococcus aureus* and *Proteus* spp. are usually not found in tea herbs.

There may be differences in the thermal resistivity of the indigenous and externally raised populations. The inherent thermal resistivity of indigenous populations is still a case for concern because of the survival rate after hot-water treatment.

The radiosensitivities of most common microbial contaminants of tea herbs are similar in various herbs. Contamination by different strains of sulfite-reducing *Clostridia*, having different radiosensitivities, is possible. The incidence and the level of contamination by *Clostridia,* however, are relatively low and are automatically taken care of by irradiation. To calculate the dose required for any desired reduction of microorganisms, it is necessary to know the initial contamination level and the radiosensitivities of the contaminating microorganisms. Commercial requirements are met with a dose usually not exceeding 10 kGy.

The yields and compositions of ethereal oils in aromatic plants apparently do not change with irradiation up to 10 kGy. Color pigments are stable at up to ten times higher doses. Total polyphenols, flavonoids, and carotenoids are also stable and the only change induced by irradiation is the loss of vitamin C. Radiation induced losses, if any, must be smaller than the errors of measurements, which are about 10% for gas chromatography and 3 to 5% for spectrophotometry. They are smaller than the natural variability of the content of plant materials or the changes resulting from natural processes.

Irradiation appears to be the method of choice for microbiological decontamination of dry products of vegetable origin rich in aromatic components, as the only safe "cold" method.

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Sunflower Protein Concentrates and Isolates Low in Polyphenols and Phytate

MOHAMMAD SAEED and MUNIR CHERYAN

ABSTRACT

Protein concentrates and isolates from sunflower (*Helianthus annuus* L.), essentially free of polyphenols and/or low in phytate, were prepared from dehulled seeds by a sequential extraction procedure using organic solvents, such as hexane for defatting and acidic butanol for removing polyphenols. Phytate was removed by aqueous extraction and separation at acidic and/or alkaline conditions, depending on the relative solubility of the protein and phytate. Reduced-phytate concentrates and isolates developed off-colors unless the polyphenol concentration was less than 0.05%. The protein solubility profile of the reduced-phytate products was much better than that of the defatted meal, especially below the isoelectric point.

INTRODUCTION

SUNFLOWER (*Helianthus annuus* L.) appears to have a tremendous potential for meeting the ever increasing demand for edible oil and protein by virtue of its high oil content, high oil yield per acre and the relatively high protein content of its meal. In terms of world production, sunflower is the fourth major oilseed produced, the third most important source of edible oil, and the fourth largest resource of food and feed protein (Lusas, 1985). Sunflower meal is used primarily in ruminant feed but its nutritional, sensory and functional properties make sunflower meal potentially useful in human foods.

As with many other oilseeds, sunflower contains several undesirable or antinutritive components which affects its utilization as a source of dietary protein. Sunflower seeds contain a substantial quantity of hull (>20%) which is sometimes difficult to remove and results in high fiber in the meal, but also imparts a dark color to the meal (Wan et al., 1979). Phenolic compounds have been a major deterrent to the use of sunflower protein in food products, due to their chromophoric properties. If the pH of sunflower flour or meal is raised above neutrality, its color progresses from a cream yellow to light green, to dark green and finally, to brown (Carter et al., 1972). Polyphenoloxidase catalyzes the oxidation of polyphenols to *o*-quinones (Pierpoint, 1969) which may bind covalently with thiol or amino groups of proteins (Loomis, 1974; Synge, 1975). Not only does this reduce the protein quality further, since lysine is the limiting amino acid in sunflower, but the new condensation compound cannot be metabolized by humans (Deshpande et al., 1984).

The phytate in sunflower meal (typically more than 3.5%) must also be reduced because of its well-documented effect on nutritional and functional properties of proteins (Cheryan, 1980). Accordingly, the overall objective in this research was to develop methods for the preparation of sunflower protein concentrates and isolates that are substantially free of polyphenols and/or phytate and to study the effect of these processing treatments on the protein solubility profile.

Authors Saeed and Cheryan are with the Dept. of Food Science, 382 A.E.S. Building, Univ. of Illinois, Urbana, IL 61801.

MATERIALS & METHODS

Raw material

The two cultivars of sunflower seeds selected for this study, Interstate 7000 and Interstate 7111, are hybrids grown for their high oil content and are resistant to common diseases. They were obtained from Interstate Seed Company (Fargo, ND). The weight per 100 seeds is about 7.0g and the average hull content is 21%.

Dehulling

Sunflower seeds were dehulled using the method of Pomenta and Burns (1971). The seeds were heated in an air-draft oven at 40°C for 2 hr. The seeds were dehulled by violent agitation using a jet of compressed air in a special apparatus. This was a 12-oz Ball freezer jar covered at the top with two layers of 16-mesh wire screen with a 0.5 cm diameter pipe inserted to a depth of 1 cm in a small hole at the edge of the wire screen. Air under high pressure (about 70 psig) was applied for 2 min. In this way about 25g seeds could be dehulled in 2 min under high air pressure. This was followed by separation of kernels and hulls in an air stream. Hulls still remaining on the seeds were manually removed.

Preparation of defatted sunflower meal (DSM)

Dehulled sunflower seeds were ground at 4°C in a Waring Blendor. This oil was extracted by shaking the flour with n-hexane using a flour-to-hexane ratio of 1:3 w/v for 16 hr at room temperature (22–26°C). The suspension was filtered under vacuum through Whatman No. 3 paper. The residue was dried under nitrogen at room temperature and ground to a 60-mesh size with a Buhler mill.

Preparation of low-polyphenol protein concentrate (LPC)

The method of Sodini and Canella (1977) was used for the preparation of sunflower protein concentrate that was substantially free of polyphenols. A schematic of the process is shown in the top half of Fig. 1. The defatted meal was suspended in acidified butanol in a ratio of 1:20 (w/v). Acidified butanol was prepared by mixing 1-butanol (T.J. Baker Chemical Company, Phillipsburg, NJ) with 0.005N HCl (92:8, v/v), and the pH was adjusted to 5.0 with 0.5N HCl. The extraction was carried out for 15 min at room temperature under constant stirring with a magnetic stirrer. The pH of the slurry was maintained at 5.0 by addition of 0.5N HCl. At the end of the extraction, the suspension was filtered through Whatman No. 3 filter paper under suction. The residue was extracted several times more under identical experimental conditions. The final residue was the protein concentrate, which was dried under nitrogen at room temperature. The extraction of the phenolic compounds was followed by measuring the absorbance of every extraction at 328 nm. The absorbance values were converted into grams chlorogenic acid extracted per 100g meal using the standard curve described later.

Preparation of reduced-phytate protein concentrate (RPC)

The defatted meal was suspended in tap water in a ratio of 1:5 (w/v) and the pH adjusted to 5.0 with 0.5N HCl. After 30 min at room temperature at pH 5.0, the slurry was centrifuged at 6000 $\times g$ for 20 min, and the pellet at the bottom of the centrifuge tube was filtered through Whatman No. 3 paper under suction. The insoluble pellet was extracted twice more under identical experimental conditions. The final residue, designated the reduced-phytate protein concentrate or RPC, was freeze-dried.

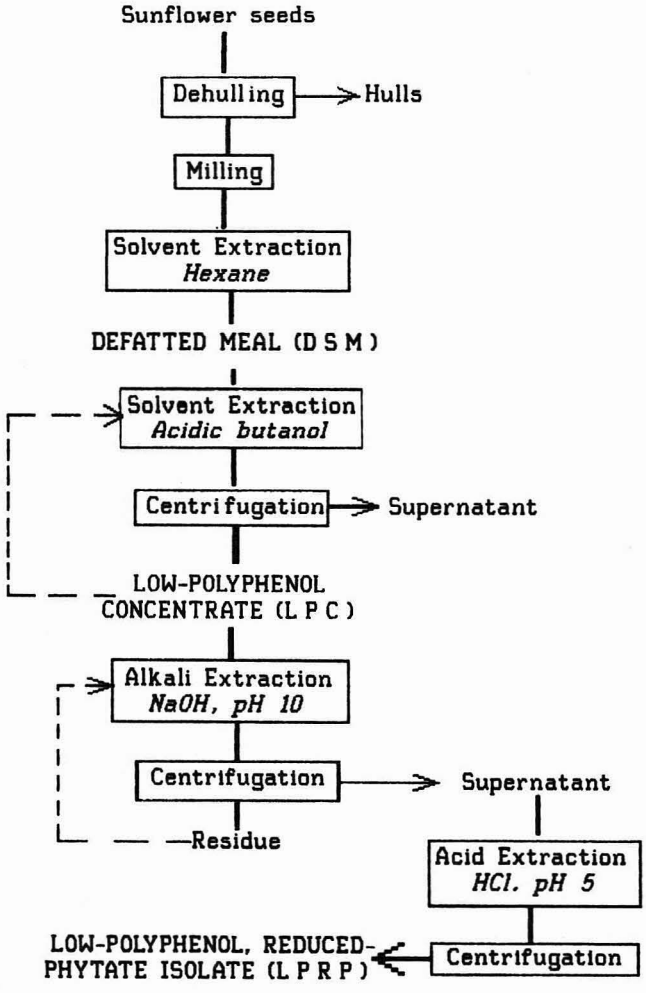

Fig. 1—Schematic for processes for preparation of defatted sunflower meal (DSM), low-polyphenol protein concentrate (LPC), and low-polyphenol, reduced-phytate isolate (LPRP).

Preparation of reduced-phytate protein isolate (RPI)

Protein isolate low in phytate was prepared by taking advantage of the differences in solubility of protein and phytic acid at certain pH values (see later discussion). The defatted meal was extracted in water, adjusted to pH 10 with 0.5N NaOH for 30 min at a flour-to-solvent ratio of 1:15 w/v. The suspension was centrifuged at 6000 $\times g$ for 20 min, and the supernatant was filtered through Whatman No. 3 paper under suction. The supernatant was saved and the residue extracted once again under identical experimental conditions. The two clear supernatants were combined. For 10g defatted meal, this would total about 280 mL of solution. This was treated with 0.5N HCl to bring the pH to 5.0 (the point of minimum solubility of the sunflower proteins: see later). The precipitate (the protein isolate) was separated by centrifugation at 6000 $\times g$ for 20 min, resuspended in 50 mL acidified water at pH 5.0, and centrifuged again at 6000 $\times g$ for 20 min. The washed residue was resuspended in 25 mL water, neutralized to pH 7.0 with 0.5N NaOH and freeze-dried.

Preparation of low-polyphenol, reduced-phytate protein isolate (LPRP)

The schematic for this process is shown in Fig. 1. Ten grams low-polyphenol protein concentrate, prepared according to methods outlined earlier, were extracted with 150 mL of water adjusted to pH 10 with 0.5N NaOH for 30 min at a flour-to-solvent ratio of 1:15 w/v. The suspension was centrifuged, filtered and the residue extracted once again under the same conditions. The two clear supernatants were combined and adjusted to pH 5.0 with 0.5N HCL. The precipitate was separated by centrifugation, washed with acidified water at pH 5.0, slurried in water, neutralized to pH 7.0 with NaOH, and freeze-dried.

Solubility profiles of sunflower products

The pH-nitrogen and pH-phytate solubility profiles of sunflower meal, protein concentrates, and protein isolates were obtained using a modification of the procedure of Gheyasuddin et al. (1970a). A sample (1g of defatted meal or 0.5g protein concentrate or protein isolate) was extracted for 1 hr with water over a pH range 2–12. The pH was maintained during extraction with 0.5N NaOH or 0.5N HCl. Clear supernatants were obtained by centrifugation for 30 min at 6000 $\times g$. The clear supernatant was filtered through Whatman filter paper (No. 3) and the filtrate analyzed for nitrogen and for phytate. The results are reported as a percentage of the total nitrogen or total phytate extracted in the filtrate.

Analytical methods

Moisture and ash were determined by a gravimetric method, fat was determined using solvent extraction, and protein was expressed as Kjeldahl nitrogen × 6.25 (AOAC, 1984).

Polyphenolic compounds were determined by the method of Dorrell (1976). Triplicate samples (500 mg each) were extracted by refluxing with 125 mL ethanol at pH 4 for 30 min. The samples were cooled, centrifuged at 2000 rpm for 5 min, and the volume was readjusted to 125 mL. The samples were properly diluted and the absorbance determined at a wavelength of 328 nm. The data are expressed in terms of chlorogenic acid equivalents (g/100g meal) using a standard curve developed using pure chlorogenic acid (Sigma Chemical Co., St. Louis, MO).

Phytate in the sunflower products was determined by the supernatant difference method (Thompson and Erdman, 1982). Phosphorus was determined by the Bartlett (1959) method. Sodium phytate (Sigma Chemical Company) was used as a reference standard.

RESULTS & DISCUSSION

Proximate composition

The proximate composition of the two cultivars reported in Table 1 show no large differences between them. The values for crude protein, oil, and ash are within the range reported by other workers for hybrid varieties (Dreher et al., 1983) and high oil varieties (Wan et al., 1979), but are lower for protein and higher for fat than the southern grown hybrid seeds reported by Robertson et al. (1971). These differences may be due to genetic differences, location, and also laboratory variability since different methods of analysis were used. Phytate data for whole sunflower seeds are rarely reported in the literature, but these results are within the range recently reported by Miller et al. (1986).

Effect of dehulling on composition

Removal of seed coat increased the lipid and protein in the kernels by 24% and 17%, respectively, while the ash was lowered by 10%. Since the hull contributes a substantial portion of the whole seed weight, and it contains relatively low levels of lipid and protein, its removal will lead to an increase of these components on a unit weight basis.

Dehulling increased the relative concentration of phytate in both cultivars by an average of 20% (Table 1). This suggested that phytate might be characteristically present in the kernel

Table 1—Proximate analysis of sunflower seed, dehulled seed and defatted meal (% dry basis)

Cultivar	Lipid	Protein	Ash	Poly-phenol	Phytic acid
Whole seed[a]					
Hybrid 7000	45.54	23.47	3.76	1.55	1.61
Hybrid 7111	47.85	21.01	3.68	1.49	1.70
Dehulled seed					
Hybrid 7000	56.86	27.27	3.35	1.68	1.96
Hybrid 7111	58.96	24.66	3.28	1.62	2.05
Defatted meal					
Hybrid 7000	1.24	62.21	7.76	3.18	3.92
Hybrid 7111	1.29	60.05	7.99	3.06	4.11

[a] Moisture (as-is): Hybrid 7000 = 7.45%, Hybrid 7111 = 7.02%. Values are means of duplicate analysis.

and very little in the hull. Since the hull contributes a substantial portion of the whole seed weight (about 21% of the whole seed is hull), removing the hull will lead to an increase in the concentration of phytate on a unit weight basis. However, dehulling may affect the assay of phytate, by improving its extraction from the sunflower seed during the assay. Phytic acid reportedly forms complexes with the seed coat fractions of other seeds (Cummings, 1976). The formation of such a complex may lead to a lower estimation of phytate in whole sunflower seed, since these complexes will be retained in the residue fraction after centrifugation.

Dehulling also increased the polyphenol concentration by about 8%. It appeared that both hull and kernel contained polyphenols, with the kernel containing slightly higher concentrations. These results are similar to those of Pomenta and Burns (1971), Brummet and Burns (1972) and Dreher and Holm (1983).

Dehulling of sunflower seeds is a major problem, both at the laboratory (bench-top) and commercial levels. With the cultivars used in this study, the hull strongly adhered to the kernel. This resulted in substantial loss of material, especially protein, during dehulling. To increase the subsequent yield of sunflower protein isolates and concentrates, a more efficient means must be developed for dehulling high-oil varieties that does not affect the yield of oil during the oil extraction process, particularly when mechanical expelling is used. Alternatively, genetic engineering and breeding of varieties with loose hulls could be undertaken to alleviate this problem.

Oil extraction

Oil extraction is an integral part of sunflower processing, especially with oil-type varieties which are grown primarily for oil production. The methods used in the industry for oil extraction include screw-pressing, solvent extraction, and a combination of screw-pressing and solvent extraction. The solvent is usually food-grade hexane. The removal of oil resulted in significant increase in all components as expected (Table 1). The oil constitutes a substantial portion of the kernel weight (56–59%). Phytate of the defatted meal averaged 4.02% while the polyphenols averaged 3.12%, representing an increase of 143% and 100%, respectively, over the whole seed. Protein and ash also increased in proportion to the amount of oil removed. The values for protein, ash and polyphenols of the defatted meal for the two varieties investigated are within the range and/or higher than the values reported for sunflower defatted meal by other workers (Rossi et al., 1985; Dreher et al., 1983; Wan et al., 1979; Sodini and Canella, 1977; Robertson et al., 1971).

Protein concentrates—removal of polyphenols

Several solvents have been investigated by other workers for the extraction of polyphenols from sunflower seeds, among them acidic ethanol, methanol, water, and NaCl solutions. Our attempts using water and acidic ethanol failed to yield a product that did not turn yellow or brown at alkaline pH. Acidic butanol, using the method shown in Fig. 1, was very successful, an observation previously reported by Sodini and Canella (1977). It took at least eight extractions to lower the polyphenols to undetectable levels (Fig. 2). There was a significant decrease in polyphenols from the first extraction to the second. However, as the number of extractions increased, the relative drop in absorbance decreased, i.e., the actual amount of polyphenol extracted decreases at every extraction step. However, less than eight extractions with acidic 1-butanol under our experimental conditions did not satisfactorily remove polyphenols from the meal. Thus, it is obvious that from at least the second extraction onwards, a process other than batch extraction, such as counter-current extraction, may be more effective and more economical in the use of solvent.

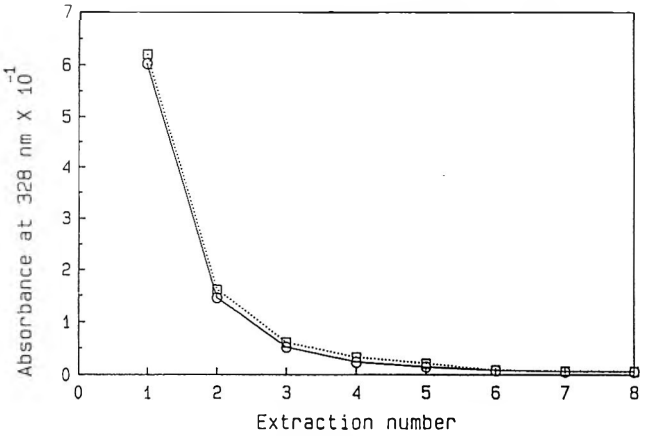

Fig. 2—Extraction of phenolic compounds from defatted sunflower meal by multiple extraction with acidic butanol: o—o Hybrid 7000 cultivar; □·····□ Hybrid 7111.

Table 2—Proximate analysis of defatted sunflower meal (DSM), low-polyphenol protein concentrate (LPC) and reduced-phytate protein concentrate (RPC), % dry basis[a]

Product	Lipid	Protein	Ash	Polyphenol	Phytic acid
DSM	0.94	61.1	7.9	3.12	4.01
LPC	0.86	69.2	8.7	N.D.	3.33
RPC	0.97	76.6	3.8	1.07	0.82

[a] Values are means of duplicate analysis of products made from both hybrid varieties listed in Table 1. N.D. = None detected.

Regardless of the variety tested, the procedure yielded a protein concentrate very low in polyphenols (<0.05%, expressed as chlorogenic acid), compared to the amount present in corresponding defatted meal (Table 2), and which was colorless under alkaline conditions. The LPC product, however, still contained large quantities of phytate. The yield of protein concentrate (on a weight basis) was approximately 80% of the corresponding defatted meal (as-is basis). The recovery of protein was over 95%.

The acidic butanol extraction involves the use of an organic polar solvent and an electrolyte with an acidic nature. The electrolyte weakens interactions between protein and phenols, whereas the weakly acidic environment increases the solubility of the phenols and serves to minimize protein-polyphenol interactions (Saeed and Cheryan, 1987), thus maximizing the extraction of chlorogenic acid. The nonreducing conditions of the solvent system are due to the presence of a high butanol concentration which assures a negligible protein solubility, preventing hydration of protein and the binding of polyphenols (Sodini and Canella, 1977).

Removal of phytate

To remove phytate from sunflower meal, we took advantage of the differences in solubility between the phytate and protein at certain pH values. As shown in Fig. 3, there are two regions where the solubility of phytate does not correspond to that of the nitrogen constituents. At pH 10, proteins are highly soluble while phytates are highly insoluble. There is also a large solubility difference at pH 5, where proteins are insoluble and phytates are soluble. Thus the reduced-phytate concentrate (RPC) was prepared from the defatted meal by adjusting a suspension to the isoelectric point and centrifuging.

The chemical composition of defatted meal from the two cultivars and the corresponding RPC are given in Table 2. The extraction process appreciably increased protein from 61% in the defatted meal to 76%, while reducing the phytate concentration from 4.01% to 0.82%. It also decreased the polyphenols to 1% in the RPC. However, this residual chlorogenic acid

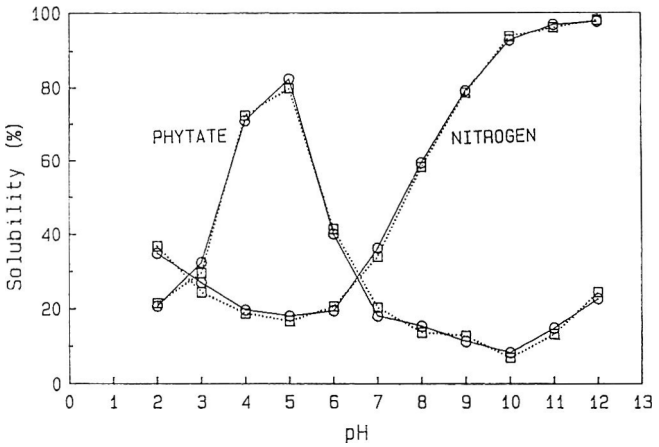

Fig. 3—Solubility profiles of phytate and nitrogen in defatted sunflower meal. ○—○ and □·····□ represent hybrid cultivars 7000 and 7111, respectively. Solubility is expressed as percent total nitrogen or phytate in the meal that is soluble at that pH.

Table 3—Proximate analysis of reduced-phytate protein isolate (RPI) and low-polyphenol, reduced-phytate isolate (LPRP), % dry basis[a]

Variety	Lipid	Protein	Ash	Polyphenol	Phytic acid
DSM	0.94	61.1	7.9	3.12	4.01
RPI	0.21	92.7	1.4	1.00	0.29
LPRP	0.23	94.1	1.4	N.D.	0.35

[a] Values are means of duplicate analysis of products made from both hybrid varieties listed in Table 1.

was too high to be acceptable, since it developed a dark brown color at alkaline pH. Ash was much lower in the protein concentrate (3.88%), compared to the 7.88% ash of the defatted meal. The yield of the RPC was only 60% of the defatted meal. The loss of protein in the extract was 23% of the total protein in defatted meal.

The reduced-phytate isolate (RPI) was prepared by extraction and centrifugation at two pH values. As seen in Table 3, RPI had very low phytate (0.29%) and higher protein (92.7%). Ash of the isolate (1.36%) was much lower than the defatted meal (7.88%). The product, however, was dark brown in color because of the binding of the oxidized products of polyphenols to protein. The yield of the protein isolate was 30% of the defatted meal.

Low-polyphenol, reduced-phytate protein isolate (LPRP)

The procedure for preparing this purified form of the isolate essentially consisted of first preparing a low-polyphenol protein concentrate (LPC) using the acidic butanol reagent, and then removing the phytate by alkaline and acid extractions. The LPRP isolate was low in phytate (0.35%) and ash (1.4%) and was essentially free of polyphenols. The product had 94% protein and did not turn brown at alkaline pH. The yield of LPRP in the bench-top experiments was very low (<30%), primarily due to losses of material during dehulling and the extractions.

Solubility characteristics of sunflower protein products

Solubility is a critical functional property of protein ingredients. In beverages, it is a prerequisite that protein ingredients remain fully dispersed and soluble. Many other functional properties depend upon the capacity of the protein to go into solution initially, e.g., gelation, emulsifying, and foaming properties. Fully solvated proteins are also necessary in such foods as comminuted meats when water adsorption and flavor binding characteristics are important (Kinsella, 1976; Mattil 1971).

Defatted meal

The nitrogen solubility profile of defatted sunflower meal (DSM) and the reduced-phytate products (RPC and RPI) are shown in Fig. 4. Each point in the figure represents the average of duplicate samples. Unlike soybean meal (Grynspan and Cheryan, 1988; Kilara et al., 1972), a sharp minimum solubility at the isoelectric point was not observed with defatted sunflower meal. Instead a broad range of minimum solubility between pH 3 and 6 was found, with the minimum solubility being 17% (average for both hybrid cultivars) at pH 5. This has been observed by others (Gheyasuddin et al., 1970b; Sosulki and Fleming, 1977; Mattil, 1971; Clark et al., 1980; Rossi and Germandari, 1982). Sunflower meal showed good extractability above pH 10, being over 90% soluble compared to 80% for soybeans (Kilara et al., 1972; Grynspan and Cheryan, 1988).

Below the isoelectric point, the nitrogen solubility of the RPC was slightly higher, and of the RPI, much higher than the defatted meal. This could be due to removal of the phytate; the reduced-phytate products were 80–90% lower in phytate than the defatted meal. Phytate has been shown to complex with proteins below their isoelectric points in soybeans (Smith and Rackis, 1957; Shen, 1976; Cheryan, 1980; Lah and Cheryan, 1980; Grynspan and Cheryan, 1988), in peanuts and cottonseed protein (Fontaine et al., 1946). Phytate-reduced soy protein isolates were substantially more soluble below its isoelectric point (Chen and Morr, 1985).

However, the difference in the phytate content *per se* between RPI and RPC are not that significant (Tables 2 and 3) to account for the large difference in solubility at pH 2. Considering that the nitrogen solubility at the isoelectric region (pH 4–6) was much lower for both products compared to the defatted meal, it appeared that sunflower contained a significant proportion of acid-soluble "whey" protein, which was removed during the acid extraction step. The reduced-phytate products are also much more soluble at pH 6–9 than the defatted meal, due possibly to removal of phytate which can form ternary complexes with proteins (Cheryan, 1980) that may be insoluble, or due to loss of alkaline-insoluble proteins during the extraction-centrifugation steps.

Low-polyphenol protein concentrate

The same broad range of minimum solubility between pH 3 and 6 is observed with LPC also (Fig. 5), but it exhibited a

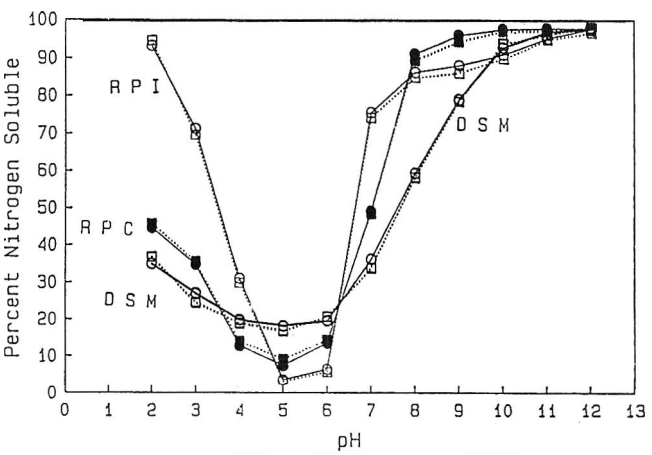

Fig. 4—Nitrogen solubility profile of defatted sunflower meal (DSM), reduced-phytate protein concentrate (RPC) and reduced-phytate protein isolate (RPI). ○—○ and □·····□ represent hybrid cultivars 7000 and 7111, respectively.

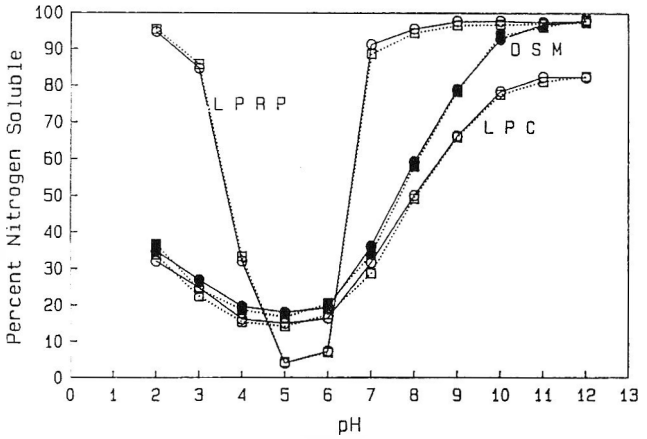

Fig. 5—Nitrogen solubility profile of defatted sunflower meal (DSM), low-polyphenol concentrate (LPC) and low-polyphenol, reduced-phytate isolate (LPRP) for both cultivars. ○—○ and □·····□ represent hybrid cultivars 7000 and 7111, respectively.

lower protein solubility than the defatted meal over the pH range examined. An organic solvent was used for the removal of the polyphenol, which may have caused some denaturation of the protein (Sodini and Canella, 1977; Rahma and Narasinga Rao, 1981).

It is frequently suggested that the broad range of minimum protein solubility is due to interaction of polyphenols with the protein. For example, aqueous extraction of chlorogenic acid at pH 5 results in an apparent increase in nitrogen solubility (Rahma and Narasinga Rao, 1981; Sosulki et al., 1973; Fan et al., 1976). Furthermore, the extracted meal has a sharp minimum solubility point, compared to a broad minimum solubility pH range for the untreated meal (Rahma and Narasinga Rao, 1979). However, those who used organic solvents to extract the polyphenols could not ascribe this phenomenon strictly to the presence of chlorogenic acid, due to the denaturing effect of the solvent. It should also be remembered that phytate is highly soluble at pH 5, and thus water extraction will also remove a significant amount of phytate. Phytate is known to interact with proteins and cause significant shifts in the solubility profiles (Cheryan, 1980; Grynspan and Cheryan, 1987; Lah and Cheryan, 1980). This confounds the interpretation of solubility data published in the literature; for example, Rahma and Narasinga Rao (1979) showed that the removal of polyphenols by extraction with acidic sodium chloride lowered the nitrogen solubility index of the extracted meal, and the isoelectric point had shifted to the right to pH 6.

Low-polyphenol, reduced-phytate protein isolate

Figure 5 also shows the nitrogen solubility profile of the LPRP isolate. At pH 2, the solubility increased from 35% for the defatted meal to 95% for the LPRP isolate. In contrast to the defatted meal and LPC, LPRP isolate had a sharp, but lower, minimum solubility point near pH 5. This could be due to the loss of water-soluble ("whey") proteins or nonprotein nitrogen (NPN) during the extraction-centrifugation steps. LPRP isolate was also much more soluble at pH 7 and above than DSM and LPC. A comparison of the solubility profiles of all five products in Fig. 4 and 5 suggests that removal or phytate or polyphenol *per se* does not have as large an effect on the solubility profile as the alkaline extraction step in the manufacturing procedure.

SUMMARY & CONCLUSIONS

DEHULLING and defatting did not appreciably reduce polyphenol and phytate. The best solvent for removal of polyphenols from defatted meal was acidic butanol at pH 5. The polyphenol concentration was reduced to an undetectable level (<.05%) with little further denaturation of the protein as evidenced from the nitrogen solubility profile of the low polyphenol protein concentrate (LPC). LPC contained 70% protein (dry basis) and had good potential in food applications as it did not turn brown at alkaline pH.

To remove phytate, the large differences in solubility of phytic acid and protein at its isoelectric point was used to prepare a reduced-phytate protein concentrate (RPC) from defatted sunflower meal with 75% protein and generally improved protein solubility. However, it had a high polyphenolic content and developed a brown color at alkaline pH. The reduced-phytate isolate (RPI) was similarly high in protein (>90%) and low in phytate (about half the phytate of RPC and one-ninth that of the defatted meal), but was brown in color because of binding of oxidized phenolic compounds.

The low-polyphenol, reduced-phytate protein isolate (LPRP) prepared by a multiple extraction process has excellent potential in food applications. Not only does it have good functional properties (good and stable color, much higher protein solubility at certain pH values), but also significantly better nutritional properties due to the removal of phytates and polyphenols. This should vastly improve its utilization in both animal feed and human food products.

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—Continued on page 1143

Enhancement of Peptidoglutaminase Deamidation of Soy Protein by Heat Treatment and/or Proteolysis

JAMEL S. HAMADA and WAYNE E. MARSHALL

ABSTRACT

The limited deamidating ability of *B. circulans* peptidoglutaminase towards soy protein was increased 27-fold by protein hydrolysis and altering soy protein conformation by moist heat. Adding disulfide bond reducing agents such as cysteine or sodium sulfite or salts such as NaCl or KCl to the reaction mixture had little or no effect on deamidation. However, heat treatment at 100°C for 15 min, both before and after Alcalase proteolysis, rendered soy protein very susceptible to amide hydrolysis by peptidoglutaminase.

INTRODUCTION

DEAMIDATION OF FOOD PROTEINS is known to improve solubility and other functional properties of proteins under mildly acidic conditions. An enzymatic approach to protein deamidation offers several advantages over a chemical approach, including reaction selectivity and milder deamidating conditions such as neutral pH and room temperature. However, enzymatic deamidation of food proteins has received little attention until recently. Gill et al. (1985) detected limited deamidating activity of *Bacillus circulans* peptidoglutaminase towards casein and whey protein hydrolyzates. Motoki et al. (1986) used guinea pig transglutaminase to catalyze the hydrolysis of amide groups of glutamine residues in casein. Kato et al. (1987) developed a method to deamidate food proteins, including soy proteins, by treatment with proteases at pH 10. They observed, however, a significant amount of proteolysis with soy globulins, concurring with deamidation. Furthermore, processing of food proteins at pH 10 is undesirable for a variety of reasons including the implication of alkali-treated foods in causing kidney damage in rats (Woodard and Short, 1973).

Hamada et al. (1988) used the peptidoglutaminase from *B. circulans* to deamidate soy peptides and proteins in phosphate buffer at pH 7.5. Peptidoglutaminase readily deamidated soy peptides but its activity toward the intact protein was small. They also suggested that limited deamidation was due to the large molecular size and/or unique conformation of soy protein. The objective of this investigation was to quantify the effects of altering soy protein molecular size by proteolysis or modifying soy protein conformation, principally by heat treatment, on soy protein deamidation using the peptidoglutaminase.

MATERIALS & METHODS

Materials

CBZ-L-glutamine was purchased from Sigma Chemical Co., St. Louis, MO. and t-BOC-L-glutaminyl-L-proline from Peptides International, Louisville, KY. Nutrisoy 7B flakes were obtained from Archer Daniels Midland Co., Decatur, IL. Alcalase 2.4 L (2.4 Anson units/g) was obtained from Novo Laboratories Inc., Wilton, CT. *Bacillus circulans* culture (ATCC #21590) was obtained from the American Type Culture Collection, Rockville, MD. Other chemicals were reagent grade or the highest purity obtainable.

Authors Hamada and Marshall are with the USDA-ARS, Southern Regional Research Center, P.O. Box 19687, New Orleans, LA 70179.

Preparation of soy protein substrate

The soy protein, used as substrate for peptidoglutaminase deamidation and as starting material for the proteolysis experiments, was a water extract of soy flakes. Nutrisoy 7B flakes were extracted with water (1:20 ratio) at 25°C for 1 hr and passed through 16 layers of chesecloth to remove insoluble materials. The freeze-dried preparation was 55.0% protein.

Preparation of peptidoglutaminase

Phosphate gel eluate was prepared according to Hamada et al. (1988), freeze-dried and stored at −5°C until needed. The lyophylized eluate contained 35.5% protein.

Peptidoglutaminase assay and measurement of deamidation

A 1 mL aliquot of the enzyme solution containing 2 mg of freeze-dried phosphate gel eluate was added to 2 mL of substrate in 0.05 M phosphate buffer, pH 7.5. The substrate concentrations were 0.25% CBZ-L-glutamine to assay peptidoglutaminase I, 0.10% t-BOC-L-glutaminyl-L-proline to assay peptidoglutaminase II and 5% soy protein or soy protein hydrolysate to study soy protein deamidation by peptidoglutaminase. Substrate concentrations were determined to be sufficient for saturation of the enzyme. Reaction mixtures were incubated at 30°C for 1 hr, and 0.3 mL of 50% TCA was added to stop the reaction. The ammonia content of the reaction mixtures and controls was measured with an Orion ammonia electrode (model 95-10) connected to an Ion Analyzer model EA 940 (Orion Research Inc., Cambridge, MA). The extent of deamidation was calculated as the ratio of ammonia released enzymatically to the ammonia released by the soy protein after amide hydrolysis with 2N HCl at 100°C for 4 hr (Wilcox, 1967).

Protein hydrolysis

The pH of the dispersions of both undenatured and heat-treated soy proteins (4g in 100 mL water) was adjusted to 8.0 and then 0.05g Alcalase 2.4L was added to start the proteolysis. The extent of protein hydrolysis was controlled quantitatively using the pH-stat method of Adler-Nissen (1986). During hydrolysis at 50°C, the reaction mixture was continuously titrated to pH 8.0 with 0.5N NaOH using an Auto Titrator (Radiometer A/S, Copenhagen). Depending on the degree of hydrolysis desired, the reation mixture consumed 0.2 to 8.3 mL 0.5N NaOH. Proteolysis was stopped by a 10 min heat treatment at 85°C. Hydrolysates were freeze-dried. The degree of hydrolysis, or percent of peptide bonds cleaved, was determined by reacting free amino groups with trinitrobenzenesulfonic acid (Alder-Nissen, 1979).

Protein treatment with heat, salt or disulfide reducing agents

Heat treatments were carried out using 5% dispersions of soy protein or soy protein hydrolysate in 0.05 M phosphate buffer, pH 7.5. A 2 mL protein suspension in culture tubes was covered with screw caps, placed in a boiling water bath and heat for 10–120 min at 100°C. Concentration of salts and reducing agents were based on 3 mL total volume of reaction mixture. Salt or reducing agent was dissolved in 0.5 mL water, added to 2 mL hydrolysate or protein dispersion, and the mixture was stirred for 15 min. Peptidoglutaminase (2 mg eluate dissolved in 0.5 mL water) was added to start the reaction.

Protein determination

The protein content of PGase was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard. The

protein content of soy protein was determined by the Kjeldahl method using 6.25 as a conversion factor.

RESULTS & DISCUSSION

THE PHOSPHATE GEL ELUATE, reported by Hamada et al. (1988) to exhibit higher peptidoglutaminase (PGase) activity towards soy peptides and proteins than the crude extract, was used throughout this investigation. The eluate was a mixture of PGase I and PGase II activities, which were measured using CBZ-L-glutamine and t-BOC-glutaminyl-L-proline, respectively. The phosphate gel eluate released 11.6 and 3.4 micromoles ammonia/mg protein/hr at 30°C from the two synthetic substrates, respectivley. No loss of phosphate gel eluate activity towards both synthetic substrates was noted upon storage at −5°C for at least 10 wk.

Susceptibility of heat-treated soy protein to PGase deamidation

Figure 1 presents the effect of soy protein heating time on protein deamidation by PGase. Heating soy protein at 100°C for 2 hr increased deamidation 5.5-fold. When soy protien was heated at intervals from 0 to 90 min before deamidation, the subsequent extent of deamidation increased in an almost linear manner before leveling off. Therefore, there is little to be gained by heating soy protein at 100°C longer than 90 min prior to deamidation with PGase. The noted increased in deamidation of soy protein upon heating may be explained by the conformational changes soy proteins undergo when denatured by heat. Wolf (1978) found that heating soy protein disrupts its native structure with the possibility of forming soluble aggregates through the interaction between the different subunits of the soy globulins.

Influence of salt and reducing agents on soy protein and its subsequent deamidation by PGase

The ability of PGase to deamidate soy protein increased slightly with the addition of less than 0.1M sodium chloride and 0.05M potassium chloride as shown in Fig. 2. At greater concentrations, both salts appeared to have an inhibitory effect. This should be taken into account in determining the amount of salt used to prepare the protein for deamidation, since NaCl is commonly used to adjust the ionic strength of enzyme reaction mixtures.

Addition of 0.001 or 0.005M cysteine or 0.002 or 0.01M sodium sulfite, two reducing agents commonly used in foods, to reaction mixtures had no effect on the deamidation of the protein substrate by PGase. However, a 6–10% improvement in deamidation was observed when 0.001M cysteine and heat treatment were used with proteins hydrolyzed to approximately 2-5% DH. Cysteine and sodium sulfite have been used at comparable concentrations to break disulfide bonds in food proteins, including soy protein (Circle et al., 1964). Breaking disulfide bonds in soy protein apparently did not render the protein more susceptible to PGase attack.

Proteolysis of soy protein and its effect on PGase activity

Proteolysis is a convenient way to reduce the molecular size of proteins by reducing the length of the amino acid chains. When soy proteins were hydrolyzed by Alcalase before deamidation, the extent of deamidation increased linearly to 10% DH and rapidly leveled off (Fig. 3A). From 0 to 20% DH, an 8-fold increase in deamidation was observed. This signficant improvement in deamidation might be due to changes in both protein conformation and molecular weight accompanying proteolysis. Whitaker (1977) noted that modification of hydrolyzed proteins was due to cleavage of specific peptide bonds, protein size and solubility. After about 10% of the total peptide bonds were cleaved, new peptide conformations might offer

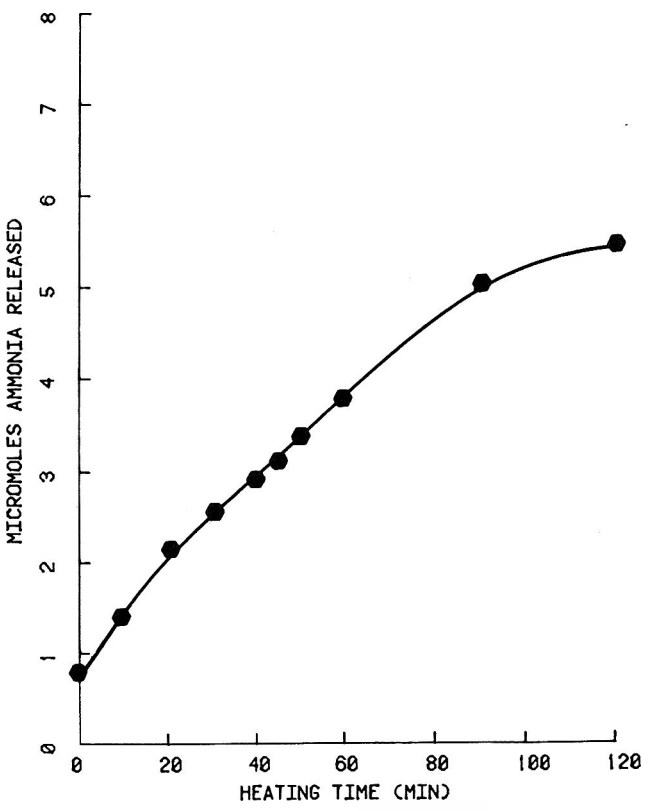

Fig. 1— Effect of heat treatment of soy protein on PGase deamidation.

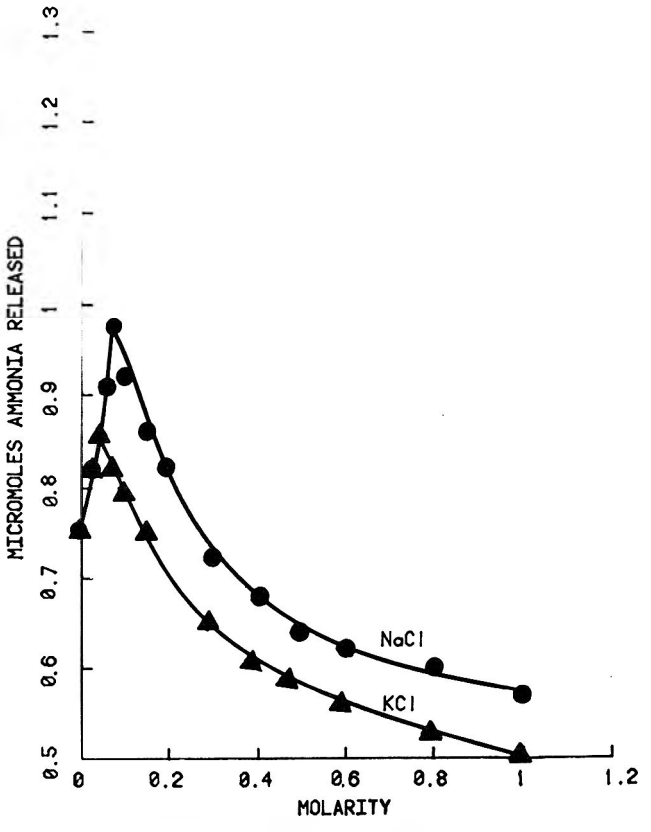

Fig. 2— Effect of NaCl and KCl concentrations on PGase deamidation of soy protein.

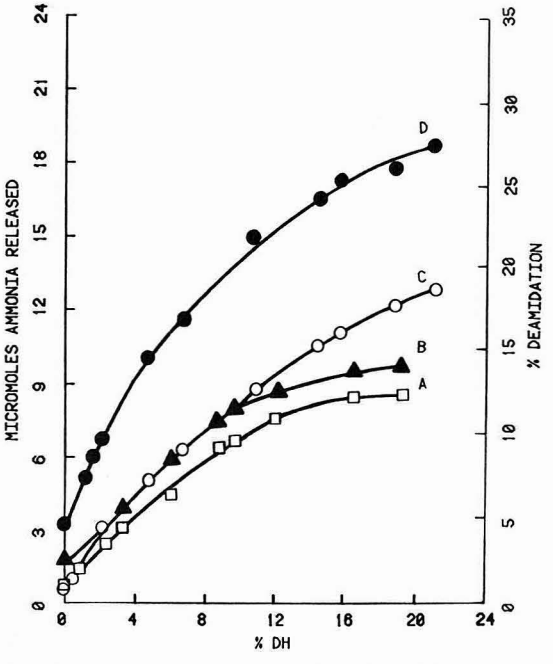

Fig. 3— Effect of Alcalase proteolysis and heat treatment on the PGase deamidation of soy protein: (A) unheated, (B) heated after proteolysis, (C) heated before proteolysis, and (D) heated before and after proteolysis.

maximum accessibility of amide groups to PGase, providing a leveling off in the quantity of deamidation that could occur.

Relatively small amounts of ammonia were released from soy protein during Alcalase hydrolysis under conditions given in Fig. 3. Ammonia concentration ranged from 0.1 to 1.4 micromoles ammonia/100 mg protein and was proportional to the DH values. Considering that soy protein contained 68.3 micromoles amide/100 mg protein, the greatest concentration of ammonia released (1.4 micromoles) was equivalent to only 2% of the total amide in the soy protein.

Effect of heat treatment and proteolysis on PGase deamidation of soy protein

Heating soy protein at 100°C for 15 min after Alcalase hydrolysis increased the rate of deamidation as a function of DH at all degrees of hydrolysis (compare Fig. 3A to Fig. 3B). Improvement in deamidation may be due to the direct effect of the heat treatment. When soy protein was heated at 100°C for 15 min prior to hydrolysis (Fig. 3C), the degree of deamidation was similar to the post-heat treatment results (Fig. 3B) at DH values below 10% but deamidation was greater at DH values above 10%. Pre- and post-heat treatment of hydrolyzed soy protein produced the greatest degree of deamidation (Fig. 3D) at all DH values. As a result of the two heat treatments, deamidation of soy protein increased more than 27-fold when compared to the deamidation of soy protein with no heat treatment and no proteolysis. Results presented in Fig. 3 illustrate that heat treatment improves soy protein susceptibility to deamidation. Heat treatment caused the protein or polypeptide to unfold and the new conformations were apparently more susceptible to PGase activity. Since heat treatment improved deamidation, even for hydrolyzates with relatively larger DH values, a portion of the polypeptide chain might still retain some secondary and tertiary structure. Evidently, this residual structure must be disrupted before glutamine residues become completely accessible to PGase. Accordingly, it may be possible to estimate the amount of secondary and tertiary structure in a soy polypeptide using heat treatment and PGase. The results presented here differ from observations of Gill et al. (1986). They found little PGase deamidation of whey and casein proteins even after protein hydrolysis. Also, when heat and other protein-denaturing conditions were used, PGase deamidation was not observed for these proteins. These differences in deamidating activity may be due to differences in the proteins used as substrates.

Combining protein substrate treatments for PGase deamidation

Table 1 presents the effect of combining heat treatment, salt, reducing agent and enzyme concentration at various degrees of soy protein hydrolysis on the deamidation of soy protein. A study on the progress of PGase deamidation of soy protein as a function of time revealed that the reaction was complete after 120 min. Accordingly, reaction mixtures were incubated at 30°C for 120 min to maximize deamidation. Doubling the concentration of enzyme in the reaction mixture had little effect on soy protein deamidation. In Table 1, only low levels of proteolysis were selected. High levels of proteolysis are normally not used in food applications because of adverse effects on flavor and physical properties of the hydrolysates. The greatest amount of ammonia released (10.8 micromoles/100 mg protein) from these soy proteins with limited proteolysis, corresponded to 16% deamidation since the untreated soy protein contained 68.3 micromoles amide/100 mg protein.

Enzymatic hydrolysis of soy proteins is usually accompanied by bitter taste. Low levels of bitterness can be obtained by restricting the degree of hydrolysis to small levels (3–5% (Adler-Nissen, 1986). At DH values of 3-5%, 10-15% of soy protein deamidation by PGase can be achieved (Fig. 3). This respectable level of deamidation should be sufficient to observe changes in the solubility and physical properties of the deamidated proteins. Furthermore, glutamate-containing peptides are thought to mask the bitterness released at larger DH values (Adler-Nissen, 1986). Accordingly, combining deamidation with proteolysis may allow hydrolysates with larger degrees of hydrolysis before bitterness is a problem.

CONCLUSIONS

HEAT TREATMENT and/or proteolysis of a soluble extract of soy flakes significantly increased the degree to which soy protein could be deamidated by *Bacillus circulans* peptidoglutaminase. With heat treatment and proteolysis of soy protein to a DH value of 20%, over 27% protein deamidation could be achieved. Heat treatment and proteolysis of soy protein were the major factors affecting deamidation. The presence of various salts and disulfide reducing agents had little effect on soy protein deamidation. It appeared that PGase deamidation was impaired by soy protein molecular size and conformation as changes in both resulted in significant protein deamidation. A wide range of soy protein deamidation can be achieved by heat or proteolysis alone or a combination of both. Control of the deamidation process will allow soy proteins with different degrees of deamidation to be evaluated for improved functionality in a wide variety of food applications.

—*Continued on page 1149*

Table 1—Deamidating activity of peptidoglutaminase towards untreated, heat-treated, or proteolyzed soy protein

	Micromoles ammonia released in reaction mixture[a]			
	2 mg PGase		4 mg PGase	
Sample % DH	Untreated	Treated[b]	Untreated	Treated[b]
0	1.0	2.9	1.0	3.0
1.4	2.0	4.5	2.2	4.7
4.8	4.7	8.5	5.4	9.5
6.7	6.3	9.5	7.5	10.8

[a] Reaction mixture contained 1.1g preheated protein (100°C, 15 min) and 2 or 4 mg PGase; incubated at 30°C for 2 hr.

[b] Soy protein was modified by heating for 30 min at 100°C in 0.05M phosphate buffer, pH 7.5 with 0.05M phosphate buffer, pH 7.5 with 0.001M cysteine and 0.1M NaCl.

Nutrient and Sensory Properties of Dry Beans (*Phaseolus vulgaris* L.) Grown Under Various Cultural Conditions

H. H. KOEHLER and D. W. BURKE

ABSTRACT

Dry beans UI-114 (Pinto) and Rufus (Red Mexican) were field-grown under presence or absence of *Fusarium* root rot, drought or optimum water, low or high nitrogen fertilization. Raw bean powder was analyzed chemically for proximate composition, minerals, vitamins and amino acids, and by *Tetrahymena pyriformis* W for protein quality. Cooked beans underwent sensory evaluation. Protein content of raw beans generally varied inversely with irrigation. Rufus beans contained significantly more thiamin than comparable UI-114 beans. High-N non-diseased soils produced beans with methionine concentrations greater than those from low-N Fusarium-infected soils. Pinto UI-114 beans were rated significantly more acceptable than Rufus beans ($P < 0.001$).

INTRODUCTION

UI-114 and Rufus are widely grown cultivars in the northwestern states (Koehler and Burke, 1981). Pinto and Red Mexican (small red) beans comprised about 42% of the total *Phaseolus vulgaris* tonnage grown in the United States in 1985 (USDA, 1986).

Dry beans grown under irrigation are subjected to several variables of management and environment which affect seed yield and may affect nutrient value and consumer acceptability of beans. Planting cultivars with disease-resistant properties is important (Morrison and Burke, 1962). A major disease problem is *Fusarium* root rot [*Fusarium solani* (Mart.) Appel and Wr. f. sp. *phaseoli* (Burkh.) Snyd. and Hans.] (Burke and Nelson, 1967). In tests of twelve commerical cultivars grown on *Fusarium*-infested and noninfested fields, Burke and Nelson (1967) reported much higher yields from the non-infested fields, but on infested fields, Pinto UI-114 and two other cultivars had less root rot and significantly higher yields than the other cultivars. Morrison and Burke (1962) cautioned that excess early irrigation may encourage root rot. Burke and Nelson (1967) found that root rot severity was not affected by level of nitrogen (N) fertilization, and concluded that root rot reduced the ability of bean plants to use N because *Fusarium*-damaged root systems were less capable of absorbing large quantities of N than were healthy root systems. Morrison and Burke (1962) stated that to produce good seed yield, 45-70 kg/Ha available N was needed on fertile soils and 90-200 kg/Ha on poor soils. Burke and Nelson (1967) found that yields of their twelve cultivars increased curvilinearly as available N increased on non-infested fields up to 240 kg/Ha and on *fusarium*-infested fields up to 150 kg/Ha. Other soil nutrients necessary for high bean seed yield are phosphorus and zinc (Morrison and Burke, 1962). Because beans are a shallow-rooted crop, sufficient and timely irrigation is a factor in producing good yields, according to Robins and Domingo (1956). They reported that visible moisture stress resulted in yield reductions of about 20% measured in terms of number of pods, number of seeds per pod and bean seed weight. In tests with bush snap beans, Middleton and Silbernagel (1977) found that seed production was less consistently reduced by infrequent irrigation than was green pod production. They reported that the percentages of seed protein increased slightly with less frequent irrigation. Leleji et al. (1972) found that yield and crude protein percentage were generally negatively correlated, that is, high-yielding segregates tended to be relatively low in protein percentage. However, they identified plants having both high seed yield and above average percentage of crude protein. Kelly and Bliss (1975) suggested using such bean strains in breeding programs to raise the nutritional contribution of beans by improving seed protein quantity and quality as well as seed yield. In a study of genetic variations and interrelationships of dry bean quality traits, Ghaderi et al. (1984) found significant cultivar differences for most of the fifteen traits measured, and highly significant location effects for all traits, although they doubted the quality differences between beans from the two locations could have been distinguished easily by a sensory panel. Seasonal effects predominated over genotypic effects in studies of yield and physico-chemical seed characteristics of 25 strains of Black Turtle Soup-class beans (Hosfield et al., 1984). They found inconsistent bean strain rankings from year to year, suggesting the need for several years of testing to assess strain performance accurately in measurements of food quality. Neither sensory evaluations nor nutrient determinations were involved in their study.

The purpose of this study was to evaluate nutrient, sensory and texture qualities of commercial dry-bean cultivars UI-114 (Pinto) and Rufus (Red Mexican) grown under several management and environmental conditions.

MATERIALS & METHODS

Cultural conditions

Cultivars UI-114 (Pinto) and Rufus (Red Mexican) were field-grown at the Washington Irrigated Agriculture Research and Extension Center (IAREC), Prosser, under a range of conditions simulating those found in commercial bean fields of Washington State, as follows:

Trial 1. *Fusarium* infestation; irrigation at 5-day intervals to insure optimum soil moisture.

Trial 2. *Fusarium* infestation; irrigation at 10-day intervals to cause drought stress.

Trial 3. No evident *Fusarium* root rot; irrigation every 5 days to provide optimum water.

Trial 4. No evident *Fusarium* root rot; irrigation every 10 days to cause drought stress.

Trial 5. Infestation of *Fusarium* root rot; low soil N (20-40 kg/ha); standard irrigation (every 5-7 days).

Trial 6. No evident *Fusarium* root rot; high soil N (>120 kg/ha); standard irrigation.

Beans were seeded in fine sandy loam in 56-cm rows. All six field plots were similar in location. Irrigation water was applied in alternate interrow ditches every five or ten days. Phosphorus, potassium and zinc were applied to obtain optimum levels according to soil tests. Nitrogen applied in Trials 1-4 was 60 kg/ha. Beans were grown a single season, cut at maturity, dried in windrows, and harvested when seed moisture content reached 10–12%. Ten pounds of seeds from each trial plot were collected randomly and stored in cloth bags at 10°C and 50% humidity for approximately 45 days.

At the Washington State University (WSU) Agricultural Research Center, beans were sealed in plastic bags and stored at 20°C. Aliquots

Author Koehler is Associate Professor, Dept. of Food Science & Human Nutrition, Washington State Univ., Pullman, WA 99164-2032. Author Burke was Research Plant Pathologist, USDA/ARS, Washington Irrigated Agriculture Research & Extension Center, Prosser, WA 99350.

from each trial were ground in a Wiley Mill with stainless steel fittings (Arthur H. Thomas Company, Philadelphia) and a mortar until all powder passed through a 60-mesh sieve. Raw bean powders were stored at 4°C in air-tight, light-proof jars until analyzed. Trials 1, 2, 3 and 4 formed a factorially designed experiment.

Nutrient composition

Bean powders were analyzed for contents of moisture, fat, ash, total nitrogen, calcium, iron, zinc, potassium, thiamin and riboflavin according to procedures of the Association of Official Analytical Chemists (AOAC, 1980). Nutrient concentrations were calculated from triplicate determinations on the basis of 100 g bean powder, dry basis. Amino acid composition of beans grown in Trials 5 and 6 was determined by the WSU Bioanalytical Laboratory according to the method of Spackman et al. (1958) using a Beckman Model 121 MB Amino Acid Analyzer. Cystine and methionine were determined after treatment with performic acid. Tryptophan was not determined since it is destroyed by acid hydrolysis. Amino acid concentrations were calculated to dry basis. Protein quality of raw bean powder (Trials 5 and 6) was evaluated using *Tetrahymena pyriformis* W. (Evancho et al., 1977). Triplicate aliquots were drawn from each triplicate bean sample. Growth of organisms was estimated after three days' incubation at 25°C by counting in a hemacytometer.

Sensory evaluation

Beans were cooked in their soak water, drained and presented hot to ten trained sensory panelists in five replications, as previously described (Koehler et al., 1987), providing 50 evaluations of each cultivar per trial. A form of descriptive analysis plus a rating scale for overall acceptability was used (Fig. 1). Shear force was measured using an Allo-Kramer instrument to shear 50 g of the cooked, drained beans that were undergoing sensory evaluation.

Statistical analysis

Nutrient composition and sensory evaluation data were subjected to Analysis of Variance and Duncan's Multiple Range Test (Steel and Torrie, 1980).

Code_____ Judge_____

Date_____

DESCRIPTIVE ANALYSIS OF COOKED DRY BEANS

		Character Notes	Intensity	Scale
Flavor	Aroma:	Fragrant, fruity	_____	0 = Not present
		brothy	_____	x = Threshold
		"green", sl. acid, sour	_____	1 = Slight
		musty, "rubbery"	_____	2 = Moderate
		rich	_____	3 = Strong
		browned	_____	
		nutty	_____	
		Other_____	_____	
	Taste:	sweet	_____	
		brothy	_____	
		"green vegetable"	_____	
		acid, sharp	_____	
		astringent	_____	
		"old rubber"	_____	
		musty	_____	
		other_____	_____	
Texture		Seed coat: toughness	_____	
		Cotyledons: hardness	_____	
		graininess	_____	
		Residue: seed coat pieces	_____	
		Residue: cotyledon graininess	_____	

OVERALL ACCEPTABILITY (including appearance)

10 9 8 7 6 5 4 3 2 1 0

excellent acceptable inedible

Fig. 1.—Score sheet used for sensory evaluation.

RESULTS & DISCUSSION

Proximate composition

Percentages of water, fat, ash and nitrogen in raw UI-114 (Pinto) and Rufus (Red Mexican) bean seeds grown under various conditions are given in Table 1. Seeds from apparently healthy plants (Trial 3) contained significantly greater concentrations of nitrogen than seeds from plants with root rot (Trial 1) when both were grown under optimum irrigation. Drought-stressed beans (Trials 2 and 4) contained significantly greater amounts of nitrogen than their optimally irrigated counterparts (Trials 1 and 3), whether from healthy or root-rot infected plants. Middleton and Silbernagel (1977) reported that percentages of bean seed protein (N × 6.25) increased slightly with less frequent irrigation. The diseased beans (Trial 2), however, had significantly greater nitrogen contents than corresponding healthy beans (Trial 4). Seeds from healthy plants grown under high-N fertilization (Trial 6) had greater concentrations of nitrogen than seeds from root-rot infected plants grown under low-N fertilization (Trial 5). In a study of dry bean yields resulting from varied rates of N application on *Fusarium*-infested and non-infested plots, Burke and Nelson (1967) reported greater yield increases with higher applications of N in the non-infested than in the infested fields. They suggested that *Fusarium*-damaged root systems are less capable of absorbing large quantities of N than are healthy root systems, but these researchers did not determine N concentrations of the seeds grown.

Minerals and vitamins

Concentrations of calcium, iron, potassium, zinc, thiamin, and riboflavin in bean seeds grown in the six trials are listed in Table 2. Drought stress resulted in greater thiamin concentration in root-rot infected seeds (Trial 2) of both cultivars than in all other trials and cultivars except UI-114, Trial 6. Greater quantities of potassium were found in seeds from healthy than from root-rot infected Rufus plants. Under conditions of drought stress, greater concentrations of iron, zinc and thiamin were found in seeds from root-rot infected plants than in beans from healthy plants of both cultivars. Rufus beans contained equal or greater calcium concentrations than UI-114 seeds from all trials except 6. Concentrations of thiamin in all Rufus beans were greater than comparable values for UI-114 seeds to a highly significant degree ($P < 0.001$). Except for these differences in thiamin concentration, variations in nutrient content are not important nutritionally, being small percentages of the Recommended Dietary Allowances (National Research Council, 1980) even though they may be statistically significant. Dry beans are an important dietary source of thiamin, iron, potassium and calcium.

Amino acids

Although seventeen amino acid (AA) concentrations were determined in bean seeds grown in Trial 5 (low-N fertilization on root rot infested plots) and Trial 6 (high-N fertilization on healthy soil), the experimental design of Trials 5 and 6 prevents valid comparisons. It can be reported, however, that seeds from healthy, high-N plants contained greater concentrations of seven of the eight AA essential for human beings (tryptophan was not determined) than quantities reported for similar beans in the literature (Evans and Bandemer, 1967; Hernandez-Infante et al., 1979; Koehler and Burke, 1981). The exception was lysine content smaller than that in beans analyzed by Hernandez-Infante et al. (1979). Methionine is the first-limiting AA in dry beans. The mean methionine concentrations of seeds from high-N soil and healthy plants (UI-114, 0.46; Rufus, 0.47 mg/100g) were 37%, 46% and 88% greater than values reported for similar beans by Evans et al. (1978), Sgarbieri et al. (1979), and Hernandez-Infante et al. (1979),

Table 1—Proximate composition of raw UI-114 (Pinto) and Rufus (Red Mexican) beans grown under various cultural conditions[a]

Cultural Conditions	Trial no.	H_2O (%)	Fat (%)	Ash (%)	N (%)
UI-114					
Optimum H_2O; root rot	1	10.13 c[b]	0.80	4.47	4.64 d
H_2O stress; root rot	2	9.80 d	1.00	4.37	5.27 a
Optimum H_2O; healthy	3	9.59 d	1.17	4.06	4.88 c
H_2O stress; healthy	4	9.24 e	1.11	4.15	5.09 b
Low Nitrogen; root rot	5	10.84 b	0.98	4.90	4.73 d
High Nitrogen; healthy	6	11.43 a	0.90	4.95	4.95 c
Rufus					
Optimum H_2O; root rot	1	10.31 C	1.03	4.24	4.55 D
H_2O stress; root rot	2	9.82 D	1.14	4.28	5.02 A
Optimum H_2O; healthy	3	10.26 C	1.34	4.05	4.62 C
H_2O stress; healthy	4	8.68 E	1.35	4.01	4.84 B
Low Nitrogen; root rot	5	11.23 B	1.23	4.79	4.60 C
High Nitrogen; healthy	6	12.08 A	1.31	4.70	4.85 B
Mean Standard Deviation		±0.09	±0.04	±0.09	±0.08

[a] Each value is the mean of triplicate determinations, dry basis.
[b] Means within a column and cultivar with the same letter are not significantly different ($p > 0.05$).

Table 2—Mineral and vitamin contents of raw UI-114 (Pinto) and Rufus (Red Mexican) beans grown under various cultural conditions[a]

Cultural Conditions	Trial no.	Ca (mg/100g)	Fe (mg/100g)	K (mg/100g)	Zn (mg/100g)	Thiamin (mg/100g)	Riboflavin (mg/100g)
UI-114							
Optium H_2O; root rot	1	179 a	9.7 b	1698 c	1.86 d	0.56 c	0.23 c
H_2O stress; root rot	2	165 cd	10.5 a	1565 e	1.95 c	0.69 a	0.25 b
Optimum H_2O; healthy	3	155 e	8.4 cd	1612 d	1.83 d	0.59 b	0.24 bc
H_2O stress; healthy	4	172 b	8.0 d	1580 de	1.73 e	0.58 b	0.25 b
Low nitrogen; root rot	5	162.d	8.1 d	1779 b	2.09 b	0.60 b	0.27 a
High nitrogen; healthy	6	168 c	9.2 c	1852 a	2.22 a	0.70 a	0.23 c
RUFUS							
Optimum H_2O; root rot	1	183 AB	7.8 C	1579 D	1.74 C	0.83 D	0.24 C
H_2O stress; root rot	2	175 BC	8.6 A	1576 D	1.83 B	0.98 A	0.21 E
Optimum H_2O; healthy	3	160 D	8.2 B	1689 B	1.86 B	0.88 B	0.25 B
H_2O stress; healthy	4	161 D	8.0 BC	1664 BC	1.76 C	0.89 B	0.22 D
Low nitrogen; root rot	5	187 A	8.5 A	1654 C	1.99 A	0.90 B	0.25 B
High nitrogen; healthy	6	168 C	8.5 A	1780 A	2.00 A	0.85 C	0.26 A
Mean standard deviation		±3.10	±0.25	±10.9	±0.02	±0.007	±0.003

[a] Values are means of triplicate analyses, except for riboflavin (duplicate analyses); dry basis. Means within a column and cultivar with the same letter are not significantly different ($p > 0.05$).

respectively. However, Lantz et al. (1958) reported highly significant differences in amino acid contents of beans attributed to location, year of production and other environmental factors. Hosfield et al. (1984) found that seasonal effects predominated over genotype in studies of physico-chemical traits. In a similar study, Ghaderi et al. (1984) reported highly significant effects of location for all traits measured. These were both nonnutritive, nonsensory dry-bean studies.

Protein quality

Measures of protein quantity and quality of UI-114 and Rufus beans grown in Trial 5 (low-N fertilization; root-rot infestation) and Trial 6 (high-N fertilization; healthy plants) are given in Table 3. Relative Nutritive Value (RNV), as estimated using *Tetrahymena pyriformis* W, compares the nutritive value of the test protein with that of casein. There were no significant differences in RNV between cultivars or cultural conditions. The greater concentrations of methionine in seeds from Trial 6 had no apparent effect on RNV. These protein quality estimations were made on raw beans. Although cooking destroys antinutritional and toxic components, lower *Tetrahymena* RNVs have been reported after cooking beans (Koehler and Burke, 1981) and lentils (McCurdy et al., 1978) than before cooking.

Sensory evaluation

Conspicuous sensory characteristics and overall acceptability scores as evaluated by descriptive analysis of UI-114 and

Table 3—Protein quantity and quality of UI-114 (Pinto) and Rufus (Red Mexican) dry beans grown with two levels of nitrogen fertilizer

Variety	Trial[a]	Protein (g/100g) (dry basis)	Protein quality[b] RNV[c]
UI-114	5 (low N, root rot)	29.6	60.7[d]
UI-114	6 (high N, healthy)	30.9	59.6
Rufus	5 (low N, root rot)	28.8	58.6
Rufus	6 (high N, healthy)	30.3	51.8

[a] Trial 5: low nitrogen fertilization (20-40 kg/ha); Trial 6: high nitrogen fertilization (>120 kg/ha).
[b] Determined using *Tetrahymena pyriformis* W; each value is the mean of three separate analyses (18 countings).
[c] $\text{RNV} = \frac{\text{no. organisms/mL of sample}}{\text{no. organisms/mL of casein}} \times 100$
[d] There were no significant differences ($P > 0.05$).

Rufus beans grown in the six trials are summarized in Table 4. Trials 1, 2, 3, and 4 were a factorial design involving optimum irrigation or drought stress, and presence or absence of *Fusarium* root rot.

Amount of irrigation (Trials 1 and 2; Trials 3 and 4) had little effect on aroma and taste characteristics of the resulting beans. When significant differences occurred, they were between the two cultivars in a trial: e.g., UI-114 beans were significantly more fragrant (Trial 2) and sweeter (Trials 1, 3 and 4) than Rufus beans.

UI-114 beans were judged to have greater intensities of fragrant aroma than comparable Rufus beans, although the difference was significant only in Trial 2. Both cultivars in Trial 1 (optimum irrigation, root rot) had significantly greater rich

Table 4—Sensory characteristics[a] and acceptability scores[b] of UI-114 and Rufus beans grown under various cultural conditions.

Trial[c]	Overall acceptability	Conspicuous sensory characteristics		
		Aroma	Taste	Texture[d]
1 UI-114	6.78 ab[e]	Fragrant, green, rich	Sweet, green, brothy	SD CT RES
Rufus	6.34 cd	Rich	Brothy	HD CT, SD CT RES
2 UI-114	704 a	Fragrant, green, browned	Sweet, green, brothy, musty	SD CT RES, GR CT RES
Rufus	6.60 bcd	NCC[f]	Green	HD CT
3 UI-114	7.09 a	Fragrant	Sweet, green, brothy, musty	GR CT, GR CT RES
Rufus	6.31 d	NCC	Brothy, musty	SD CT RES
4 UI-114	7.00 ab	Fragrant, green, browned	Sweet, green, brothy, musty	SD CT RES, GR CT RES
Rufus	6.75 bc	Browned	Green	HD CT
5 UI-114	6.33 AB	Fragrant, browned	Green, musty, rubbery	GR CT, SD CT RES
Rufus	5.82 B	Rich, browned	Musty, rubbery	HD CT, GR CT, SD CT RES, GR CT RES
6 UI-114	6.53 A	Fragrant, green	Sweet, green, brothy, musty	GR CT, SD CT RES
Rufus	6.70 A	NCC	Sweet, brothy	NCC

[a] Based on means of 50 evaluations of each culitvar per trial using an intensity scale of 0 = not present to 3 = strong.
[b] Means of ratings on a scale from 10 (excellent) to 0 (inedible).
[c] Trial 1: Optimum H_2O, root rot; Trial 2: H_2O stress, root rot; Trial 3: optimum H_2O, healthy; Trial 4: H_2O stress, healthy; Trial 5: Low N, root rot; Trial 6: high N, healthy.
[d] HD CT = hard cotyledons; GR CT = grainy cotyledons; SD CT RES = seed coat residue; GR CT RES = grainy cotyledon residue
[e] Beans in the same column and group having the same letters are not significantly different ($P>0.05$).
[f] NCC = No conspicuous characteristics.

aroma than beans in all other trials. In evaluation of taste characteristics, UI-114 beans were judged sweeter and brothier than corresponding Rufus beans. In previous descriptive analysis of cooked beans, fragrant, rich, browned, nutty, sweet and brothy flavor characteristics were considered desirable but musty, rubbery and astringent ones were regarded as less desirable (Koehler and Burke, 1981).

Among texture characteristics, UI-114 beans in Trial 4 had conspicuously the most seed coat residue (substance remaining after swallowing) and the corresponding Rufus beans the least. Differences in shear-force measurements between cultivars or among cultural conditions were not significant. Analysis of variance for ratings of overall acceptability (scored on completion of flavor and texture judgments; Fig. 1) showed that panelists preferred UI-114 Pinto beans over Rufus Red Mexican beans to a highly significant degree ($P < 0.001$). Previous studies (Koehler and Burke, 1981; Koehler et al., 1987) have shown a similar preference.

In Trials 5 and 6, beans in Trial 5 (low N, root rot) had greater browned aroma and rubbery taste, and were less sweet and brothy, than beans grown in Trial 6 (high N, healthy plants). Pinto UI-114 beans had greater intensities of fragrant aroma and sweet and green tastes than Rufus beans. Rufus beans from Trial 5 were judged to have significantly greater intensities of hard cotyledons, grainy cotyledons, seed-coat residue and grainy cotyledon residue than Rufus beans from Trial 6. These textural judgments were borne out by the shear-force measurements in which Rufus beans from Trial 5 required more force to shear than Rufus beans from Trial 6. Both cultivars produced in Trial 6 were significantly more acceptable to the panel than Rufus beans grown in Trial 5.

In summary, frequency of irrigation affected both N and thiamin concentrations in the resulting beans. Optimum irrigation resulted in greater percentages of N in healthy seeds than in corresponding root-rot infected seeds. Under drought stress root-rot infected beans contained greater N quantities than comparable healthy beans, and both healthy and diseased beans had greater N concentrations than comparable beans grown under optimum irrigation. Drought stress also resulted in greater thiamin concentrations in root-rot infected beans from both cultivars than in all healthy beans except UI-114 in Trial 6. All Rufus bean seeds contained thiamin concentrations significantly greater than those in all UI-114 beans, a nutritionally important difference. Healthy seeds grown with high-N fertilization contained greater methionine concentrations than root-rot infected seeds from low-N soils, but this difference did not influence protein quality as estimated using *Tetrahymena*. Sensory evaluations identified significant cultivar differences, UI-114 beans being judged to have greater intensities of fragrant aroma and sweet and brothy tastes than corresponding Rufus beans. All UI-114 beans were rated significantly more acceptable than all Rufus beans, though the difference would probably not be important commercially. These results are from one season only, and replications would have been valuable in view of reports by Hosfield et al. (1984) and others of significant seasonal and location effects on instrumentally measured quality traits. Because sufficient irrigation is necessary for the high seed yield required by growers, and on the other hand breeders are attempting to improve the nutritional value of beans, it is important to consider and balance the effects of irrigation frequency, root-rot control, N fertilization, and cultivar selection.

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Nutritional Quality of Great Northern Bean Proteins Processed at Varying pH

N. J. EICHER and L. D. SATTERLEE

ABSTRACT

Heating whole dry beans at pH well above or below their average protein isoelectric point of 6.3, results in significant increases in protein digestibility, as measured by the rat NPR assay. Yet the nutritional quality of these pH-adjusted beans was lower than that for conventionally cooked dry beans due to the loss of cysteine/cystine (Cys) from the beans during the long pH adjustment step as well as the increased sensitivity of Cys to heat at alkaline pHs. In contrast pH adjusted bean flour slurries, after rapidly drying (40 sec) on a double drum drier, yielded products having a high protein digestibility (>80%), low trypsin inhibitor content (<10mg TI/g protein) and showing little or no loss of Cys.

INTRODUCTION

DRY EDIBLE BEANS are a common crop worldwide and their importance as part of the world's protein supply is evident. However, there are several problems that currently limit the usefulness of dry beans. These include the low levels of the sulfur amino acids in the bean proteins (Bressani, 1975; Molina et al., 1975), and the low digestibility of unheated bean proteins (Chang and Satterlee, 1981), high levels of antinutrients (Liener, 1976) and the hard-to-cook phenomenon which can develop during bean storage (Antunes and Sgarbieri, 1979 and Molina et al., 1976). The last three problems can only be overcome by long cooking times.

The work of Chang and Satterlee (1981) demonstrated that a large amount of heat energy could be saved when heating the isolated major storage protein of Great Northern beans at pH values away from its isoelectric point. The presence of antinutrients such as trypsin inhibitor has long been known to be a problem in dry beans. There appears to be a significant relationship between pH and trypsin inhibitor (TI) inactivation. Miyoshi et al. (1978) and Marquez and Lajolo (1981) have demonstrated that about 75% of the TI remains active in varieties of *Phaseolus vulgaris* beans after heating for 1 to 4 hr at 100°C under acidic conditions. Under alkaline conditions the TI was easily destroyed. The relationship between TI content and protein quality is not clear. Several authors (Liener, 1976; Marquez and Lajolo, 1981; Jaffe, 1975) could not find a direct correlation between TI content of beans and protein quality. It has been estimated by Kakade et al. (1973) that 40% of the growth inhibition in rats fed raw soybeans is due to the beans' TI content.

Several processing methods have been used in an attempt to increase the availability of protein in dry beans. Rockland and Metzler (1967) devised a process for quick-cooking lima beans. Bakker-Arkema et al. (1967) used a drum drier and Kon et al. (1974) used steam heat to produce a high quality bean product. Carvalho and Jansen (1977) and Yadav and Liener (1978) both prepared pre-cooked, instant bean products by dry roasting.

The use of alkali in processing has come into close examination in recent years. It is a controversial processing technique, due to the reported levels of protein damage found in alkali processed foods. Several authors (Feeney, 1980; Masters and Friedman, 1980) state that alkali can cause detrimental protein changes such as hydrolysis of susceptible amide and peptide bonds, racemization of amino acids, and formation of crosslinked products such as lysinoalanine. These changes lower nutritional quality due to loss of availability of amino acids, particularly the essential amino acids.

It is the purpose of this study to: (1) determine whether the pH of whole Great Northern beans and bean flours can be altered by soaking in acid or alkaline solutions, and (2) see if bean protein denaturation (a factor responsible for increases in protein digestibility) will occur with minimal heat treatment.

Author Satterlee is with the Dept. of Food Science, Pennsylvania State Univ, University Park, PA 16802. Author Eicher, formerly with the Dept. of Food Science & Technology, Univ. of Nebraska, is now with General Mills, James Ford Bell R&D Laboratory, Minneapolis, MN.

MATERIALS & METHODS

Control beans

The beans used in this study were *Phaseolus vulgaris* cv Great Northern UT 59. Conventional home-style beans were prepared in order to have a standard product to compare with the various processed bean products. These were prepared by soaking in distilled, deionized water at a 1:20 bean:water (w/v) for 18 hr, followed by cooking over medium heat for 2 hr. The cook water was discarded, and the beans were freeze-dried, ground to a flour and stored at −20°C until needed.

Studies on whole beans

To adjust the pH away from the isoelectric point of whole bean protein (pH 6.3), beans were hydrated with acidic and basic solutions under an alternating pressure/vacuum soaking cycle. The hydration apparatus utilized a water aspirator attached to the inlet valve of a sealed 4L stainless steel pressure/vacuum chamber. The outlet valve of the chamber was attached to a nitrogen tank which provided the needed pressure. The chamber could then be alternately evacuated and pressurized.

The hydration protocol was as follows: the beans were placed in the chamber in pH 12.5 NaOH. The chamber was evacuated to 20 in. mercury, followed by 40 psi nitrogen pressure. The dry bean:water ratio was kept at 1:10 (w/v) during the pH adjustment and soaking process, with pH adjustment taking 22 hr. Starting at time zero, and for the first 10 hr, the beans were alternately pressurized for periods of 1 hr and 55 min and held under vacuum for 5 min. The frequency of the pressure-vacuum cycles was then reduced to just once at Hour 16 and once at Hour 20, for a total of eight cycles over the 22 h period. A portion of the beans was treated with pH 1.0 water, then repeatedly evacuated for 5 min and pressurized for 55 min, over a 3 h period. The pH of the beans was determined by slicing the bean in cross section and pressing the sliced surface against narrow range pHydrion paper (vivid color coded, Micro Essential Laboratory).

Bean proteins were further denatured by applying heat to the rehydrated beans in an autoclave for periods of from 1 to 15 min at temperatures ranging from 80° to 100°C. The beans were spread in a single layer on the autoclave trays. Timing was started when the desired temperature was achieved within the autoclave. Following the heating, the beans were immediately removed and cooled on ice. The heated bean samples were freeze dried, ground in the analytical mill and stored at −20°C until needed.

Drum drying pH adjusted flours

A double drum-drier (Buflovak Atmospheric No. D-2802) was used to both heat and dry bean flour slurries (1:2 w/v flour to water) ad-

justed to pH 6, 7, 8, and 9 with HCl or NaOH. The pressure of the steam entering the drums was held at 25 psi, which corresponds to a drum temperature of 130°C. The gap between the counter-rotating drums was 0.10 mm (0.004 inch) and the drum speed was varied from 0.5 to 2 revolutions per min (rpm). After drum drying, all samples were ground to a powder and stored at −20°C until needed.

Scanning electron microscopy

Physical changes that accompanied the pH shift in the whole beans were studied using Scanning Electron Microscopy, according to the procedure of Bair and Snyder (1980). Procedural modifications included the omission of paraformaldehyde for the fixation step and the use of gold-palladium (60:40) to coat the samples in a vacuum evaporator. The samples were examined on a Cambridge Stereoscan S4-10 Model Scanning Electron Microscope.

Nutritional quality assays

Rat feeding trials (net protein ratio (NPR) and in vivo protein digestibility) were used to evaluate the in vivo protein nutritional quality of the whole bean proteins (Bender and Doell, 1957). All diets were formulated using the guidelines for biological evaluation of protein quality (AOAC, 1985) which calls for a 10% protein (N × 6.25) level in the diets. Individual rat weight gain and feed consumption values were recorded throughout the study. The in vitro protein digestibility of the various bean flour samples were determined by the procedure of Satterlee et al. (1981).

The trypsin inhibitor, (TI) activity of the bean samples was determined by the procedure of Hamerstrand et al. (1981). The presence of oxidized methionine and cystine was determined using the procedure of Chang et al. (1985).

All amino acid analyses were performed using the hydrolysis procedures of Satterlee et al. (1981), followed by separation and quantitation of each amino acid on a Beckman 120°C amino acid analyzer. Available cystine/cysteine (Cys) and methionine were determined using procedures described by Chang et al. (1985).

Statistical analysis

Statistical comparisons for difference were determined using the CMS statistical programs available from the University of Nebraska Computer System.

RESULTS & DISCUSSION

Processing whole beans

Initial studies were performed to determine the resistance of whole beans to pH adjustment. All attempts to soak the beans at atmospheric pressure in water maintained at varying pH levels failed, even with long exposure times (24-36 hr) at pH 12.5. It was apparent that at very high pH under atmospheric pressure, only the outer layer of the bean absorbed the [OH^-]. However, a pH 12.5 solution, under the alternating pressure-vacuum conditions, was successful in altering the pH of the entire bean. At pH levels lower than 12.5, the [OH^-] were absorbed and neutralized by components within the bean. When beans were soaked in pH 9, 11, and 12 solutions in the pressure/vacuum chamber, an advancing water front could be seen in the beans, but the pH of the hydrated and unhydrated areas within the bean were not different. Only the pH 12.5 solution had the capacity of altering the pH of the cellular constituents of the bean.

Feeney (1980) and Masters and Friedman (1980) have cited that lysinoalanine formation and amino acid racemization were associated with the processing of foods at alkaline pHs. Because of a desire to obtain an edible product at or near neutrality, an attempt was made to readjust the whole beans from an alkaline pH (12.5) back to approximately pH 7.0. Soaking pH 12.5 beans in water at pH levels ranging from 6.0 to 2.0 were unsuccessful in lowering the pH. The difficulty in neutralizing the beans could be due to the bean's barrier to water penetration as well as the high buffering capacity of the bean. When the beans were soaked under pressure-vacuum with pH 1 water, they attained a pH 1-2 after 3 hr. There appeared to be no intermediate pH state during the adjustment, and, secondly, a distinct textural difference was observed between the alkaline and the acidic pH beans (at alkaline pHs, the beans were very soft, and at acidic pHs, the beans were hard and rubbery).

Soaking whole beans in alkaline (pH 12.5) and alkaline + acid (pH 1) water under vacuum/pressure conditions decreased trypsin inhibitor content and increased *in vivo* protein digestibility as well as protein nutritional quality (NPR) (Table 1). The increase in protein digestibility for uncooked beans treated at pH 12.5 alone or at pH 12.5 and pH 1, was very large. The increased digestibility, can be attributed to the loss of TI from the beans as well as the partial denaturation of the bean's storage proteins, thus enhancing protein degradation by proteases. All three bean samples had at least an 80% reduction in TI activity (Table 1). The slightly higher TI level in the acidic beans could be predicted, since Miyoshi et al. (1978) and Marquez and Lajolo (1981) found that TI is most stable at low pHs and very unstable at high pHs.

A sample of the soaking water from uncooked acidic and basic cooked beans was freeze dried to recover the dissolved solids, which were assayed for protein and TI content. The TI activity of this soaking water was 160.1 mg TI/g protein, which indicates that a substantial amount of TI leached out of the beans into the alkaline soaking water. Therefore, the low level of TI in the basic and acidic beans could be due to the loss of TI to the soaking water.

Even though the protein digestibilities were comparable between the pH treated beans and conventionally cooked beans, the overall protein nutritional quality of the cooked beans was significantly greater. This difference in quality was due to the diminished level of Cys in the pH treated beans as well as the superior ability of heat over pH to destroy antinutrients.

Amino acid analysis

Concentrations of all amino acids in the various samples were comparable to the concentrations found in the raw bean protein, with the exception of Cys for the pH treated beans. Since the bioavailability of the oxidized forms of the sulfur amino acids is not certain, the availability of Cys and Met were determined. The Cys assay is specific for unaltered cysteine and cystine and is assumed to be equivalent to available cysteine/cystine (Cys) for the four samples listed in Table 2. The conventional cooked beans lost only 13% of their reactive

Table 1—Trypsin inhibitor (TI) activity, apparent protein digestibility, and net protein ratio (NPR) of treated and untreated whole beans

Treatment	mg TI / g Bean protein	% Reduction of TI from raw sample	% Apparent protein Digestibility (*in vivo*)	NPR
Casein (control diet)	---	---	93.6	4.5
Raw Beans	57.9[a]	0.0	20.8[a]	−0.8[a]
Basic (pH 12.5) Uncooked Beans	2.7[b]	95.2[b]	78.2[b]	1.9[c]
Acidic (pH 1) Uncooked Beans	9.5[c]	83.6[c]	79.7[b]	2.2[c]
Conventional Cooked Beans[d]	1.6[b]	97.2[b]	77.2[b]	2.7[b]

[a,b,c] Values within a column and with the same letter superscript are not significantly different (p = 0.05).

[d] Beans soaked in distilled water at atmospheric pressure and then cooked.

Table 2—Available Cys levels of treated and untreated whole bean samples

Bean treatment	g Available Cys / 16g Nitrogen	% Loss available Cys during processing
Raw	0.68	0.0
Conventional cooked beans	0.59	13.2
Basic beans	0.26	61.8
Acidic beans	0.40	41.2

Cys as compared with a 62% loss of Cys for the basic beans. Alkaline treatments are known to adversely affect the bioavailability of Cys. A second reason for the low Cys levels could be the loss of the Cys-rich trypsin inhibitor, which leached into the soaking water. The TI from cultivars of *Phaseolus vulgaris* contain up to 14% Cys by weight, (Pusztai, 1966; Miyoshi et al., 1978).

No oxidized forms of cystine or methionine were found in any bean sample. The lysine level of all samples remained constant, and no lysinoalanine (LAL) was detected in any sample. It would appear that the only amino acid partially lost during pH adjustment was Cys.

Scanning electron microscopy

A scanning electron photomicrograph of a typical cell within the raw, dry bean cotyledon is shown in Fig. 1a. The rigid cell wall was presumably composed of pectic substances and cellulose in a matrix formation with divalent cations such as calcium and magnesium (Rockland, 1978 and El-Shimi et al., 1980). The large round spheres are starch granules. The fluffy, honeycomb structure appears to be the supporting matrix or the cytoplasmic network, composed of carbohydrate and protein. The small bodies contained within this network are the protein bodies (Varriano-Marston and De Omana, 1979).

A soaked bean cotyledon, in which the cytoplasmic network has disappeared, possibly by dissolving in the soaking water as suggested by Wolf and Baker (1975) or by becoming part of the swollen cellular material, is shown in Fig. 1b. The left side of the photomicrograph shows that the cell wall had lost rigidity and that there was a greater degree of cellular separation. The surface of the cell was much rougher, showing increasing signs of deterioration, possibly due to the sodium hydroxide treatment. Rockland (1978) suggested that sodium caused the divalent cations (calcium and magnesium) to leach out, thus softening the cell wall and allowing for cellular separation and collapse. Varriano-Marston and De Omana (1979) found that the degree of cellular separation was much greater in salt soaked beans than in water soaked, an observation that is confirmed by the SEM photographs in Fig. 1b and 1c). Rehydration filled the otherwise dry intercellular spaces with water, improving heat transfer and increasing the rate and extent of protein denaturation.

In a cell from a bean first treated with pH 12.5 water, and then pH 1 water (Fig. 1c), the cell wall appeared to be much more rigid than with the other treatments (Fig. 1a and 1b). The cement-like material between cells appears rough, and an increase in the degree of intra-cellular degradation is apparent. These SEM observations could explain why the basic beans (Fig. 1b) were soft, while the acidic beans (Fig. 1c) were very rigid.

Processing of bean flours

To avoid the problems of protein damage in whole beans, caused by extreme alkalinity, as well as for practicality, the flours were adjusted to pH levels 6.0, 7.0, 8.0, and 9.0 with HCl or NaOH. The pH-adjusted samples were then dried on a

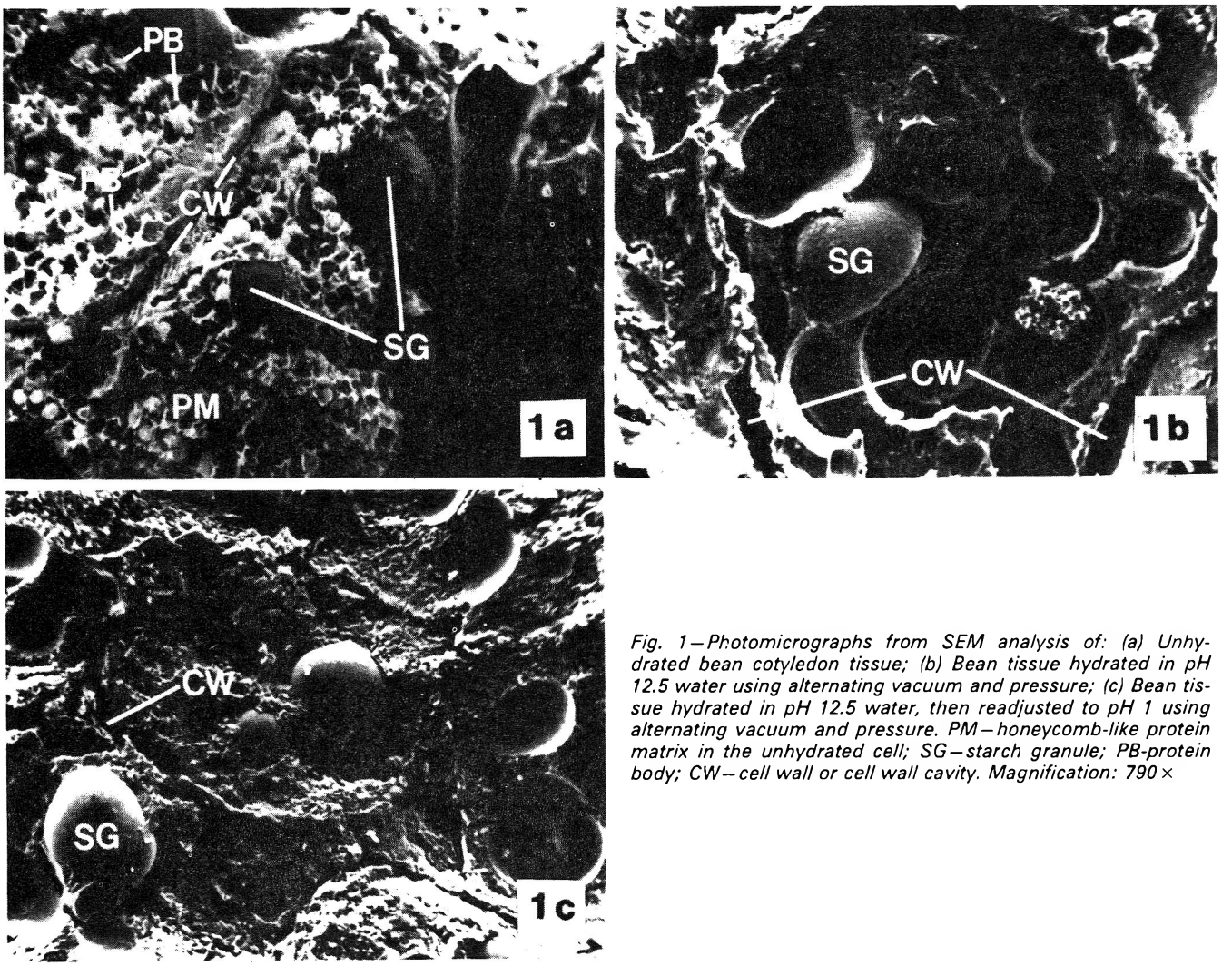

Fig. 1—Photomicrographs from SEM analysis of: (a) Unhydrated bean cotyledon tissue; (b) Bean tissue hydrated in pH 12.5 water using alternating vacuum and pressure; (c) Bean tissue hydrated in pH 12.5 water, then readjusted to pH 1 using alternating vacuum and pressure. PM—honeycomb-like protein matrix in the unhydrated cell; SG—starch granule; PB-protein body; CW—cell wall or cell wall cavity. Magnification: 790×

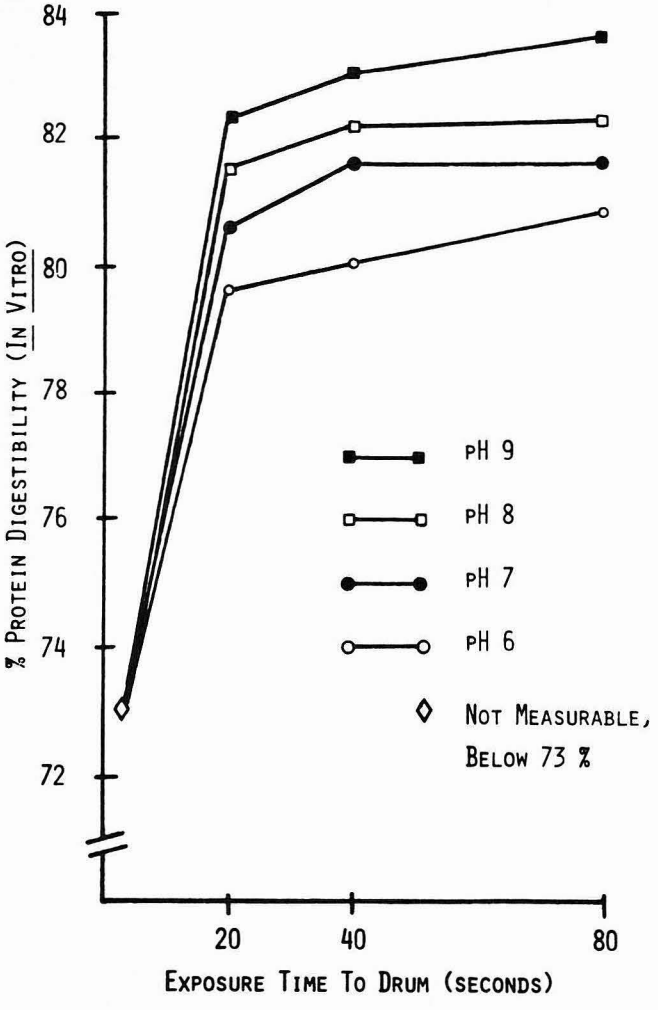

*Fig. 2—Percent protein digestibility (*in vitro*) of flour samples exposed for varying times on the drum-drier.*

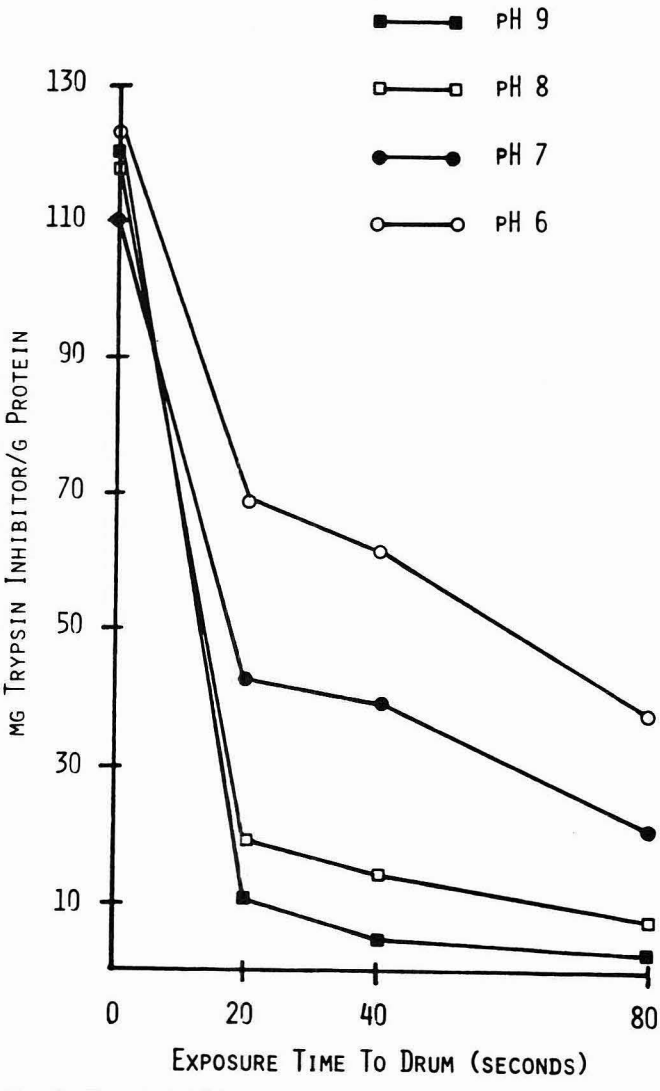

Fig. 3—Trypsin inhibitor content of flour samples exposed for varying times on the drum-drier.

drum drier at drum speeds of 0.5, 1.0, and 2.0 rpm, which correspond to 80, 40, and 20 sec, respectively, of sample contact time on the drums. The initial sharp rise in protein digestibility in the drum dried, pH treated bean flours directly corresponded to their initial rapid drop in TI content. However, it has been shown by Kakade et al. (1973) that only 40% of the growth inhibition of raw soybeans could be attributed to TI content. Since it was shown that whole beans can have a range of TI levels and still possess relatively high *in vitro* protein digestibilities (Table 1), other factors, such as protein structure change as heat is initially applied and which can directly affect protein digestibility.

As stated above, protein denaturation could possibly play a major role in the sharp rise in *in vitro* protein digestibility. The bean flour sample adjusted to pH 9 was superior in terms of its low TI content and high protein digestibility. However, the flour samples adjusted to pH 7 and 8 are more practical for use in food products, and still possess acceptable TI activities and protein digestibles (Fig. 2, 3).

The availability of Cys was determined for each bean flour sample in order to determine if Cys destruction occurred with increasing exposure to the drum drier. The results (Table 3) indicate that during exposure to neutral or only slightly alkaline conditions and high drying temperatures, very little Cys was lost. Complete amino acid analysis of the samples indicated that 1) no lysinoalanine (LAL) or methionine sulfoxide were formed during processing, 2) no differences in amino acid content of the pH and heat treated flours, when compared to the values for raw beans.

The pH 7 and 8 bean flour samples drum-dried for 40 sec were chosen as being optimal, since they required less heat and possessed similar digestibilities and TI activities, when compared to samples dried for 80 sec.

Table 3—Available Cys content of bean flour samples drum dried at pH 7 and 8

Flour treatment	Contact time with drum surface (sec)[a]	g Available Cys / 16g Nitrogen
pH 7.0	0	0.78
	20	0.85
	40	0.84
	80	0.83
pH 8.0	0	0.82
	20	0.81
	40	0.81
	80	0.72

[a] Drum temperature = 130°C.

CONCLUSIONS

THE AVAILABLE METHIONINE levels in both whole beans and bean flours remained very high throughout alkaline and acid (pH 12.5 and 1). Whole beans lost from 40-60% of their available Cys during soaking, while pH treated and drum dried bean flour samples lost little or no Cys. The extensive destruction of Cys in the treated whole beans could be due to: (1)

long exposure time to extreme pH, and (2) the leaching of Cys rich trypsin inhibitors into the soaking media.

Even though TI is difficult to inactivate in the flour samples, especially at acidic pHs, its contribution as a protein, to the overall cysteine content of the bean is important. The use of the drum drier on the pH adjusted flours, heating, drying and TI inactivation was accomplished in one step. This process resulted in a bean product which required a much lower heat input and less severe pH treatment needed for treated whole beans to achieve the same degree of protein denaturation and digestibility.

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Comparison of Oligo- and Polysaccharides Formed from Starch During Processing of Sweet Potato Puree by Endogenous and Exogenous Enzyme Treatments

P.L. CHANG-RUPP and S.J. SCHWARTZ

ABSTRACT

Hydrolysis patterns of starch were monitored during production of sweet potato puree (Jewel variety) using high performance size exclusion chromatography. A wide range of polysaccharides from large molecular size to simple sugars was observed. Lye peeling, prior to pureeing, induced partial hydrolysis by native enzymes due to gelatinization of starch from heat and alkali. Native enzymes were inactivated by steam injection (105°C for 5 min) and the puree treated with *Bacillus subtilis* alpha-amylase and Tenase, a thermostable alpha-amylase. *Bacillus subtilis* alpha-amylase produced a narrower molecular size range of polysaccharides during hydrolysis relative to Tenase. The average molecular size distribution of amylolysis end products from the native and exogenously treated systems differed. Specific lower molecular weight oligosaccharides formed during the final stages of amylolysis.

INTRODUCTION

SWEET POTATOES are a nutritious vegetable which provide energy and appreciable amounts of vitamins and minerals. The sweet potato is receiving increased consumer attention as a new and different commodity, due to its nutritious character and versatility. The potential for using sweet potatoes in the development of new products is being realized by food processing industries. Processing techniques, such as pureeing, have been developed to preserve sweet potatoes as a puree or a drum-dried flake for later reconstitution into consumer products.

Hoover (1966) reported a process for pureeing sweet potatoes into drum-dried flakes. Starch conversion and flake quality were found to be directly related to time, temperature, and enzyme concentration. Variations in the native amylase activity occur due to differing storage conditions, harvest dates, and sweet potato cultivars. This makes it difficult to control starch hydrolysis (Ikemiya and Deobald, 1966; Deobald et al., 1969, 1971; Hoover, 1967). Variation in the amylase activity of roots and different starch hydrolytic products are detrimental factors resulting in inconsistent purees and poor quality flakes.

Gelatinization occurs during heating of the puree, allowing the starch to become more susceptible to attack by endogenous enzymes present in the roots. The result of this process is dextrinization of the starch. A similar process occurs during baking, converting 72–99% of the starch to maltose and dextrins of unspecified chain length (Walter et al., 1975). Detailed information on the specific hydrolytic products formed during baking and puree production is not known.

To overcome the problem of inconsistent purees, Szyperski et al. (1986) developed a process where puree, previously processed by steam injection to inactivate the native amylases, was treated with exogenous alpha-amylase. The hydrolyzed puree was then blended with untreated portions to produce a puree with consistent viscosity.The polysaccharides formed via these exogenous treatments may differ in composition relative to those produced in the native puree system.

The objectives of this study were: (1) to investigate the composition of starch products formed during manufacture of sweet potato puree and (2) to compare these to the hydrolysis patterns and end products formed using a commercial thermostable alpha-amylase, Tenase, and a *Bacillus subtilis* alpha-amylase.

Authors Chang-Rupp and Schwartz are with the Dept. of Food Science, Box 7624, North Carolina State Univ., Raleigh, NC 27695-7624.

MATERIAL & METHODS

Raw material

Jewel cultivar sweet potatoes were obtained during the 1985 growing season from the North Carolina State University Agricultural Experiment Station. Roots were harvested during October, cured for 7 days at approximately 30°C and 85% relative humidity, then stored at 14°C and 90% relative humidity for 4 months until processed.

Preparation of puree

Cured roots were hand-peeled, trimmed and cut into 2.5 cm diameter cubes, then comminuted in a Waring Blendor until uniform. This mixture was pureed with a Wiley mill and immediately heated in a water bath to 75°C with constant stirring (Hoover, 1967). Puree was also produced by the process described by Szyperski et al. (1986). The roots were washed, peeled in 10% sodium hydroxide solution for 5 min at 102°C and then rinsed under a cold water spray to remove peel and excess lye. Roots were trimmed to remove ends and surface defects and comminuted in a Fitzmill comminutor fitted with a 1.52-mm mesh screen. The puree was thoroughly mixed, heated to 105°C by direct steam injection and held at this temperature for 5 min to inactivate the native enzymes and to gelatinize the starch. The puree was cooled and stored at 4°C until used. These conditions were sufficient to provide the required enzyme inactivation.

Preparation of enzyme solutions

Crystalline *Bacillus subtilis* alpha-amylase (approximately 1000 units per mg protein) was purchased from Sigma Chemical Company (St. Louis, MO). Twenty milligrams of alpha-amylase were dissolved in 2 mL of 0.1M acetate buffer (pH 5.5), placed in a collodion bag (Fisher Scientific) and 19 mL of acetate buffer added. The solution was concentrated to 0.5 mL at 44°C, diluted to 20 mL with buffer and stored at 4°C for use.

A commercial liquid alpha-amylase, Tenase (340,000 MWU/g), was obtained from Miles Laboratories, Inc. (Elkhart, IN). Tenase was used without further purification.

Enzyme-treated puree

The puree was divided into two lots (250g each) for separate studies with different enzymes. The lots were heated in a water bath to 70°C and 40°C (optimum temperature for each enzyme) and treated with Tenase (0.5 mL) and *Bacillus subtilis* alpha-amylase (0.5 mL, 10 mg/mL IU), respectively. All purees were stirred continuously during hydrolysis. Aliquots (1 mL) were removed from the heated puree at specified time intervals (every min up to 5 min, then every 5 min up to 65 min) and immediately placed into centrifuge tubes (15 mL) containing 10 mL 95% ethanol.

Sample extraction

A schematic of the sample extraction procedure is depicted in Fig. 1. The samples were extracted with ethanol (10 mL, 3X) to remove sugars, followed by petroleum ether (10 mL, 3X) to remove lipid soluble components. All samples were mixed after each solvent addition. After each extraction, the samples were centrifuged and the extract decanted. Five mL of 99% dimethyl sulfoxide (DMSO) was added to the residue, heated 5 min in a boiling water bath to "solubilize" the starch and centrifuged (5 min, 3000 xg). Starch left in the residue from centrifuging was measured after treatment with 35 units amyloglucosidase (from *Aspergillus niger,* Sigma) in 0.1M acetate buffer, followed by determination of glucose using a glucose oxidase-peroxidase procedure (No. 510, Sigma Chemical Co., St. Louis, MO). An aliquot (2 mL) of the supernatant solution was transferred to test tubes. Acetone (8 mL) was added to precipitate the starch polymers and the mixture was centrifuged. The precipitate was dissolved in 1 mL DMSO by heating in a boiling water bath for 5 min prior to high-performance size-exclusion chromatography (HPSEC) analysis.

HPSEC analysis

A method similar to that described by Kobayashi et al. (1986) was used to analyze the starch components and their hydrolysis products. A Waters Associates (Milford, MA.) series liquid chromatograph system, equipped with a model 510 pump, U6K injector, and a Waters model 410 differential refractometer, was used. The detector signal was electronically recorded and integrated by a Apple II Plus computer, equipped with an ADALAB data acquisition/control card and CHROMATOCHART chromatography software for peak area integration (Interactive Microware, State College, PA).

Two DuPont (Wilmington, DE) Zorbax PSM 60S HPSEC columns (6.2 mm × 30 cm) were connected in series. The columns were protected with a guard column (Waters Assoc., 3.9 mm × 2.5 cm), packed with Co:Pell ODS (Whatman Inc., Clifton, NJ). The columns were immersed in 40°C water bath. The refractometer was also maintained at 40°C with its internal temperature heater. Detector sensitivity was set at 30 and scale factor of 20. Samples were injected and eluted using an isocratic solvent system of 99% DMSO:1% H_2O (DMSO, Fisher certified ACS) at a flow rate o 0.4 mL/min. All commercially available DMSO contained 0.1–0.4% H_2O, but was adjusted to 1%. Distilled and deionized water was used. The water and DMSO were filtered through a fritted disk funnel (pore size, 10—15 μm) and degassed at room temperature. All other chemicals used were chemically pure grade.

Average molecular sizes ($\bar{M}_{50}$) of hydrolytic products were estimated from retention times obtained at the mean (50% area) of each chromatogram. The columns were calibrated using a series of dextran standards (Blue dextran-2000, T_{500}, T_{70}, T_{40}, T_{10}; Pharmacia, Piscataway, NJ) and also from linear alpha-(1,4) fragments obtained by debranching amylopectin with isoamylase (Chang-Rupp and Schwartz, 1988).

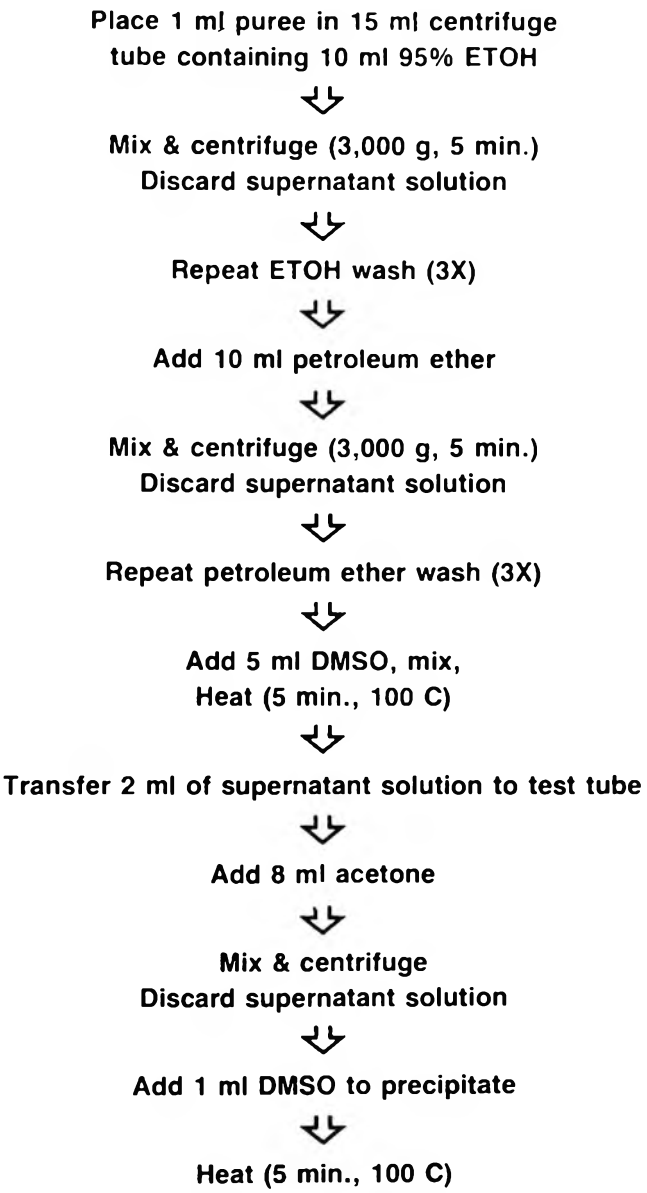

Fig. 1.—Schematic of sample extraction procedure for high performance size-exclusion chromatography analysis.

Oligosaccharide analysis

The ethanol extract form enzyme-treated purees was analyzed for sugar and oligosaccharide content by HPLC using a DuPont Zorbax NH_2 column (4.6 mm × 25 cm), and protected with a guard column (2-mm ID × 2 cm) packed with ETH BH631 DuPont Permaphase packing. An aliquot (2 mL) of the ethanol supernatant solution was concentrated under reduced pressure and dissolved in 0.5 mL water. Samples were filtered through a 0.45 μm membrane. Samples were injected (10μL) and eluted using an isocratic solvent system of 70:30 acetonitrile:water (CH_3CN:H_2O) at a flow rate of 2.0 mL/min. Detector sensitivity was set a 16 and scale factor of 10. Standards of maltose, maltotriose, maltotetraose, maltopentaose, maltohexaose, maltoheptaose and raffinose were purchased from Sigma Chemical Co. (St. Louis, MO). Glucose, fructose and sucrose were obtained from Fisher Scientific Co. (Raleigh, NC). One percent (w/v) standards were prepared to assist in identifying sugars and oligosaccharides present in puree extracts.

RESULTS & DISCUSSION

THE EXTRACTION PROCEDURE outlined in Fig. 1 removes monosaccharides and short chain oligosaccharides and "solubilizes" the starch polysaccharides in DMSO. Residual starch remaining after extraction with DMSO was insignificant. Chromatograms of DMSO extracts of hand-peeled roots (Fig. 2A) showed amylopectin (Ap) and amylose (Am) in the expected ratio (70:30, respectively) similar to chromatograms of sweet potato starch (Chang-Rupp and Schwartz, 1988). These results indicated that the extraction procedure coupled with the

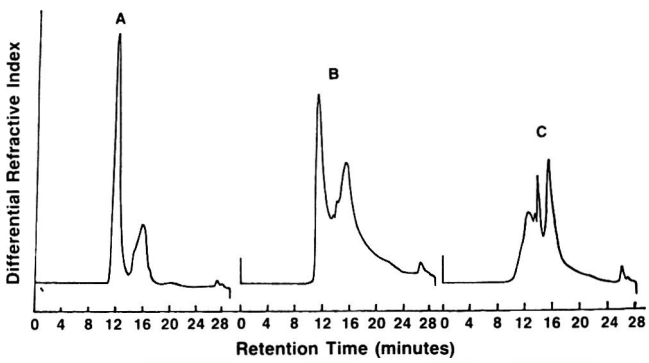

Fig. 2.—Chromatograms of starch polysaccharides from processed pureed sweet potatoes: (A) hand-peeled roots, (B) lye-peeled roots, (C) lye-peeled roots after steam injection.

HPSEC method, provided chromatograms representative of the starch polysaccharides present in sweet potato puree.

Comparison of peeling method

Hand-peeled and lye-peeled sweet potatoes were pureed, then steam-injected and their carbohydrates analyzed to determine if lye peeling and steam injection initiated starch hydrolysis relative to untreated roots (hand-peeled). The conditions used were characteristic of commercial sweet potato puree processing. Profiles (Fig. 2) illustrate typical HPSEC chromatograms of starch polysaccharides in the puree system subjected to the different heat and alkali treatments. Figure 2B, taken immediately after pureeing lye-peeled sweet potatoes, reveals a significantly different profile than that of the hand-peeled puree (Fig. 2A). The heat and alkali conditions used in lye peeling caused gelatinization of some starch and initiated hydrolysis. This is indicated by the formation of lower molecular weight products and a broadening of the molecular weight distribution. Further hydrolysis occurred during steam injection (Fig. 2C), necessary for inactivating the native enzymes. As anticipated, these results suggested that enzyme inactivation did not occur instantaneously, allowing for the formation of lower molecular weight hydrolysis products. Presumably, starch gelatinization and enzyme inactivation occur simultaneously during steam injection until inactivation is complete, allowing no further enzyme action.

The outer layers of a representative sample of lye-peeled sweet potatoes were carefully removed and separated from the center material. These separated layers were analyzed individually. The centers, where little or no heat penetration occurred, resembled the profile of hand peeled sweet potatoes (Fig. 2A). The lye-peeled outer layer showed a distinctively different profile, revealing considerable amylase action at the surface (data not shown). The starch of this outer layer was partially gelatinized due to the alkali and heat treatments from the lye-peeling process and thus, was susceptible to the action of native amylases. Walter and Schadel (1982) found that lye peeling contributed from slight to complete gelatinization of the starch at depths 3–15 mm from the surface. The major effect of heat penetration from peeling was restricted to the tissue ≤ 2.5 cm from the peeled surface, with the maximum effect centered in the outer 1.5 cm. Lye peeling is a typical and critical step in the commercial processing of sweet potatoes.

Native enzyme-hydrolyzed puree

Heating puree (prepared form hand-peeled sweet potatoes with no previous heat treatment) to the activation temperature of 75°C for starch gelatinization, resulted in the series of carbohydrate patterns shown in Fig. 3 (native enzymes). The puree reached 75°C after 12 min heating. There is a definite initial hydrolysis of starch prior to the actual activation temperature being reached (Fig. 3: 3 min and 7 min). Gelatinization causes the starch to become more susceptible to attack by alpha-amylases and increases as the extent of granular expansion increases (Coulter and Potter, 1972; Slack and Wainwright, 1980). As hydrolysis continued, distinctive peaks from degradation products were formed over a wide molecular weight range. Two distinctive low molecular weight peaks (Fig 3, native: 120 min), coeluted with spiked standards of maltotriose (retention time = 29 min) and maltose (retention time = 30.5 min), respectively, and increased as hydrolysis proceeded. No further hydrolysis was noted after 120 min.

Tenase-treated puree

Figure 3 illustrates a series of HPSEC profiles which characterize the breakdown of polysaccharides over time, using the commercial alpha-amylase, Tenase, at 70°C. Time 0 (T_0) refers to the chromatographic profile of the puree obtained prior to the addition of the enzyme. The lower molecular weight fragments shown, relative to native starch, occurred due to processing of the lye-peeled puree. The profile of T_0 was used as the reference for the series of chromatograms illustrated in Fig. 3. One minute after the addition of Tenase, there was considerable degradation of the starch. Distinctive peaks were formed initially after the addition of Tenase, and hydrolysis continued from higher to lower molecular weight compounds. There were specific peaks formed in the lower molecular weight range. From 5 min to the end of hydrolysis, there was considerable broadening of the molecular weight distribution. At 55 min of hydrolysis, more defined peaks emerged in specific molecular size regions. No further hydrolysis was apparent after 60 min. Addition of Tenase and/or water to the puree at 60 min did not result in additional hydrolysis.

Bacillus subtilis alpha-amylase-treated puree

Figure 3 illustrates the series of chromatograms characterizing the breakdown pattern of sweet potato puree using *Bacillus subtilis* alpha-amylase at 40°C. Time 0 (T_0) refers to the starch profile prior to enzyme addition and was used as a reference for this series of chromatograms. One minute after the addition of the alpha-amylase, hydrolysis had occurred. By 5 min of hydrolysis, a specific peak (retention time = 17 min), increased in area. This peak predominated and broadened, as hydrolysis continued, to a wide range of molecular weight products. Hydrolysis ceased after 65 min even after additional enzyme and/or water was added.

A comparison of the profiles by Tenase and *Bacillus subtilis* alpha-amylase showed unique similarities and differences. Both enzymes exhibited classical alpha-amylase hydrolysis patterns of dextrinization, where there was a patterned breakdown from higher to lower molecular weight compounds. This accounted for the broad peak areas where a variety of lower molecular weight products existed. In addition, specific peaks appeared to concentrate and emerge from the broad distribution, designating particular molecular size regions. A portion of these compounds could possibly be the resulting alpha-limit dextrins. Alpha-limit dextrins are the alpha (1,6)-linked branch points remaining from alpha-amylolysis, ranging in size from Dp 2 - Dp 7 and higher (Robyt, 1984). Robyt and French (1963) found that the action of amylase on amylose and amylopectin components on starch predominantly formed products of Dp 3 and Dp 6, although other low molecular weight compounds were also produced (Dp 2, 4, 5, 7). These may be the broad peak areas spread over the latter part of the HPSEC chromatograms (retention time 24 to 29 min).

Tenase, a thermostable commercial *Bacillus subtilis* alpha-amylase preparation, appeared to form a broad distribution of products after initial hydrolysis from addition of the enzyme. The *Bacillus subtilis* alpha-amylase had a more specific region and narrower range of molecular weight products upon addition of enzyme. As hydrolysis proceeded, there was no gradual shifting of the molecular weight region but an increase in specific hydrolysis products.

Microscopic examination of the puree under polarized light after steam injection revealed numerous birefringent fragments. Despite the severe heating conditions, total gelatinization of the starch granules was not complete. This could be one of the reasons why enzymatic hydrolysis is slow and leaves high molecular weight material, since starch granules are relatively resistent to amylases in comparison to the degradation of soluble substrates (Kruger and Lineback, 1987). Adding additional enzyme and/or water to the puree mixture caused no further detectable hydrolysis. Thus, water and/or enzyme were not limiting factors. There have been reports of proteins which inhibit alpha-amylase activity (Strumeyer and Fisher, 1983). These inhibitor proteins, however, are not effective against microbial enzymes and are naturally-occurring in plants or produced by microorganisms (Buonocore et al., 1977). An-

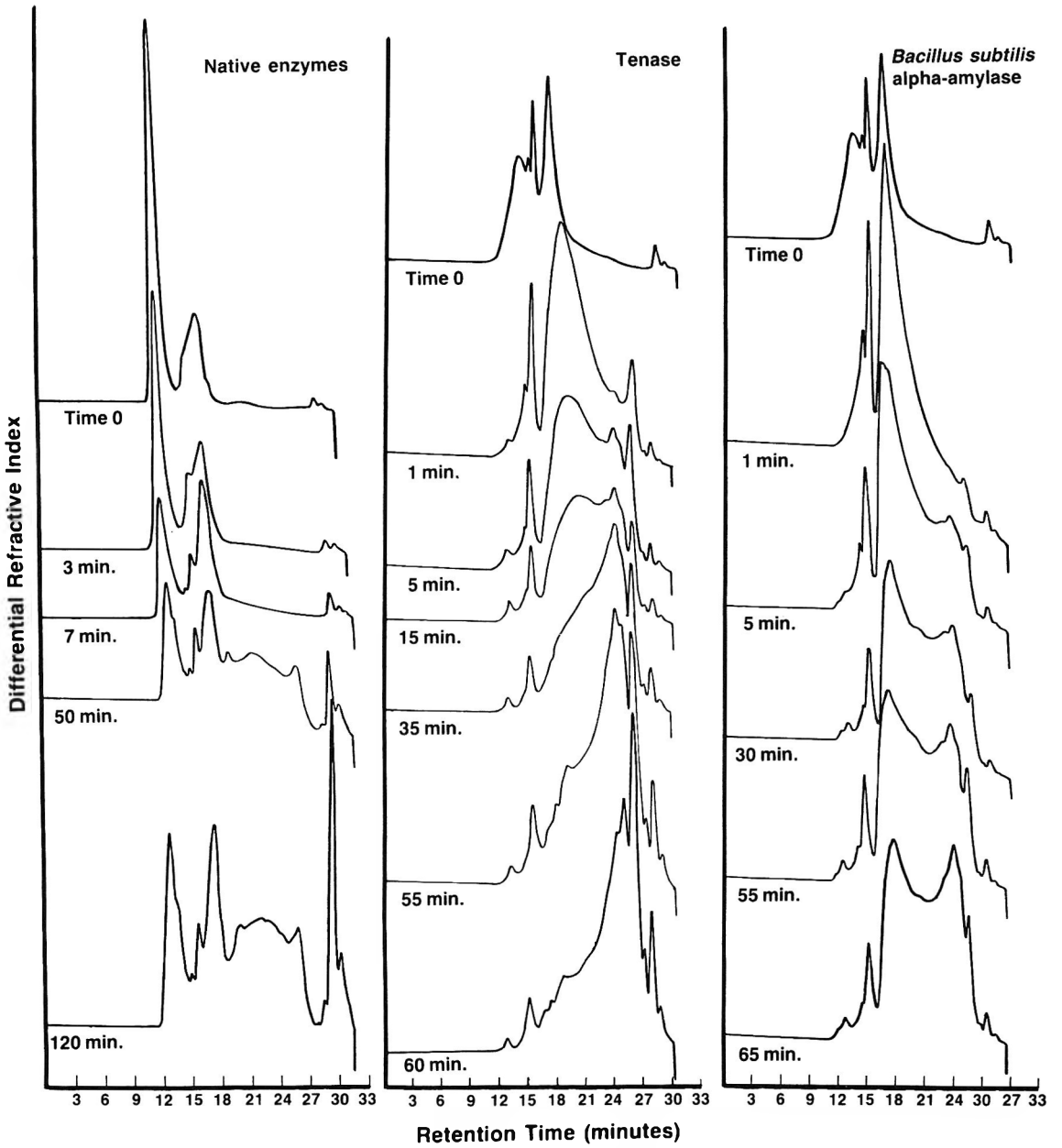

Fig. 3.—HPSEC profiles of sweet potato puree during starch hydrolysis by native enzymes, Tenase and B. subtillus alpha-amylase at specific time intervals.

other possibility exists that the oligosaccharide and polysaccharide end products of hydrolysis may inhibit further enzyme action.

Each chromatogram of Fig. 3 was separated into four fractions on the basis of molecular size (fraction A < 14.3 min ($\overline{M}_{50} > 3.0 \times 10^5$), fraction B = 14.3 to 18.3 min ($\overline{M}_{50} \sim 3.0 \times 10^5$ to 1.1×10^4), fraction C = 18.3 to 22.3 min ($\overline{M}_{50} \sim 1.1 \times 10^4$ to 1.4×10^3), fraction D > 22.3 min ($\overline{M}_{50} < 1.4 \times 10^3$) and each of their percent areas was calculated as hydrolysis continued. The diagrams in Fig. 4 illustrate the change in the area of each fraction of the chromatograms over time for the native, Tenase- and alpha-amylase-treated purees, respectively. In all series of hydrolysis, there was a similar pattern of progressive increasing (▲) and decreasing (▼) percent areas as hydrolysis of the higher to lower molecular weight compounds continued. The trend for each molecular size fraction was identical (A ▼, B ▲, then ▼; C ▲; D ▲). Thus, components of $\overline{M}_{50}$ 3.0×10^5 to 1.1×10^4 (fraction B) are formed predominantly as intermediate products during hydrolysis.

It is interesting to note that the distribution of end products are all different at the end of each hydrolysis (Fig. 4). The native enzyme system (120 min) revealed almost equivalent amounts of each fraction. The final percent areas of each fraction of the Tenase system (60 min) were all quite different. Fraction A began as the highest percent area and ended as the lowest. Fraction D began as the lowest percent area and ended as the highest. The alpha-amylase system (65 min) resulted in almost equal areas of products in fractions B and C.

Further detail in the high molecular weight region can be shown by subdividing fractions A and B into A_1, A_2, and B_1, B_2, (A_1 = 10.3 to 12.3 min ($\overline{M}_{50} \sim 2.8 \times 10^7$ to 2.5×10^6), A_2 = 12.3 to 14.3 min ($\overline{M}_{50} \sim 2.5 \times 10^6$ to 3.0×10^5), B_1 = 14.3-16.3 min ($\overline{M}_{50} \sim 3.0 \times 10^5$ to 5.0×10^4), B_2 = 16.3 to 18.3 min ($\overline{M}_{50} \sim 5.0 \times 10^4$ to 1.1×10^4). Figure 5 illustrates the changes in the subfractions. The change in molecular size for subfractions of the Tenase and alpha-amylase diagrams are similar. Subfractions A_1, A_2, and B_1 rapidly decreased in area, while B_2 increased, then finally decreased over time. This similarity in hydrolytic pattern was expected in the initial stages of hydrolysis since both enzymes are derived from *B. subtilis*. How-

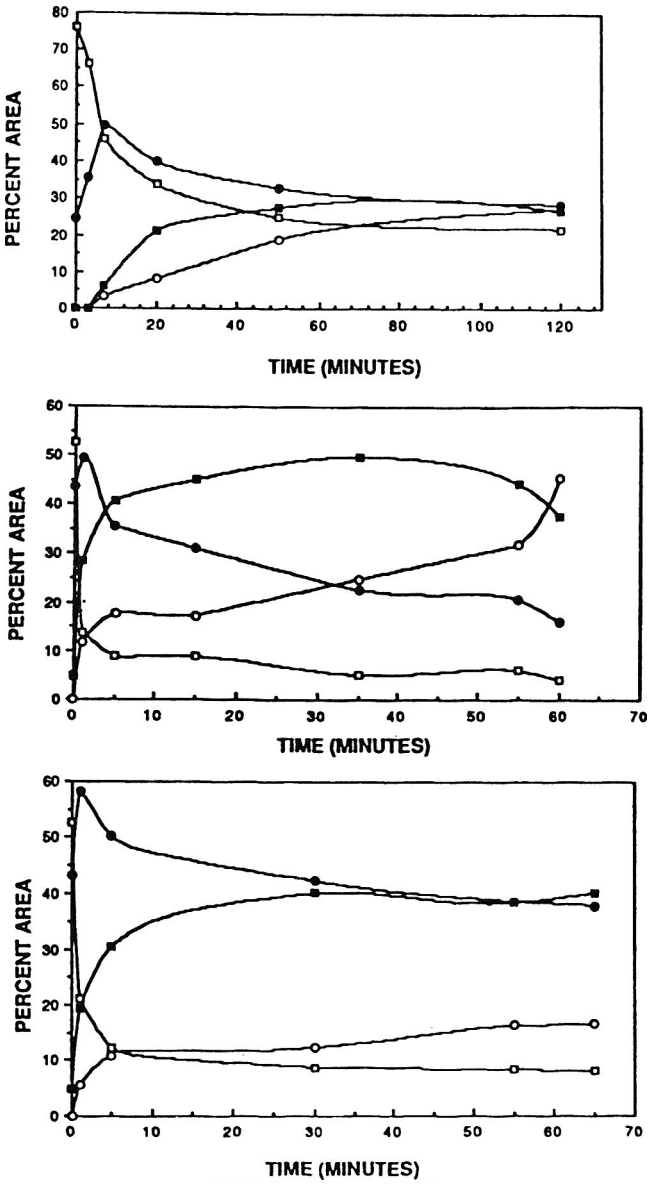

Fig. 4.—Change in the distribution of molecular size versus time for the native- (top) Tenase- (center), and alpha-amylase- (bottom) treated purees. Fractions A = (□, $\overline{M}_{50}$ > 3.0 × 10⁵), B = (●, $\overline{M}_{50}$ ~ 3.0 × 10⁵ to 1.1 × 10⁴), C = (■, $\overline{M}_{50}$ ~ 1.1 × 10⁴ to 1.4 × 10³), D = (○, $\overline{M}_{50}$ < 1.4 × 10³).

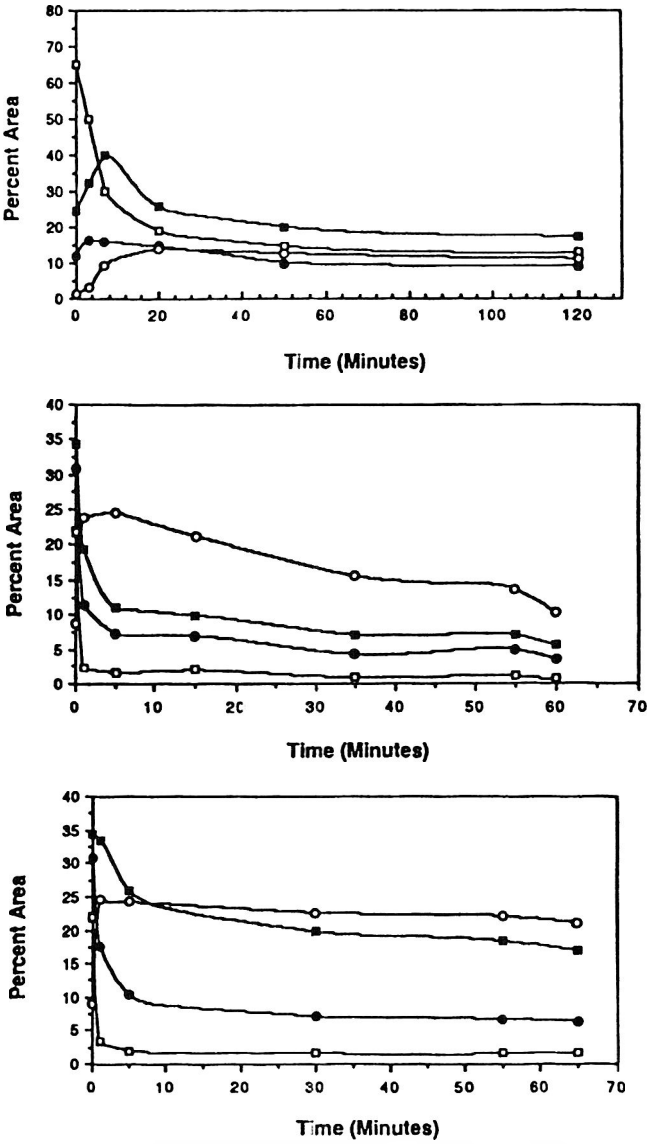

Fig. 5.—Change in the distribution of molecular size versus time for the native (top), Tenase- 'center), and alpha-amylase (bottom) treated purees. Fractions A_1 = (□, $\overline{M}_{50}$ ~ 2.8 × 10⁷ to 2.5 × 10⁶), A_2 = (●, $\overline{M}_{50}$ ~ 2.5 × 10⁶ to 3.0 × 10⁵), B_1 = (■, $\overline{M}_{50}$ ~ 3.0 × 10⁵ to 5.0 × 10⁴), B_2 = (○, $\overline{M}_{50}$ ~ 5.0 × 10⁴ to 1.1 × 10⁴).

ever, the percent area diagrams of the native system in the high molecular size region were very different from that of Tenase and alpha-amylase. Both fractions A_2 and B_1 ($\overline{M}_{50}$ ~ 2.5 × 10⁶ to 5.0 × 10⁴) increased then decreased in area over time. These different patterns probably reflected the molecular size differences at time 0 prior to enzyme treatment versus the higher $\overline{M}_{50}$ at time 0 for the native system.

Oligosaccharide determination

Figure 6 illustrates a typical chromatogram of mono- and oligosaccharides obtained from ethanol extracts of Tenase- and *B. subtilis* alpha-amylase-treated purees. Identification of each sugar was based on HPLC retention times and confirmed by coelution of 1% sugar standards. A large solvent (ethanol-water) peak eluted before the monosaccharides, disaccharides and oligosaccharides, respectively. Higher molecular size oligosaccharides, other than maltotriose - maltoheptaose were not found in ethanol extracts. It was assumed these were included in the DMSO extracts. The results of this study agree with those reported by Walter et al. (1975) who identified maltose and dextrins of unspecified chain length accumulating in processed sweet potato. Van Den et al. (1986) identified several short chain oligosaccharides in sweet potato extracts similar to those identified in this study.

The profiles of polysaccharides and oligosaccharides shown in this study were obtained using a single batch of sweet potatoes harvested from one growing season. Variability would be expected depending upon growing conditions and harvest dates. However, a preliminary study using roots obtained from retail markets revealed profiles with similar hydrolysis patterns. Replicate analyses of samples also gave reproducible results. Thus, similar trends would be expected when using roots obtained from different growing seasons, particularly when native enzymes have been inactivated and processed with exogenous amylases. However, some variability in the quantity of saccharides formed would be expected when utilizing the native amylases for starch conversion.

This study revealed definite differences in the starch polysaccharides of sweet potato due to different amylolytic treatments. More research is needed to determine the relationships

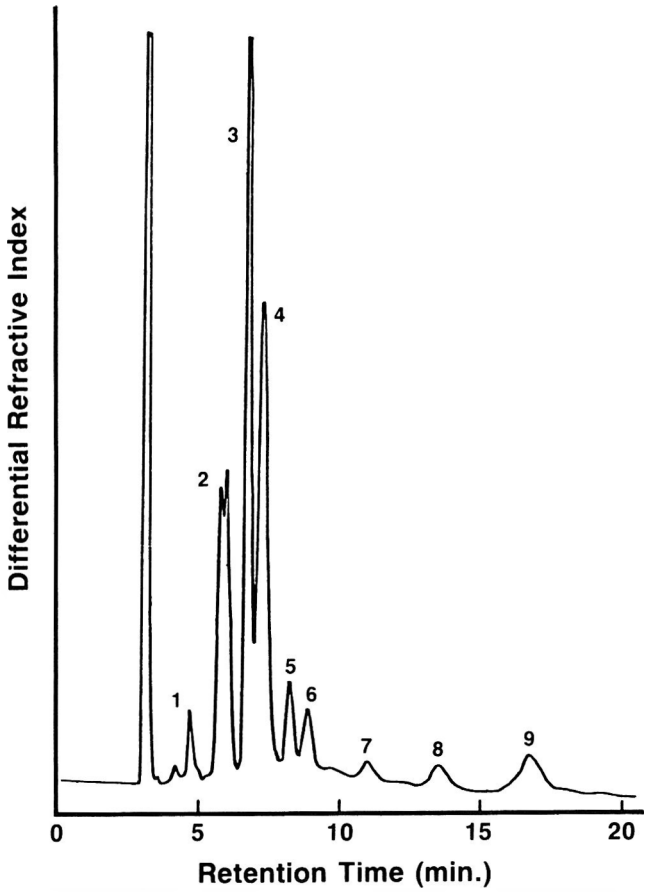

Fig. 6.—Typical chromatogram of mono-and oligosaccharides in ethanol extracts of hydrolyzed sweet potato puree. Peaks: (1) fructose; (2) glucose (α- and β-anomers); (3) sucrose; (4) maltose; (5) maltotriose; (6) maltotetraose; (7) maltopentaose; (8) maltohexaose; (9) maltoheptaose.

between these starch components and their functional properties within the final product.

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Moisture Sorption Characteristics of Several Food Fibers

ANN-MARIE CADDEN

ABSTRACT

Adsorption behavior of microcrystalline cellulose, guar gum, wheat bran and oat bran were compared at 4, 25, and 37°C. Guar gum adsorbed the most water and microcrystalline cellulose adsorbed the least. Adsorption abilities of oat bran and wheat bran were similar at low and intermediate water activity levels. However, wheat bran was able to adsorb more moisture in the high water activity regions of the isotherm. Of the four fibers studied, particle size reduction had the greatest effect on sorption properties of wheat bran, in part, due to the reduction of surface area available for binding at the monolayer.

INTRODUCTION

THE PROPHYLACTIC VALUE attributed to certain food fibers in regulating colonic function is, in part, determined by that fiber's ability to bind water (Health and Welfare Canada, 1985; Hill, 1983; Staub et al., 1983). However, the absolute amount of water held may be less important than the manner by which that water is held (Eastwood et al., 1983). Strongly bound water has been found to have no effect on stool weight, whereas loosely associated water readily increases stool weight (Robertson and Eastwood, 1981). Processes that alter the physical characteristics of certain food fibers can affect the total amount of water held by the fiber, the manner by which that water is held (Cadden, 1987), and the ability of that fiber to regulate colonic function. The addition of finely ground wheat bran to a low- fiber diet has been reported to cause constipation in human subjects (Wrick et al., 1983).

The maximum amount of water that a fiber may hold is a function of the fiber source, the method of measurement and the mode of fiber preparation (Rasper, 1982; Robertson and Eastwood, 1981). In a previous study (Cadden, 1987), the effects of particle size reduction on physical structure and water binding properties of wheat bran, oat bran, microcrystalline cellulose and guar gum were investigated. Water binding was measured by centrifugation techniques and by a dialysis procedure against known osmotic potentials. The fiber matrix structure of wheat bran collapsed during the grinding procedure used and water binding properties were impaired. Physical structure of the commercial oat bran collapsed during manufacture and further grinding had limited effect. Physical structure of microcrystalline cellulose and guar gums did not differ between product types. The amount of water held by oat bran and microcrystalline cellulose was increased by reducing particle size; however, water binding properties of guar gum were not affected by particle size.

Water binding is of concern to the food industry when selecting ingredients and/or processing steps to improve ingredient functionality, product yield and shelf stability (Labuza, 1985). Sorption isotherms, that plot the functional relationship between water activity and the equilibrium water content of a food at constant temperature and pressure, are used extensively to study the water binding properties of food materials. The typical sigmoidally-shaped sorption isotherm consists of three regions— an initial convex region up to a water activity of approximately 0.1–0.2, a linear portion from a water activity of 0.2 to approximately 0.5 and a final concave region for water activity levels greater than 0.5 (Mazza, 1980; Van den Berg and Bruin, 1981). Water in the initial "monolayer region" is held by strong hydrophillic bonds on polar sites in the food solid. A high water uptake in this region usually reflects high levels of hydrophillic macromolecular proteins and polysaccharides. In the second region, the water is more loosely held by hydrogen bonds within the pores or matrix structure of the fiber. This "multilayer region" can be considered a transition phase between the initial and final regions of the isotherm. The least firmly bound water occurs when the water activity level rises above 0.5. In this region, "condensed water" is mechanically entrapped within the void spaces of the food, and has many of the characteristics of liquid water. A high level of "condensed water" indicates that major system components are being dissolved.

Sorption data for several food fibers have been reported by Chen et al. (1984) and Wallingford and Labuza (1983). In its simplest form, the sorption isotherm allows the water content of two foods to be compared at a constant water activity; the water being adsorbed by passive diffusion. However, more information can be obtained. Adsorbed water can be partitioned into its "monolayer", "multilayer" and "condensed" forms. The solid surface area of the sample and the energy required for the sorption process can be calculated. Mathematical models such as the Guggenheim-Anderson-DeBoer (GAB) equation (Bizot, 1983) have been developed which enable the sorption behavior of water in foods to be better understood. This approach allows fiber types to be selected that increase water binding in the gastrointestinal tract and still satisfy the requirements of the food industry.

The purpose of this study was to investigate the effects of altering particle size and fiber structure on the moisture sorption characteristics of microcrystalline cellulose, guar gum, wheat bran and oat bran, and to relate any changes to the known physiological effects of these food fibers.

MATERIALS & METHODS

Preparation of fiber samples

Fiber preparation procedures have been described by Cadden (1987). Two microcrystalline celluloses (Avicel PH101 and PH105; FMC of Canada Limited, Food and Pharmaceutical Products, Dorval, Quebec), and two guar gums (Nutriloid Pretested Coarse and Fine Powders; TIC Gums, Inc., New York), were used without further preparation as purified sources of dietary fiber. Particle size of AACC certified hard red spring wheat bran (American Association of Cereal Chemists, St. Paul, MN) and oat bran (The Quaker Oats Company of Canada Limited, Peterborough, Ontario) was controlled by determining the particle size distribution by screen analysis (Ensor et al., 1970; Heller et al., 1977) and plotting the logarithm of the cumulative percentage weight of the sample passing through each sieve against the logarithm of the sieve aperture (Ensor et al., 1970). The geometric mean particle diameter was defined as the aperture through which 50% of the sample passed; the geometric standard deviation was determined from particle diameters at 16, 50, and 84% undersize. Particles falling within the size range corresponding to the mean ± one standard deviation (50% ± 34% undersize) were selected for further study. Particles corresponding to a screen size greater than 16% or less than 84% undersize were discarded. Half of the particles in the selected fraction (50% ± 34% undersize) were ground in a Wiley mill through a 1 mm screen such that the majority of particles were smaller in diameter than the smallest particles in the unground fraction. All ground particles were retained to ensure that the "selected" and "ground" wheat and oat bran fractions differed only in particle size and not in product composition.

Author Cadden is with the Dept. of Food Science, Univ. of Alberta, Edmonton, Alberta, Canada T6G 2P5.

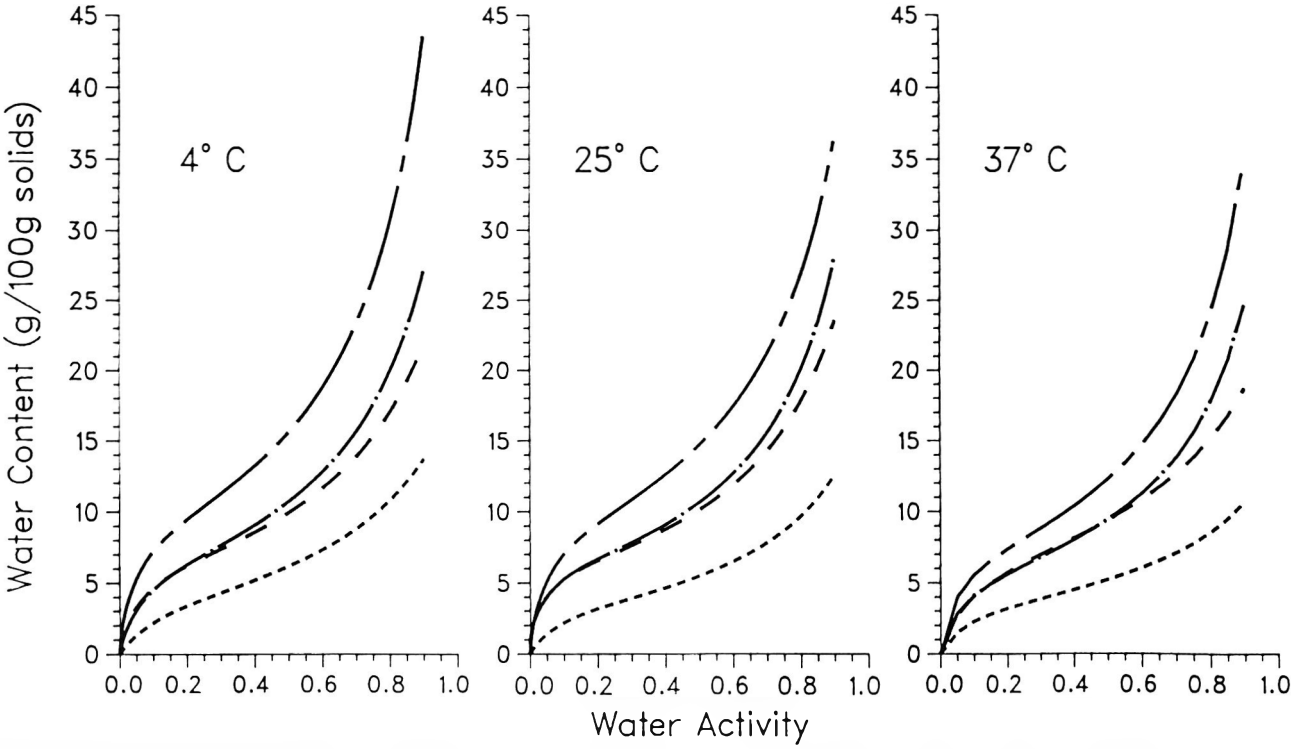

Fig. 1—Adsorption isotherms of different fibers at 4°, 25°, and 37°C: - - - - Microcrystalline cellulose (Avicel PH101), —·— Guar gum (coarse), —·—·— AACC wheat bran, — — — Oat bran.

Preparation of sorption isotherms

Adsorption isotherms were determined at 4°C, 25°C, and 37°C for the two microcrystalline celluloses, two guar gums, the packaged AACC wheat bran and oat bran, and the "selected" and "ground" wheat and oat bran fractions. Duplicate samples were dried to constant weight over phosphorous pentoxide, then placed over saturated salt solutions in desiccators at constant temperature (4°C, 25°C, 37°C) to provide water activities in the range 0–0.90 (Karel, 1975; Labuza, 1984). Final moisture contents were determined when moisture equilibrium had been obtained (after 4–6 weeks) by drying the samples overnight at 100°C under vacuum (AOAC, 1980). Microbial growth was inhibited by placing small vials of toluene in each desiccator that provided water activities greater than 0.60. The moisture content of the samples (g/100g dry solids) was plotted against water activity for each of the three temperatures tested.

Interpretation of data

The effects of water activity, temperature, and particle size/form on the experimental moisture content of each food fiber were assessed by analysis of variance and treatment means were compared using the Student-Newman-Keul's Test. In addition, isotherm data was fitted to the Guggenheim-Anderson-DeBoer (GAB) model as outlined by Bizot (1983). The form of the equation used was:

$$W = \frac{(Wm\ C\ k\ a_w)}{(1 - k\ a_w)(1 - k\ a_w + C\ k\ a_w)} \quad (1)$$

where W was the water content of the sample on a dry basis; Wm was the water content corresponding to the saturation of all primary adsorption sites by one water molecule (equivalent to the BET "monolayer"; Brunauer et al., 1938); C was the Guggenheim constant = $c'\exp(H1 - Hm)/RT$, and H1 = the total heat of sorption of the first layer in primary sites; Hm = total heat of sorption of the multilayer (which differs from the heat of condensation of pure water); k was a factor correcting properties of the multilayer molecules with respect to the bulk liquid. $k = k'\exp(Hm\text{-}He)/RT$ where He was the heat of vaporization of pure water vapor; and, a_w was the water activity. Estimates of the GAB constants (Wm, C and k) were obtained by transforming Eq. (1) to the quadratic form:

$$(a_w/w) = \alpha a_w^2 + \beta a_w + \gamma \quad (2)$$

with $\alpha = (k/Wm)[(1/C) - 1]$; $\beta = (1/Wm)[(1/C) - 1]$; $\gamma = 1/(WmCk)$ and solving for the original parameters. Asymptotic standard deviations were obtained directly by entering the calculated GAB constants into a BMDP nonlinear regression routine (Ralston, 1983). The reported GAB values are from the nonlinear regression and not from the quadratic equation. Asymptotic standard deviations were used to evaluate the effect of temperature and particle size on each GAB constant for each fiber source. The quality of the fit of the GAB model was judged from the value of the relative percent root mean square (%RMS):

$$\%RMS = 100\sqrt{\frac{\sum[Wi - Wi^*]^2}{N}} \quad (3)$$

where N was the number of experimental points; Wi was the average experimental water content; Wi* was the calculated water content.

Once the moisture content at the "monolayer" (Wm) was known, the solid surface area of the fiber samples could be determined (Mazza and Le Maguer, 1978):

$$A = Wm\ (1/{}^{M}H_2O)(N)({}^{\wedge}H_2O) = (3.5 \times 10^6)\ Wm \quad (4)$$

where A was the solid surface area (m^2/kg solid); Wm was the monolayer moisture content (kg H_2O/kg solid); ${}^{M}H_2O$ was the molecular weight of H_2O (18 kg/kmole); N was Avogadro's number (6×10^{26} molecules/kmole); ${}^{\wedge}H_2O$ was the area of a water molecule (10.6 Å $= 10.6 \times 10^{-20}$ m^2).

Finally, heats of sorption (Qs), the amount of energy required to remove water from the substrate in excess of the amount of energy required for normal water vaporization, were derived from the Clausius-Clayperon equation (Labuza, 1984):

$$\ln(a2/a1) = (Qs/R)[(1/T1) - (1/T2)] \quad (5)$$

where a2 = water activity at temperature, T2 (°K); a1 = water activity at temperature, T1 (°K); Qs = isosteric heat of sorption (kJ/kg H_2O); R = gas constant (0.46188 kJ/kg H_2O).

Isosteric heats of sorption (Qs) were calculated at several moisture levels from the slopes of the isosteres of the natural logarithms of water activity versus the reciprocal absolute temperature.

RESULTS & DISCUSSION

ADSORPTION ISOTHERMS for the four food fibers possessed the characteristic sigmoidal shape described by Mazza

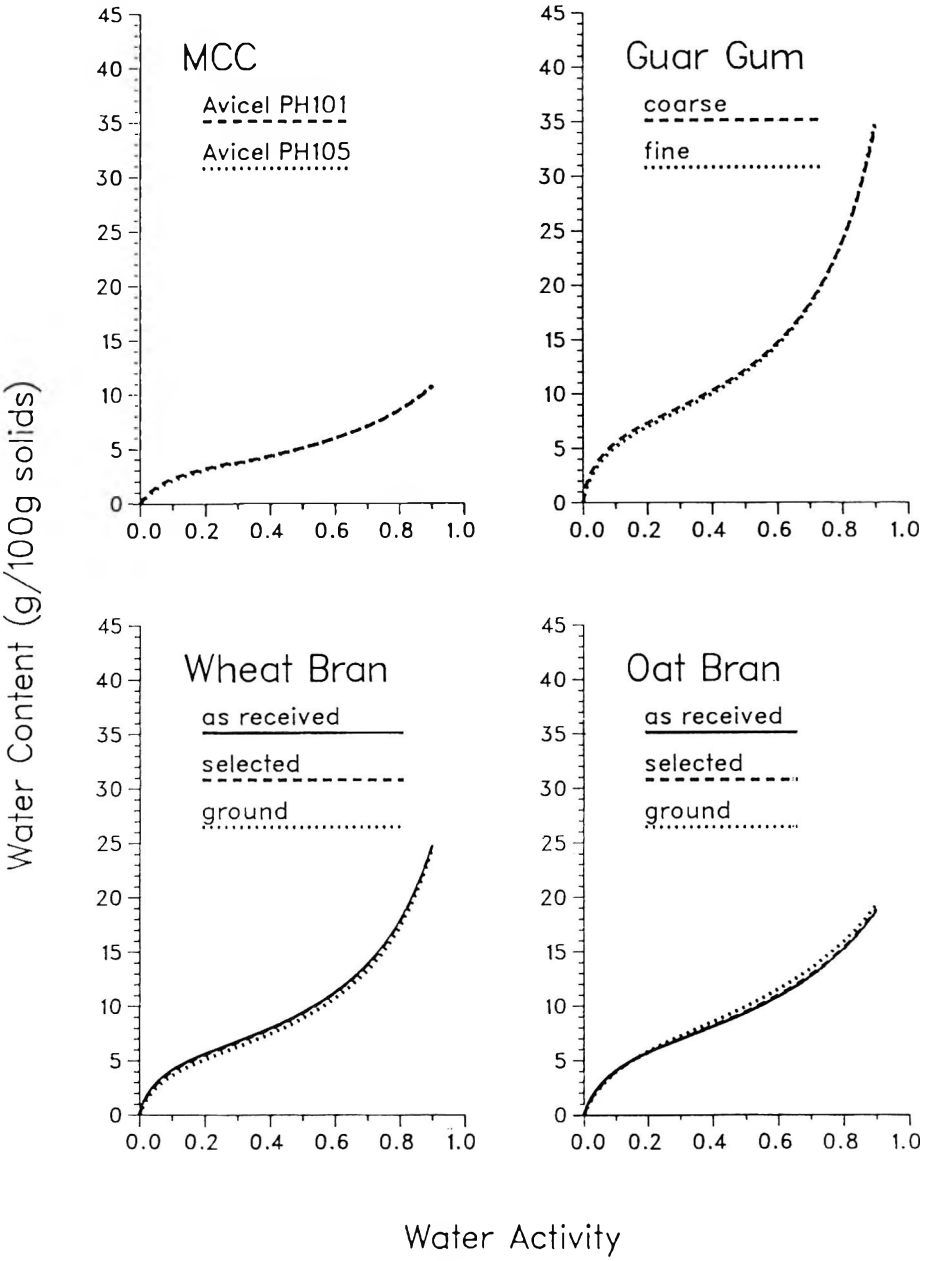

Fig. 2—Effect of particle size on adsorption isotherms at 37°C. MCC = microcrystalline cellulose.

Table 1— Analysis of variance: Effects of water activity, temperature, and physical form/size on moisture adsorption of four food fibers

Source of variation	MCC		Guar gum		Wheat bran		Oat bran	
	df	Mean square	df	Mean square	df	Mean square	df	Mean square
Water activity (W)	8	251.481*	8	3996.200*	8	1130.820*	8	613.791*
Temperature (T)	2	39.998*	2	453.388*	2	96.899*	2	53.038*
Physical form (P)	1	0.246*	1	0.382	2	4.466*	2	0.816*
W × T	16	4.309*	16	60.158*	16	15.308*	16	8.438*
W × P	8	0.123	8	0.933*	16	0.7[illegible]2*	16	0.362*
T × P	2	0.288*	2	1.553*	4	0.587*	4	0.733*
W × T × P	15	0.236*	15	0.713*	32	0.241	32	0.305*
Error	56	0.035	56	0.323	82	0.177	82	0.102

* F ratio calculated from treatment mean square and error mean square is significant (P = 0.05).

(1980) and Van den Berg and Bruin (1981). Each isotherm could be partitioned into an initial "monolayer region" (water activity levels less than 0.2), an intermediate "multilayer region" (water activity levels of 0.2–0.7) and a final "condensed water region" (water activity levels greater than 0.7). The excellent water adsorption properties of guar gum, the poor adsorption properties of microcrystalline cellulose and the intermediate adsorption properties of the nonpurified wheat and oat brans were compared in Fig. 1 and 2. Guar gum was able to adsorb more water than any of the other fibers in all isotherm regions. Water adsorption properties of wheat and oat bran were similar in the "monolayer" and "multilayer" regions; however, wheat bran was able to adsorb more "condensed water" than oat bran.

The equilibrium moisture content of each fiber could be influenced by its environmental temperature and physical form/

Table 2—Moisture adsorption of microcrystalline cellulose (MCC) and guar gum as determined by the Guggenheim-Anderson-DeBoer (GAB) model

		MCC-Avicel		Guar gum	
		PH101	PH105	Coarse	Fine
%RMS	4°C	1.2	3.3	1.3	2.1
	25°C	1.3	3.2	2.5	2.6
	37°C	0.8	0.4	2.3	2.2
GAB constants[a]					
Wm	4°C	4.42 ± 0.09	5.04 ± 0.41	9.15 ± 0.10	8.66 ± 0.28[c]
	25°C	3.65 ± 0.08	3.21 ± 0.13[b]	8.98 ± 0.38	8.11 ± 0.36[c]
	37°C	3.55 ± 0.04	3.61 ± 0.03	7.19 ± 0.16	7.05 ± 0.15
c	4°C	10.1 ± 0.7	7.1 ± 1.4[b]	26.9 ± 2.7	23.3 ± 6.5
	25°C	14.2 ± 1.5	21.7 ± 6.4[b]	27.7 ± 10.6	30.8 ± 15.4
	37°C	18.6 ± 1.2	14.6 ± 0.5[b]	25.2 ± 5.5	20.7 ± 3.9
k	4°C	0.768 ± 0.007	0.717 ± 0.030[b]	0.880 ± 0.003	0.887 ± 0.009
	25°C	0.798 ± 0.007	0.830 ± 0.012[b]	0.839 ± 0.013	0.877 ± 0.012[c]
	37°C	0.751 ± 0.005	0.751 ± 0.003	0.882 ± 0.006	0.890 ± 0.005
Solid surface area[a] (m^2/g)					
	4°C	154.5 ± 3.2	176.3 ± 14.4[b]	320.1 ± 3.4	303.2 ± 9.8[c]
	25°C	127.8 ± 2.8	112.3 ± 4.5[b]	314.2 ± 13.2	283.9 ± 12.6[c]
	37°C	124.3 ± 1.5	126.3 ± 1.0	251.7 ± 5.6	246.7 ± 5.4

[a] Mean ± asymptotic SD.
[b] Particle size had a significant effect on the GAB constant and/or the solid surface area of MCC (P = 0.05).
[c] Particle size had a significant effect on the GAB constant and/or the solid surface area of guar gum (P = 0.05).

Table 3—Moistrue adsorption of AACC wheat bran as determined by the Guggenheim-Anderson-DeBoer (GAB) model

		As received	Selected	Ground
% RMS	4°C	2.1	1.8	1.6
	25°C	2.0	1.6	1.7
	37°C	1.4	1.4	1.6
GAB constants[a]				
Wm	4°C	6.67 ± 0.19	6.36 ± 0.16[b]	5.95 ± 0.15[c]
	25°C	6.28 ± 0.19	6.25 ± 0.17	5.47 ± 0.18[c]
	37°C	5.75 ± 0.10	5.72 ± 0.13	5.43 ± 0.16[c]
c	4°C	17.8 ± 3.2	21.9 ± 4.3	14.9 ± 2.4
	25°C	34.9 ± 12.8	38.2 ± 12.8	48.5 ± 27.9
	37°C	20.6 ± 2.9	19.4 ± 3.4	16.8 ± 3.4
k	4°C	0.842 ± 0.009	0.861 ± 0.008[b]	0.893 ± 0.007[c]
	25°C	0.864 ± 0.009	0.857 ± 0.008	0.822 ± 0.008
	37°C	0.855 ± 0.005	0.859 ± 0.006	0.869 ± 0.008[c]
Solid surface area[a] (m^2/g)				
	4°C	233.5 ± 6.7	222.6 ± 5.7[b]	208.3 ± 5.3[c]
	25°C	219.8 ± 6.8	218.7 ± 5.9	191.5 ± 6.4
	37°C	201.3 ± 3.6	200.2 ± 4.6	190.0 ± 5.5[c]

[a] Mean ± asymptotic SD.
[b] The GAB constant/solid surface area for the selected bran particles differed significantly from the value for the original bran particle (P = 0.05).
[c] Reducing particle size had a significant effect on the GAB constant and/or solid surface area of ground wheat bran compared to selected wheat bran (P = 0.05).

Table 4—Moisture adsorption of oat bran as determined by the Guggenheim-Anderson-DeBoer (GAB) model

		As received	Selected	Ground
%RMS	4°C	2.0	2.1	1.9
	25°C	2.0	1.6	1.4
	37°C	1.5	1.6	1.1
GAB constants[a]				
Wm	4°C	6.38 ± 0.21	6.85 ± 0.24	7.31 ± 0.34[c]
	25°C	6.18 ± 0.16	6.96 ± 0.25[b]	6.83 ± 0.16
	37°C	6.52 ± 0.23	6.66 ± 0.26	7.31 ± 0.31[c]
c	4°C	23.8 ± 5.2	21.3 ± 4.0	16.4 ± 3.2
	25°C	39.4 ± 11.4	24.1 ± 5.6	25.3 ± 4.1
	37°C	17.6 ± 2.9	16.1 ± 2.8	13.3 ± 1.7
k	4°C	0.793 ± 0.013	0.753 ± 0.015[b]	0.753 ± 0.019
	25°C	0.822 ± 0.008	0.755 ± 0.014[b]	0.771 ± 0.008[c]
	37°C	0.734 ± 0.014	0.731 ± 0.015	0.708 ± 0.020[c]
Solid surface area[a] (m^2/g)				
	4°C	223.2 ± 7.4	239.6 ± 8.3	255.9 ± 11.7[c]
	25°C	216.2 ± 5.9	243.6 ± 8.8[b]	239.1 ± 5.5
	37°C	228.2 ± 8.1	233.1 ± 9.1	256.0 ± 10.7[c]

[a] Mean ± asymptotic SD
[b] The GAB constant/solid surface area for the selected bran particles differed significantly from the value for the original bran particle (P = 0.05).
[c] Reducing particle size had a significant effect on the GAB constant and/or solid surface area of ground oat bran compared to selected oat bran (P = 0.05).

size as well as by water activity (P = 0.05, Table 1). Most foods become less hygroscopic as temperature is increased (Iglesias and Chirife, 1982). In the case of microcrystalline cellulose, moisture content was reduced by increasing the temperature from 4°C to 25°C to 37°C. Guar gum, wheat bran and oat bran became less hygroscopic when the temperature

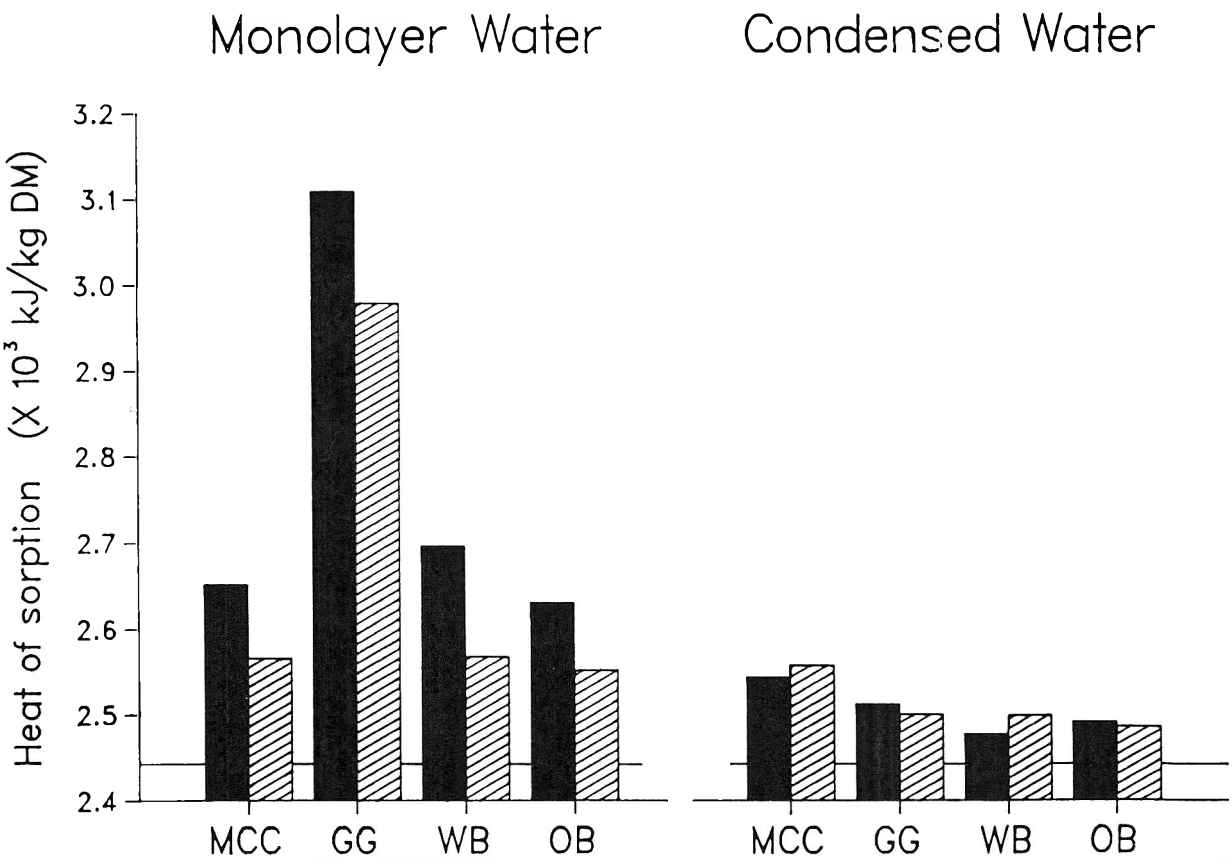

Fig. 3—Isosteric heats of adsorption for monolayer (Wm) and condensed water (aw = 0.90–0.95) layers. The horizontal line refers to the heat of vaporization of water at 25°C. MCC = microcrystalline cellulose, GG = guar gum, WB = wheat bran, OB = oat bran. ■ = larger sized particles of each product; ▨ = smaller sized particles.

was increased from 25°C to 37°C. According to the analysis of variance table, the physical form/size of the fiber affected sorption data for all fibers except guar gum ($P = 0.05$, Table 1). However, the similarity of the isotherm curves (Fig. 2) and the numerous significant interactions in the analysis of variance table ($P = 0.05$, Table 1) suggest that the effects of particle size reduction were limited and not consistent over each isotherm region.

Low %RMS values confirmed that the GAB equation was a good model for experimental data (Tables 2-4). Trends determined by analysis of variance (Table 1) were, in general, consistent with trends determined by the examination of the three GAB constants.

Moisture content of microcrystalline cellulose decreased when temperature was increased from 4° to 37°C (Fig. 1). No consistent particle size effects could be demonstrated (Table 1, Fig. 2).

Moisture content of guar gum decreased as temperature increased. Isotherm curves for the coarse and fine guar gums were similar (Fig. 2). However, monolayer values (Wm) for the coarse guar gum were larger than for the fine guar gum at 4° and 25°C ($P = 0.05$, Table 2).

Equilibrium moisture contents of all wheat and oat bran samples at water activity levels lower than 0.4–0.5 tended to be larger at 25°C than at 4°C or 37°C (Fig. 1). The "c" constant from the GAB equation was larger at 25°C for these samples (Tables 3-4). Although most foods do tend to become less hygroscopic at higher temperatures, some sugar-containing foods become more hygroscopic due, in part, to their sugar components partially dissolving in water (Iglesias and Chirife, 1982). Since wheat and oat brans contain components other than dietary fiber, the slightly higher moisture contents occurring at 25°C may be due, in part, to solubility differences for nonfibrous components between 4°C and 25°C.

Sorption isotherms for the three types of wheat bran particles (as received, selected, ground) were similar (Fig. 2). However, experimental values for moisture contents of the ground wheat bran particles tended to be lower than for the selected wheat bran particles, especially at low and intermediate water activity levels. This had the effect of reducing "Wm" and increasing the value of the "k" constant in the GAB equation ($P = 0.05$, Table 3).

Oat bran adsorbed similar amounts of water as wheat bran at low and intermediate water activities, but less water at water activities greater than 0.5 (Fig. 1). Experimental values for moisture contents of the ground oat bran particles were slightly higher than those for selected oat bran particles in the intermediate and high water activity regions. The reduction of particle size increased "Wm" at 4° and 37°C ($P = 0.05$, Table 4).

Cadden (1987) reported that physical structure and theoretical surface area played an important role in determining the water binding properties of dietary fiber. The solid surface area of each fiber was calculated to further explore this relationship (Tables 2–4). Since the required calculation is a function of "monolayer water" which can be influenced by temperature, solid surface area values should decrease with increasing temperatures. At 37°C, the temperature of physiological interest, the solid surface area of the two guar gums and the two microcrystalline celluloses did not differ between product types ($P = 0.05$, Table 2). Solid surface area of the ground wheat bran was smaller than that of the selected wheat bran. However, solid surface area of the ground oat bran was larger than that of the selected oat bran. This was consistent with the results of the earlier water binding study.

"Solid surface area" refers to the total surface area available for hydrophillic binding. It differs from "theoretical surface area" by taking fiber porosity as well as particle size reduction

into account. If the structure of the fiber remained unchanged by the reduction of particle size, the surface area available for binding would either remain unchanged or increase. This occurred in the case of microcrystalline cellulose, guar gum and oat bran ($P = 0.05$, Tables 2, 4). Grinding the selected wheat bran particles collapsed the matrix structure and reduced fiber porosity (Cadden, 1987). This resulted in ground wheat bran having a smaller surface area available for hydrophillic binding ($P = 0.05$, Table 3).

The energy required by the different fibers to adsorb moisture is also of physiological interest (Robertson and Eastwood, 1981). Isosteric heats of sorption (Qs) are differential quantities that vary inversely with the amount of water vapor adsorbed by the solid (Mazza, 1980); the higher the moisture content, the less energy is required to remove water molecules from the solid. That is, water bound in the "monolayer region" is more tightly bound than water occurring in the "condensed water regions" of the isotherm. Since increasing the temperature from 4° to 25°C did not affect sorption data for guar gum, wheat bran, or oat bran ($P = 0.05$, Tables 1–4), the typical Qs values reported in Fig. 3 should be considered as estimates and only used to show trends amongst the different samples.

Guar gum held water molecules more tightly than other fibers at the monolayer (Fig. 3). This was consistent with known physiological data (Poksay and Schneeman, 1983; Robertson and Eastwood, 1981). Heats of sorption at the monolayer for other fibers were similar, that is, in the order of 2500–2700 kJ/kg water. Within each fiber type, sorption heats at the monolayer were slightly higher for the product with the larger particle size. Heats of sorption of "condensed water" were similar for all fibers—approximately 2500 kJ/kg water.

In a physiological situation, much of the adsorbed water associated with dietary fiber would be in the "free" or "liquid" state. As the dietary fiber and its associated water progressed through the gastrointestinal tract, the osmotic pressure would increase (Labuza, 1985; Robertson and Eastwood; 1981) resulting in decreased water activity levels (Labuza, 1984). Free water would be released from the fiber surface leaving the "multilayer" and "monolayer" water. If fiber porosity were affected by the processing treatment, water adsorption in the "multilayer region" of the isotherm would also be influenced. Alterations in particle size would affect water adsorption in the "monolayer region" if physical structure of the food were modified.

CONCLUSIONS

THE ADSORPTION ISOTHERMS of the different food fibers were more influenced by the type of fiber than by that fiber's particle size. However, structural changes can and do modify water binding properties of certain fibers. Therefore, physical structure must be monitored when water-binding studies of different fibers are being conducted, especially if the results are to be used to predict the physiological efficiency of those food fibers.

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Thermal Degradation of Flavor Enhancers, Inosine 5′-monophosphate, and Guanosine 5′-monophosphate in Aqueous Solution

TERUYOSHI MATOBA, MARIKO KUCHIBA, MASARU KIMURA, and KIYOZO HASEGAWA

ABSTRACT

The mechanism of thermal degradation of inosine 5′-monophosphate (IMP) and guanosine (GMP) was investigated kinetically in aqueous solution as a function of pH and temperature. The degradation of IMP and GMP followed first order kinetics. The rate constants were considerably affected by pH and temperature. The half-life times at 100°C were: IMP, 8.7 hr (pH 4.0), 13.1 hr (pH 7.0), 46.2 hr (pH 9.0); GMP, 6.4 hr (pH 4.0), 8.2 hr (pH 7.0), 38.5 hr (pH 9.0). These times were shortened to about one-third by raising the temperature 10°C. The predominant degradation products were nucleosides and phosphoric acid, indicating that the main reaction of the thermal degradation was the hydrolysis of the phosphoric ester bond in the nucleotides.

INTRODUCTION

SINCE ANCIENT TIMES sauces and soups have been utilized to improve the taste quality of food. In Japan, for a long time, the extracts from dried sea tangle and dried bonito have been widely used for the broth for traditional dishes. In 1908, Ikeda (1909) found that monosodium glutamate (MSG) was a palatable taste substance and tentatively named this characteristic tase "umami". Subsequently, 5′-ribonucleotides such as inosine 5′-monophosphate (IMP) extracted from dried bonito and guanosine 5′-monophosphate (GMP) from dried mushroom (*Lentinus edodes* Sing.) were found to be substances having the umami taste (Kodama, 1913; Kuninaka, 1960). Since then some Japanese people have considered "umami taste" as a fifth basic taste in addition to the four basic tastes (sweet, bitter, salt, and sour) (Henning, 1916), and MSG, IMP and GMP have become important enhancers in improving the palatability (or flavor) of broth cooked at home and in commercial foods. This trend has spread not only within Japan but also to China and other Asian countries, with the result that consumption of these flavor enhancers has increased rapidly. In Europe and the United States, these compounds which are permitted as food additives, have been available as flavor potentiators. Recently, many scientists have extensively investigated these flavor enhancers focusing on various aspects such as physiology, nutrition, food science and psychology (Kawamura and Kare, 1987; Maga, 1983). Of these enhancers IMP and GMP seem to be less stable thermally than MSG, because these nucleotides have weak chemical bonds such as glucosidic and ester linkages. When these enhancers are utilized in home cooking and by the food industry, they may be degraded by heat treatment. Therefore, it is important to investigate their thermal stability. Kuriyama et al. (1965), Fujita et al. (1965) and Davidek et al. (1972) reported the profile of thermal degradation of IMP and GMP through analysis by enzymatic, paper electrophoretic and conventional ion-exchange (Dowex 50W) chromatographic methods. Recently, Shaoul and Sprons (1987) examined the degradation of 5′-ribonucleotides by high performance liquid chromatography. However, the mechanisim of degradation was not fully clarified. The purpose of this investigation was to elucidate the mechanisim of thermal degradation of IMP and GMP at pH 4, 7 and 9 kinetically and to predict the thermal stability of these flavor enhancers during cooking or processing.

Authors Matoba, Kuchiba, and Hasegawa are with the Dept. of Food Science & Nutrition, Faculty of Home Economics, and author Kimura is with the Dept. of Chemistry, Faculty of Science, Nara Women's Univ., Nara 630, Japan.

MATERIALS & METHODS

Materials

Inosine 5′-monophosphate (IMP), guanosine 5′-monophosphate (GMP), ribose 5-phosphate, O-phosphorylethanolamine, β-glycerophosphoric acid, sodium acetate, 3-(N-morpholino)-propanesulfonic acid (MOPS), and 2-(cyclohexylamino)-ethanesulfonic acid (CHES) were purchased from Nakarai Chemicals, Ltd., Kyoto. Inosine, hypoxanthine, guanosine and guanine were obtained from Wako Pure Chemical Industries, Ltd., Osaka.

Thermal reaction system

The 5′-ribonucleotide was dissolved in buffer (0.01 M sodium acetate at pH 4.0, 0.01M MOPS at pH 7.0, 0.01M CHES at pH 9.0), and the solution was incubated in a sealed Pyrex tube at 80–100°C. After incubation, the tube was cooled immediately in an ice bath to stop the reaction. The reacted sample was analyzed by thin-layer chromatography (TLC) and high performance liquid chromatography (HPLC).

TLC analysis

TLC was used to identify the thermal reaction products of 5′-ribonucleotides on Kieselgel 60 F_{254} (Merck), with a solvent system consisting of ethanol and 1M ammonium acetate (2:1 by v/v). The spots on the chromatograms were detected by UV-light.

HPLC analysis

A Shimadzu LC-6A liquid chromatograph, equipped with a UV detector was used to determine 5′-ribonucleotide, nucleoside and the purine base in the reacted sample. The detector was set at 254 nm. The column used was Cosmosil $5C_{18}$-P (ODS type: 4 × 250 mm), which was purchased from Nakarai Chemicals, Ltd., Kyoto. An integrator C-R3A (Shimadzu) was used for the calculation of peak areas. The mobile phase was methanol-0.02M KH_2PO_4 (15:85, v/v). The flow rate was 0.5 mL/min; the temperature of the column was 25–30°C. Under these conditions, the retention times of 5′-ribonucleotide (IMP, GMP), nucleoside (inosine, guanosine) and base (hypoxanthine, guanine) were 6.5, 10.5, and 8.5 min, respectively. Free phosphoric acid was determined by the method of Fiske and Subbarow (1925).

RESULTS & DISCUSSION

Process of the thermal degradation of 5′-ribonucleotides

After heating IMP for 1 hr at 100°C and pH 4, inosine and hypoxanthine were detected on the chromatogram under UV-light (Fig. 1), indicating that the thermal degradation products from 5′-ribonucleotide contained at least nucleoside and base. This result suggested that phosphoric acid and ribose (or ribose 5-phosphate) also were in the degradation products. The qual-

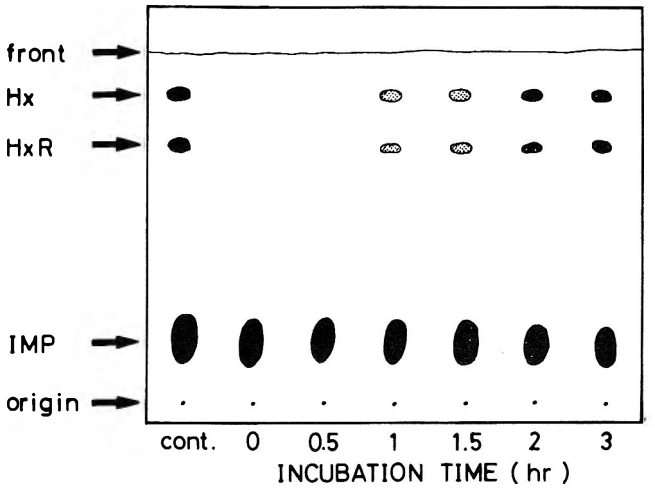

Fig. 1—Thin layer chromatogram of heated IMP. IMP solution was incubated at 100°C and pH 4.0. Hx; hypoxanthine, HxR; inosine, IMP; inosine 5'-monophosphate.

itative profile of GMP degradation on TLC was similar to that of IMP.

Figures 2a and 2b show the changes in concentration of IMP, GMP and their degradation products (nucleoside and base) during heating as a function of time (0–200 hr) at pH 4 and 100°C. The decrease in IMP concentration was observed immediately after heating, with most of the IMP degraded in 30 hr (Fig. 2a). The concentration of inosine, which had increased initially upon heating, began to decrease after heating for 24 hr. The concentration of hypoxanthine continued to increase with heating and then reached a plateau after 150 hr. In the case of GMP, the change in GMP, guanosine and guanine during heating was similar to that of IMP although there was a quantitative difference between GMP and IMP (Fig. 2b). These results suggested that 5'-ribonucleotides were first degraded to nucleosides and phosphoric acid, and then the resulting nucelotide was further hydrolyzed to purine and ribose. During the heating process of both nucleotides, the sum of the concentrations of nucleotide, nucleoside and purine up to 50 hr heating was equivalent to the initial concentration of the corresponding nucleotide before heating, indicating that only two types of cleavages of the N-glucosidic bond between base and ribose and the ester bond between ribose and phosphoric acid occurred during the heating treatment. However, the sum of the concentrations of these compounds after 100 hr was about 95% of the initial concentration of nucleotide, suggesting that a reaction occurred other than the above mentioned cleavages. In fact, trace amounts of an unidentified peak were observed on the HPLC chromatogram (data not shown). Davidek et al. (1972) examined the time dependence of degradation of IMP, inosine and hypoxanthine by using a conventional ion-exchange (Dowex 50W) chromatographic method, which showed that the degration of these compounds was more susceptible than that of the present study (Fig. 2.).

Next, the process of the thermal degradation of the nucleotide was compared with that of nucleoside. The processes of the thermal degradation of IMP and inosine as a function of time (0-7.5 hr) at pH 4 and 100° C are shown in Fig. 3a and 3b. The amount of hypoxanthine produced from IMP after heating for 7.5 hr (Fig. 3a) was about 60% that from inosine (Fig. 3b). But the amount of inosine produced from IMP was small, while the ratio of hypoxanthine to inosine in Fig. 3a was considerably higher than that in Fig. 3b. This evidence suggested that hypoxanthine was also produced directly from IMP; that is, the base was formed not only from the nucleoside but also from the nucleotide. The process of the thermal degradation of GMP with that of guanosine was also examined as shown in Fig. 4a, 4b, which suggest that guanine is produced from both GMP and guanosine as in the case of IMP. Fujita et al. (1965) suggested that the N-glucosidic bond might be cleaved at a lower pH range (pH 2–3.5), but in this study this bond was cleaved even at pH 4.0.

Kinetic parameters for the thermal degradation of 5'-ribonucleotide

To determine the kinetic parameters for the thermal degradation, a logarithm of the amount of nucleotide remaining againt heating time was plotted. The semi-log plots for the thermal degradation of IMP and GMP at pH 4.0 and 100°C gave straight lines (Fig. 5), suggesting that the degradation of both nucleotides proceeded according to the first order reaction with the law of $-d[IMP]/dt = k_{obsd}[IMP]$ or $-d[GMP]/dt = k_{obsd}[GMP]$ where k_{obsd} is the observed first-order rate constant. Labuza (1979) stated that in many degradative reactions of food, if the data statistically fitted a straight line on a semi-log plot through two half-lives for a total of 75% loss, then the reaction was first order, since the difference in reaction order (zero order or first order) was small at earlier times. In the present experiment, the behavior of IMP and GMP in the degradation experiment, followed first order kinetics until both IMP and GMP were completely degraded.

The first order rate constants for the degradation of IMP and GMP as functions of pH (4–9) and temperature (80°C – 100°C) were obtained from the slopes of semi-log plots for the reac-

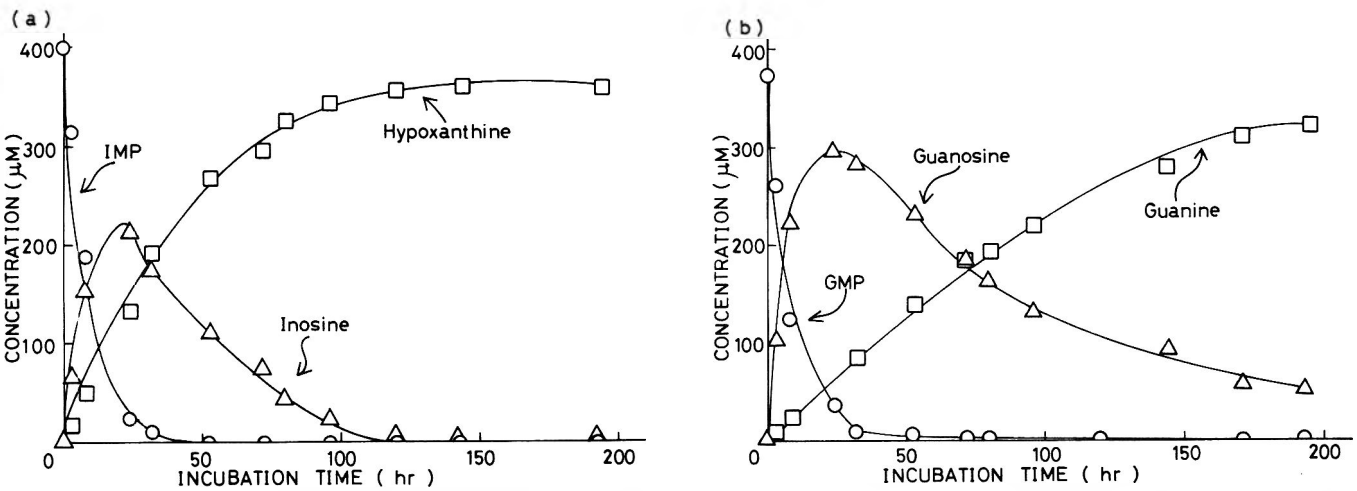

Fig. 2—Thermal degradation curve of 5'-ribonucleotide at 100°C and pH 4.0. (a) Inosine 5'-monophosphate (IMP); (b) Guanosine 5'-monophosphate (GMP).

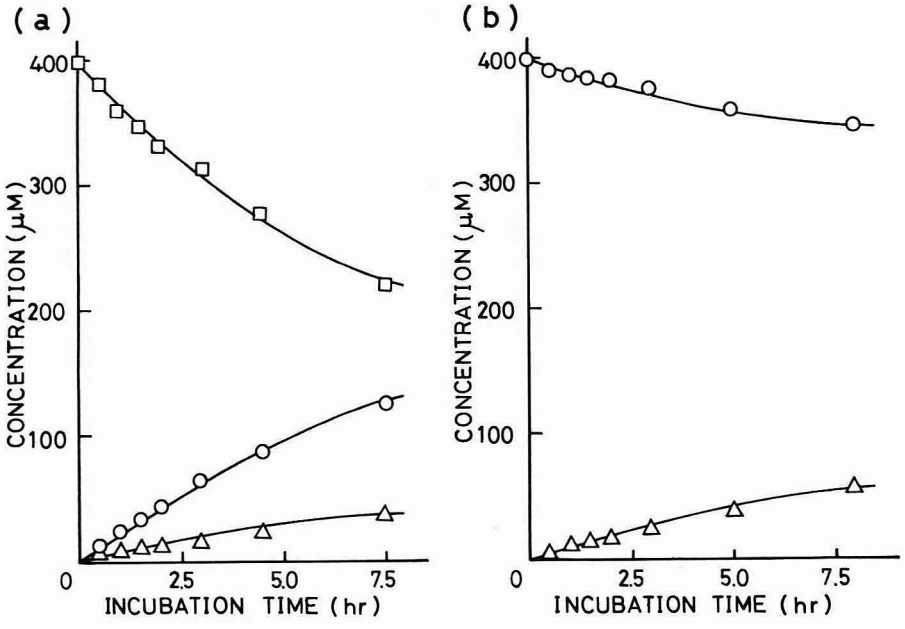

Fig. 3—Thermal degradation curve of inosine 5'-monophosphate (IMP) and inosine at 100°C and pH 4.0. (a)IMP; (b)inosine; □—□, IMP; ○—○, inosine; △—△, hypoxanthine.

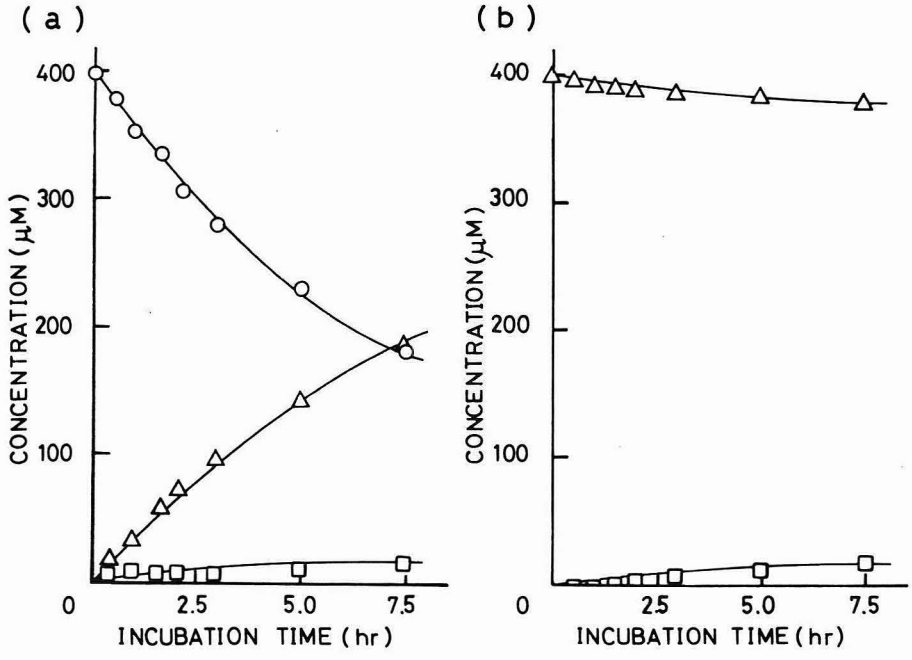

Fig. 4—Thermal degradation curve of guanosine 5'-monophosphate (GMP) and guanosine at 100°C and pH 4.0. (a) GMP; (b) guanosine; ○—○, GMP; △—△, guanosine; □—□, guanine.

tions. The activation energy of the degradative reactions was also calculated from the rate constants at 80°C, 90°C, and 100°C by using the Arrhenius' equation ($\ln k_{obsd} = -E_a/RT + \ln C$). The obtained kinetic parameters, observed rate constants (k_{obsd}), half-life times ($t_{1/2}$) and activation energies (E_a), are summarized in Tables 1 and 2. For both nucleotides, the degradtion rates increased with lower pH, and the half-life times were shortened to about one third by increasing the temperature 10°C. The activation energies in both IMP and GMP at each pH examined were similar and were about 1.3×10^5 J·mol^{-1} (30 kcal·mol^{-1}). At each pH (4–9) examined, GMP was degraded much faster than IMP.

Lee (1979) reported that the maximum rate of IMP decomposition was observed near pKa_2 (pH 5.87). However, in the present study, the reaction rate of decomposition of IMP and GMP increased with lower pH as reported by Kuriyama et al. (1965), Fujita et al. (1965), Davidek et al. (1972), and Shaoul and Sporns (1987). Fujita et al. (1965) reported that the first-order reaction rate constants for degradation of both IMP and GMP at pH 7.0 were the same, but the rate for IMP at pH 2–3 was larger than that for GMP. Kuriyama et al. (1965) showed that GMP was more susceptible to destruction than IMP at pH 5–7, while IMP was more susceptible than GMP at pH 3. On the other hand, Shaoul and Sporns (1987) reported than GMP was more susceptible than IMP at pH 3–8, in agreement with the results of this study.

Thermal degradation of phosphoric esters

To compare the degradative rates for the O-phosphoryl bond in nucleotides with those in phosphoric esters other than nucleotides, the kinetic parameters for the thermal hydrolysis of the esters were determined according to the method described above the measuring the amount of phosphoric acid released. The behavior of the esters in the hydrolysis also followed first order kinetics as seen for IMP and GMP. The kinetic parameters, rate constants and activation energies are shown in Table 3. Among all the esters, the hydrolysis rates were increased by lowering the pH and the activation energies were similar (about 1.3 J·mol^{-1}). Cox and Ramsey (1964) reported that

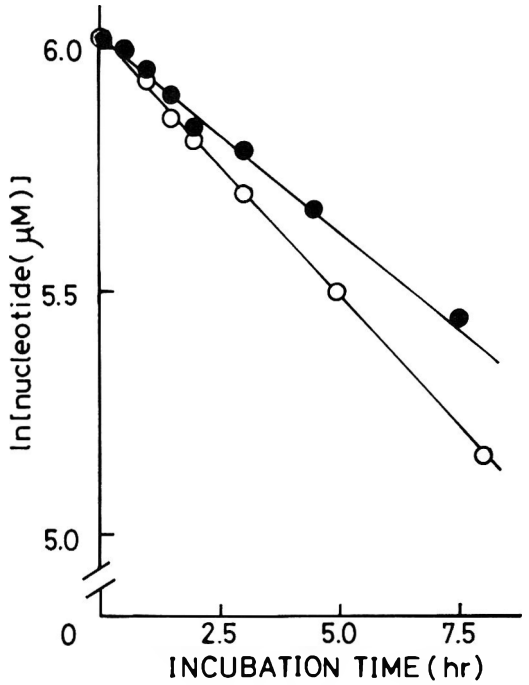

Fig. 5—First order plot for the thermal degradation of 5'-ribonucleotide at 100°C and pH 4.0. ●—●, IMP; ○—○, GMP.

Table 1—Kinetic parameters for thermal degradation of inosine 5'-monophosphate (IMP) at various pH values and temperatures[a]

pH	Temp °C	k_{obsd}[b] ($\times 10^{-2}hr^{-1}$)	$t_{1/2}$[c] (hr)	E_a[d] ($\times 10^5 Jmol^{-1}$)
4	100	8.0±0.12	8.7	1.1
	90	2.7±0.12	25.7	
	80	1.0±0.05	69.3	
7	100	5.3±0.002	13.1	1.3
	95	2.9±0.06	23.9	
	90	1.7±0.09	40.8	
9	100	1.5±0.08	46.2	1.6
	95	0.7±0.05	99.0	

[a] IMP solution (0.4×10^{-3} mol dm^{-3}) was incubated for 0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, and 7.5 hr.
[b] Observed rate constant.
[c] $t_{1/2}=0.693/k_{obsd}$.
[d] Activation energy.

Table 2—Kinetic parameters for thermal degradation of inosine 5'-monophosphate (IMP) at various pH values and temperatures[a]

pH	Temp °C	k_{obsd}[b] ($\times 10^{-2}hr^{-1}$)	$t_{1/2}$[c] (hr)	E_a[d] ($\times 10^5 Jmol^{-1}$)
4	100	11.0±0.11	6.4	1.2
	90	3.7±0.03	18.7	
	80	1.3±0.04	53.3	
7	100	8.5±0.02	8.2	1.1
	90	3.0±0.01	23.1	
	80	1.1±0.06	63.0	
9	100	1.8±0.09	38.5	1.2
	95	1.0±0.07	69.3	
	90	0.6±0.05	108.3	

[a] GMP solution (0.4×10^{-3} mol dm^{-3}) was incubated for 0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, and 7.5 hr.
[b] Observed rate constant.
[c] $t_{1/2}=0.693/k_{obsd}$.
[d] Activation energy.

phosphoric mono esters were most liable to hydrolysis at around pH 4. Both rates and activation energies in the esters at each pH (Table 3) were the same as those for IMP and GMP (Table 2). Furthermore, the observed rate constants (k_{obsd}) of the esters (Table 3) were considerably higher than the N-glucoside

Table 3—Kinetic parameters for thermal degradation of phosphoric esters at various pH values and temperatures[a]

Esters[b]	pH	Temp °C	K_{obsd}[c] ($\times 10^{-2}hr^{-1}$)	E_a[d] ($\times 10^5 Jmol^{-1}$)
	4	100	8.3±0.12	1.2
A		90	3.2±0.04	
		80	1.1±0.02	
	4	100	10.4±0.06	1.3
		90	3.6±0.003	
B		80	1.1±0.02	
	7	100	3.3±0.03	1.3
		95	1.8±0.01	
		90	1.1±0.02	
	4	100	9.6±0.02	1.3
		90	3.0±0.07	
		80	0.9±0.04	
C	7	100	6.8±0.04	1.6
		90	2.0±0.004	
		80	0.5±0.08	
	9	100	1.5±0.01	1.2
		95	0.9±0.01	

[a] The ester solution ($2–3\times10^{-3}$ mol dm^{-3}) was incubated for 0, 0.5, 1.0, 1.5, 2.0, 3.0, 5.0, and 7.5 hr.
[b] A: Ribose 5'-phosphate, B: O-Phosphorylethanolamine, C: Glycerophosphoric acid.
[c] Observed rate constant.
[d] Activation energy.

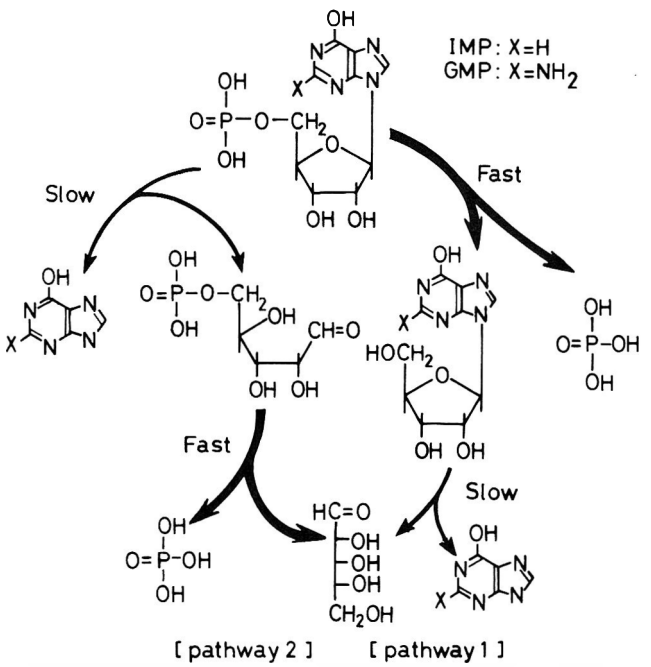

Fig. 6—Possible mechanism of thermal degradation of 5'-ribonucleotides.

bond cleavage rate constants in nucleosides (the cleavage rate constants at pH 4.0; $1.8\times10^{-2}hr^{-1}$ for inosine and $0.6\times10^{-2}hr^{-1}$ for guanosine, calculated from the data in Fig. 3 and 4). These results suggested that the observed activation energies for the thermal degradation of 5'-ribonucleotides reflected the cleavage of the phosphoric ester bond and that the main reaction of the thermal degradation was the hydrolysis of phosphoric ester bond in the nucleotides.

Possible mechanisim of thermal degradation of nucleotides

From the evidence described above, a possible process of the thermal degradation of nucleotides is illustrated in Fig. 6. The degradation of nucleotides follows two pathways: (1) the nucleotide is decomposed into nucleoside and phosphoric acid by hydrolysis of the phosphoric ester bond, and then the nu-

—Continued on page 1170

Effect of Chilling Exposure of Tomatoes During Subsequent Ripening

T.-S. CHENG and R.L. SHEWFELT

ABSTRACT

Mature green tomatoes were stored at 4°C, ≥ 90% RH, for up to 39 days and transferred to 21°C, ≥ 90% RH, for further ripening. After 15 days of chilling exposure, stimulation of C_2H_4 production and increased susceptibility to decay were observed when the fruit was ripened at ambient temperatures. As chilling exposure increased, C_2H_4 production upon removal to 21°C returned to the level of the control. Irreversible inhibition of color development was observed in fruit exposed to 4°C for 34 days and an increase in CO_2 production was noted upon warming of tomatoes chilled for 39 days.

INTRODUCTION

THE TWO MOST clearly documented symptoms of chilling injury in tomato fruit are increased susceptibility to decay (McColloch and Worthington, 1952; Hall, 1961; Tompkins, 1963; McColloch et al., 1966; Risse et al., 1980) and alteration of ripening pattern as evidenced by inadequate color development (McColloch et al., 1966; Risse et al., 1980; Hobson, 1987). Although the tomato frequently has been used as a model to study cellular and subcellular response to chilling stress (Moline, 1976; King and Ludford, 1983; Autio and Bramlage, 1986), little attention has been devoted to characterizing the response in the whole fruit. A better understanding of the outward response in the fruit is needed to properly interpret changes within the cell. Monitoring of the appearance of the visible damage due to cold temperature can also help in locating the period during which the irreversible cellular and subcellular changes might have occurred in cold-stored tomatoes. Tchalukova and Krivoshiev (1978) have described color development in ripening tomatoes during storage at 4–20°C and found that no change in color index occurred during storage below 6°C. Autio and Bramlage (1986) suggested that chilling injury in tomato fruit may be brought about by two separate mechanisms—one affecting mature green fruit and the other affecting tomatoes after they reach the 'pink' stage of maturity. The objective of this study was to characterize the color development and gaseous evolution rates of tomato fruit during ripening after different lengths of chilling exposure at the 'mature green' stage.

MATERIALS & METHODS

TOMATOES (*Lycopersicon esculentum* cv. 'Flora-Dade') were purchased from suppliers at the Atlanta Terminal Market in the fall of the year. All fruit used in the experiment were from the same shipping lot and had been treated with ethylene for one day using the procedure described earlier (Cheng et al., 1988). Mature green tomatoes were visually selected for this study by comparison with the USDA tomato color chart (USDA, 1975). To avoid the effect of desiccation during chilling, both the control and treatment groups were stored under high relative humidity (≥ 90% RH). Mature green tomatoes stored at 21°C in a dark cabinet served as the control group. Color development, rates of respiration and ethylene biosynthesis were monitored during ripening. Mature green tomatoes were chilled at 4°C also in the dark. After 15, 21, 27, 34, and 39 days of cold storage 13 tomatoes randomly selected were transferred to 21°C and ≥ 90% RH dark environment for further ripening.

The color of tomatoes was measured using a Gardner colorimeter (XL-845) (Pacific Scientific, Bethesda, MD) adjusted with a pink standard tile (L = 68.75, a = +23.32, b = +9.43). Eight approximately equidistant points around the equatorial surface of a tomato were measured for color. At least eight fruits were measured for each treatment. Lightness (L) and hue angle ($\tan^{-1}b/a$) differences were analyzed statistically by standard analysis of variance techniques and means separations obtained by Duncan's multiple range test (SAS, 1985).

Carbon dioxide and ethylene were measured on three tomatoes from each treatment with a gas chromatograph (Hewlett Packard 5790A, Mt. View, CA) using a method developed in our laboratory and referred to previously (Yang and Chinnan, 1987). Each tomato was placed in a hermetically-sealed glass jar and allowed to accumulate gases for 1–2 hr. The incubation was at 25°C regardless of its previous storage temperature. If the tomato had been chilled it was allowed to equilibrate at 25°C for 3–4 hr before being placed into the jar. If the tomato had been at 21°C, no equilibration was carried out. Gaseous samples were removed from the headspace via a syringe inserted through a rubber septum. A helium carrier gas with flow rate of 100 mL/min was used for both CO_2 and C_2H_4 measurements. For CO_2 analysis a CTRI Column (Alltech Associates, Deerfield, IL 60015) was used. The column temperature was 33°C and detection was by thermal conductivity. Ethylene was measured using a flame ionization detector with column temperature of 90°C. The nickel column (0.125 inch, 25 inches long) was packed with acid washed aluminum oxide (Merck, Rahway, NJ). Only control, 15 day, 27 day and 39 day cold-stored tomatoes were measured for gaseous evolution.

RESULTS & DISCUSSION

A CLIMACTERIC RISE in respiration rate was observed in all treatment groups after the transfer from 4°C to 21°C (Fig. 1.) The climacteric peak was not appreciably higher than the control except for tomatoes previously stored at 4°C for 39

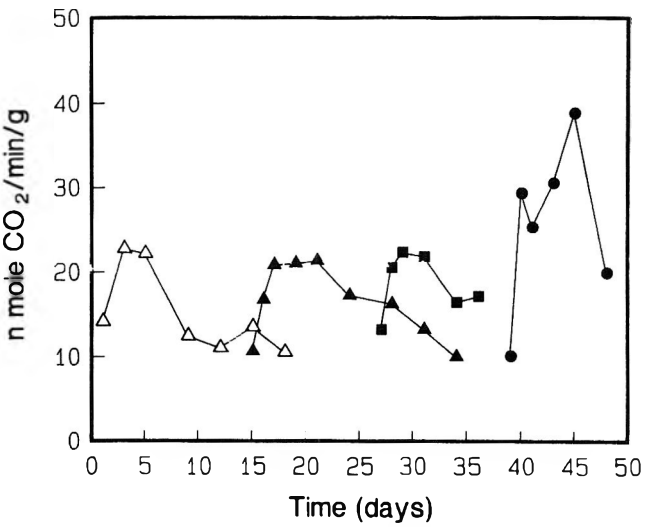

Fig. 1.—CO_2 evolution rate of tomatoes (measured at 25°C) after transfer from 4°C storage for 0 days (Control) (△), 15 days (▲), 27 days (■), and 39 days (●) to 21°C for further ripening.

Authors Cheng and Shewfelt are with the Dept. of Food Science & Technology, Univ. of Georgia, Griffin, GA 30223-1797.

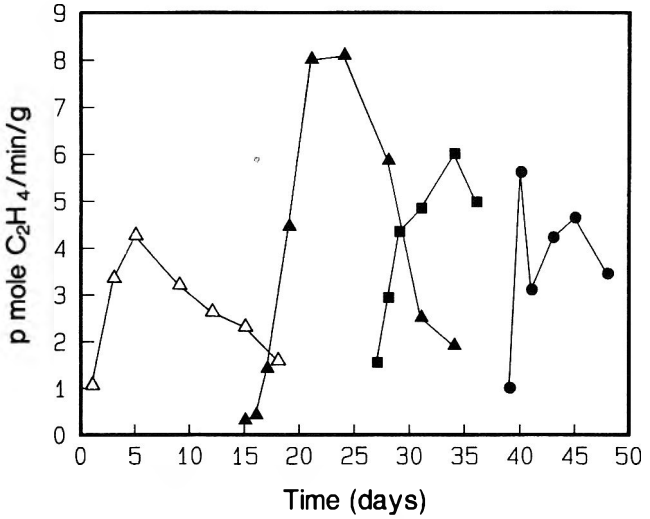

Fig. 2.—Ethylene evolution rate of tomatoes (measured at 25°C) after transfer from 4°C storage for 0 days (Control) (△), 15 days (▲), 27 days (■), and 39 days (●) to 21°C for further ripening.

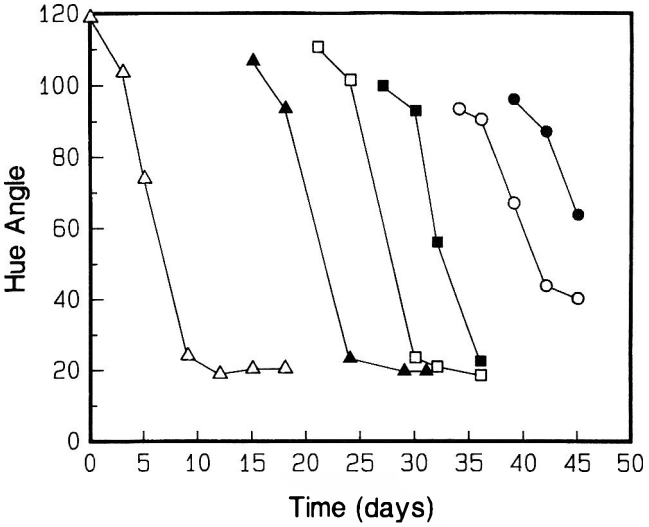

Fig. 3.—Change in hue angle of tomato color during ripening at 21°C after transfer from cold storage at 4°C for 0 days (Control) (△), 15 days (▲), 21 days (□), 27 days (■), 34 days (○), and 39 days (●).

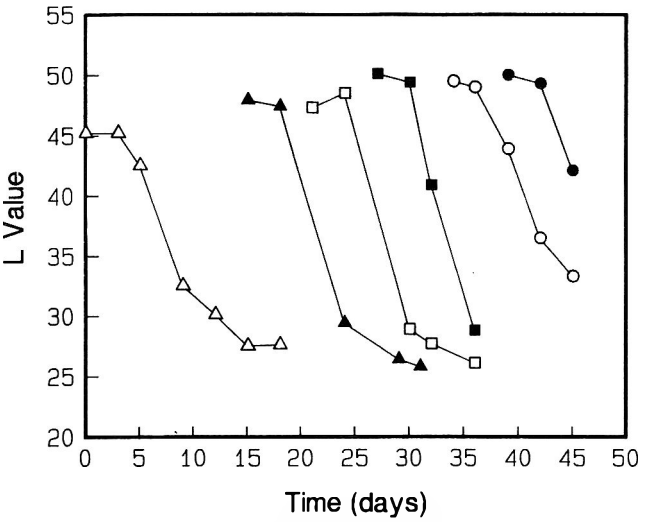

Fig. 4.—Change in L value of tomato color during ripening at 21°C after transfer from cold storage at 4°C for 0 days (Control) (△), 15 days (▲), 21 days (□), 27 days (■), 34 days (○), and 39 days (●).

days. Ethylene evolution showed a rise during subsequent ripening (Fig. 2.) Although the peak was higher for tomatoes stored at 4°C for 15 days when compared with the control group, the magnitude of the peak began to decrease as the time of chilling was prolonged. The bursts of CO_2 and ethylene production by tomatoes upon transfer to higher temperatures from the chilling environment have been reported (Crooks, 1985; Autio and Bramlage, 1986). This study demonstrates an increase in CO_2 stimulation and a lower peak of ethylene evolution as responses to the protracted chilling storage. The increase in CO_2 burst after a longer chilling period could be attributed to the greater accumulation of oxidizable intermediates (Eaks, 1980) at the warmer temperature. A decrease in ethylene stimulation by a longer chilling period was previously reported on a non-climacteric fruit, i.e. cucumber (Wang and Adams, 1982) as a result of the chilling damage to the system which converts ACC (1-aminocyclopropane-1-carboxylic acid) to ethylene. If the ehtylene-forming system resides in tonoplasts (Mayne and Kende, 1986), the results suggest that tonoplasts are more susceptible to chilling temperature than mitochondrial membranes which were still functioning, as suggested by higher CO_2 evolution after 39 days of chilling.

The change in the hue angle of tomato color appeared to proceed in three phases (Fig. 3). Following a 2–3 day lag period the hue decreased rapidly before leveling off. There was no significant difference in the changing rate of hue angle during the initial lag phase among all the treatments. No significant differences were observed in the slope of the second phase (rapid color change) between the control and chilling treatment for up to 27 days. When the chilling period was prolonged to 34 days and longer, however, the rate of change in hue angle during this phase was significantly retarded when compared with the control at the 0.05 level. The ultimate hue angle in treatments exposed for 34 and 39 days of chilling was higher than that of the control demonstrating inadequate color development and suggesting that irreversible damage had occurred between 27 days and 34 days. Decay was so pronounced in fruit chilled for 34 and 39 days that no color measurements could be made past day 45.

Change in L value of tomato color (Fig. 4) was also exhibited in three phases. However, the change in L during rapid color development with more severely chilled tomatoes (34 and 39 days of chilling) behaved in a way more similar to that of the control than the pattern seen in tomatoes chilled for a shorter period (15, 21, and 27 days of chilling). It is concluded that the hue angle of tomato color is more indicative of chilling damage to tomato color development than is the lightness index (L).

Both inhibition of color development and increased susceptibility to decay are the major symptoms of tomato chilling injury (McColloch and Worthington, 1952; Hall, 1961; Tompkins, 1963; McColloch et al., 1966; Risse et al., 1980, Hobson, 1987). Our study indicates the susceptibility to decay is more vulnerable to chilling temperature than the inhibition of color development. The decay percentage was increased to more than 50% for the tomatoes stored for 15 days (Fig. 5), while the rate of color development as expressed by the change in hue angle was not significantly lower at 21°C until the tomatoes had been stored for 34 days at 4°C. An ultrastructural study of tomato fruit by Moline (1976) revealed that chilling can interfere with the conversion of chloroplasts to chromoplasts. This damage to chloroplasts by chilling occurred before the damage to mitochondria was observed in terms of ultrastructural change (Moline, 1976). Our results at the tissue level also suggested the irreversible interference with the conversion of chloroplasts to chromoplasts might have occurred between

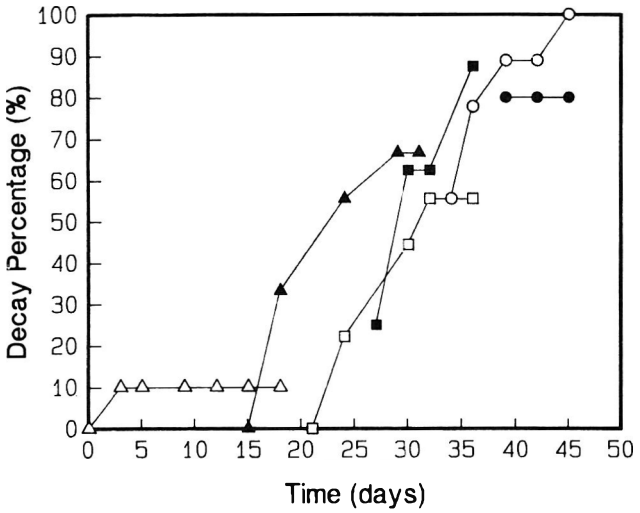

Fig. 5.—Decay percentage of tomatoes during ripening at 21°C after transfer from cold storage at 4°C for 0 days (Control) (△), 15 days (▲), 21 days (□), 27 days (■), 34 days (○), and 39 days (●), based on a sample size of approximately 10 fruit per data point.

27 da and 34 da of chilling storage, and the damage to mitochondria might not have been obvious due to the high CO_2 evolution observed even after the tomatoes had been stored for 39 days. An increase in membrane permeability has been considered an early response of plant tissue to chilling temperature (Sasson and Bramlage, 1978; Christiansen, 1979). Although an increase in susceptibility to decay is of rather complex mechanism, increased plasma membrane permeability probably plays an important role (Markhart, 1986). The rapid increase in decay percentage with the tomatoes stored for 15 days (Fig. 5) suggested an irreversible damage to the plasma membrane might have occurred by the end of 15 days at 4°C. These results suggest that either decay susceptibility and inhibition of ripening proceed by different pathways during chilling or that decay susceptibility is a much earlier manifestation of the chilling response mechanism than color inhibition.

SUMMARY & CONCLUSIONS

IN SUMMARY, a pronounced increase in decay percentage during ripening occurred to tomatoes previously stored at 4°C for 15 days; stimulation of ethylene production by previous cold storage started to decline if the tomatoes had been stored for 27 days or longer; retardation of color development began to appear in tomatoes stored for 34 days; and a post-chilling climacteric peak of CO_2 evolution began to increase upon extended chilling storage for as long as 39 days at 4°C. These results suggest plasma membrane, vacuoles, chloroplasts, and mitochondria to be in decreasing order of vulnerability to chilling temperature.

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Effects of Processing Temperature and Added Antimicrobial Agents on Keeping Quality of Mexican-Style Sauce

SIEW LIAN CHUNG, KAREN V. JORGENSEN, and RALPH L. PRICE

ABSTRACT

Mexican-style hot sauce (salsa) was prepared and heated to 63°C, 71°C, and 79°C. Seven combinations of antimicrobial agents, utilizing potassium sorbate, 900 ppm; sodium benzoate, 700 ppm; and EDTA, 50 ppm were incorporated into the salsa. Samples of the salsa with a pH ranging from 3.7–3.95 and stored at 31°C (88°F) were taken initially and monthly for 96 days; microbial growth in each was determined using five culture media. Microbes decreased significantly in all samples during the storage period. Increasing the temperature of processing to 79°C and the low pH were most important in reducing microbial numbers.

INTRODUCTION

MEXICAN-STYLE HOT SAUCE (salsa) is an integral part of the cuisine of Mexico and in recent years has increased in popularity in the United States. Many dishes are considered incomplete without the addition of the salsa. Historically it has been prepared fresh without heating shortly before consumption using tomatoes, onions, chilies, vinegar and certain combinations of spices. Heating of the product, which occurs during its commercial preparation, changes the physical properties of the onion chili pieces which are characteristic of the salsa. Decreasing the heat treatment during processing would be desirable if spoilage could be prevented and safety assured.

Draughon et al. (1981) determined that there was no potential health problem associated with Mexican-style salsas, enchilada and taco, because of the low pH resulting from using vinegar as an ingredient, which prevented the growth of foodborne pathogens. They also identified the dominant microorganisms from spoiled taco and enchilada salsas as the Bacillus species. Potential spoilage of the salsa is an important consideration because most spices contribute a large number of microorganisms, including spoilage types, to a food product (Julseth and Deibel, 1974).

The objective of this study was to determine effects of processing temperatures and antimicrobial agents on the keeping quality of a commercial salsa and to determine if the shelf life of salsa with minimal or no heat treatment could be extended by combinations of antimicrobial agents.

MATERIALS & METHODS

Samples

Salsa was prepared at a commercial factory in Tucson, AZ, in a 180L batch with the following ingredients: tomatoes, onions, green chilies, water, jalapenos, vinegar, salt, garlic, oregano, pepper and parsley. The salsa had an initial pH of 3.7–3.95.

Glass bottles, 120 mL each, were sanitized by soaking them in sodium hypochlorite solution (50 ppm free residual chlorine). After 15 min, they were removed from the solution, inverted and allowed to drain. Immediately prior to filling, aqueous concentrated solutions of potassium sorbate, sodium benzoate or ethylenediamine tetraacetic acid (EDTA) were added to the empty bottles to give 900, 700, and 50 ppm, respectively, in the final product. Inhibitory action against colony formation was studied in seven combinations: (1) potassium sorbate; (2) sodium benzoate; (3) EDTA; (4) potassium sorbate and sodium benzoate; (5) potassium sorbate and EDTA; (6) sodium benzoate and EDTA; (7) potassium benzoate, sodium benzoate and EDTA. A control sample containing no additional antimicrobials was tested along with the treated samples.

Processing temperatures were: no heating, 63°C, 71°C, and 79°C. Salsas were first made by mixing the necessary ingredients with an electric stirrer without heat treatment in a closed stainless steel steam kettle. Salsas were then dispensed into each bottle using a commerical filler. For the first batch, the bottles were thoroughly shaken after filling for complete mixing of the treatment solution with salsa. The second batch of salsa was processed to 63°C and then immediately bottled. The third and fourth batches of samples were processed using temperatures of 70°C and 79°C, respectively, and bottled in the same manner. Heated samples were air cooled to approximately 30°C before being placed in the incubator.

Samples were taken for microbiological analysis at four intervals: zero-time (within 3 hrs after salsas were made) and after 35, 70, and 96 days of storage in a 31°C incubator. At each interval, 64 samples were tested for total microbial counts.

Microbial counts

The pH of each sample was first recorded. Eleven grams salsa were added to 99 mL of 0.1% sterilized peptone water. Decimal dilutions were prepared. Plate counts were made in duplicate by the pour plate method on the following Difco media (Detroit, MI): (1) Plate Count Agar (PCA); (2) Tomato Juice Agar (TJA); (3) Violet Red Bile agar, (VRBA); (4) Malt Extract Agar, (MEA); and on (5) Potato Dextrose Agar (acidified with tartaric acid) by the spread-plate method (PDA).

PCA amd TJA plates were incubated at 32°C for 24 hr. MEA and acidified PDA plates were incubated at 24°C for 5 days to determine yeasts and molds.

Taste preference study

A taste panel consisting of 20 university students who liked Mexican salsa were asked to compare an uncooked, bottled salsa which had been stored at approximately 25°C for 18 months (the pooled, untreated control from the experiment after extended storage) with a fresh sample of salsa of the same recipe and to evaluate each on a 0 to 9 hedonic scale (Neter and Wasserman, 1974).

Statistical analysis

Analysis of variance was done using the Statistical Analysis System (SAS, 1982) program at the University of Arizona Computer Center. Duncan's new multiple range tests were used to test for statistically significant differences at the 5% level.

RESULTS & DISCUSSION

THE NUMBER of microorganisms in all samples of Mexican-style salsa was reduced during storage at 31°C. Increasing the temperature to 79°C during processing significantly lowered microbial numbers; however, the addition of antimicrobial agent(s), either alone or in several combinations, caused a significant reduction of microbial numbers only in the unheated salsa at the initial sampling period. The salsa had a pH range of 3.70–3.95, with or without the addition of antimicrobial agents. This pH range is common for salsa produced in southern Arizona (Jorgensen and Price, 1986). Only slight variations in pH were found during the three-month investigation.

Five media were used to detect bacteria, yeasts and molds. There were no coliforms found using VRBA. This was prob-

Authors Chung, Jorgensen, and Price are with the Dept. of Nutrition & Food Science, Univ. of Arizona, Tucson, AZ 85721.

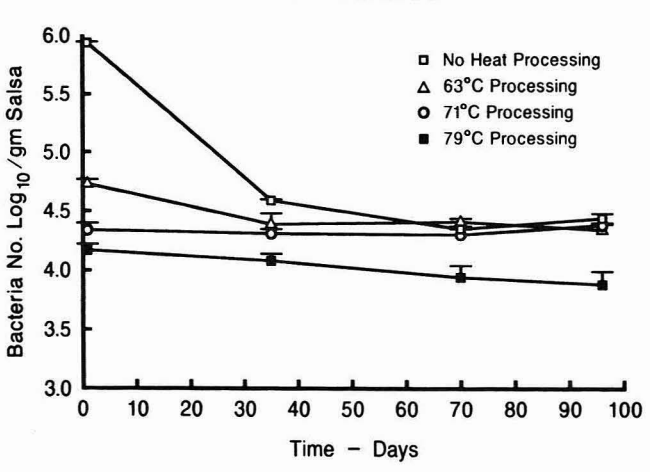

Fig. 1—Microbial numbers per gram of Mexican salsa with no added antimicrobial chemicals as affected by heating and storage time.

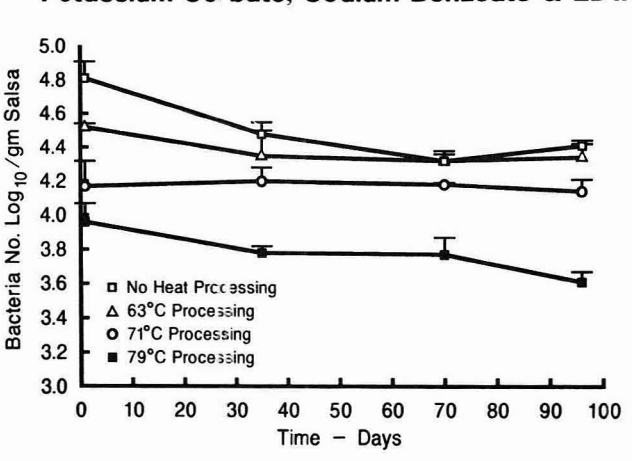

Fig. 2—Microbial numbers per gram of Mexican salsa as affected by heating, storage time and addition of potassium sorbate (900 ppm), sodium benzoate (700 ppm), and EDTA (50 ppm).

ably due to the low pH of the product at which coliforms could not survive. Molds were detected in two samples which received no heat treatment nor antimicrobial agent(s), a sample containing EDTA alone and no heat treatment, and in a sample heated to 63°C to which no antimicrobial agent was added. Molds were absent in all other samples. Yeasts were not found in any of the samples.

The total counts per gram of salsa on PCA are presented in Fig. 1 and 2. Similar results were obtained using TJA. During the experiment, certain microorganisms growing on TJA tended to spread over the entire plate. This caused difficulty in counting the colonies. The Bacillus species, which has been found previously in Mexican-style salsas (Draughon et al., 1981) has the characteristic mentioned above (Collins, 1967). Sticky spreading was seldom encountered in using PCA. Because of the counting difficulties with TJA, data are shown using only the results from PCA.

Effect of antimicrobials

In the presence of antimicrobial agent(s), the initial population of microorganisms was greatly reduced (Fig. 1). Although seven combinations of antimicrobial agents were used, the differences among the agents or among any combination of agents were not significant. Since all figures illustrating bacterial numbers in salsa with added antimicrobial chemicals were nearly identical throughout the study, only that figure representing the combination of all antimicrobials is shown (Fig. 2). Antimicrobial agent(s) acted synergistically with temperature to depress the initial microbial numbers. Because of the expansion of cell walls and damages to cytoplasmic membranes at elevated temperatures, antimicrobial agents penetrate more easily through the permeable cell walls, exert their action and kill the cells (Frazier, 1967; Russell and Harries, 1968).

Effect of heating

Increasing the processing temperature was more effective than the addition of an antimicrobial agent. After storage for 96 days, there was a decrease in the bacterial numbers of all samples when compared to the initial numbers. When the processing temperature was increased to 79°C, microbial numbers were significantly reduced. However, there was no significant reduction due to heating the salsa at lower temperatures. Samples with no heat treatment or antimicrobial(s) had the highest plate counts.

Effect of storage

During 35 days of storage at 31°C, the microbial population of the salsa containing no antimicrobials decreased in an apparently exponential manner. However, during additional storage, the microbial population reached a steady state.

The color and odor of the salsas were observed at each interval. After the first month of storage, a slight darkening appeared near the surface of the salsa. After 96 days storage, a slight darkening was apparent throughout each sample. It was more noticeable in those samples with no heat treatment and no added antimicrobial agent(s). This could be due to chemical or enzymatic oxidation depending on whether or not the sample was heat-processed.

Taste panel

Although each of the samples in this experiment was not tested by a taste panel, the two extremes, uncooked salsa which had been stored for 18 months and fresh salsa, were compared for acceptability by a small, untrained panel. There were no differences; eleven preferred the fresh and nine the stored. Although individual panelists noted differences between the samples, the difference between the overall means (6.07-fresh vs 6.05-stored) was not significant. This would indicate that after this prolonged storage the flavor and acceptability of the product were still satisfactory.

The results of this study indicate that Mexican-style salsa with a pH of 3.7–3.95 could be produced commercially with minimal or no heat treatment and with no antimicrobial agent(s) added.

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Role of Pulp Content and Particle Size in Yield Stress of Apple Sauce

C-G. QIU and M.A. RAO

ABSTRACT

A paddle mixer in conjunction with a rotational viscometer was used to determine the yield stress and the shear rate-shear stress data of apple sauce. Magnitudes of yield stress obtained by extrapolation of the shear rate-shear stress data according to Casson, Herschel-Bulkley, and Mizrahi-Berk models were considerably lower than the experimental values. The experimental values were closer to the Bingham yield values obtained by extrapolation of the straight line portion of the shear rate-shear stress data. Pulp content and average particle size of the solids affected the magnitudes of the yield stress.

INTRODUCTION

MANY FOODS of commercial importance, such as apple sauce and tomato pastes, are concentrated dispersions of insoluble matter in aqueous media (Rao, 1987). Their rheological properties are of interest in practical applications such as handling and mouthfeel. Because of separation of phases and the consequent slip at solid boundaries, errors can occur in capillary and rotational viscometers (Rao, 1975; Steffe and Ford, 1985). For this reason, mixers have been employed in some studies on food dispersions (Rao, 1975; Steffe and Ford, 1985). They also were found to be useful in the study of fermentation broths (Bongenaar et al., 1973; Roels et al., 1974; Klemblowski and Kristiansen, 1986).

Mixers are used for obtaining shear rate-shear stress data assuming that the former is proportional to the rotational speed and that the power law model (Eq. 1) describes the data. Procedures for determining the proportionality constant, k_s, between rotational speed and shear rate can be found in the studies cited above:

$$\sigma = K \dot{\gamma}^n \quad (1)$$

where, $\dot{\gamma}$ is the shear rate (sec^{-1}), σ is the shear stress (N/m^2), K is the consistency index ($N\ sec^n/m^2$), and n is the flow behavior index (−). Yield stress is also an important property of many foods whose determination requires considerable care (Rao, 1977). In one method, known as the relaxation method, it is determined using rotational viscometers by recording at a low rpm the shear stress level at which no stress relaxation occurs on reducing the rpm to zero (Mizrahi and Berk, 1972; Vitali and Rao, 1984; Tanglertpaibul and Rao, 1987a). This method, however, is time consuming and requires great care to obtain reliable results. Other experimental methods also have been proposed (Lang and Rha, 1981), but for the most part magnitudes of yield stress are determined by extrapolation of shear rate-shear stress data according to several flow models such as those of Casson (Eq. 2). Herschel-Bulkley (Eq. 3), and Mizrahi-Berk (Eq. 4) (Rao and Cooley, 1983; Rao et al., 1981):

$$\sigma^{0.5} = K_{0c} + K_c \dot{\gamma}^{0.5} \quad (2)$$

$$\sigma = \sigma_{0HB} + K_H \dot{\gamma}^{n_H} \quad (3)$$

$$\sigma^{0.5} = K_{0M} + K_M \dot{\gamma}^{n_M} \quad (4)$$

Authors Qiu and Rao are with the Dept. of Food Science & Technology, Cornell Univ., Geneva, NY 14456. Author Qiu is on leave from Huazhong Agricultural Univ., Wuhan, China.

In the above equations, K_c, K_H, and K_M are constants, and σ_{OHB} is yield stress predicted by the H-B model. From Eq. (2) and (4), the magnitudes of yield stress can be calculated as either ($\sigma_{0CA} = K_{0c}^2$) or ($\sigma_{0MB} = K_{0M}^2$). Dzuy and Boger (1983) employed a mixer viscometer for the measurement of yield stress of a concentrated non-food suspension and called it the "vane method." This method is relatively simple because the yield stress can be calculated from the maximum value of torque recorded at low rotational speeds. The vane method was found by these authors to be satisfactory for one suspension containing 37.3% solids and another containing 64–67%.

It is important to understand the role of composition of foods with respect to rheological properties and for this reason recent studies (Rao et al., 1981, 1986; Tanglertpaibul and Rao, 1987a, b, c) on food dispersions were conducted on samples that were especially prepared and whose composition was well characterized. In particular, knowledge about the insoluble solids (pulp) is useful because it affects the magnitudes of the rheological properties (Rao, 1987; Metzner, 1985).

Michaels and Bolger (1962) proposed that in flocculated suspensions, the basic flow units are small clusters or aggregates, which at low shear rates give the suspensions a finite yield stress. At high shear rates, the aggregates are broken down into individual flocs, and the model predicts a straight-line relationship between shear stress and shear rate. They also pointed out that in addition to the yield stress (σ_0) that is obtained by extrapolation of shear rate-shear stress data to zero shear rate, one can obtain a Bingham yield stress (σ_B) by extrapolation of the straight line portion of the shear rate - shear stress data. Duran and Costell (1982) determined magnitudes of σ_0 and σ_B for four samples of apricot puree and found the latter to be higher than the former.

In an earlier study on apple sauce, Rao et al. (1986) studied the influence of apple cultivar and firmness on the flow behavior and consistency indexes of the power law model, and determined the effect of pulp content on the apparent viscosity of apple sauce at a shear rate of 100 sec^{-1}. The present study was initiated specifically to investigate the mixer viscometer technique; the apple sauce samples used were made specifically for this study. The objectives of the present study were: (1) to determine whether the vane method was suitable for determining the magnitudes of yield stress of apple sauce, (2) to compare the magnitudes determined by the vane method with those obtained by extrapolation of shear rate-shear stress data according to the Casson, HB, and MB models, and (3) to examine the role of pulp content and particle size on the magnitudes of yield stress.

MATERIALS & METHODS

Preparation of apple sauce samples

Apple sauce samples were prepared from Rhode Island Greening (RIG), Golden Delicious (GD), and Macintosh (MI) apples as described in detail before (Rao et al., 1986). The procedures will be described only in brief here. RIG apples with two different firmnesses of 37.4 and 74.8N were employed. The firmness of GD and MI apples, determined with an Effegi Fruit Tester (Model FT 327), were 36.9 and 34.3N, respectively. The samples were prepared in the pilot plant of the Dept. of Food Science & Technology. Finisher screens with holes of diameter 1/16, 3/32, and 1/8 in. (1.6, 2.4, and 3.2 mm, respectively) were used and the finisher was operated at 500, 700,

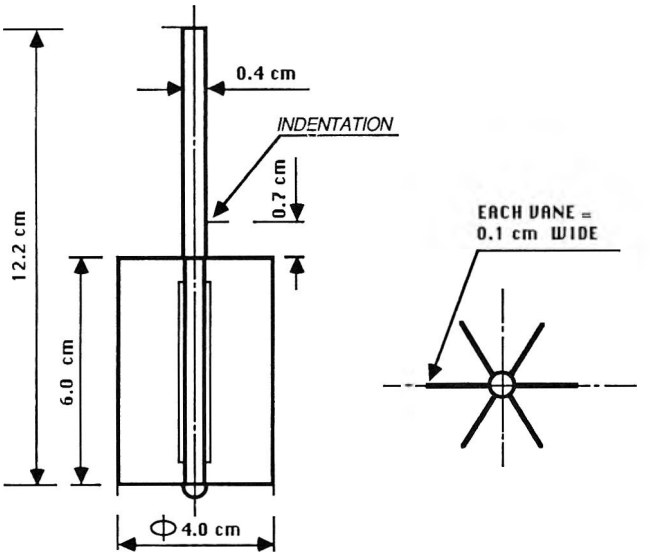

Fig. 1—Schematic diagram of Impeller I2.

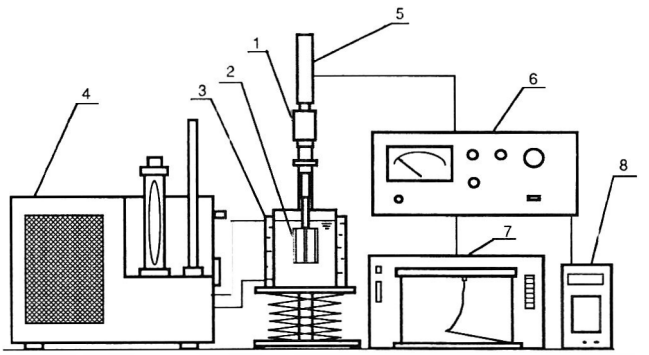

Fig. 2—Schematic diagram of equipment for measuring yield stress by the vane method. The numbers identify the components: (1) torque head, (2) impeller, (3) jacketed vessel, (4) constant temperature bath, (5) motor drive, (6) control console of Haake viscometer, (7) strip chart recorder, (8) voltmeter to check viscometer rpm.

and 900 rpm in an effort to obtain particles with different sizes and size distribution. The °Brix of all samples were adjusted to 17.0 ± 0.2. These samples, called normal pulp (NP) samples, were used for the most part in this study.

Samples with different pulp content. To obtain samples with a wide range of pulp content, the pulp was separated from sauce made from RIG apples, firmness 37.4N, 2.4 mm screen and finisher speed of 700 rpm, by centrifugation at 13,400×*g* in a Sorvall RC-5 centrifuge (Ivan Sorvall, Inc., Norwalk, CT). Seven samples with different pulp content were prepared by mixing the requisite amounts of serum and pulp. These samples will be designated as pulp-adjusted (PA) samples.

Pulp content and particle size

The pulp content of the NP samples was determined by centrifugation at 360×*g* for 10 min. For the PA samples, the pulp content was determined as the ratio: weight of pulp over the total weight of the sample. The latter method was used because the former was not satisfactory for samples with very high pulp content.

Particle size distribution of the NP samples was determined by wet sieving using five sieves with screen openings: 0.105, 0.149, 0.250, 0.420, and 0.840 mm, as described previously (Kimball and Kertesz, 1952; Rao et al., 1986).

Determination of rheological properties

A mixer was used in conjunction with a Haake RV2 (Haake Inc., Saddle Brook, NJ) viscometer to determine the shear rate and shear stress data as described before (Rao, 1975). The star impeller described in detail in a previous study (Rao and Cooley, 1983) was used in these experiments; in this study, it is designated as Impeller II. Briefly, it had eight vanes 0.079 cm wide, 5.8 cm diameter, and 4.4 cm high.

Yield stress was determined according to the method of Dzuy and Boger (1983) using the impeller II and another impeller designated as I2 illustrated in Fig. 1. Figure 2 is a schematic diagram of the experimental apparatus. Briefly, the test apple sauce sample was placed in a jacketed vessel (i.e. 7.14 cm, 11.7 cm) and was brought to temperature with the aid of a constant temperature circulator (Lauda K/2, Brinkman Instruments). All tests were conducted at 25±0.2 °C. The impeller was immersed in the test sample up to a fixed depth. Magnitudes of torque from the Haake viscometer were recorded on a strip-chart recorder (Model 196, Honeywell inc., Minneapolis, MN) as a function of time at 0.4 and 1.0 rpm for a period of 4–5 min.

The maximum recorded torque (T_m) value and the diameter (D_v) and the height (H) of the vane were used to calculate the yield stress (σ_v) according to the equation:

$$T_m = \frac{\pi D_v^3}{2}\left(\frac{H}{D_v}+\frac{1}{3}\right)\sigma_v \qquad (5)$$

Equation (5) can be derived by conducting a torque balance on the surface of the impeller (Dzuy and Boger, 1983).

Yield stresses according to the Casson model σ_{0CA} were calculated as the square of the intercept from linear regression analysis of the square roots of shear rate and shear stress. Yield stresses (σ_{0HB} and σ_{0MB}) according to the HB and MB models (Eq. 3 and 4, respectively) were calculated using the nonlinear regression analysis program of Niebergall et al. (1971) as described in detail earlier (Rao and Cooley, 1983). The HB and the MB models were selected because they provided consistent magnitudes of yield stress in our earlier study. Initial values for the nonlinear analysis were selected from the Casson yield stress and the power law parameters. The computations were performed on a Prime 9750 computer.

RESULTS & DISCUSSION

BECAUSE MOST of the experiments were conducted with the NP samples, unless stated otherwise, the discussion in this section is based on the results of the normal pulp (NP) samples.

Pulp content and particle size

It must be noted that because the pulp content of the NP samples was determined at a relatively low centrifugal force (360g), its magnitude was relatively high: 86.8% to 99.3%. Figure 3 illustrates the variation in the weight average particle size distribution among three samples of RIG apples. The data shown are averages for three different finisher speeds. One general observation is that the use of lower firmness fruit resulted in a higher percentage of particles in the range 0.63–1.26 mm. The weighted average particle size of the insoluble solids in the samples ranged from 0.534 to 0.946 mm.

Shear rate-shear stress data

The proportionality constant, k_s, between rotational speed and shear rate for impeller I2 was found to be 11.6; for impeller II, its magnitude was determined earlier (Rao and Cooley, 1984) to be 19.7. The approximate shear rate range of the data for all the samples was 0.03–21.01 sec^{-1}; this range was not as wide as that obtained with a concentric cylinder geometry. The flow behavior index of the power law model (Eq. 1) was in the range 0.15–0.24 indicating that the samples were highly shear-thinning fluids. The consistency index of the samples ranged from 40.6 to 76.9N sec^n/m^2. These magnitudes are in the range of the values reported for apple sauce in our earlier study (Rao et al., 1986). The consistency and the flow behavior indices were linearly related as found in our earlier study (Rao et al., 1986):

$$K = A - Bn \qquad (6)$$

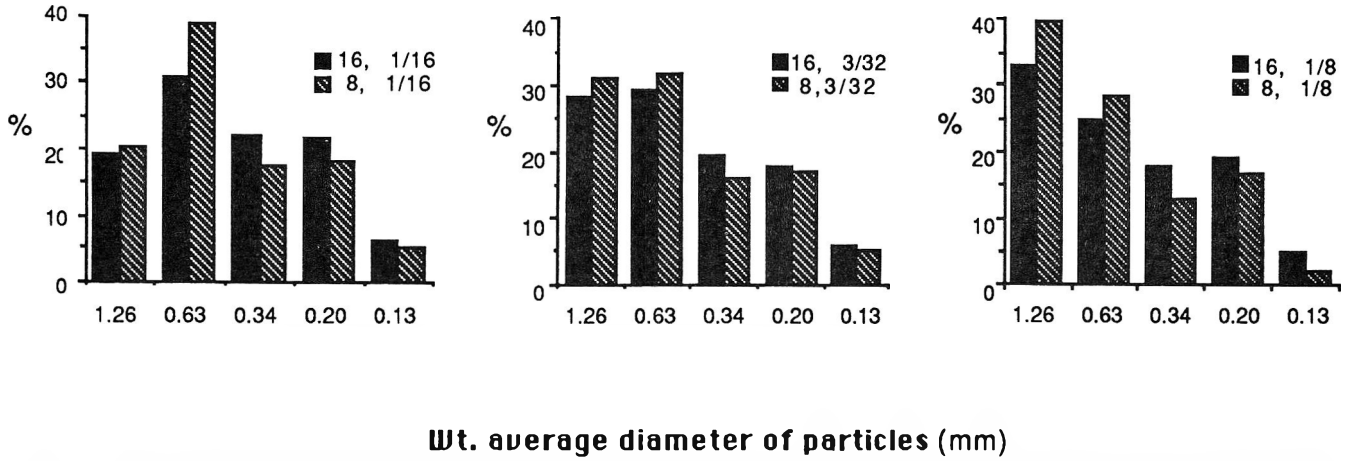

Fig. 3—Particle size (mm) distribution in sauce samples made from Rhode Island Greeining apples having two firmnesses: 8 bf (37.4N) and 16 lbf (74.8N) with three finisher screens: 1/16, 3/32, and 1/8 in. (1.6, 2.4, and 3.2 mm) averaged for three finisher speeds (500, 700, and 900 rpm).

Magnitudes of the slope, B, were nearly the same for the cultivars studied.

Yield stress from flow models

Typical magnitudes of yield stress calculated by regression analysis of the shear rate-shear stress data according to the Casson, H-B, and M-B models, are in Table 1. Linear regression analysis was used with the Casson model, while nonlinear regression analysis was used with the H-B and the M-B models. The magnitudes of R^2 of the correlations ranged from 0.995 to 0.999. The three models predict magnitudes of yield stress that are comparable to each other. However, the magnitudes predicted by the H-B model were lower than those by the Casson and the M-B models. Rao and Cooley (1983) found that for tomato concentrates, the H-B and the M-B models predicted comparable magnitudes of the yield stress. The anomalous behavior of the H-B model in the present study is attributed to the different shear rate range of the experimental data. On the other hand, based on earlier (Rao and Cooley, 1983) and the present studies, the M-B model appears to be capable of predicting consistent magnitudes of yield stress for data over different ranges of shear rate.

Yield stress by the vane method

Figures 4 and 5 illustrate typical time-torque curves recorded at 0.4 and 1.0 rpm with impellers Il and I2, respectively. With both impellers, the maximum torque value increased with rotational speed over the range 0.1–2.0 rpm. However, the increase in the magnitudes of the maximum torque with impeller I2 appeared to be less than those with Il. Typical magnitudes of yield stress with both impellers at 0.4 and 1.0 rpm are in Table 2. In general, magnitudes of yield stress were higher with Il at 1.0 rpm than with I2 at 0.4 rpm. However, the data in the four columns in Table 2 show similar trends. These data point out that for the purpose of comparing the magnitudes of yield stress of several food samples determined by the vane method, one must employ not only a specific impeller but also a specific rpm.

Table 1—Magnitudes of yield stress from extrapolation of the flow models of Casson, Herschel-Bulkley (H-B), and Mizrahi-Berk (M-B)

Sample[a]	Casson Model	H-B Model	M-B Model
RGA25	38.9	36.2	38.7
RGA27	38.7	35.6	38.1
RGA29	50.7	46.4	50.1
RGA45	48.6	45.0	48.2
RGA47	48.3	44.8	48.0
RGA49	39.6	37.2	39.4
RGB25	31.7	25.7	29.1
RGB27	31.8	24.5	29.2
RGB29	35.8	31.4	35.2
RGB45	26.6	18.4	25.5
RGB47	32.2	30.1	30.5
RGB49	39.3	37.4	39.3

[a] Sample code: RG stands for Rhode Island Geening cultivar; A indicates firmness of 37.4N, B for 74.3N; the first number indicates the finisher screen opening size: 2 for 1.6 mm screen, 4 for 3.2 mm screen; the last number indicates the finisher speed (500, 700, or 900 rpm.)

Comparison of yield stresses from flow models and vane method

Magnitudes of the yield stresses from the vane method are much higher than those from the flow models; in a few instances, the former are more than twice the latter. In an attempt to understand the difference between the two types of yield stresses, we turn to the model presented by Michaels and Bolger (1962). Because the underlying assumptions of the model have been reviewed by others (Metz et al., 1979; Duran and Costell, 1982), only the pertinent aspects of the model will be covered here.

In the model, the stress necessary to produce deformation at a constant rate is divided into three parts:

$$\sigma = \sigma_n + \sigma_{cr} + \sigma_{vi} \quad (7)$$

where, σ_n is the stress required to break the aggregate network, σ_{cr} is the stress required to break the structural bounds, and σ_{vi} is the stress required to overcome the purely viscous drag. At zero shear rate the network yield stress σ_n equals σ_0, the yield stress obtained by extrapolation of the shear rate-shear stress data to zero shear rate. Another yield stress, the Bingham yield stress (σ_B) can be obtained by extrapolation of only the linear portion of the shear rate versus shear stress diagram to zero shear rate (Michaels and Bolger, 1962). The Bingham yield stress is part of the total stress at high shear rates. In addition to the two yield stresses, the viscosity at infinite shear rate (η_{oo}) also has been shown to be important. The principal contribution of the model of Michaels and Bolger (1962) is that for σ_0, σ_B, and η_{oo}, the role of particle concentration and properties have been identified. Also, it is known that the magnitude of σ_B is higher than that of σ_0.

Magnitudes of σ_B were determined from plots of shear rate-shear stress obtained with impeller Il as the intercept as shown in Fig. 6. A linear regression analysis program was used to estimate σ_B in a systematic manner. The magnitudes of σ_B so determined are also listed in Table 2. In comparison to the magnitudes of the yield stresses obtained by extrapolation of

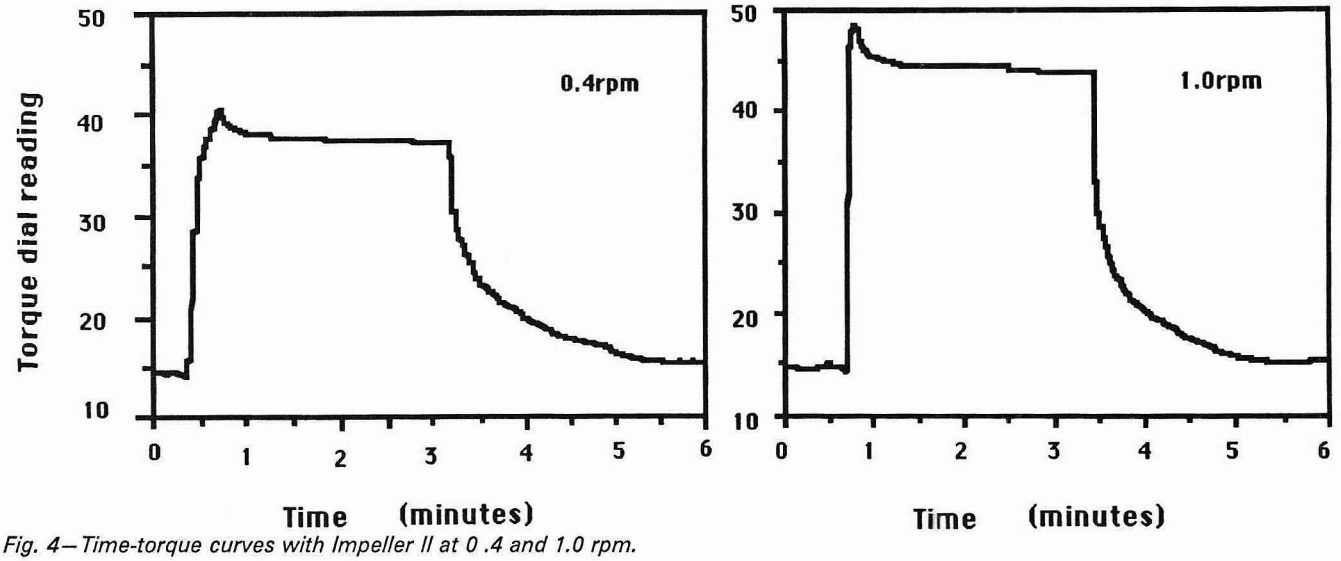

Fig. 4—Time-torque curves with Impeller II at 0 .4 and 1.0 rpm.

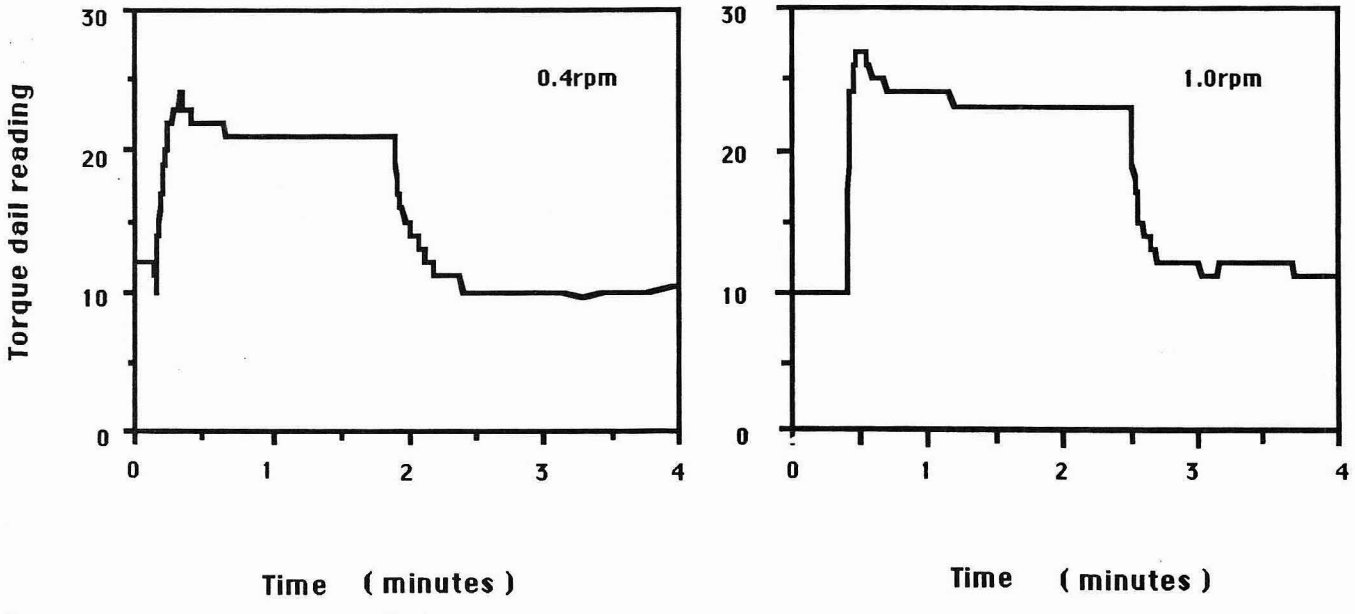

Fig. 5—Time-torque curves with Impeller I2 at 0.4 and 1.0 rpm.

Table 2—Magnitudes of yield stress determined using the vane method and extrapolated (Bingham) values

Sample[a]	Impeller[b] and rpm I1 1.0 rpm	I1 0.4 rpm	I2 1.0 rpm	I2 0.4 rpm	Bingham yield value
RGA25	63.9	55.8	66.1	58.0	70.6
RGA27	58.9	53.1	55.9	51.8	66.2
RGA29	87.1	76.0	84.1	74.2	81.6
RGA45	83.7	73.7	80.9	76.9	81.0
RGA47	86.0	72.1	87.6	81.4	82.2
RGA49	55.8	52.0	54.5	51.3	73.1
RGB25	56.6	46.5	56.1	47.2	70.8
RGB27	57.4	48.1	54.0	45.9	69.2
RGB29	67.0	57.0	68.8	62.0	71.6
RGB45	51.5	45.8	52.6	47.5	62.6
RGB47	58.9	49.3	54.0	48.0	69.4
RGB49	76.0	65.1	72.3	64.2	74.4

[a] Sample code as explained in Table 1.
[b] I1 is the impeller described by Rao and Cooley (1984), I2 is described in Fig. 1.

shear rate-shear stress data to zero shear rates (Table 1), the magnitudes of σ_B are much higher. Further, while they are close, they are still higher than the magnitudes of yield stresses determined by the vane method. Nevertheless, considering the uncertainties involved in obtaining σ_B and the range of values that can be obtained with the vane method, it appears that the vane method gives magnitudes that are very close to σ_B.

Correlation among the different yield stresses

It would be of interest to determine the relationship between the different yield stresses. One can suspect that linear relationships exist between them because they all are measures of similar properties of a material. The linear equations with σ_B as the dependent variable and the yield stresses from the vane method with impeller I2 at 0.4 rpm (σ_{2v4}) and extrapolation of shear rate shear stress data according to the M-B model (σ_{0HB}) as the independent variable were:

$$\sigma_B = 47.0 + 0.45\sigma_{2v4}; \qquad R = 0.895 \qquad (8)$$

$$\sigma_B = 47.5 + 0.67\sigma_{0MB}; \qquad R = 0.887 \qquad (9)$$

The high magnitudes of R did confirm that σ_B and the said variables were linearly related. The main difference in the correlations with the yield stress using the vane method and the M-B model was that the slope with the latter was higher, being equal to 0.67 instead of 0.45, the average value for the former.

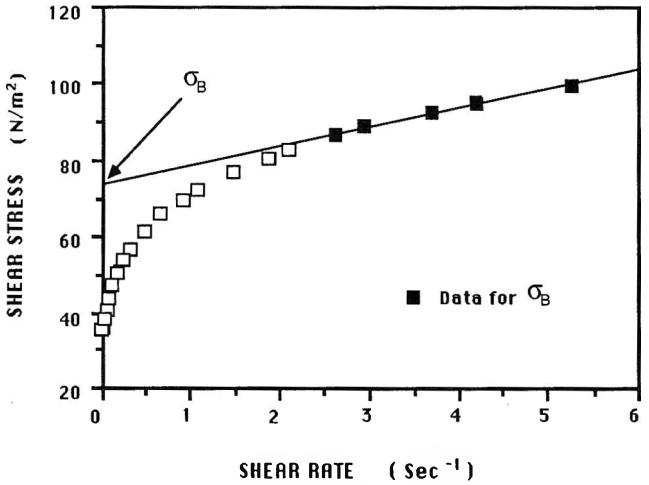

Fig. 6—Estimation of Bingham yield stress (σ_B) from shear rate-shear stress data.

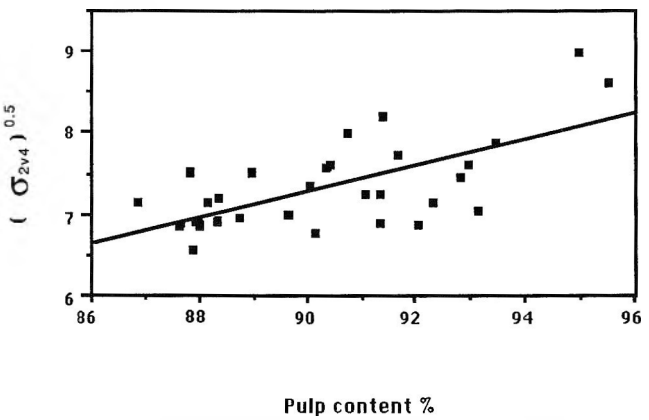

Fig. 7—Effect of pulp content (%) on yield stress of normal pulp samples determined by the vane method (σ_{2v4})—plot of pulp content (%) vs $(\sigma_{2v4})^{0.5}$.

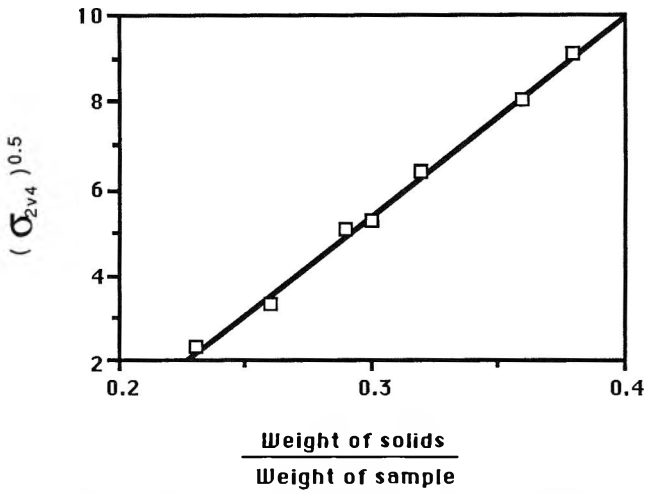

Fig. 8—Effect of pulp content (%) on yield stress of pulp adjusted samples determined by the vane method (σ_{2v4})—plot of pulp content (weight of pulp/total weight of sample) versus $(\sigma_{2v4})^{0.5}$.

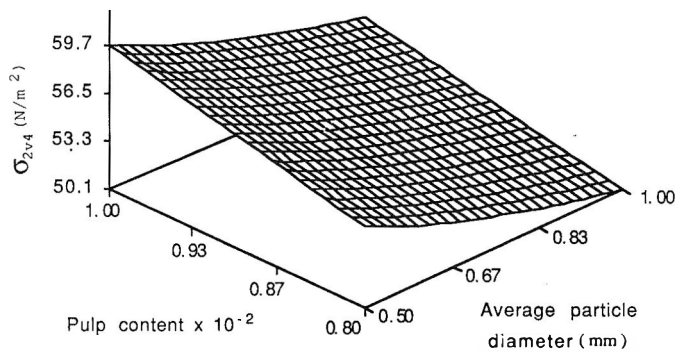

Fig. 9—Three-dimensional plot illustrating the effect of pulp content (%) and weight average diameter of particle on yield stress determined by the vane method σ_{2v4}.

Influence of pulp content on yield stress

One result of the work of Michaels and Bolger (1962) is the prediction that σ_0 is proportional to the volume fraction of solids raised to the power of three and that σ_B is proportional to the square of the volume fraction of solids. To verify the applicability of the above observation, linear regression analyses were carried out between the square root of the yield stress of NP samples determined with Impeller I2 at 0.4 rpm (σ_{2v4}) as the dependent variable and the pulp content as the independent variable. However, the magnitude of R for all the data (36 data points) was low (0.424). Deleting six points that deviated considerably resulted in a plot (Fig. 7) with a R value of 0.64. A high R value (0.825) was obtained for samples of RIG cultivar only. This might indicate the important role of particle size distribution as affected by the apple cultivar and firmness.

Michaels and Bolger (1962) confirmed their model's predictions with data on dispersions that had magnitudes of σ_B less than 0.9 N/m². The apple sauce samples of the present study were highly concentrated dispersions with some magnitudes of σ_B of about 80 N/m². In addition, the pulp content determined by centrifugation at 360g had a relatively narrow range of magnitudes. For this reason, additional verification of the Michaels-Bolger model was obtained with data on the PA samples (Fig. 8), where pulp content was expressed as: weight of pulp/total weight of sample. Because of the high correlation coefficient for these data (R = 0.999), it appeared that pulp content expressed as: weight of pulp/total weight of sample was a better indicator of the insoluble solids of concentrated suspensions than the percent of volume at a centrifugal force 360g.

Correlation of yield stress with pulp content and particle size

One can expect the magnitudes of yield stress to be dependent on both the pulp content and on the particle size. Because data were also obtained on the pulp content and particle size distribution of the NP apple sauce samples, an attempt was made to determine correlations between yield stress on one hand and the pulp content (PC1) and an average particle diameter (WTD) on the other. The correlation forms tested were:

$$\sigma_{2v4} = 23.8 + 30.5(PC1)^{0.5}(WTD)^{-0.21}; \quad R^2 = 0.823 \quad (10)$$

$$\sigma_{2v4} = 11.5(PC1)^{1.38} + 43.0(WTD)^{-0.16}; \quad R^2 = 0.823 \quad (11)$$

$$\sigma_{2v4} = 4.8 + 22.1(PC1) + 23.6(WTD)^{-0.26}; \quad R^2 = 0.823 \quad (12)$$

A parameter estimation program in the statistical package GENSTAT (Rothamsted Experiment Station, UK) was employed to determine the coefficients in the equations. It is worth noting that all the correlation forms yielded high magnitudes of R^2 equal to 0.823. It appeared that one form was not better than the others in correlating the effects of pulp content and particle size. Also, the exponent of the particle diameter was negative, indicating that the magnitude of σ_{2v4} increased with decrease in particle diameter over the range of experimental variables employed in this study.

It is emphasized that the effects of pulp content and particle

size are valid within the range of the experimental variables employed in the study. Even though high magnitudes of R^2 were obtained for the three equations (Eq. 10 to 12), improved correlation forms were likely to result from the use of more extensive data than employed in this study.

The correlation Eq. (11) was used to generate magnitudes of σ_{2v4} for different magnitudes of pulp content and WTD. Figure 9 illustrates the relationship between the variables, and it can be seen that the highest magnitude of the yield stress can be obtained at a particle diameter of 0.5 mm and 100% pulp content, while the lowest magnitude is obtained at WTD of 1.0 mm and 80% pulp content.

CONCLUSIONS

MAGNITUDES of yield stress determined by the vane method were higher than those obtained by extrapolation of the Herschel-Bulkley and the Mizrahi-Berk flow models; they were nearly equal to the magnitudes of Bingham yield stresses obtained by extrapolation of the linear portion of the shear rate-shear-stress data. The yield stress magnitudes appeared to be proportional to the square of the pulp content as predicted by the model of Michaels and Bolger and also increased with increase in pulp content and decreased with size of the insoluble solids.

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DEGRADATION OF IMP AND GMP. . . From page 1159

cleoside is further decomposed to the purine and ribose by the cleavage of the N-glucosidic bond (pathway 1); (2) the nucleotide is decomposed into the purine base and ribose 5-phosphate by cleavage of the N-glucosidic bond, and ribose 5-phosphate is further decomposed into ribose and phosphoric acid by hydrolysis of the phosphoric ester bond (pathway 2). In these pathways, the cleavage rate of the N-glucosidic bond is much slower than that of the phosphoric ester bond. As shown in Fig 2, the degradative rate of nucleotide up to 24 hr heating is almost the same as the formation rate of nucleoside, and furthermore, after heating for 24 hr, the degradative rate of nucleoside is almost the same as the formation rate of the purine. These results indicated that most of the nucleotides were degraded according to pathway 1, i.e., pathway 1 was the main degradation process of the nucleotides. On the other hand, some of the nucleotide is degraded by pathway 2, since the base is produced directly from nucleotide as shown in Fig. 3 and 4.

The present paper has provided evidence that the main mechanism of thermal degradation of 5'-ribonucleotide during the usual processing and cooking of foods (heat treatment within 5 hr) was the hydrolysis of the phosphoric ester bond, i.e., the formation of nucleoside from nucleotide. GMP was more susceptible to thermal degradation than IMP. The difference in the functional group ($-H$, $-NH_2$) at the 2-position in purine base may have caused the difference in degradation rate. However, this is not clear at the present time.

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Solubilization of Cell Wall Bound, Thermostable Pectinesterase from Valencia Orange

L. WICKER, M.R. VASSALLO, and E.J. ECHEVERRIA

ABSTRACT

Complete solubilization of citrus pectinesterase is not effected by alkaline salt buffers but is partially enhanced after degradation of the cell wall by pectinase and cellulase. After heating at 70°C for 2 min, the pectinesterase fractions solubilized after enzymatic cell wall degradation possessed three times the residual activity of the pectinesterase fractions solubilized by alkaline salt buffer. Isoelectric focusing prints of pectinesterase activity and SDS polyacrylamide gel electrophoresis suggest increased solubilization of a heat stable pectinesterase isozyme after cell wall degradation.

INTRODUCTION

CITRUS PECTINESTERASE (PE) is reported to be exclusively a cell wall bound enzyme (Jansen et al., 1960b), solubilized only by alkaline pH, high ionic strength buffers (MacDonnell et al., 1945). Since sequentially smaller increments of PE were solubilized after repeated washings, Jansen et al. (1960b) concluded that nearly complete extraction of citrus PE was achieved after three washes. Citrus PE has been purified with the use of alkaline salt buffers, and two (Evans and McHale, 1968; Korner et al., 1980) to twelve (Versteeg et al., 1978) isozymes have been identified and characterized. Versteeg et al. (1980) described the heat stability of three isozymes of citrus PE. Two were shown to be relatively heat sensitive while a third, which represented 5-30% of the total activity (Rombouts et al., 1982), was shown to be rapidly inactivated only at temperatures greater than 90°C.

Localization of enzymes within the cell influences the chromatographic, electrophoretic, kinetic, and stability characteristics of isoenzymes (Gkinis and Fennema, 1978; Haard, 1973; Thomas et al., 1981; McLellan and Robinson, 1984). The absence of a soluble PE, the capability of the citrus cell wall to bind quantities of PE up to 15 times the amount present naturally, and the incomplete solubilization of PE (Jansen et al., 1960b) suggests that a fraction of PE may be tightly associated with the wall and could conceivably account for a portion of the thermal stability of one of the PE isozymes described by Versteeg et al. (1980). The objectives of this study were to determine the relative solubility of citrus PE and to evaluate the effect of localization within the cell on thermostability of PE fractions.

MATERIALS & METHODS

Solubilization of pectinesterase

Valencia finisher pulp was collected following juice extraction during April, 1986 at the Citrus Research and Education Center, Lake Alfred, Florida. An FMC 291 B-100 extractor and an FMC Model 35 finisher with 0.020 mesh screens and 46 psi pressure were used. Small aliquots were frozen at −10°C until needed. Juice pulp was homogenized with the appropriate buffer in a 1:5 ratio at 4°C at high speed in four, 15 sec bursts in a blender (Astramixer, Model M-100, Plainview, NY). The homogenate was centrifuged at 16,000 × *g* for 25 min at 4°C. The supernatant was collected, and the pellet was reextracted in the appropriate buffer or water. Supernatants and pellets constituted the soluble and bound PE, respectively.

The authors are affiliated with the Univ. of Florida, IFAS, Citrus Research & Education Center, 700 Experiment Road, Lake Alfred, FL 33850.

Cell wall degradation and solubilization of PE

Frozen pulp was thawed and homogenized in 0.25M Tris-Cl, 0.3M NaCl, 1 mM NaN_3, pH 8.0 buffer (Tris/NaCl, pH 8.0) as described above to release the readily soluble PE. The pellet was divided and half was extracted again for 1 hr at room temperature as the control fraction with Tris/NaCl, pH 8.0 (1.:5). The second half of the pellet was extracted with 0.1M sodium citrate, 1 mM NaN_3, pH 5.6, 2% pectinase (Sigma Chemical Co., St. Louis, MO, Lot: 25F-0193), 1% cellulase (Calbiochem, San Diego, CA, Lot: 605571) for 1 hr at room temperature. After collection of the respective supernatants by centrifugation, the control and cell wall degraded pellets were extracted a third time in Tris/NaCl, pH 8.0 buffer (1:5). Fractions 1, 2 and 4 and fractions 3 and 5 represent the solubilized PE of the controls and the cell wall degraded samples, respectively. Pellets 1, 2, and 4 and pellets 3 and 5 represent the residual bound PE of the control and cell wall degraded samples, respectively.

Pectinesterase assays

Pectinesterase activity was determined by the titrimetric assay of Rouse and Atkins (1955) at pH 7.0 and 30°C. Pectinesterase units (PEU) are expressed as the microequivalents of ester hydrolyzed per minute per mL extract or per gram residue for soluble or bound PE, respectively. Pectinesterase activity was determined in the unextracted pulp, supernatants, and pellets of the buffer and water extractions. Duplicate samples differed by less than 10% error.

Biochemical characterization of pectinesterase

The extracts were concentrated by precipitation with solid $(NH_4)_2SO_4$ to 75% saturation. The pectinesterase was collected by centrifugation at 16,000 × *g*, 4°C, 25 min. The pellet was resuspended in a small amount of 10 mM potassium phosphate, 0.1M NaCl, 1mM NaN_3, pH 7.0, and sonicated twice for 10 sec at 50 cycles/sec at 4°C using a Heat Systems-Ultrasonics Model W200 R (Plainview, NY) sonicator. Protein concentration was determined by the Bradford (1976) assay. SDS polyacrylamide gel electrophoresis was conducted on 12% gels according to Laemmli (1970) with BioRad (Richmond, CA) low molecular weight standards. Isoelectric focusing was conducted for 4.5 hr at a constant power of 8 W according to Rombouts et al. (1982). The Delincee (1976) print technique was used to detect PE isozymes.

Heat stability

The heat stability of the concentrated extracts was determined by heating at the indicated time at 70°C. The buffer, 10 mM potassium phosphate, 0.1M NaCl, 1 mM NaN_3, pH 7.0, was equilibrated for 15 min at 70°C prior to the rapid addition of 0.98 mL of the respective fraction, vortexed, replaced in the 70°C water bath, and timing initiated. The heated extracts were rapidly cooled in an ice bath. The total volume was 4.48 mL. The residual activity of duplicate samples was determined by comparison to an unheated control.

RESULTS

Solubilization and heat stability of ionically bound PE

The presence of a heat stable pectinesterase isozyme in extracts of Florida Valencia juice pulp was confirmed and estimated to represent about 1% of the total activity based on residual activity after heating for 2 min at 70°C in 10 mM

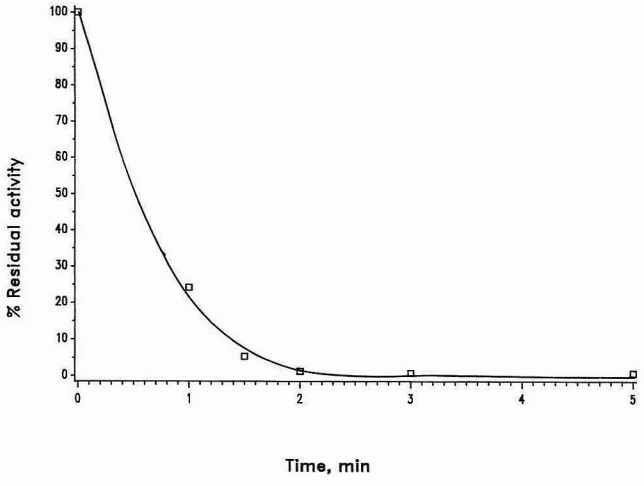

Fig. 1—Effect of heating at 70°C on pectinesterase activity.

Table 1—Effect of extraction medium on solubilization of pectinesterase from Valencia finisher pulp[a]

Buffer	Buffer extract[b]	Water extract[b]	Pellet[c]
0.25M Tris, 0.3M NaCl, pH 8.0	17.66	–	11.93
0.25M Tris, 10 mM EGTA, pH 8.0	28.29	3.19	16.93
0.25M Tris, 50mM $CaCl_2$, pH 8.0	22.26	8.72	12.43
0.25M Tris, 1% Triton, pH 8.0	22.06	5.09	17.74
1M Tris, pH 8.0	22.54	11.32	21.57
1M urea	NA[d]	0.73	31.49
0.1M boric acid, 0.3M NaCl, pH 9.0	14.95	7.49	13.23
0.1M CAPS, 0.3M NaCl, pH 10.0	27.48	18.37	21.28
0.1M CAPS, 0.3M NaCl, pH 11.0	3.25	2.74	NA[d]

[a] Replicate values differed by less than 10%.
[b] PEU = microequivalents of ester hydrolyzed per min per mL extract.
[c] PEU = microequivalents of ester hydrolyzed per min per g wet residue.
[d] NA = no activity.

potassium phosphate, 0.1M NaCl, 1 mM NaN_3, pH 7.0 as described in the Methods section. Under these conditions, 95–98% of the pectinesterase activity was lost in less than 2 min with negligible decreases in enzyme activity observed between 2 and 5 min (Fig. 1).

The PE remaining detectable but unextractable after three washes has been observed to be quite variable and high relative to the amount of activity detected in the starting material. Since 0.25M Tris-Cl^-, 0.3M NaCl, pH 8.0 was slightly more effective at solubilization of citrus PE than 0.44M boric acid, 0.11M borax, 0.3M acetate, pH 8.0 (MacDonnell et al., 1945), Tris/NaCl was used in subsequent extractions. Absence of stimulatory or inhibitory effects by Tris was confirmed using Sigma PE as a control.

Efforts to solubilize unextracted PE from finisher pulp not released by Tris/NaCl are summarized in Table 1. Increasing the Tris concentration to 1M had a minimal effect on solubilization of PE. Similar results were observed with Triton X-100. The addition of 50 mM $CaCl_2$ did not increase PE solubilization, although calcium has been shown to stimulate PE activity (MacDonnell et al., 1945). In the presence of 1M urea, pH 5.4, no PE was detected in the extracts even after exhaustive dialysis. A slight improvement in the solubilization of PE was observed with the use of 10 mM EGTA. An incremental increase in the pH of extraction did not greatly improve the efficiency of extraction and had a detrimental effect on enzyme

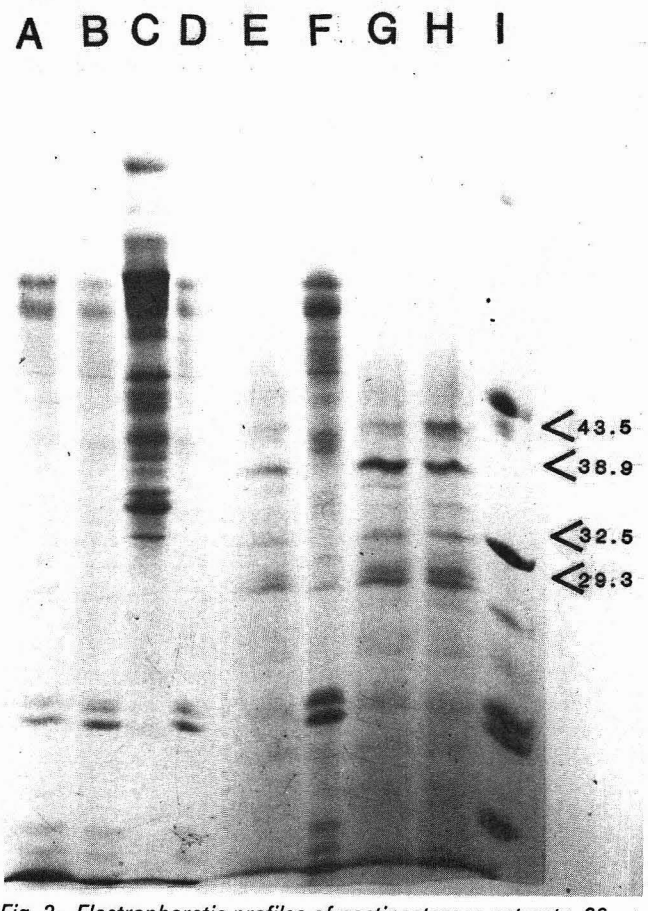

Fig. 2—Electrophoretic profiles of pectinesterase extracts. 30 μg of protein of each extract was layered on the stacking gel; Lane A, Fraction 5; Lane B, Fraction 3; Lane C, Pectinase; Lane D, Cellulase; Lane E, Fraction 4; Lane F, Replicate Fraction 3; Lane G, Fraction 2; Lane H, Fraction 1; Lane I, BioRad standards.

recovery at pH 11.0. These results contrast with those of King et al. (1986) who observed that an increase in pH from 3.5 to 8.5 to 10.0, at constant ionic strength of 0.3M NaCl, increased the percentage of PE solubilized from lime from 38% to 70% to 90%, respectively.

Driselase, a multi-enzyme preparation from Sigma consisting of laminarinase, xylanase, and cellulase, was used to evaluate the extractability of PE after digestion of the cell wall. After extraction of the pulp in 0.25M Tris, 0.3M NaCl, pH 8.0 for 1 hr, 27.87 PEU/mL were detected. The pellet was washed in water and re-extracted for 2 hr at 32°C in 0.1M citric acid at pH 4.5 containing 1.6% Driselase. The water and Driselase extracts contained 5.74 and 3.10 PEU/mL, respectively, whereas 8.00 PEU/g remained bound in the pellet. Treatment of the cell wall with 0.33% lysozyme, pH 6.2 also resulted in incomplete solubilization of PE.

Solubilization and heat stability of covalently bound PE

The total activity detected in the pulp before each extraction increased with storage time (November, 1986 through April, 1987) and was determined to be 45.01, 61.22, and 131.81 PEU/g, for Replicates 1, 2, and 3, respectively. Similarly, the total PE activity solubilized mirrored the trend observed for unextracted pulp, i.e., the higher the activity detected in the initial pulp, the higher the total amount of activity extracted. The values for three separate experiments are reported in Table 2. Within experiments, duplicate enzyme assays differed by less than 10%. Although the absolute PE activity increased with time, the relative solubilization and heat stability of the five fractions was similar in the three experiments.

Sequentially smaller increments of total activity are recovered with repeated alkaline salt buffer extractions as reported by Jansen et al.

(1960b). Comparison of the bound PE of Fractions 4 and 5 show that the amount of tightly bound PE is not markedly different in the cell wall degraded extracts than in the control extracts. However, the total activity extracted after cell wall degradation is higher than in the control samples. Extraction in either the control or in the presence of cellulase and pectinase for long times (18 hr) at room temperature resulted in a loss of PE activity. Triton (1%) was used in the presence of 0.25M Tris, 0.3M NaCl, pH 8.0 to evaluate the heat stability of PE solubilized by 1% Triton, but the extractions were not as effective as with cell wall degrading enzymes.

A substantial amount of PE is solubilized in the presence of 0.1M sodium citrate, pH 5.6, 1% cellulase, 2% pectinase (Table 2). Previous work in our lab has shown a marked decrease in solubilization of PE unless the pH was maintained at pH 8.0. Apparently, PE can be solubilized at lower pH values and at lower ionic strengths if the cell wall is degraded. At concentrations used to degrade the cell wall, no PE activity was detected in cellulase or pectinase by the titrimetric assay.

The major difference between the controls, Fractions 2 and 4, and the cell wall degraded samples, Fractions 3 and 5, is the higher residual activity of the latter two fractions after heating at 70°C for 2 min. Following equilibration of buffer at a constant pH, ionic strength, and volume, the residual activity of the control PE fractions (1, 2, and 4) was close to 1% whereas the residual activity of PE extracted after cell wall degradation is at least three times the respective control in all replicates (Table 2).

Biochemical characterization of PE

To further characterize the PE fractions, SDS-PAGE was conducted on 12% gels (Fig. 2). The typical profiles of the three control PE extracts are shown in Lanes E, G, and H. Four major protein bands stain Coomassie blue with apparent molecular weights of 43.5, 38.9, 32.5, and 29.3 kdaltons. Pectinase (Lane C) and cellulase (Lane D) were run as standards. Although there is considerable carry-over of the added enzymes into Fractions 3 (Lanes B and F) and 5 (Lane A), it appears that the 29.3 kd protein band appears in greater quantity in Fractions 3 and 5 extracts as well as a doublet near 20 kd. Although the 20 kd is present in cellulase as well, it is in apparently less quantity.

To more conclusively evaluate the potential solubilization of a heat stable isozyme after degradation of the cell wall, pH gradient focusing followed by the Delincce (1976) print technique for PE was conducted. A similar characteristic electrophoretic profile for each of the five fractions was in all replicates. A representative print of ten focusing experiments is given in Fig. 3. There was one main band observed in the IEF profile of Fractions 1, 2, and 4 with numerous, smaller quantities of PE isozymes at lower pH values. In Fractions 3 and 5, a second band was also observed that was present in nearly the same quantity as the main band in the controls. In pH gradient electrofocusing, it is impossible to determine and average Rf value of PE isozymes. In this specific replicate, Rf values of 0.43 and 0.37 were observed for the main bands in the control and cell wall degraded fractions, respectively. The added cellulase and pectinase were run as controls and were negative for the presence of PE by the Delincee (1976) print technique. The molecular weight of the two major PE bands from IEF is unknown.

DISCUSSION

BINDING OF PECTINESTERASE to the cell wall pectin has been described as electrostatic binding similar to an enzyme-substrate complex. In citrus, high pH and high ionic strength buffers are unsuccessful at complete solubilization of PE from the cell wall matrix (Jansen et al., 1960b). Attempts to release the tightly bound, insoluble PE were not completely successful. The enhanced solubilization of PE with EGTA suggests that PE is entrapped in a cell wall matrix stabilized by divalent cation bridges (Powell et al., 1982) or PE is bound to the pectin via divalent cation bridges. It is not likely that PE became bound and irreversibly associated with the cell wall as a result of the rapid pH drop during juice extraction, since maintaining the bulk pH greater than 8.0 during extraction did not improve solubilization of the bound PE. An entire orange was homogenized in 1M Tris, 0.3M NaCl and the pH maintained at 8.0. Approximately 20.62 and 15.30 PEU were detected in the extract and pellet, respectively. Although this suggests that there is non-electostatic interaction involved in the binding of PE to the cell wall, the pH of the microenvironment of the PE binding site may have been low and promoted electrostatic binding. Triton is typically used to solubilize membrane bound enzymes and was used in this case to evaluate the potential association of PE with the hydrophobic domains described in pectin by Oakenfull and Scott (1985). Scott and O'Neill (1984) compared the extraction of cell wall polypeptides with salts vs. detergents from carrot suspension culture cells and observed that the solubilization was similar but salts provided technical advantages. No clear increase in complete solubilization of PE at higher extraction buffer pH values was observed. To avoid possible loss activity by extended times at alkaline pH values, the extraction buffer pH of 8.0 was retained.

Ultimately, none of the treatments were successful at complete solubilization of PE from the cell wall. The phenomenon of irreversibly bound cell wall enzymes has been previously reported (Pressey, 1986; Hultin and Levine, 1963; Yamasaki and Konno, 1987). Complete solubilization of citrus PE may not be feasible or possible.

Solubilization and purification of citrus PE is somewhat unusual in that the yield of PE extracted in the first wash was variable and several hundred-fold over the total units detected in the unextracted pulp. The total units extracted in Replicates 1, 2, and 3 approximated 400%, 910%, and 506% respectively of the amount detected in the respective initial pulp. Furthermore, the absolute amount of PE that was solubilized increased with storage time as well. The nature of the apparent increase in yield with subsequent extractions is unknown but may reflect error in estimating the total initial activity. By use of solubilized citrus PE and isolated cell walls, Jansen et al. (1960b) reported that PE was inactive at pH 4.5 when bound to cell wall pectin. It is conceivable that partitioning of a tightly bound fraction of PE within the cell wall matrix precludes accurate assessment of the true total activity. The increase in total activity with storage time suggests that physicochemical changes are occurring during frozen storage that enhances solubilization and consequently detection of citrus PE. Nonenzymatic disruption of the pulp size by comminution has been observed to result in higher PE activities (Roger Waters, personal com-

Table 2—Solubilization of tightly bound pectinesterase isozymes and relative heat stability

	Extraction 1			Extraction 2			Extraction 3		
Frac	Solb[a]	Bd[b]	Rsdl Act[c]	Solb[a]	Bd[b]	Rsdl Act[c]	Solb[a]	Bd[b]	Rsdl Act[c]
1	48.02	82.44	1.34	107.18	171.97	0.80	132.37	152.60	1.02
2	20.22	28.18	0.76	40.02	MD[d]	0.70	47.28	58.10	0.23
3	21.17	22.81	2.81	44.91	MD[d]	2.48	50.68	47.31	3.71
4	5.89	10.48	0.97	9.52	20.70	1.12	8.12	28.42	1.23
5	8.42	6.52	4.52	20.34	21.78	3.38	21.86	19.37	5.03

[a] Solb = pectinesterase activity (PEU/mL) of the supernatant collected after centrifugation.

[b] Bd = pectinesterase activity (PEU/G) detected in the pellet after centrifugation.

[c] Rsdl Act = pectinesterase activity after heating 0.98 mL PE extract in 3.50 mL 10 mM potassium phosphate, 0.1M NaCl, 1 mM NaN_3, pH 7.0 at 70°C for 2 min relative to an unheated control. Heating was conducted after concentration of the respective soluble fraction with $(NH_4)_2SO_4$ to 75% and resuspension of pellet in 10 mM potassium phosphate, 0.1M NaCl, 1 mM NaN_3, pH 7.0.

[d] MD = missing data.

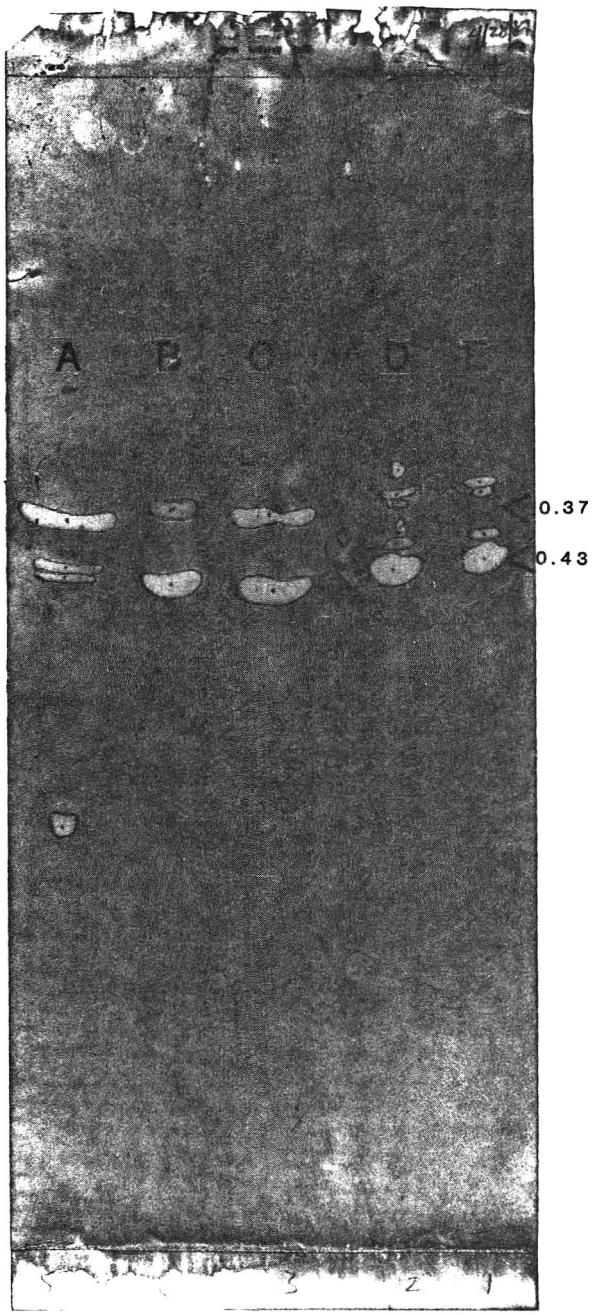

Fig. 3—pH gradient isoelectric focusing of pectinesterase extracts. Ten PEU of each extract was layered on filter paper on the gel bed; Lane A, Fraction 5; Lane B, Fraction 4; Lane C, Fraction 3; Lane D, Fraction 2; Lane E, Fraction 1.

munication). Alternatively, the increase in activity could conceivably be due to an inactivation of an inhibitor during frozen storage. Preparation of antibodies to PE isozymes would definitively identify an actual increase in active isozymes or a decrease in an endogenous inhibitor. A project is in place to study these possibilities.

Wasserman and Guilfoy (1984) summarized the extraction of peroxidase fractions by solubility differences: soluble peroxidase is extracted by low ionic strength buffers, ionically bound peroxidase is extracted by high ionic strength buffers, covalently bound peroxidase is that residual activity after extraction in the latter buffer. Covalently bound peroxidase, solubilized by use of added cellulases and pectinases has been reported to be more heat stable than soluble or ionically bound peroxidases (Gkinis and Fennema, 1978; Haard, 1973; Thomas et al., 1981; McLellen and Robinson, 1983).

The relative heat stabilities of extracts prepared after disruption of the cell wall were evaluated in more detail to determine the feasibility of enzymatic solubilization of the tightly bound citrus PE. The combination of cellulase and pectinase has been shown to be effective to prepare citrus juice sac protoplasts (Echeverria, 1987) and was used here to degrade the citrus cell wall to solubilize a potentially thermostable covalently bound PE. Residual activity of ionically bound citrus PE after heating at 70°C for 2 min is due to a PE isozyme that requires a temperature of 90°C to rapidly inactivate (Versteeg et al., 1980). Although the amount of the thermostable isozyme has been estimated by pH gradient electrofocusing to range from 5–30% of the total activity (Rombouts et al., 1982), the residual activity after heating for 2 min at 70°C will vary with the experimental protocol. Under the conditions described in the Methods section, approximately 1% residual activity was observed in the control PE fractions compared to at least 3% residual activity in the cell wall degraded PE fractions. It was assumed that under the conditions of this study that the cell wall was completely degraded (Echeverria, 1987) and the covalently bound PE was soluble. The increased heat stability of the PE solubilized after degradation of the cell wall relative to the control PE may be due to the preferential selection of heat sensitive PE isozymes by alkaline, salt buffers.

SDS-PAGE and pH gradient focusing profiles suggest that a greater quantity of an isozyme is released after degradation of the cell wall. The 38.9 kd protein on SDS-PAGE gels is most likely the heat sensitive isozyme, PE-I, described by Versteeg (1979) as having a molecular weight of 36.2 kd . They reported the heat stable isozyme to have a molecular weight of 54 kd based on gel filtration. No bands in this region were observed in the control lanes and due to the carry-over from pectinase and cellulase in this molecular weight range, it was impossible to determine if a greater quantity of 54 kd component was present in the cell wall degraded extracts. Purification of the covalently bound PE must precede definitive statements on the identity of other protein bands observed on SDS-PAGE gels. The coincidence of a higher quantity of a less positively charged PE isozyme and increased thermostability suggests that the heat stable isozyme is present in higher amounts than previously observed and previous extraction protocols have been selective for heat sensitive isozymes.

CONCLUSIONS

A FRACTION OF PE is not released by alkaline salt buffer but is partially released after degradation of the cell wall by pectinase and cellulase. After heating to 70°C, the fractions of PE solubilized after cell degradation have about three times the residual activity of the control.

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Molecular Mobilities of Instant Starch Gels Determined by Oxygen-17 and Carbon-13 Nuclear Magnetic Resonance

SHELLY J. RICHARDSON

ABSTRACT

The molecular mobility of both the water component and the starch component of three instant starches was determined by proton decoupled oxygen-17 (^{17}O) and proton decoupled carbon-13 (^{13}C) Nuclear Magnetic Resonance (NMR) spectroscopy. The effect of starch concentration and various storage conditions were studied. The molecular mobility of the water associated with each of the instant starches over all concentrations studied as measured by ^{17}O NMR transverse relaxation rates was not much slower than that of free or bulk water. The effect of storage conditions on the molecular mobility and conformation of the starches, as monitored by proton decoupled ^{17}O and ^{13}C NMR, was in most cases small, despite the observed differences in retrogradation among the starches.

INTRODUCTION

MODIFICATION OF STARCHES to alter their functional properties is now a field of industrial importance. Instant starches are modified to hydrate in cold liquid systems, producing an instant gel or paste without heating. There are two basic instant starch types: (1) precooked or pregelatinized starch and (2) cold water-soluble (CWS) starch. The major difference is that in case of pregelatinized starch, the raw granules have been fragmentized during manufacture, while the CWS starch process leaves the granules intact (Luallen, 1985). No basic research has been reported on the molecular mobilities of both the starch and the water in a soluble starch-water system. This can best be done by use of Nuclear Magnetic Resonance (NMR) spectroscopy. The mobility of water can be studied by oxygen-17 (^{17}O) NMR and the mobility of the carbohydrate polymer by carbon-13 (^{13}C) NMR.

Oxygen-17 NMR has been previously used to examine the mobility of water in a variety of systems such as proteins (Halle et al., 1981; Lioutas et al., 1986), wheat flour (Richardson et al., 1986) and starch (Richardson and Steinberg, 1987). In studying water mobility with NMR, ^{17}O has become the nucleus of choice due to the recent problems with interpretation of proton NMR data (Halle et al., 1981). One concern with employing ^{17}O NMR is that the ^{17}O transverse relaxation is influenced by proton exchange broadening, especially for the pH range of approximately 5.5 to 8.5 (Meiboom, 1961; Rabideau and Hecht, 1967). Proton exchange broadening can be greatly decreased by using deuterium oxide as a solvent (Richardson and Steinberg, 1987) and can be eliminated in pure water and model systems by means of an instrumental technique which decouples the protons (Earl and Niederberger, 1977).

Carbon-13 NMR spectroscopy has been extensively used to study the structure and molecular dynamics of carbohydrates, both in solution and more recently in the solid state (Perlin and Hamer, 1979; Jennings and Smith, 1980; Dais and Perlin, 1982; Perlin and Casu, 1982; Rees et al., 1982; Casu, 1985; Jane, 1985; Marchessault et al., 1985). Information on the intra- and inter-molecular environment of each atom can be extracted from the parameters associated with the NMR signal, such as chemical shift, coupling constants and relaxation rates. As applied in this study, the ^{13}C NMR spectra served to monitor the mobility of the carbon backbone of the starch molecule.

Employing one nucleus to probe the water component and another nucleus to probe the starch component has been reported by only one investigator (Callaghan et al., 1983). They used both ^{1}H and ^{13}C NMR to investigate wheat starch pastes with contrasting rheological properties. The ^{1}H NMR spin-spin and spin-lattice relaxation rates were characterized by a single-time constant, indicating that a two-state model with fast exchange may be adequate to account for such observations. However, they could not relate these ^{1}H relaxation measurements to the different rheological properties of the starches. The ^{13}C NMR measurements indicated that approximately 60% of the starch existed as a mobile liquid-like polymer, while the remaining 40% was in an immobile crystalline form.

The objective of this research was to investigate by ^{17}O and ^{13}C NMR the molecular mobilities of both the water and the instant starch components as affected by concentration and storage conditions.

MATERIALS & METHODS

Materials

Three soluble or instant starches were investigated: Dura-Jel, crosslinked, hydroxypropylenated and pregelatinized waxy corn starch; Mira-Gel, high-temperature, aqueous alcohol modified common dent corn starch; and Mira-Thik, crosslinked, hydroxypropylenated, high-temperature, aqueous alcohol modified common dent corn starch. These starches were provided by A.E. Staley Manufacturing Co. (Decatur, IL).

Moisture content of the three starches was 7.5%, 5.7%, and 5.4%, respectively, as determined by vacuum oven method (AOAC, 1980) using 60°C and 29.8 in Hg vacuum for 24 hr.

NMR spectroscopy

^{17}O NMR. Dura-Jel, Mira-Gel and Mira-Thik starches at selected concentrations, ranging from 0.5 to 9.0% starch wet basis (or 0.08 to 1.7g dry starch/mole H_2O), were prepared by mixing in an Omnimixer (Ivan Sorwall Inc., Norwalk, CT) the appropriate amount of distilled water with starch for 1.0 min at 8500 rpm at room temperature (23°C).

A laboratory-assembled NSF-250 multinuclear NMR spectrometer operating at 33.912-MHz ^{17}O NMR resonance frequency was used for the ^{17}O NMR measurements. A low frequency multinuclear 12 mm probe was used. Single-pulse proton decoupled ^{17}O NMR experiments were done in duplicate at 20°C. The 90 degree ^{17}O pulse width of 100 msec and a recycling time of 0.21 sec were used. A WALTZ-16 decoupling sequence was used for proton decoupling. The proton decoupling frequency was 250.2 MHz with a 90 degree proton pulse of 250 msec and decoupling power of 0.8 watts. The number of scans was 2000 and the samples were spun at 15 ± 2 Hz. The spectra were retained in an 8K point array using a Nicolet NMC-1280 computer system.

^{17}O NMR spectra of freshly made (ca 4 hr old) starch samples at each concentration were taken; then, the 5% starch samples were divided for storage and freeze-thaw stability experiments. The storage stability of the starches was monitored by measuring the ^{17}O NMR transverse relaxation rate, R_2 (sec^{-1}). The samples were stored under two conditions: room temperature (23 ± 2°C) and low temperature

Author Richardson is with the Univ. of Illinois, Div. of Foods & Nutrition, 367 Bevier Hall, 905 South Goodwin Ave., Urbana, IL 61801.

(4 ± 1°C). Samples were removed from their particular storage condition (at 6 and 12 days), allowed to equilibrate (for ca 4 hr) to 20°C and measured.

Freeze-thaw stability was also monitored by ^{17}O NMR. The starch samples were put through six cycles of freezing and thawing; 12 hr of freezing at −15°C, followed by 12 hr of thawing at room temperature (23°C). After completion of the last cycle, samples were allowed to equilibrate (for ca 4 hr) to 20°C and the ^{17}O NMR spectra remeasured.

The line shape for the ^{17}O NMR Fourier transform spectra at each concentration and condition (fresh, stored and freeze-thawed) was analyzed using the NMC-1280 data reduction routine (Nicolet, 1982). This program evaluated the line shapes in terms of the equation for a Lorentzian peak:

$$Y = A/\{1-[2(x-T)/W]^2\} \quad (1)$$

where A is the amplitude at x = T, T is the center frequency, W is the line width at A/2 and x is the frequency. Good agreement, as evaluated by standard deviation calculations, was obtained between the experimental and fitted peaks (height, half-width and frequency for fitted peaks were all within 95% that of experimental). Therefore, R_2 (sec^{-1}) was calculated by the following (Dwek, 1973):

$$R_2\ (sec^{-1}) = \pi\ \Delta\nu_{obs} = \frac{1}{T_2} \quad (2)$$

where $\Delta\nu_{obs}$ is the line width at half-height for each spectrum. For comparison of the ^{17}O R_2 values measured for the storage and freeze-thaw stability studies, the net or differential transverse relaxation ratio (ΔR_2, sec^{-1}) was calculated by subtracting the line width of liquid water ($\Delta\nu_{free}$) from the line width of the sample ($\Delta\nu_{obs}$) before the multiplying by π (Lioutas et al., 1986):

$$\Delta R_2\ (sec^{-1}) = \pi\ (\Delta\nu_{obs} - \Delta\nu_{free}) \quad (3)$$

^{13}C NMR. Dura-Jel, Mira-Gel and Mira-Thik starches at 5% starch concentration were prepared by mixing in an Omni-mixer as described above. In addition, 10% of the distilled water was replaced with 99.8% deuterium oxide (Sigma Chemical Corp., St. Louis, MO) for field-frequency locking purposes.

An NT-360 NMR spectrometer (Nicolet Technologies, Inc., Madison, WI), operating at 90.546 MHz ^{13}C NMR resonance frequency, was used for the ^{13}C NMR measurements. Single-pulse fully proton decoupled carbon experiments were done at 20°C. A 90 degree pulse of 4 msec and a recycling time of 1.41 sec were used. The proton decoupling frequency was 360.061 MHz. The number of scans was 15,000 and the samples were spun at 30 ± 2 Hz. The spectra were retained in a 16K point array which was shown to provide adequate resolution.

^{13}C NMR spectra of freshly made starch samples (ca 4 hr old) were taken. Then these samples were divided for storage and freeze-thaw stability experiments. Storage stability of Dura-Jel and Mira-Gel starches was monitored by ^{13}C NMR. Samples were subjected to the same two storage conditions described under the ^{17}O NMR section. Freeze-thaw stabililty of Dura-Jel, Mira Gel and Mira-Thik starches also was monitored by ^{13}C NMR. Samples were subjected to the same six-cycle freeze-thaw described above.

For all ^{13}C NMR experiments, DSS was used as the external standard (Perlin and Casu, 1982).

RESULTS & DISCUSSION

Oxygen-17 NMR

Starch concentration. The variation of the ^1H decoupled ^{17}O NMR transverse relaxation rate (R_2, sec^{-1}) with increasing instant starch concentration is shown in Fig. 1.

The ^1H decoupled ^{17}O R_2 for Dura-Jel showed a linear behavior with increasing starch concentration from a thin suspension (0.08g dry starch/mole H_2O) to a very thick paste (1.7g dry starch/mole H_2O). This linear behavior may be explained by the isotropic two-state model with fast exchange (Zimmerman and Brittin, 1957). This model predicts a linear relationship between the observed relaxation rate (R_{obs}) and concentration, if no additional contributions to relaxation are present. This relationship is based on a population weighted

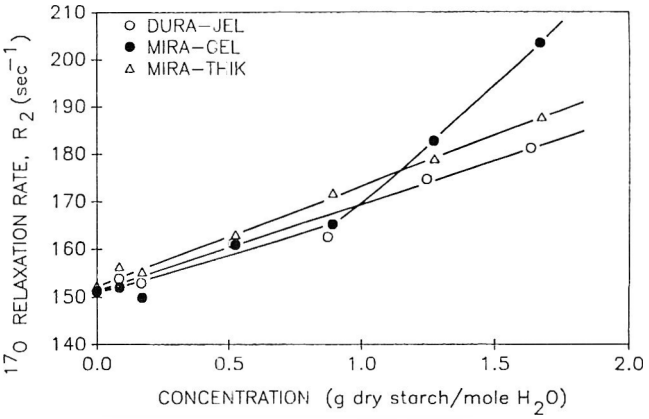

Fig. 1—Dependence of ^1H decoupled ^{17}O NMR transverse relaxation rates (Eq. 2) on instant starch concentration.

average between bound (R_E) and free (R_F) water, expressed as:

$$R_{obs} = P_B\ R_B + P_F\ R_F \quad (4)$$

where P_B and P_F are the fractions of "bound" and "free" water in the system, respectively, expressed as a decimal. The relatively small increase in R_2 from 151.2 sec^{-1} for free water (0.0g dry starch/mole H_2O) to 181.2 sec^{-1} for the highest Dura-Jel starch concentration (1.64g dry starch/mole H_2O) indicates a very small decrease in the water mobility over this concentration range. Thus, the water in the thick starch gel was only slightly less rotationally mobile than free water. Thus, the water was most likely trapped in the gel matrix rather than chemically bound to individual groups on the starch molecules.

At low starch concentrations, the Mir-Gel (Fig. 1) also showed a linear relationship between R_2 and concentration. However, at concentrations greater than 0.9g dry starch/mole H_2O, a departure from linearity was observed. This departure indicates that either additional water "states" must be considered or new interactions were present or both.

Similar departures from linearity have been reported for a variety of biopolymers from proteins to wheat flour (Oakes, 1976; Halle et al., 1981; Derbyshire, 1982; Richardson et al., 1986). According to these reports, both the explanations for the deviation from linearity, as well as the concentration at which the departure occurred, were unique for the specific system.

Mira-Thik exhibited a linear relation between R_2 and increasing concentration (Fig. 1) throughout the concentration range studied. Thus, in this respect, Mira-Thik (alcohol modified, crosslinked and hydroxypropylenated common dent corn starch) was similar to Dura-Jel (pregelatinized, crosslinked and hydroxypropylenated waxy corn starch) but different from Mira-Gel (alcohol modified common dent corn starch). This comparison was difficult to explain because of similarities and differences in composition and modification. Evidently, the crosslinking and substitution were the important treatments here.

Temperature and time. The variation of the ^1H decoupled ^{17}O NMR differential transverse relaxation rate (ΔR_2, sec^{-1}) of the three instant starches at 5% concentration in water under the various time-temperature conditions is given in Table 1. Every starch under every condition, except one (Mira-Gel under the 6-day room temperature storage condition), showed an increase in ΔR_2.

Before these measurements were collected, it was hypothesized that if a starch exhibited syneresis the ^{17}O NMR R_2 (Eq. 2) or ΔR_2 (Eq. 3) would significantly decrease due to the increase in the mobility of the water. Conversely, a starch which did not exhibit syneresis would show no change in R_2 (or ΔR_2). Based on visual observation, the only starch that

Table 1—^1H decoupled ^{17}O NMR ΔR_2 (Eq. 3) of Dura-Jel, Mira-Gel and Mira-Thik corn starches at 5% concentration in water under various time-temperature conditions

	ΔR_2 (sec^{-1}) under various conditions			
Starch type	Fresh	Room temp storage (6/12 days)	Low temp storage (6/12 days)	Six freeze-thaw cycles
Dura-Jel	4.56	23.23/17.85	14.27/21.05	15.03
Mira-Gel	17.13	16.80/19.29	29.83/31.23	37.22
Mira-Thik	8.50	20.35/20.67	17.22/22.42	23.78

exhibited syneresis was the Mira-Gel starch. This starch showed increasing syneresis with time (to 12 days) and with a decrease in temperature. These visual observations were consistent with literature reports (del Rosario and Pontivero, 1983; Kitamura et al., 1984; Eliasson, 1985; Guilbot and Mercier, 1985; Miles et al., 1985; Nakazawa et al., 1985). Thus, according to the hypothesis, the R_2 (or ΔR_2) for the Mira-Gel starch should decrease with time, whereas the R_2 for the Dura-Jel and Mira-Thik starches should remain constant. However, as previously stated, all starches under all conditions except one showed an increase in ΔR_2. This indicates that there was a consistent decrease in water mobility in all three starches compared to the increase expected in the Mira-Gel starch (showed syneresis), and the constant mobility expected in the other two starches.

Since ΔR_2 is the weighted average of the mobility of all the water in these systems (i.e., trapped and free), this increase in ΔR_2 may be translated into a variety of physical models. For example, in the case of Mira-Gel, the free water due to syneresis would contribute to an increase in mobility. However, there may have been a simultaneous increase in the trapped water portion with the trapped overshadowing the mobile portion, resulting in a higher ΔR_2. On the other hand, for the non-retrograded starches, where no syneresis water was observed, the decrease in mobility can be attributed to an increased trapping of all the water.

As can be seen from Table 1, no specific trends in mobility were observed for either time or temperature of storage.

Freeze-thaw stability. The ^1H decoupled ^{17}O NMR of the three instant starches under six freeze-thaw cycles is given in Table 1. All three starches exhibited an increase in the ΔR_2 after the freeze-thaw process.

Before these measurements were collected, it was hypothesized that a starch which was freeze-thaw unstable (i.e., exhibited syneresis) would show higher water mobility, reflected in a lower ΔR_2 value. Conversely, a freeze-thaw stable starch would show no change in ΔR_2. The definition of freeze-thaw stability proposed by Albrecht et al. (1960a) was employed to visually evaluate the stability of the three instant starches subjected to the six-cycle frees-thaw treatment. The definition states that a system that exhibits "freeze-thaw stability is one which does not become lumpy, grainy or spongy, is free from liquid separation (syneresis), and remains homogeneous after it has been frozen." The Dura-Jel and Mira-Thik starches did not exhibit any of these characteristics, whereas the Mira-Gel did exhibit extensive syneresis and become very sponge-like. Therefore, according to the proposed hypothesis, the ^{17}O NMR ΔR_2 for the Mira-Gel starch should show a decrease in ΔR_2 (i.e., increased water mobility) and the other two starches should show no change in ΔR_2. However, as stated previously, all three starches give a larger ΔR_2 value after the freeze-thaw treatment than when measured fresh. This indicates that the average mobility of the water decreased slightly after freeze-thawing. The same explanation given above under temperature and time can be invoked here.

Ablett et al. (1976), studying freeze-thawing of gel forming polysaccharides by ^1H spin-lattice relaxation (R_1), reported that the R_1 relaxation became non-exponential in gels which exhibited syneresis (i.e., agarose gel) but remained a single component in a gel which exhibited no syneresis (i.e., iota carrageenan). However, no R_1 values were given, so comparison with the present work cannot be made.

Lelievre and Creamer (1978) employed both ^1H NMR spin-lattice and spin-spin relaxation rates to characterize the mobility of water during the formation and syneresis of renneted milk gels. They found that when distinct signs of syneresis were present in the milk gels, two relaxation times were observed; one relaxation time was associated with the protons in the gel, while the other was due to the protons in the liquid expelled from the gel phase. These results are similar to those reported by Ablett et al. (1976).

Carbon-13 NMR

Temperature and time. Because the ^{17}O data did not lead to specific conclusions, the starches were analyzed by ^{13}C NMR. The ^{13}C fully ^1H decoupled NMR spectra of Dura-Jel and Mira-Gel corn starches at 5% concentration and fresh and after storage at room temperature (23°C) and low temperature (4°C) are shown in Fig. 2. Proposed assignments of the major ^{13}C NMR sugar ring carbon resonances are given for the fresh samples in Table 2. These assignments are based on the ^{13}C NMR chemical shifts previously reported for amylose and amylopectin from wheat starch (Dais and Perlin, 1982), modified corn starch (Mora-Gutierrez and Baianu, 1987) and the representative ^{13}C chemical shift ranges proposed by Perlin and Casu (1982).

The overall attributes of the ^{13}C NMR spectra for both Dura-Jel and Mira-Gel starches after both room- and low-temperature storage remained similar to that of the fresh spectrum, despite the fact that the Dura-Jel starch did not exhibit any syneresis, while the Mira-Gel showed extensive syneresis. The Mira-Gel starch spectrum after storage, however, exhibited slight linebroadening over the fresh Mira-Gel spectra, especially in the carbon region 2–5. Also, more broadening is observed in the low temperature storage than in the room temperature. This line-broadening suggests a slight decrease in polymer mobility; however, the broadening was not large enough to suggest a major change in conformation or mobility of the starch. Thus, while there was a visually observable transition from the soluble gel-state to the insoluble retrograded state, little to no conformation change was observed in the ^{13}C NMR spectra. A proposed physical explanation for this observation is that the Mira-Gel starch chains became tangled in the retrogradation process to give insoluble starch aggregates; however, there remained a sufficient number of water molecules surrounding (i.e., "hydrating") the Mira-Gel starch chains in the retrograded state to retain the same chain conformation and mobility as in the soluble gel-state. Similar findings for amylodextrin were reported by Jane (1985). It was found that the ^{13}C NMR spectrum of retrograded amylodextrin was nearly identical to that of amylodextrin in solution. Jane (1985) attributed this similarity in spectra to little conformational change about the glycosidic bond between the two states, as well as sufficient hydration of the amylodextrins in both solution and retrograded conditions.

Freeze-thaw stability. The ^{13}C fully ^1H decoupled NMR spectra of Dura-Jel, Mira-Gel and Mira-Thik corn starches at 5% concentration, fresh and after six freeze-thaw cycles, are given in Fig. 3. The proposed assignments of the major ^{13}C NMR sugar ring carbon resonances for Mira-Thik are given in Table 2.

The ^{13}C NMR spectra for the frozen and thawed starch systems show two basic patterns. For the starch that did not exhibit freeze-thaw stability (Mira-Gel), a large change in the spectrum between fresh and freeze-thawed conditions was observed. However, for the two starches which did exhibit freeze-thaw stability (Dura-Jel and Mira-Thik), no changes in the ^{13}C NMR spectra were observed. The observed changes in the nonfreeze-thaw stable starch are attributed to polymer conformational and chemical changes during freezing, giving rise to

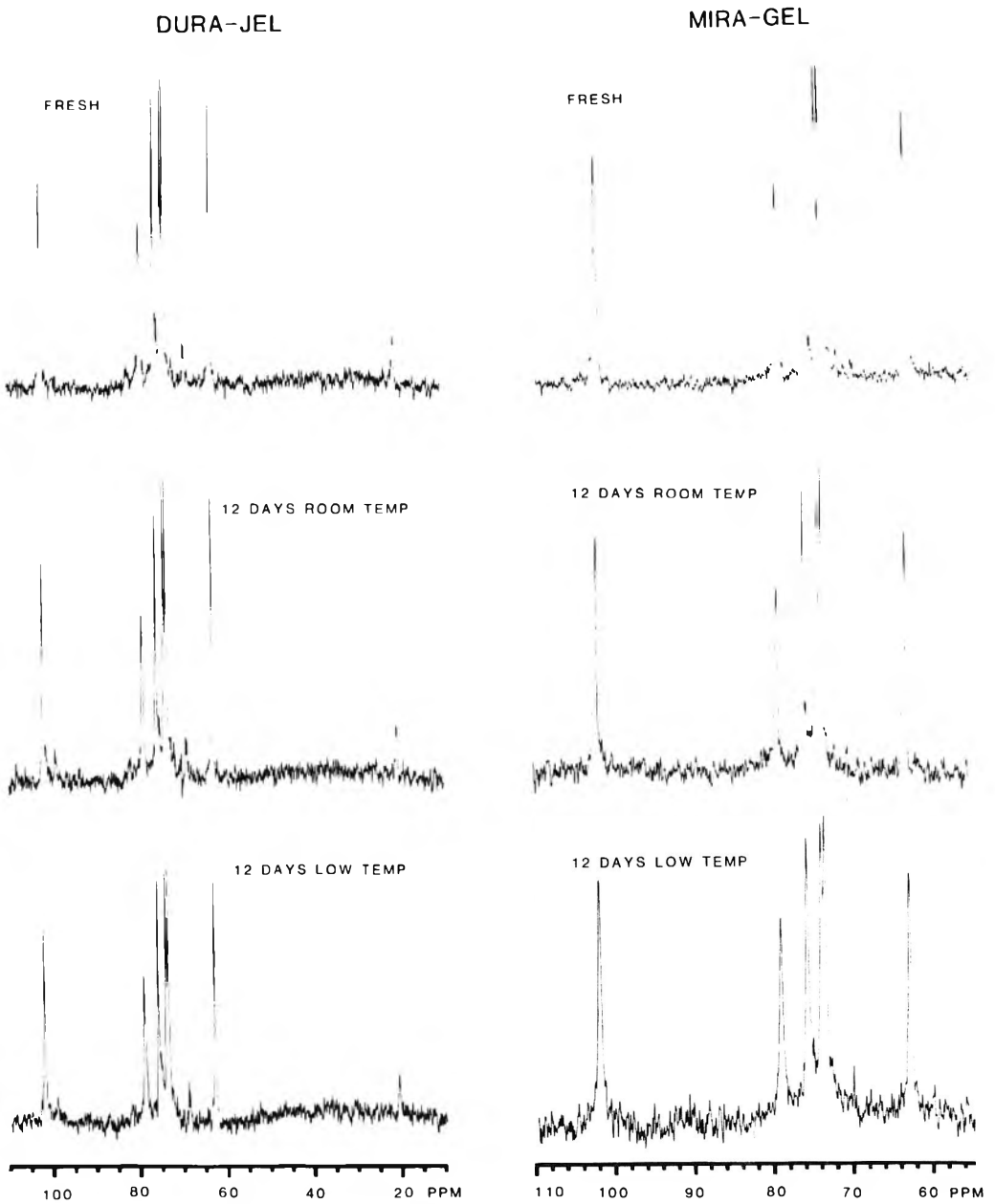

Fig. 2—^{13}C fully ^{1}H decoupled NMR spectra of Dura-Jel and Mira-Gel corn starches at 5% concentration fresh and after storage at room temperature (23°C) and low temperature (4°C) for 12 days.

the observed spectral changes in all of the carbon environments except carbon 6.

Of special interest is the appearance of two additional resonance peaks in the anomeric carbon (carbon-1) region at 98.28 and 94.40 ppm (Fig. 3). Based on chemical shift data from the literature, these resonances cannot be ascribed to simple α to β conformational changes, since the β carbon-1 conformation is shown to resonate at higher fields than the α carbon-1 conformation (Colson et al., 1974; Perlin and Casu, 1982; Casu, 1985). Therefore, these resonances most probably arise from complex chemical changes in the starch upon freezing. A detailed investigation of these changes is currently underway.

During the freezing process, the water in the starch-water system progressively forms ice crystals. In turn, this causes an increase in the effective starch concentration and results in the promotion of starch chain associations (Dea, 1979). These starch chain associations can be either reversible or irreversible in nature upon thawing. If the majority of the associations are reversible, the starch will exhibit freeze-thaw stability, that is, the associations "redissolve" on thawing. However, if the majority of the associations are irreversible, the starch is not freeze-thaw stable. The reversibility or irreversibility of the associations can be influenced by several factors such as pH, water content or water activity, ionic strength, conformation or structure of the chains and introduction of other components (Dea, 1979). In the two starch systems exhibiting freeze-thaw stability in this study, the reversibility of the chain-chain associations is due to modification of these starches by substitution (hydroxypropylenated). The addition of these groups results in weak or reversible chain-chain associations which "redissolve" upon thawing. On the other hand, the non-substituted starch (Mira-Gel) developed relatively strong or irreversible chain-chain associations which did not "redissolve" upon thawing.

Ablett et al. (1976), studying the freeze-thaw behavior of gels using ^{1}H NMR, suggested a similar mechanism to explain the syneresis observed in agarose but not in iota carrageenan. They attributed the lack of syneresis to the presence of charged sulphate groups which prevented aggregation of the iota car-

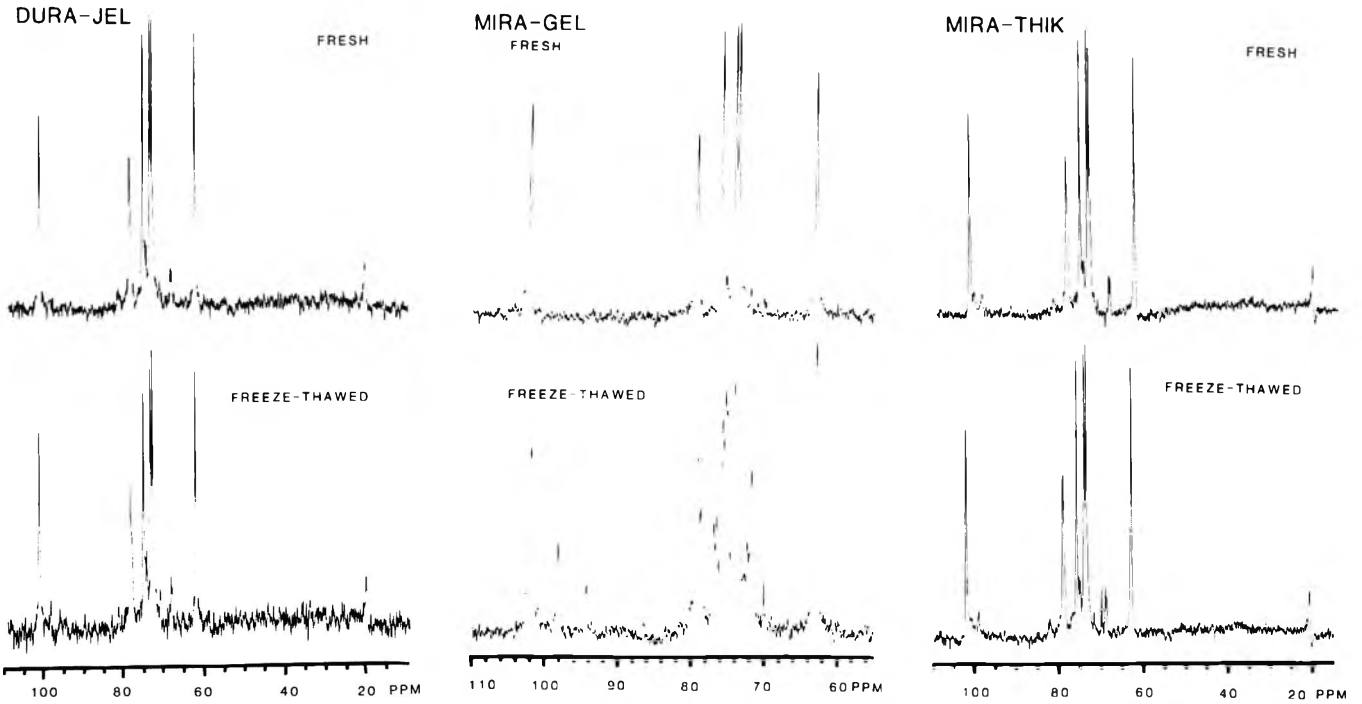

Fig. 3—^{13}C fully ^{1}H decoupled NMR spectra of Dura-Jel, Mira-Gel and Mira-Thik corn starches at 5% concentration under fresh and after six freeze-thaw cycles.

Table 2—Major ^{13}C NMR resonances in 360 MHz spectra of fresh Dura-Jel, Mira-Gel and Mira-Thik in water

Major carbon assignment	Chemical shift (γ)		
	Dura-Jel	Mira-Gel	Mira-Thik
	In ppm, relative to DSS as an external standard		
C-1	102.15	102.15	102.09
C-4	79.27	79.29	79.23
C-3	75.94	75.94	76.00
C-2	74.14	74.14	74.18
C-5	73.69	73.70	73.72
C-6	63.03	63.05	63.08
Substitution resonance	20.74	—	20.66

rageenan helices on freezing or prevented their disruption upon thawing. They attributed the syneresis to the extensive irreversible aggregation of the uncharged agarose. Dea (1979), investigating interaction phenomena in hydrocolloids, also presented a similar explanation for the freeze-thaw behavior of the gel systems he studied.

It is interesting to note the difference in the ^{13}C NMR spectra of Mira-Gel under the two storage conditions (room and low temperature) versus the spectrum for the freeze-thawed condition. All three conditions exhibited visual retrogradation and syneresis; however, only under the freeze-thaw condition did the spectrum show conformational changes. This suggests that either the proposed chain-chain associations can only form during the freezing process or that the associations take longer to develop than 12 days. Schoch (1968) suggested that the physical changes which occur in starch pastes during prolonged cold storage at low temperatures (i.e., 4°C) parallel the changes caused by freezing and thawing. However, he reported that the changes which may occur in one cycle of freezing and thawing may take several weeks to occur in the low temperature sample. If this is true for the chain-chain associations reported here for the Mira-Gel freeze-thaw sample, we should expect similar developments in the low temperature spectra after several weeks of cold storage.

Only a limited number of investigations were found in the literature on the freeze-thaw behavior of biological systems (Albrecht et al., 1960a,b; Whistler et al., 1967; Schoch, 1968; Hood and Seifried, 1974; Ablett et al., 1976; Chan and Toledo, 1976; Lelievre and Creamer, 1978; Lynch and Webster, 1979; Dea, 1979; Katayama and Fujiwara, 1980; Dreher et al., 1983; Cameron et al, 1985; Strauss et al., 1986). Only three of these studies included a food-related component as well as employing NMR as the major instrumental method (Ablett et al., 1976; Lelievre and Creamer, 1978; Katayama and Fujiwara, 1980) and none used ^{13}C. Thus, there is a great opportunity and need for continued research in this area, exploring further aspects of the phenomena reported here, such as the specific nature of the proposed chain-chain associations.

The observation of ^{13}C as well as ^{17}O nuclei gave a complete profile of the molecular mobility of the system. These data showed both the starch and water components remained highly mobile under all storage conditions, despite the syneresis shown by the Mira-Gel starch.

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Starch Transformation During Banana Ripening: The Amylase and Glucosidase Behavior

ELISABETH GARCIA and FRANCO M. LAJOLO

ABSTRACT

Banana starch disappearance during ripening originated at the central portion of the fruit radiating, afterwards, to the surface; the amylose/amylopectin ratio remained constant during the process. The amyloplast was investigated by light and scanning electron microscopy. The surface of starch granules was smooth, and the unique modification observed during ripening was the reduction of the granule dimensions; at advanced ripening stages, some striations were detected on the surface of both small and large granules. Several amylolytic enzymes were followed during banana ripening. Seven amylases were detected at all stages, presumably three α- and four β-amylases and, like glucosidases, their activity increased at the climacteric phase; only β-amylase activity increased before the onset of the respiratory peak. For the first time α-1, 6-glucosidase activity was detected in banana.

INTRODUCTION

THE RAPID STARCH disappearance during banana ripening is one of the most evident transformations observed. In spite of the importance of this change either in fruit physiology or in consideration of its eating and technological qualities, little research has been done on the mechanisms involved since the first observations of Barnell (1940) and Loesecke (1980).

In general, starch degradation in vegetables can be accomplished by amylase and glucosidase action on α-(1→4)- and α-(1→6)-linkages and also by the action of phosphorylases (Ap Rees, 1974; De Fekete and Vieweg, 1974; Manners, 1974).

In bananas, probably more than one enzyme and more than one unique pathway are operative during the climacteric period which characterizes the accelerated ripening phase of the fruit. In a previous paper (Arêas and Lajolo, 1981), the behavior of phosphorylase was studied and found that its activity increased just before the onset of starch degradation and that this increase was not reduced by infiltration of protein synthesis inhibitors. In a subsequent paper, Terra et al. (1983) observed that the accumulation of sucrose in the fruit preceeded the increase of glucose and fructose as the starch content decreased concomitant with ripening. At the same time, sucrose synthetase activity increased, and incorporation of ^{14}C-glucose 1-phosphate into sucrose demonstrated that the direct transformation of starch to sucrose via phosphorolysis was operative during ripening of bananas. The increase in activity could be reduced by infiltration of protein or nucleic acid synthesis inhibitors in tissue before the climacteric period.

The involvement of hydrolases cannot be ruled out. Young et al. (1974) and Mao and Kinsella (1981) detected, by electrophoresis, several starch hydrolases in banana fruit, raising the possibility of an additional pathway.

Another important aspect to be considered is the physicochemical characterization of the starch granules of bananas during ripening as a way for improved understanding of the enzymatic action. Unfortunately, the information available in the literature is scarce and mostly related to investigation of the functional properties of starch with interest in its industrial utilization.

This paper describes investigations conducted to establish the possible involvement of several types of hydrolases, including debranching enzymes, on starch degradation and to characterize starch granules during ripening, including electron microscopy studies.

Authors Garcia and Lajolo are affiliated with Depto. Alimentos e Nutrição Experimental Faculdade de Ciências Farmacêuticas-USP, Caixa Postal 30, 786, CEP 01051, São Paulo, Brasil.

MATERIALS & METHODS

THE BANANAS used (*Musa cavendish*) were harvested when the central diameter of the middle fruit of the second bunch was 33–34 mm. The fruits were detached from the bunch and washed with a 1% sodium hypochlorite solution, weighed, labeled and stored in a respiration chamber at 20°C, as described by Arêas and Lajolo (1981).

Enzymes extraction

Approximately, 12g banana pulp was sliced into 18 mL cold 0.1M phosphate buffer (pH 7.0) containing 1% polyvinylpyrrolidone (MW 20,000) and 0.02M cysteine hydrochloride freshly neutralized (Baijal et al., 1972). The tissue was homogenized in a Potter-Elvejhem homogenizer at 4°C; the suspension was centrifuged at 25,000×*g* for 2 hr at 4°C. The supernatant (crude extract) was used as source of the enzymes.

Assay of α-amylase and glucosidase

The chromogenic substrate Amylochrome® (Cibachron blue F3GA-amylose) was purchased from F. Hoffmann La Roche. An aliquot (1.5mL) of crude extract was pre-incubated at 37°C for 1 hr at either one of three different conditions: with 0.5 mL previously neutralized stock solutions to give final concentration of 1×10^{-4} $CaCl_2$, or 3×10^{-2}M EDTA or 0.15M TRIS. Then 2 mL enzyme extract were mixed with 30 mg dry substrate Amylochrome and incubated at 37°C for 2 hr. The reaction was interrupted by the addition of 3 mL 0.1N HCl; the resulting suspension was centrifuged at 4,500×*g* for 15 min, and the supernatant was used for estimating enzyme activity after reading absorbance at 620 nm. The enzyme units calculation was based on a standard curve prepared with Cibachron blue® (Ciba-Geigy). One enzyme unit was expressed as equivalent to the liberation of 1μg Cibachron blue/min/g banana pulp.

α-Amylase activity was calculated by the difference between the extract pre-incubated with $CaCl_2$ (total hydrolytic activity) and the extract pre-incubated with EDTA (α-amylase inhibitor). Glucosidase activity as calculated by the difference between extracts pre-incubated with $CaCl_2$ and TRIS (glucosidase inhibitor).

Assay of β-amylase

This assay was run using soluble starch as substrate and measuring the production of reducing sugars. The crude enzyme extract was first dialyzed against 0.1M phosphate buffer (pH 6.0) to remove soluble sugars present in the banana. The dialyzed extract was pre-incubated either with aliquots of stock solutions, first equilibrated at pH 6.0, to give final concentration of 1×10^{-2}M cysteine or 2×10^{-3}M $HgCl_2$ (Pech et al., 1973) for 1 hr at 37°C. Then 1 mL extract was incubated with 0.5 mL 2% starch suspension for 8 min at 37°C; the reaction was stopped by boiling in a water bath for 5 min, followed by the addition of 4.5 mL ethanol for the extraction of soluble sugars. The ethanolic suspension was centrifuged at 12,000×*g* for 20 min at 4°C; the supernatant was used to determine sugars (Dreywood, 1957); a standard curve was prepared with maltose. The enzyme activity was estimated by the difference between the extracts pre-incubated with cysteine and $HgCl_2$ (SH-dependent β-amylase inhibitor). One unit corresponded to the liberation of 1 μM maltose/min/g banana pulp.

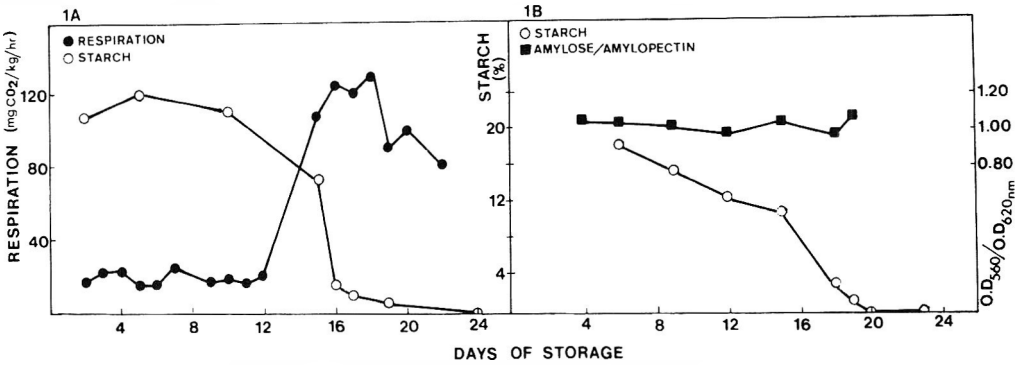

Fig. 1—(A) Starch hydrolysis during the ripening of bananas; (B) Amylose/amylopectin ratio during banana starch breakdown.

Assay of α-1,4-glucosidase activity

The enzyme assay was conducted using p-nitrophenyl-α-D-glucopyranoside and measuring the liberated p-nitrophenol. The crude extract was first dialyzed against 0.1M phosphate buffer (pH 6.5) containing 8 mM cysteine (stock solution equilibrated at pH 6.5). One milliliter dialyzed enzyme extract was mixed with the substrate solubilized in phosphate buffer (pH 6.5) to give a final concentration of 5 mg substrate/mL enzyme extract and incubated at 37°C for 30 min. The reaction was interrupted by the addition of 2mL 0.4M sodium carbonate/bicarbonate buffer (pH 10.0). The suspension was centrifuged at 3,000×*g* for 10 min; the supernatant was used for absorbance reading at 400 nm. A standard curve was prepared with p-nitrophenol at the same assay conditions. One enzyme unit corresponded to the liberation of 1 μg p-nitrophenol/min/g banana pulp.

Assay of α-1,6-glucosidase activity

The α-1,6-glucosidase activity was determined using isomaltose as substrate and an enzyme extract previously dialyzed (0.1M phosphate buffer pH 6.5 containing 12.5mM cysteine). One milliliter dialyzed extract was incubated with an aliquot of isomaltose stock solution in phosphate buffer pH 6.5 to give a final substrate concentration of 3.5mM and then incubated at 37°C for 15 min. The reaction was stopped by heating in a boiling water bath for 5 min. The suspension was centrifuged at 12,000×*g* for 10 min, and the supernatant was used for the enzymatic determination of glucose produced with the glucose oxidase/peroxidase technique, according to Bergmeyer and Bernt (1974). One enzyme unit corresponded to the liberation of 1μg glucose/min/g pulp.

Starch determination

Banana pulp (1g) was extracted with 4 mL 0.5N NaOH in a Potter-Elvejhem homogenizer; the flask was repeatedly washed with water; the extract was neutralized with 4mL 0.5N acetic acid, and the volume completed to 100mL with water. Starch was enzymatically determined as already described by Aréas and Lajolo (1981).

Amylose/amylopectin ratio

This ratio was determined in aliquots of starch extracts (as obtained above) containing 400 μg starch using excess aqueous I_2/KI (0,2/2.0%) solution. The A_{560}/A_{620} ratio was calculated from absorbances.

Starch location in banana slices

Transversal slices of the central part of peeled banana fruit were fixed, dehydrated (Silva and Luh, 1978) and dyed with I_2/KI (0.2/2.0%) aqueous solution.

Starch granules size determination

Starch granules were isolated according to Carson (1971), and suspensions were prepared with I_2/KI (0.2/2.0%) and 50% glycerol over microscope lamina. A light microscope was used to observe and count 500 granules of each sample; the small diameters of the granules were measured using a graduated occular.

Scanning electron microscopy

Transversal slices of banana fruit from several ripening stages were fixed in 0.1M phosphate buffer (pH 7.0), containing 3% glutaraldehyde for 24 hr and then dehydrated (Silva and Luh, 1978). Isolated starch granules were also examined after adhesion to a double-face band. The samples received a gold-palladium coating and were photographed with a Cambridge Stereoscan S4-10 scanning electron microscope using a Telford camera and Kodak PXP 120 film.

Electrophoresis

Electrophoresis was conducted as described by Aréas and Lajolo (1981) except the amylopectin concentration was changed to 0.1%. To detect hydrolase activity, the gels were incubated; for 12 hr at 37°C in either one of the three media: 0.1M citrate buffer (pH 6.5) containing 0.02M glucose 1-phosphate, 2×10^{-4}M $CaCl_2$, 8×10^{-3}M TRIS (first neutralized) and 0.01M cysteine (first neutralized); or replacing $CaCl_2$ by 0.04M EDTA (to inhibit α-amylase); or replacing cysteine by 4×10^{-3} mM $HgCl_2$ (to inhibit β-amylase). To detect the bands, the gels were immersed in a I_2/KI (0.2/2.0%) solution; the clear zones corresponded to amylase bands.

Paper chromatography of sugars

Chromatography was conducted with Whatman No. 1 paper and butanol: acetic acid: water (12:3:5) as descending solvent. Detection was according to Trevelyan et al. (1950).

RESULTS & DISCUSSION

Changes in starch during ripening

Starch degradation began during the preclimacteric phase (Fig. 1A) several days before the onset of the respiratory peak, suggesting a dissociation of the control of both phenomena. The rate of degradation was slow initially but increased as the climacteric period was reached confirming previous results (Aréas and Lajolo, 1981; Terra et al., 1983). Total tissue starch dropped but the amylose to amylopectin ratio did not change (Fig. 1B), indicating the existence of an enzyme system capable of hydrolyzing both α-(1→4) and α-(1→6)-glucosidic linkages and that the enzymes involved had a coordinated action since there was no accumulation of either of the polymeric constituents of starch granules.

Previous reports from the literature (Loesecke, 1980; Mao and Kinsella, 1981) suggested that ripening starts at the ends of banana fruits but our results did not confirm this. In this study of starch disappearance in situ in tissue slices (Fig. 2), starch degradation started at the central core of the fruit advancing toward the outer borders as ripening proceeded. During the early preclimacteric phase, starch was well distributed in the tissue (Fig. 2a, b). Slice c showed the situation at the commencement of starch degradation during the preclimacteric. In slices d, e, and f, starch content had already decreased as a ripening advanced from the central part of the fruit to the surface. Finally, in slice h, corresponding to a postclimacteric, no starch was detected, and the process was completed. The

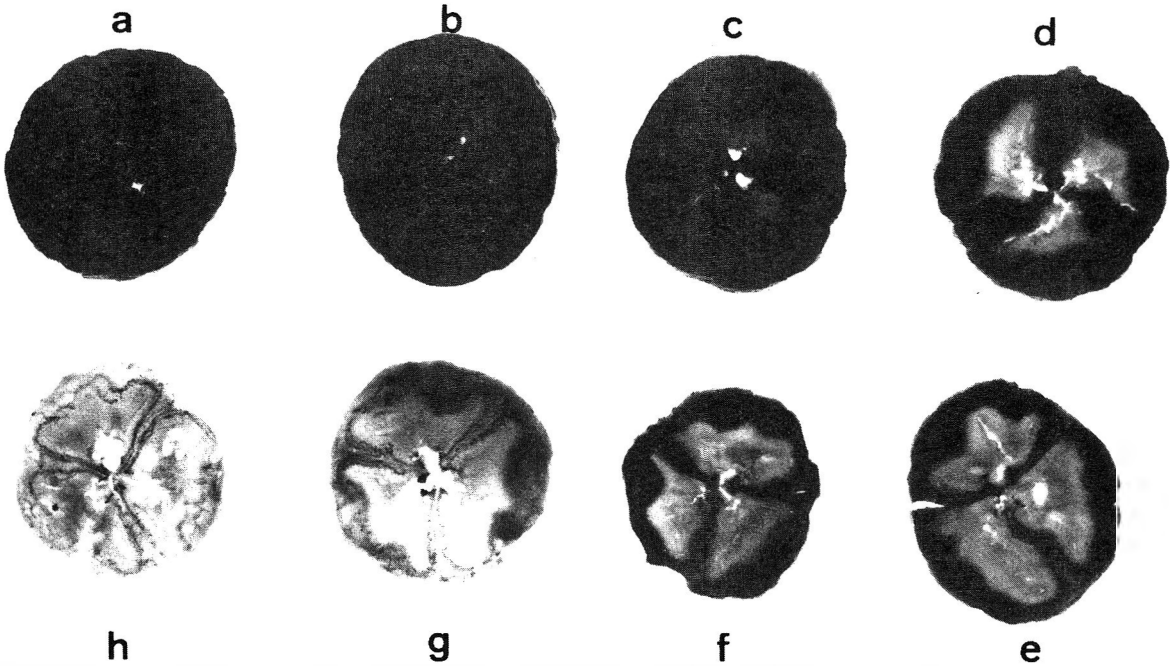

Fig. 2—Transversal slices of banana dyed with iodine. a, b, c preclimacteric fruit samples; d, e, f slices from climacteric bananas; g, h postclimacteric bananas.

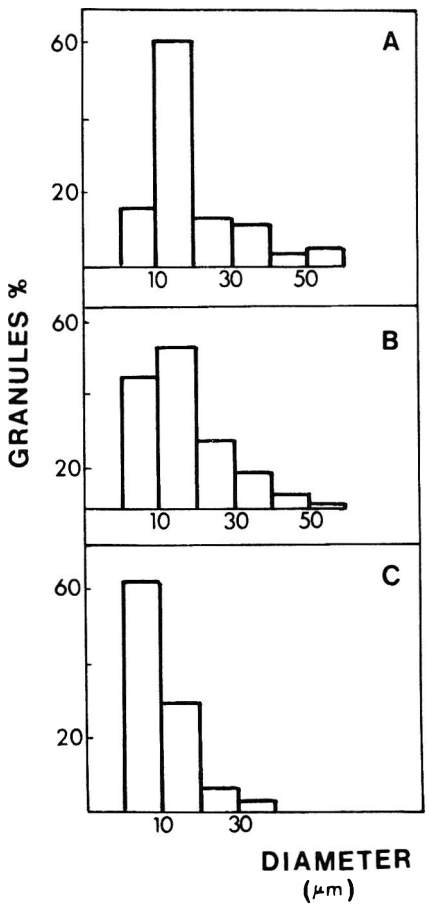

Fig. 3—Starch granules size distribution. Granules isolated from bananas with different starch content: (A) 18.4%; (B) 12.6%, (C) 3.2%.

same pattern was seen in the middle section of the fruit and at 2 cm from both ends. These results also have technological significance relative to processing such as drying fruits and water activity.

The isolated starch granules, observed with the light microscope, showed an irregular shape with predominance of spheroid and oval or elongated forms. The size was also variable; the granule size distribution from three different ripening stages is shown in Fig. 3. In green bananas (18.4% starch), about 60% of the granules were 10–20μ in size; only 15% were smaller (< 10μ). As ripening proceeded, there was an accumulation of smaller granules (less than 10μ) with a great reduction of those with 10–20μ diameter. Similar results were obtained previously by Lii et al. (1982) but at different stages. They reported the disappearance of small granules (< 15μ) when starch content had been reduced by 50% and the attack of the granules in the 20μ range in advanced stages of ripening (less than 2% residual starch). The differences in the two studies could have been related to different methodologies used for the starch granules isolation. Lii et al. (1982) sedimented the granules from the filtered starch slurry; in this work, the slurry was centrifuged. In the Lii et al. (1982) study, some fraction of the small granules could have remained in suspension especially from ripe banana extracts (low starch content).

Scanning electron microscopy of banana tissue (Fig. 4) showed that in the green fruit the dehydrated cells constitute an envelope containing only starch granules; in the ripe fruit, starch decreased, and there were several membrane folds which made the observation of the granules difficult. Eventual superficial granule alteration could be detected only after starch isolation. Isolated starch granules (Fig. 5) from green banana had a smooth surface, while several granules from ripe banana showed striations as observed by Fuwa et al. (1979) and Kayisu et al. (1981).

The electron microscopy observations and especially the size distribution study of the granules indicated that the attack of starch granules by the enzymes was probably an exo-corrosion process from those more susceptible to attack; during the process they were transformed into smaller granules which tended to accumulate before final transformation. More studies are needed to prove this hypothesis.

Changes in enzyme activity

The presence of several hydrolytic enzymes was demonstrated using selective enzyme inhibitors; α- and β-amylases

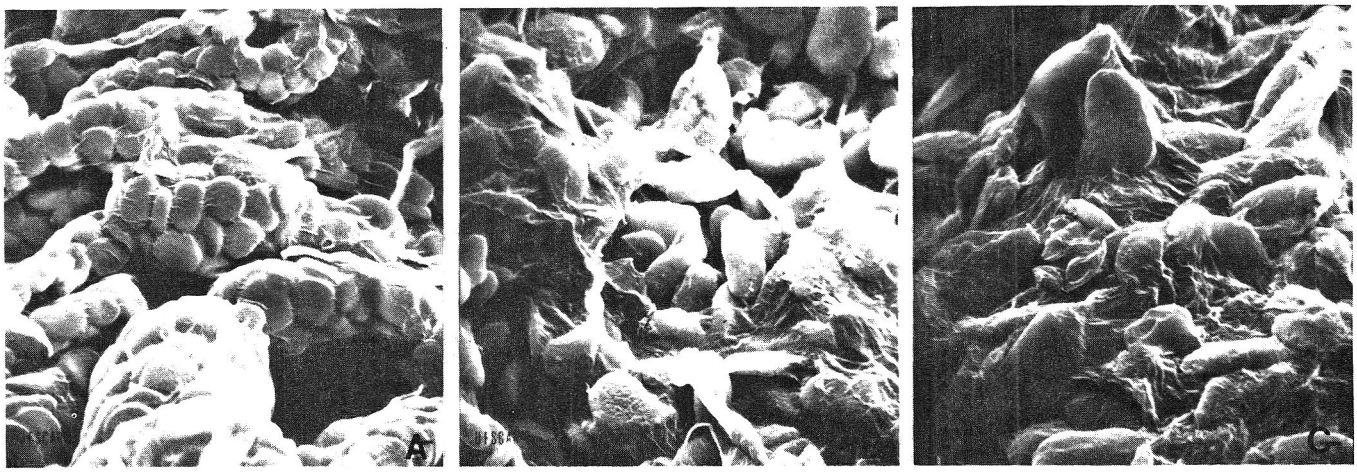

Fig. 4—Microphotographs of banana pulp tissue. (A) Banana with 10% starch (900×); (B) Starch content not determined (800×); (C) Banana with 1% starch (900X).

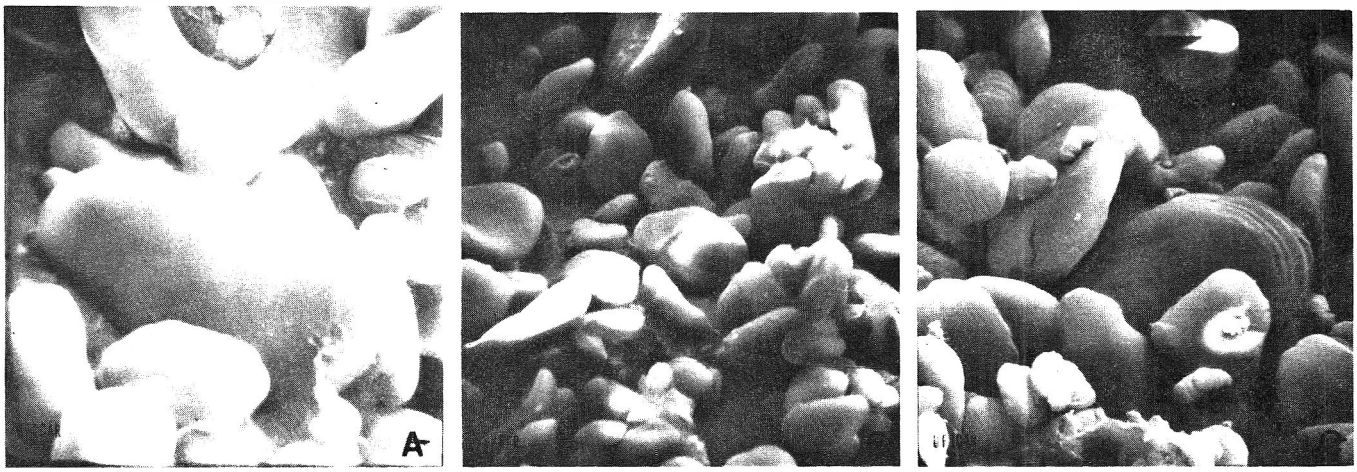

Fig. 5—Microphotographs of banana starch granules. (A) Starch granules isolated from green banana with 18.4% starch (1200×); (B) Starch granules from ripe banana with 3.2% starch.

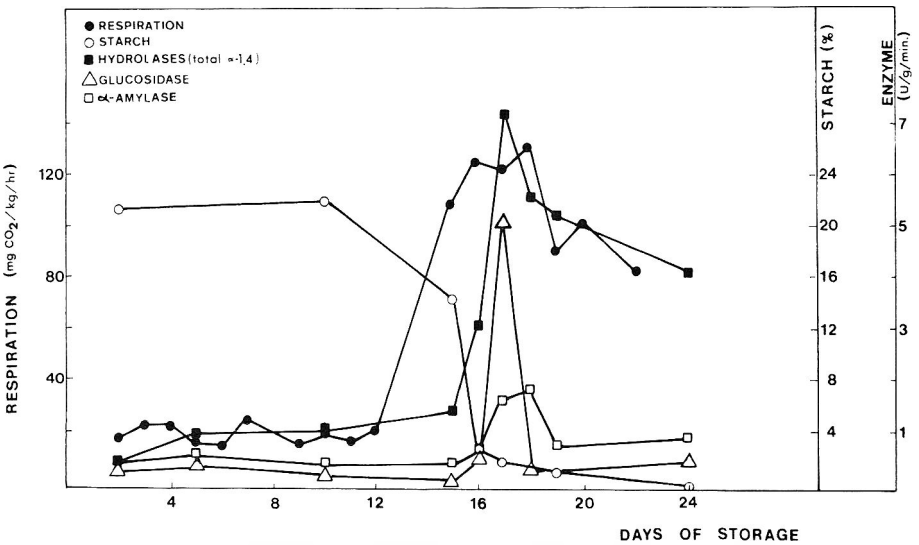

Fig. 6—Starch hydrolytic activity and starch disappearance during the ripening period of bananas.

and also glucosidases showed pronounced changes during ripening. Figure 6 shows one peak of total hydrolytic (α-1,4) activity coincident with the respiratory peak. The maximum α-amylase activity (inhibited by EDTA) preceeded the increase of glucosidase activity (inhibited by TRIS). This difference in timing is logical and can result from previous action of α-amylase-releasing products which could then be substrate for the glucosidases and help to induce them. The sum of α-amylase and glucosidase activities is lower than the total hydrolytic activity (Fig. 6), which suggests that other enzymes not inhibited by EDTA or TRIS can act on Amylochrome or that the concentration of these inhibitors was not enough for total inhibition. We presume that this latter hypothesis could be discarded once the appropriate inhibitor concentration was optimized.

Young et al. (1974) obtained different results detecting an increase of α-amylase activity during the climacteric period. They also found that starch breakdown only started when respiration had already reached two-thirds of the climacteric peak, but the respiration curve they had recorded was flat, suggesting

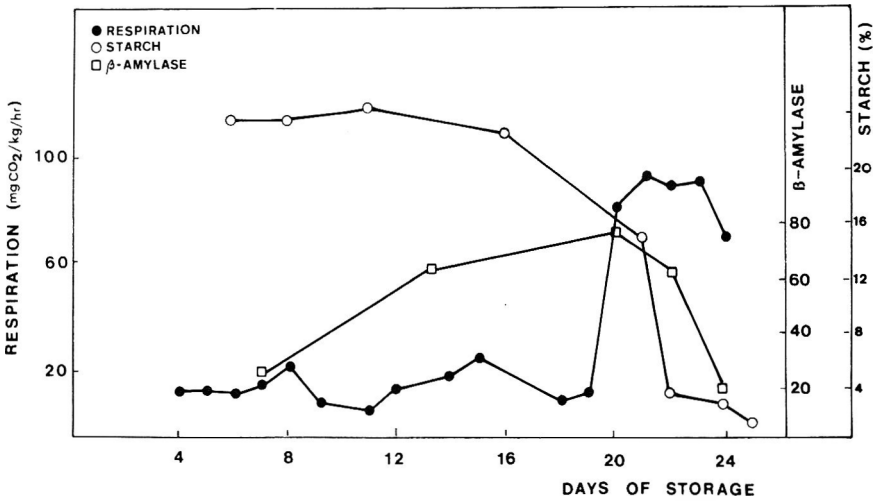

Fig. 7—β-Amylase activity during the ripening period of bananas.

heterogenity of the samples in relation to the ripening stage. Difference in varieties or the fruit history could also be another explanation.

β-Amylase activity was low in green banana (Fig. 7), but it increased significantly (3.5×) simultaneously with starch hydrolysis which started during the preclimacteric phase. The presence of β-amylases was confirmed by paper chromatography of the sugars released from starch (Fig. 8). Maltose was detected in higher concentration than other oligosaccharides (such as maltotriose, maltotetraose) contrary to the results observed with α-amylase reactions which produce maltotriose and other carbohydrates (with more than 3 glucose units) at higher concentration than maltose. The observation that β-amylase activity increased before other hydrolytic enzymes had not been reported previously and was an indication of the importance of this enzyme in the process.

The products resulting from α- and β-amylase activity can probably be attacked by glucosidases which showed increased activity when starch disappearance was more pronounced (Fig. 6). Comparing total glucosidase activity on Amylochrome (Fig. 6) with its activity on p-nitrophenyl-α-glucopyranoside (Fig. 9) indicated the presence of α-1,4-glucosidases with different substrate specificities. The study of the involvement of glucosidases in banana starch breakdown has been neglected since more importance has been given to the amylase and phosphorylase. The presence of glucosidases in bananas has not been mentioned before in the literature, except in a reference to maltase (Glass and Rand, 1982).

Banana starch has around 80% amylopectin (Kayisu and Hood, 1981; Ling et al., 1982; Patil and Magar, 1974), suggesting that debranching enzymes must be present to break α-

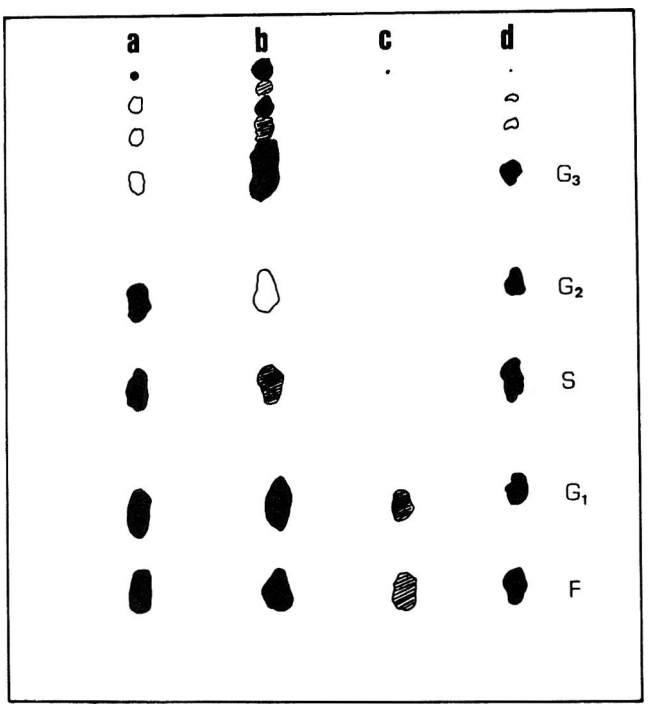

Fig. 8—Sugar chromatogram (a) Released sugars after incubation of enzyme extract (containing EDTA, TRIS and cysteine) with starch suspension; (b) idem with enzyme extract preincubated with TRIS and cysteine; (c) control (sugars present in the enzyme extract); (d) sugar standards; (F: fructose; G_1 = glucose; S = sucrose; G_2 = maltose; G_3 = maltotriose).

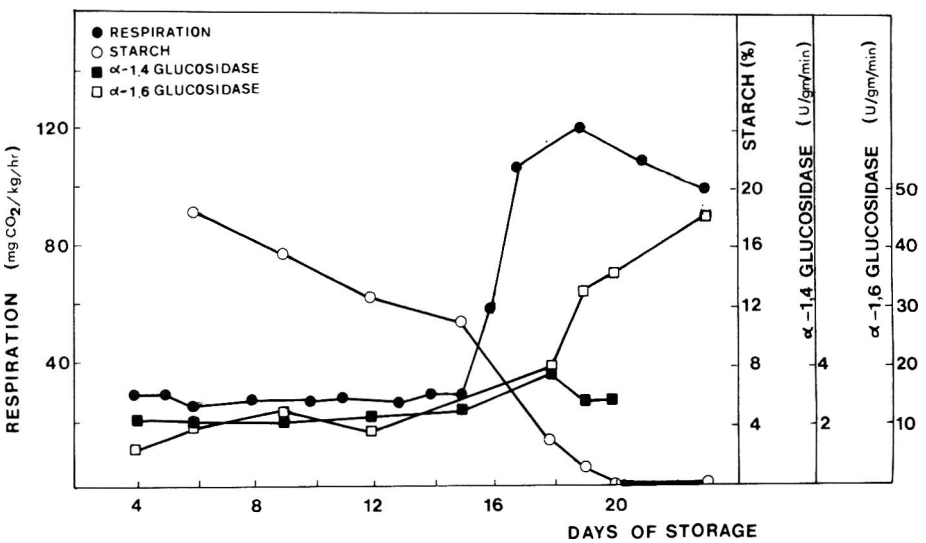

Fig. 9—α1,4- and α-1,6-Glucosidase activity during the ripening of bananas.

1,6-glucosidic linkages. Isomaltose activity (Fig. 9) was detected for the first time in banana fruit. Probably other α-1,6-glucosidases important in limit dextrins hydrolysis also exist.

By gel electrophoresis, it was possible to detect seven bands with starch hydrolytic activity (Fig. 10). All the gels were incubated in citrate buffer in the presence of glucose 1-phosphate to avoid phosphorylase activity in the direction of starch degradation. Young et al. (1974) have described seven enzymes, including two α-amylases and two β-amylases. The electrophoretic pattern (Fig. 10) indicates that the number of enzymes (bands) didn't change during ripening and also that there was an increase in activity of all bands. Data are insufficient to conclude if the increase in activity during ripening was due to amylase synthesis or de-inhibition of pre-existent enzymes.

Probably, two degradative pathways are active during starch breakdown: one in the preclimacteric phase contributing to the main decrease of starch and, the second in the climacteric period, concomitant with all the other well known changes that characterize this phase and that either phosphorolysis or hydrolysis probably takes place at different times.

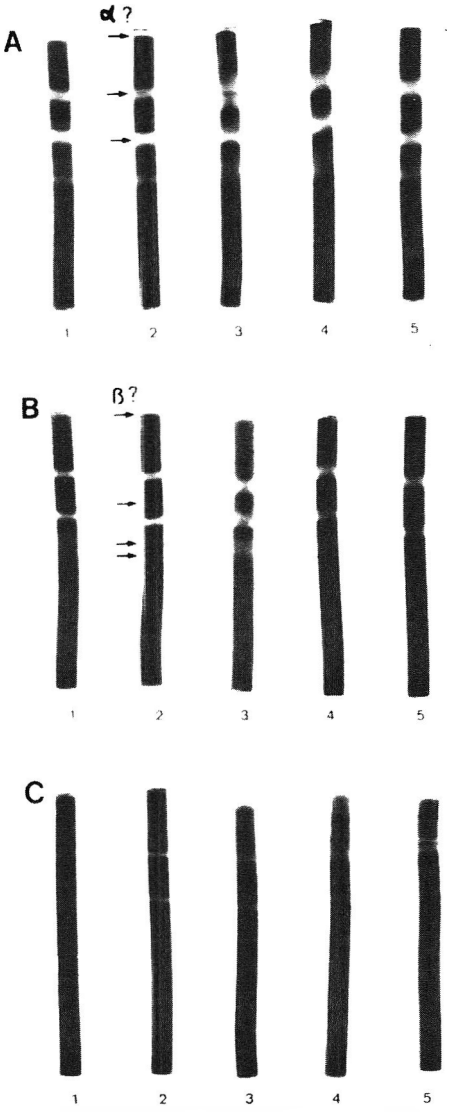

Fig. 10—Acrylamide gel electrophoresis of banana enzyme extracts from different ripening stages: column 1 preclimacteric banana; columns 2 and 3 climacteric fruit; columns 4 and 5 postclimacteric banana. (A) Gels incubated in media which allowed the development of total hydrolytic activity on amylopectin; seven bands were detected. (B) Gels incubated in the presence of EDTA to avoid α-amylase activity; four bands can be observed. (C) Gels incubated in the presence of $HgCl_2$ to avoid β-amylase activity, three bands are active.

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Effect of Blanching and Drying on Pectin Constitutents and Related Characteristics of Dehydrated Peaches

AHARON LEVI, NOACH BEN-SHALOM, DAVID PLAT, and DAVID S. REID

ABSTRACT

The effects of sulfuring, blanching, dehydration, and storage (of dehydrated fruits), on the pectic constitutents and other characteristics of peaches are reported. Adequate blanching stabilized pectins of the dehydrated peaches so higher rehydration capacity and lower rehydration losses were observed after 5 min blanching. Increase in degradation was observed in nonblanched or 15 min heat-treated fruits, which resulted in lower rehydration capacity. A significant correlation was found between the contents of total pectin and protopectin fractions and the firmness or rehydration ratio of the peaches. Thus, pectin, one of the major cell-wall and intercellular tissue components, plays a significant role in determining the textural-structural characteristics of dehydrated fruits.

INTRODUCTION

WHEN PLANT TISSUE is heat treated during an industrial process, (blanching, cooking, dehydration, etc.), some physicochemical changes of the structural constituents of the cell wall (CW) and intercellular (IC) tissue are observed. The resulting textural and other behavioral characteristics of the processed tissue, would be different from those of the untreated plant tissue.

Pectins, hemicellulose, and cellulose are among the main water-binding components of the plant CW in particular and plant tissue in general. The degradation and molecular configuration of the pectins could cause structural-textural breakdown, resulting in variations in firmness, water liberation or binding, tissue disintegration, etc. Pectins are also the most susceptible to enzymatic and heat-induced degradation and configuration changes. Natural differences or induced changes in their physicochemical characteristics would affect the plant CW and IC tissues, including their water-holding capacity (Parrott and Thrall, 1978).

Buescher and Furmansky (1978), reported that reduced pectinesterase and polygalacturonase activities, were associated with reduced juiciness, reduced level of soluble pectins, poor texture and enhanced levels of insoluble pectins in peaches ripened after storage at 1°C. Luh et al., (1978) reported changes in firmness of canned apricots during storage, due to pectin degradation catalyzed by added polygalacturonase (PG) of mold origin. Levi et al. (1980) reported variation in water removal, with increasing blanching time, during dehydration of sulfited bananas. Heat treatments also affect the permeability of the cellular membranes and the CW (Rostein and Cornish, 1978), parallel with the water-binding capacity of plant fibrous materials (Parrot and Thrall, 1978). Levi et al. (1983) found that the dehydration velocity of papaya was affected by blanching and by the dehydration temperature. Mrak and Perry (1948) reported that blanched peaches took less time to dehydrate.

The amount of the pectic substances in fresh peaches was reported to be about 0.45% (Vidal-Valerde et al. 1982). The soluble pectin fraction in the peach tissue increased dramatically during ripening (Pressey et al., 1971). PG activity was not found in unripe peaches, but developed during ripening, parallel to the formation of soluble pectins whose molecular weight decreased progressively during the ripening (Pressey and Avants, 1978). According to Shewfelt (1965), pectinesterase (PE) was present in the peaches at all stages of ripening, therefore, PE activity did not appear to be a limiting factor for pectin solubilization during ripening. Postmayr et al. (1956), found that heat processing (canning for 18 min or more) affected both the texture and the pectin content of the canned peaches. Assessing the pectic fractions in the homogenized slurry of canned freestone peaches, they reported a decrease of the total pectin (TP) and the protopectin (PP), and an increase of the water soluble pectin (SP) as a percentage of the TP.

The basic commercial process steps for freestone peach dehydration in Israel are: washing; halving (or cutting in 4); removal of stones; drying—sun or forced air (3 hr at 70°C + 8–12 hr at 50°C); or combination of both, until the desired moisture content is reached. The process does not include heat-pretreatment (blanching). The producers claim that no clear advantage of the 'blanch' is expected, while a loss of solids is observed, reducing the production yield. On the other hand, adequate blanching would improve some quality characteristics of the dry product, facilitating water removal and inactivating undesirable enzymes.

The objective of this study was to assess the effects of heat pretreatment, sulfuring, dehydration and storage on the content of the major fractions of pectin, the rehydration capacity and the rehydration losses of dehydrated peaches, and the firmness of blanched peaches.

MATERIALS & METHODS

Pretreatments and dehydration

About 5 kg firm-ripe peaches cv 'Sommerset' (freestone), were cut in half and their stones removed manually, for each experimental treatment. The peach halves were subjected to heat pretreatment (blanching) in a steam blancher at atmospheric pressure for 0 to 15 min, and then immediately cooled to 30°C with gentle tap water sprays. The peach halves were sulfured in a sulfuring (SO_2 fumes) cabinet for 2½ hr. Dehydration was done in a cabinet-dryer with flow-through air, on perforated aluminum trays for 3 hr at 70 ± 5°C, followed by 7–10 hr at 50 ± 5°C, to >82% dry matter (DM).

One part (about 250g) was analyzed within 2 weeks of preparation and the rest was stored for one year at ambient temperature.

The experiments were repeated three times with peaches of the same variety, and the results represent the trends observed in all the experiments.

Rehydration and cooking

The rehydration capacity of the dehydrated peaches was assessed as follows: 50g dehydrated peach halves were cut in small (1 cm^3) pieces and submerged in 500 mL distilled water for 24 hr at room temperature (23 ± 2°C). The residual water was removed, the adhering water absorbed carefully with tissue paper and the peaches weighed. The rehydration weight was expressed as the percent of the initial weight of the dehydrated peaches (expressed as 100%). Data are expressed as the arithmetic average values of three replications for each blanching time.

Authors Levi, Ben-Shalom, and Plat are affiliated with the Dept. of Food Science, Agricultural Research Organization, The Volcani Center, P.O. Box 6, Bet-Dagan 50250, Israel. Author Reid is affiliated with Dept. of Food Science & Technology, Univ. of California, Davis, CA 95616.

The significance of the difference between the rehydration weights (vs blanching time) was assessed by the range method (Kramer and Twigg, 1962).

To assess the rehydration effect on cooking, the dehydrated peaches were submerged for 30 min in boiling water and treated as above.

The DM losses during the rehydration, were calculated by the following equation:

$$DML\ (\%) = \frac{W_o DM_o - W_1\ DM_1}{W_o DM_o} \times 100$$

where W_o = Weight of dehydrated sample; W_1 = weight of rehydrated sample; DM_o = DM content of dehydrated sample; DM_1 - DM content of rehydrated sample; DML = Dry matter loss during rehydration.

The significance of the difference between the rehydration losses was assessed as described above (for the rehydration weights).

Firmness

Five cubes (1 × 1 × 1 cm each) were compressed at the same time with an Instron model TM 1026 instrument to 50% of their height, at 5 cm/min velocity. The instrument was calibrated with a full scale load of 50 kg. The force applied to compress the peach cubes was calculated at the peak of the compression curve in Newton-force units. Data are expressed as the average of three separate compressions for each experimental treatment. The linear correlation between the firmness, blanching, and pectin content (r = correlation coefficient: p = significance of correlation) was assessed with a Texas Instrument TI-55-II calculator (built-in program).

Pectin fractionation and analysis

Alcohol insoluble solids (AIS) were prepared within 1 hr from the fresh, or treated nondehydrated peaches, and within 1–2 weeks or one year from the dehydrated ones. The AIS were extracted by homogenizing 250–300g nondried or 50g dried peaches in a Waring Blendor, four times in succession, with 300 mL 70% ethanol each time, followed by a fifth extraction with 100 mL 100% acetone at room temperature (23 ± 2°C). The AIS content of the peaches was calculated by the following equation:

$$AIS_t = \frac{AIS_i \times 100}{W_1}$$

AIS_t = g alcohol insoluble solids on 100g analyzed sample; AIS_i = AIS of the sample; W_1 = weight of the sample, in grams. When stated, the AIS content was calculated on a fresh matter basis. The soluble pectin (SP) was extracted at room temperature (23 ± 2°C) from the AIS as follows: 0.2g AIS was vigorously stirred for 10 min with 20 mL distilled water, then centrifuged at 27,000 × *g* for 20 min. This procedure was repeated three to seven times with the pellet until no galacturonic acid (GA) was detected (see below) in the supernatant. The supernatants were pooled. The pellet was subjected to calcium pectate (CaP) extraction, as follows: 25 mL of a solution containing 0.1M buffer TRIS/HCL and 0.2% EDTA at pH 6.2 were added to the pellet, stirred, and centrifuged 4 to 7 times until no GA was detected in the last supernatant. The supernatants were collected for CaP determination. The final pellet was extracted for protopectin (PP) with 0.05 N NaOH (pellet:NaOH = 1:50) and the supernatant analyzed for GA. The amount of each pectic fraction was assessed colorimetrically (Blumenkrantz and Asboe-Hansen, 1973) and calculated as GA (on the AIS content of 100g sample) by the following equation:

$$PF = \frac{PF_1 \times AIS_t}{0.2}$$

PF = content of pectic fraction in 100g fruit tissue, PF_1 = content of pectic fraction in 0.2g AIS, analyzed; AIS_t—see above. When assessing the correlation between the rehydration weights, the weight losses and the firmness vs the pectin content, the actual pectin contents of the peaches (fresh or dehydrated) are given. The pectin fractions are expressed as a percentage of the total pectin (TP) content of the fresh (untreated) peach halves. Total pectin (TP) is the added value of the PP + CaP + SP fractions.

The significance of the difference (p) between the means (arithmetic average values of two duplicates for each experimental condition) of the pectin constituents of each pectic fraction after the blanching (0, 5, 15, min) and the treatments (I, II, III) was calculated by Analysis of Variance (Kramer and Twigg, 1962).

The PE activity was determined by the method of Levi et al., (1980).

No PE activity was detected after blanching of the peaches for 5 min or longer.

The linear correlation (r) between the pectin content and the respective RW (rehydration weight) was calculated as described for the firmness.

Table 1—Effect of blanching and sulfuring[a] on the dry matter (DM) and alcohol insoluble solids (AIS) contents of peaches

Blanching time (min)	Dry matter (%)	Alcohol-insoluble solids[b] (%)
0 A[d]	17.2	2.24
0 B	17.8	2.20
5 A	15.2	2.42
5 B	15.9	2.48
15 A	14.2	2.19
15 B	14.5	2.12
r (correlation coefficient)	0.926[c]	0.39[c]
p (significance of correlation)	0.01	NS

[a] After blanching
[b] On fresh fruit basis
[c] Between blanching time and DM or AIS respectively
[d] A and B – samples from different batches of peaches

RESULTS

Pectins

Water and alcohol soluble solids (ASS), such as sugars, acids, GA monomers, short oligomers, etc', were removed from the tissue during the blanching-cooling and sulfuring (dripping was observed during the sulfuring) process steps. The DM and AIS in two of the experiments, are shown in Table 1. A significant decrease ($p = 0.01$) in the DM content (with the increase of the blanching time) was caused by the above removal as well as by water penetration into the fruit tissue during the cooling, while nonsignificant changes in the AIS, were observed in the blanched sulfured peaches. Although no significant difference ($p > 0.05$) was found between the blanching or the DM and the AIS, the highest value observed was that of the 5 min blanched peaches.

The arithmetic average (two replicates) of the pectin content determination of one of the batches after the different pretreatments, dehydration, and storage are given in Fig. 1 and the mean values and statistics in Table 2. The total pectin content (TP) (Fig. 1) of fresh untreated peaches was 509 mg/100g, expressed in Fig. 1 as 100%, which is the base for all the contents given in Fig. 1. Similar trends although of different magnitude were observed in the other experiments.

The effect of the blanching and/or the various treatments (I, II, III) on the TP was highly significant ($p < 0.01$). A highly significant difference ($p < 0.01$) was observed between the TP of the treatments (I vs II vs III), as well as of the differently blanched (0 vs 5 vs 15 min) peaches, probably because of enzymatic and/or heat induced degradation.

Water-soluble pectin (SP), was the major pectic fraction found in the peaches, comprising ca 60% of the TP of the fresh fruit.

Blanching and/or treatments significantly affected ($p < 0.01$) the SP. Enzymatic, and heat (drying) induced-degradation was probably the reason for the decreasing values ($p < 0.01$) due to the treatments of the nonblanched (0 min) peaches (I vs II vs III). Heat induced degradation was probably the reason for the significant decrease ($p < 0.01$) in the SP after dehydration (II) of the blanched (5 or 15 min) fruits, while the increase ($p < 0.05$) after storage (III) of the 5 min blanched (Fig. 1), could be as a result of degradation of other pectic fractions (Cap, PP) to SP. A significant decrease ($p < 0.01$) in the SP was observed due to the blanching (Table 2).

Postmayr et al. (1956) found an increase in the SP after canning of peaches, while a decrease in SP was now observed.

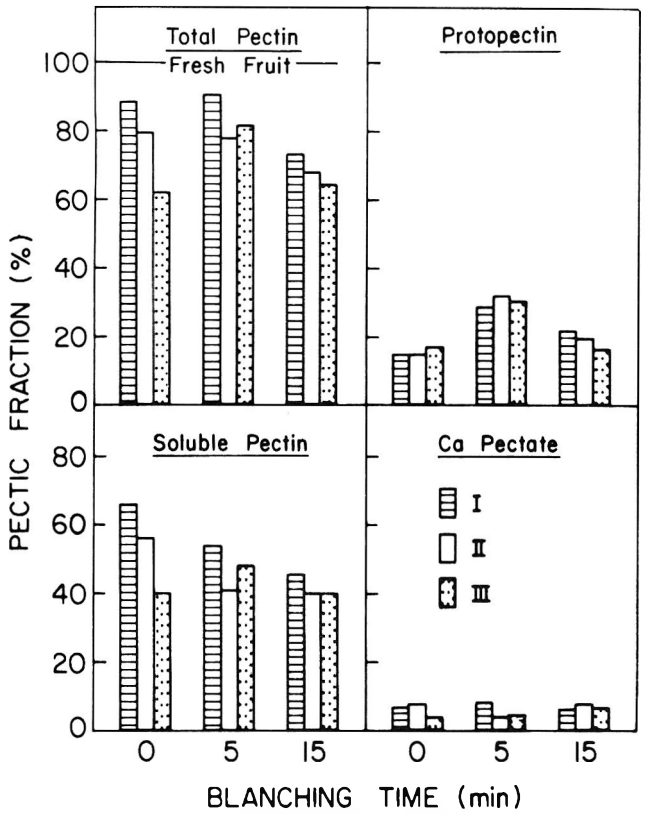


Fig. 1—Effect of blanching time, sulfuring, dehydration, and storage on the pectin content of dehydrated peaches (on a fresh matter basis; TP of fresh untreated fruit = 509 mg/100g fresh tissue). I, Sulfured (no dehydration); II, I + dehydration; III, II + storage for 1 year.

Table 2—Effects of blanching, sulfuring, dehydration, and storage on the pectin[a] content of dehydrated peaches—statistical data

	Mean Values (mg/100g fresh)		
	Soluble pectin	Protopectin	Total pectin
Treatments	(LSD[b] = ±11.5)	(LSD = ±5.5)	(LSD = ±12.9)
I-Sulfured (after blanching)	284.2[c]	110.5[c]	430.1[c]
II-I + dehydration	232.9[d]	113.9[c]	380.8[d]
III-II + storage	217.6[e]	108.9[c]	357.5[e]
p-significance of correlation	< 0.01	> 0.05	< 0.01
Blanching time-min			
0	275.4[c]	79.8[e]	392.7[d]
5	246.5[d]	153.0[c]	426.7[c]
15	212.8[e]	100.3[d]	348.5[e]
p-significance of correlation	< 0.01	< 0.01	< 0.01

[a] Total pectin in fresh untreated peaches—509 mg/100g. No significant difference was observed between the CaP content after the above treatments and/or blanching. (See also Fig. 1.)
[b] Least significant difference at the 5% level.
[c,d,e] Values with different superscripts are significantly different at the 5% level.

This apparent contradiction could be explained by: (a) the different analytical procedure used; (b) the SP extracted by the sugar syrup (on canning) was included in the homogenized slurry, but some of it could have been removed during the blanching-cooling and the dehydration (dripping) steps.

CaP (Fig. 1C) was found to be a minor fraction of the fresh peaches; about 16% of the TP. Only sulfuring caused a marked ($p<0.01$) decrease in its content (0 min I) probably as a result of pectolytic enzyme activity.

The effect of the blanching and/or the other treatments (I vs II vs III) on the CaP was not statistically significant. Dehydration and storage (II and III vs I) caused a marked decrease in the 5 min blanched but an increase in the 15 min blanched

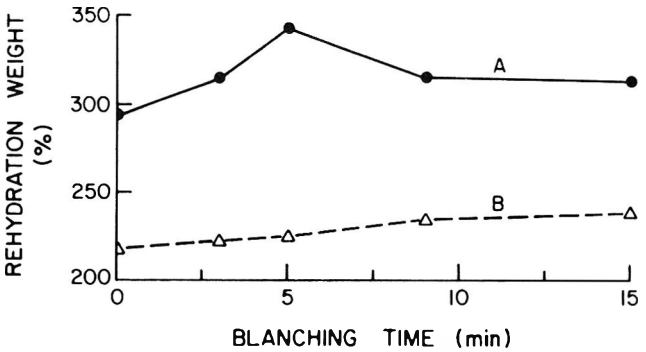


Fig. 2—Effect of blanching time on rehydration at room temperature (A) and cooking in boiling water (B) of sulfured dehydrated peaches (Treatment II - see Fig 1). ($p_A < 0.05$: $SD_A = \pm 0.84$; $LSD_A = \pm 3.44$; $p_B < 0.01$; $SD_B = \pm 1.025$; $LSD_B = \pm 3.61$).

(vs the 5 min). CaP formed between degradation products of the other pectic fractions, with exogenous (cooling tap water) or endogenous Ca, could be the reason for the nonsignificant increase observed in this case.

The PP fraction is probably the most stable and it is also interconnected with hemicellulose in the fruit tissue. It represented about 25% of the TP in the fresh peaches.

Blanching significantly affected the PP fraction ($p<0.01$). The PP content of the 5 min blanched peaches was significantly higher than that of the 0 or the 15 min blanch. Storage (I, II vs. III) did not affect ($p>0.05$) the PP content.

The PE activity in different batches of the fresh peaches was between 3.3 and 4.5 μmol/g min and in dehydrated unblanched fruits between 14.2 and 17.4 μmol/g min.

Water-absorption capability

The effect of blanching duration on the rehydration weight in one of the experiments (100% = weight of dry peaches before rehydration) at room temperature and on cooking is shown in Fig. 2. Similar trends were observed in the other experiments. The only sulfured (0 min) dry peaches absorbed about twice their weight in water.

The effect of the blanching on the rehydration weight (RW) at room temperature (23±2°C) (Fig. 2A) was significant ($p<0.05$). The 5 min blanched dehydrated peaches absorbed significantly ($p<0.01$) more water than those from the other treatments. No significant ($p>0.05$) difference was found between the RW of the 3, 9 and 15 min blanch. Those were significantly ($p<0.05$) higher than the RW of nonblanched (0 min) peaches. The lower rehydration values following cooking (Fig. 2B) were probably due to heat-caused disintegration of the peach tissue and an increase in the diffusion of the water-soluble solids to the surrounding medium (water). An increase in the blanching time caused a significant ($p<0.05$) increase in the RW. No significant difference ($p>0.05$) was observed between the RW of 9 or 15 min blanch or between 0, 3, and 5 min blanch. Different behavior, both at room and at boiling temperature (cooking) rehydration was observed in the dry peaches stored for 1 year (Fig. 3). In any event, the adequately (5 min) blanched peaches absorbed more water ($p<0.01$) than peaches from the other treatments. Lower water absorption was observed in the nonblanched (0 min) peaches (active enzymes) and in those after 9 or 15 min blanch, probably due to (the heat-induced) pectin degradation during storage.

The integrity of the peach tissue, as well as the diffusion of non-bound soluble solids from it to the surrounding medium, also affects the total solids (DM) losses during rehydration (Fig. 4).

The DM loss on rehydration of the 5 min blanched dry peaches was significantly lower ($p<0.01$) than that of the non-

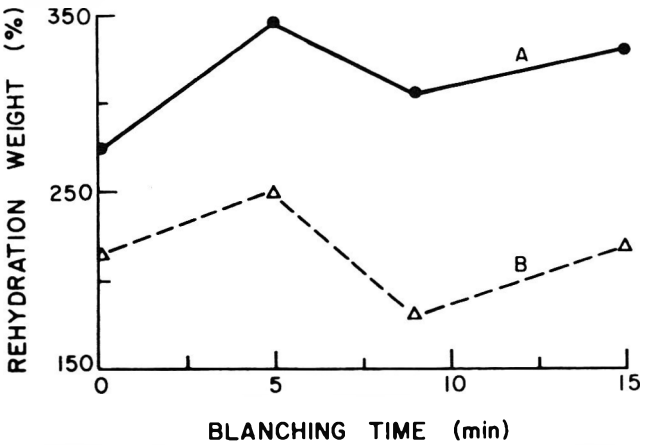


Fig. 3—Rehydration capacity after 1 year storage of blanched, sulfured, dehydrated peaches. (Treatment III - see Fig 1). ($p_A<0.01$; $SD_A = \pm 1.73$; $LSD_A = \pm 6.57$; $p_B<0.01$; $SD_B = \pm 2.25$; $LSD_B = \pm 8.5$). A-Rehydration at room temperature; B-Rehydration in boiling water for 30 min.

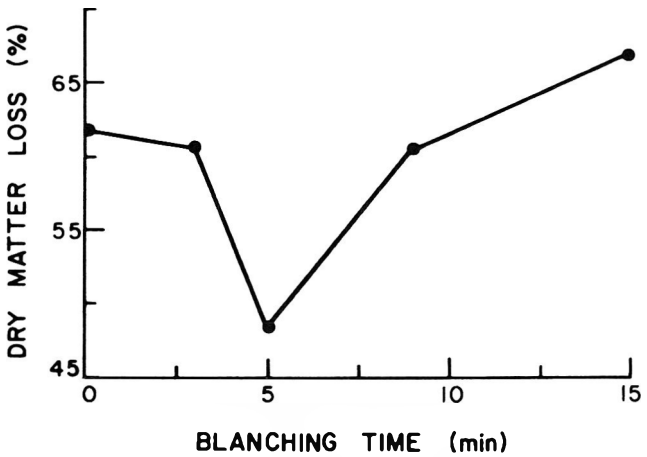


Fig. 4—Dry matter losses during rehydration at room temperature of (treatment II-see Fig 1) blanched, sulfured, dehydrated peaches (weight of dehydrated peaches = 100%). (p = 0.05; SD = ±4.025; LSD = ± 13.5).

blanched (0 min), or longer time (9 or 15 min) blanched. The 15 min blanched peaches lost significantly more ($p<0.05$) DM, than the 0, 3 or 9 min blanched.

Similar trends, although of different magnitudes were observed in both the RW and DM loss in all the experiments. Higher RW and lower DML were observed in the 5 min blanched dehydrated peaches.

Pectin, rehydration, and firmness

In order to determine whether pectins indeed play a significant role in the rehydration capacity of dried fruits, the correlation between the rehydration weight and the pectin content of the dehydrated peaches (Fig. 1, treatment II) was assessed (Table 3). Significant correlation was observed between the TP or PP content and the rehydration weight at both room temperature (23±2°C) and boiling water (100°C). No significant correlation ($p>0.05$) was found with the CaP or SP, indicating that pectins play a major role in the rehydration capability of the dehydrated fruits, while PP is the major fraction affecting the rehydration.

A decrease in firmness due to heat treatments (blanching) of plant tissue is well documented. A significant ($p<0.05$) correlation was found between blanching time and firmness, and between firmness and TP content of the blanched peaches

Table 3—Rehydration weight and pectin content of sulfured dehyorated peaches

Blanching time[a] (min)	Rehydration wt[b] (%)		(1) Total pectin (mg/100g)	(2) Protopectin (mg/100g)
	(a) Room[c]	(b) 100°C[d]		
0 A	277(50.6)[e]	–	1780[f]	450[f]
15 A	294(53.7)	218(39.8)	2428[g]	512[g]
15 B	314(66.7)	220(46.7)	2482[g]	593[h]
5 A	330(60.3)	–	2628[h]	731[i]
5 B	347(73.7)	250(53.1)	2781[i]	1135[j]
	(a) vs (1)	*(a) vs (2)*	*(b) vs (1)*	*(b) vs (2)*
r-(correlation coefficient)	0.9097	0.9282	0.9975	0.9979
p-(significance of correlation)	<0.05	0.01<p<0.05	0.05	0.05

[a] A and B – samples from different batches of peaches.
[b] Weight of non-rehydrated fruits – 100%.
[c] After 24 hr at room temperature (23±2°C).
[d] After 30 min in boiling water.
[e] In brackets are the percent recoverec weight on fresh fruit weight basis.
[f-j] Means with different superscripts are significantly different ($p<0.05$).

Table 4—Pectin degradation and firmness of blanched (non sulfured) peaches

Blanching time (min) (1)	Firmness (Newtons) (2)	Total pectin[a] (mg/100g fruit tissue) (3)
0	21.6[d]	510[d]
5	15.5[b]	454[b]
15	7.8[c]	349[c]
	(1) and (2)	*(2) and (3)*
r (correlation coefficient)	0.9992	0.9984
p (significance of correlation)	0.05	0.05

[a] Actual content in nondried peaches.
[b,c,d] Means with different superscripts are significantly different ($p<0.05$).

(Table 4). This indicates that softening of peach tissue caused by heat treatments is probably connected with degradation of pectin.

Discussion

Pectic substances are more susceptible to enzymatic or heat induced chemical degradation, than other polysaccharide constituents of the CW and the IC tissue. They influence the water-holding/absorbing capacity of plant tissue, forming gels with sugar, acid, and water under certain conditions. The natural content, configuration, and enzymatic or heat induced changes of the pectins affect the structural-textural characteristics of processed plant tissue, in general, and of the pretreated and dehydrated peaches in this case.

Pectolytic enzymes are responsible for degradation of pectins. Exo-PG would remove terminal GA monomers and oligomers from their macromolecule, thereby reducing the respective contents of different pectic fractions, while endo-PG degrades the macromolecular pectin structure very effectively—to smaller fractions. Similar degradation, although by different mechanism, would occur after heat treatment. The pectin degradation should lead to a weakening of the CW, with consequent changes in tissue behavior. The degradation of the PP, would also cause transfer to SP. Reactions of SP fractions with endogenous, or exogenous (from the cooling tap water, for example) Ca, would form CaP. Therefore the separated major fractions of SP and CaP after blanching, sulfuring, dehydration, and storage (Fig. 1), are not necessarily the same observed in the fresh peaches. Column fractionation of each of the major pectin fractions confirmed the above; the results are being prepared for publication.

The main degradation to monomers and oligomers (which are not AIS), was probably caused by pectolytic enzymes (0 min blanch), which reduced significantly the PP and the TP (Fig. 1). A short (5 min) blanch inactivated the PE, without causing significant heat-induced degradation to ASS, therefore

—Continued on page 1203

Cherimoya (*Annona cherimola* Mill,) Polyphenoloxidase:Monophenolase and Dihydroxyphenolase Activities

MARINA MARTINEZ-CAYUELA, LUIS SANCHEZ DE MEDINA, MARIA JOSE FAUS, and ANGEL GIL

ABSTRACT

Polyphenoloxidase (PPO) from cherimoya epicarp catalyzed the hydroxylation of monphenols like L(-)tyrosine, tyramine, and p-cresol (monophenolase activity), and oxidation of o-dihydroxyphenols like catechol and L-DOPA (dihydroxyphenolase activity). The hydroxylation of monophenols occurred after a lag period which was shortened by diphenols. The Km values indicated a low affinity of PPO to the substrates. Aliphatic mono- and dicarboxylic acids and sugars did not show any inhibitory effect on PPO cherimoya epicarp. Cysteine and mercaptoethanol, but not ascorbic acid, appeared to be protective agents of PPO cherimoya epicarp.

INTRODUCTION

ENZYMATIC BROWNING of fruits is due to the appearance of colored pigments originated by PPO enzyme action (Weaver and Charley, 1974; Cheftel and Cheftel, 1976). PPOs catalyze two different reactions: (1) monophenol hydroxylation to diphenols, in the presence of oxygen and a reducing agent (monophenolase activity) and (2) corresponding diphenol oxidation, also in the presence of oxygen, to o-quinones (dihydroxyphenolase activity); the spontaneous polymerization of quinones produces the dark-colored melanins (Marbach and Mayer, 1975; Cheftel and Cheftel, 1976; Kahn and Pomerantz, 1980). PPOs from several plant sources show both monophenolase and dihydroxyphenolase activities; however, in other vegetable tissues only the latter activity is present (Montgomery and Sgarbieri, 1975; Mayer and Harel, 1979).

Monophenol hydroxylation requires a reductant; in the absence of an exogenous reductant, this reaction is characterized by an initial lag period (Kahn and Pomerantz, 1980; Kahn, 1983). Ascorbate, cysteine and NADH have been used as reducing agents because they can either reduce or abolish the lag period (Mayer and Harel, 1979; Golan-Goldhirs and Whitaker, 1984); diphenols also have a similar effect (Mayer and Harel, 1979).

There are several compounds in plant tissues that could, naturally, modulate PPO activity; some catechins, for instance, are phenolic derivatives, found in great quantity in ripe fruits interfering with the natural substrates of PPOs (Van Buren, 1971). Organic acids and sugars can also be implicated in the *in vivo* activity of fruit PPO, since they decrease and increase, respectively, during the ripening, of fruits (Ulrich, 1971; Whiting, 1971).

Preincubation of some PPO in the absence of substrate with thiols or reducing compounds, sometimes causes inactivation of these enzymes (Golan-Goldhirs and Whitaker, 1984). These observations suggest that these compounds may be used in the prevention of food browning instead of sodium bisulfite considered the adverse side effects of the latter (Cash et al., 1976; Kahn, 1983).

Cherimoya (*Annona cherimola* Mill.) is a climateric fruit, named "custard apple" in Anglo-Saxon countries and "guanabana," "anon", and "atemoya" in various South American regions, of economical importance in Spain. Cherimoya is growing in restricted areas of the South coast of Andalucia, being exported to all Europe and other developed countries. One of the most important problems in the marketing of this delicate and soft fruit is the rapid ripening and browning occurring a few days after harvesting.

The aim of this work was to study the two activities of PPO from cherimoya epicarp. The influence of small amounts of diphenosis on the monophenol hydroxylation of cherimoya PPO was also studied, and the effects of some organic acids and sugars on its activity were determined. Finally, the influence of some thiol and reducing compounds, such as ascorbate, cysteine, and mercaptoethanol, on cherimoya PPO activity were evaluated.

Authors Martinez-Cayuela, Sanchez de Medina, Faus, and Gil are with the Depto. de Bioquimica y Biologica Molecular, Facultad de Ciencias, Univ. of Granada, Poligono Fuentenueva, s/n, 18071 Granada, Spain.

MATERIALS & METHODS

PPO purification

Cherimoya fruits, of the *impressa* cultivar, picked at the ripening stage, were immediately used for PPO extraction.

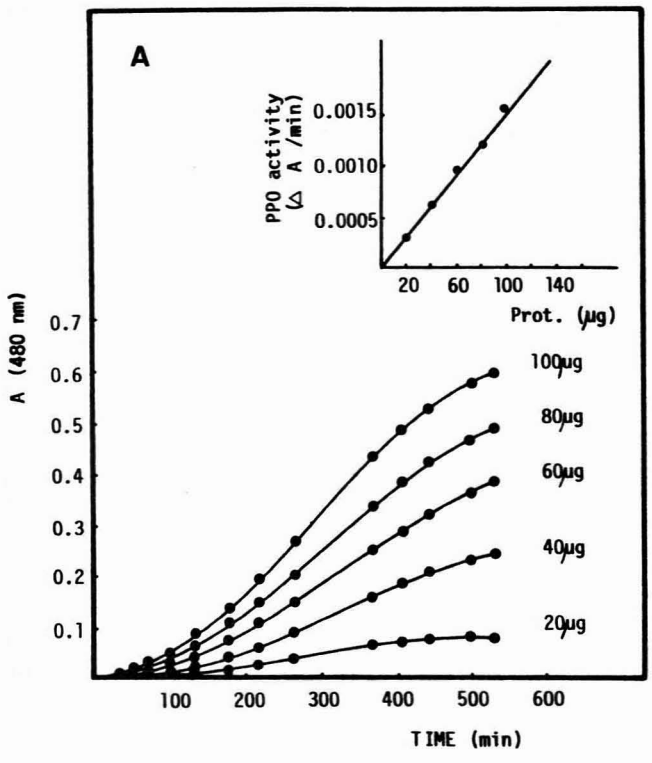
Twenty grams of cherimoya epicarp were homogenized with 200 mL cold acetone (−20°C) in a Sorvall Omni-Mixer for 5 min at high speed (Pos. 8). The homogenate was immediately filtered on a filter paper and washed with 200 mL cold acetone (−20°C) and 200 mL cold ethyl ether (−20°C); the resultant residue was dried and stored at 4°C. The PPO was extracted from epicarp acetone powders, 25g of which was resuspended into 250 mL 0.1M pH 6.5 sodium phosphate buffer, containing 0.1% cysteine and homogenized for 5 min in a Sorvall Omni-Mixer at medium speed (Pos. 6). The extract was centrifuged at 10,000 × *g* for 15 min at 4°C, and the supernatant was fractionated with ammonium sulfate; the 40–75% fraction was resuspended into 9–15 mL 50 mM pH 6.5 sodium phosphate buffer. Protein solution was dialyzed for 24 hr at 4°C against 25 mM pH 6.5 sodium phosphate buffer and further fractionated through a Sephadex G-200 column (3.8 × 100 cm) equilibrated with the resuspension phosphate buffer. A peak was found with a high PPO activity which remained stable at 2–4°C for at least 10 days. Partially purified PPO increased its activity by 70 compared to that of the crude extract. Recovery of purified PPO was 45%. The enzyme appeared as a pure protein (Pm 160000) showing some isoenzyme bands in polyacrylamide gel electrophoresis. Recovery and degree of purity was similar to those reported by other authors (Mayer and Harel, 1979). Protein was determined by the method of Lowry et al. (1951).

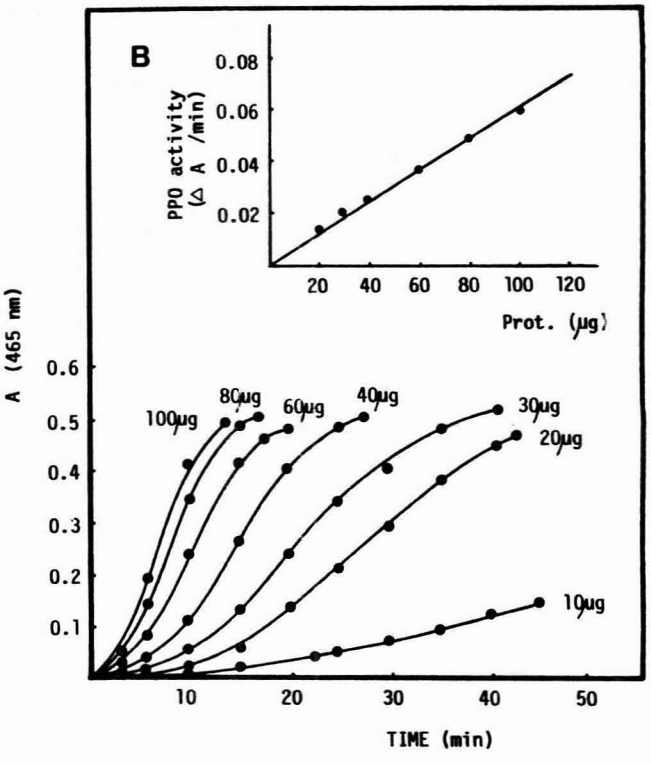
Preincubation of PPO Cherimoya epicarp with ascorbic acid, cysteine or mercaptoethanol

Two mL enzyme extract were incubated with 2 mL ascorbic acid, cysteine or mercaptoethanol solution prepared in 50 mM sodium phosphate buffer (pH 6.5) to reach the final concentration at 4°C. Enzyme extract, incubated in the cited buffer was considered as control. Seven days later samples were dialyzed against 25 mM sodium phosphate buffer (pH 6.5) for 15 hr at 4°C. Monophenol hydroxylase activity was determined as in Fig. 3, without catechol in the assay medium and was computed in the linear portion of the activity curve as ΔA_{465}/min. Diphenol oxidase activity was determined as monophenol hydroxylase activity but using catechol as substrate and 1 μg/mL protein, this activity was computed during the first 2 min of reaction as ΔA_{410}/min.

Enzymic assays

Monohydroxyphenolase and o-dihydroxyphenolase activities were assayed spectrophotometrically as described by Kahn (1975). When





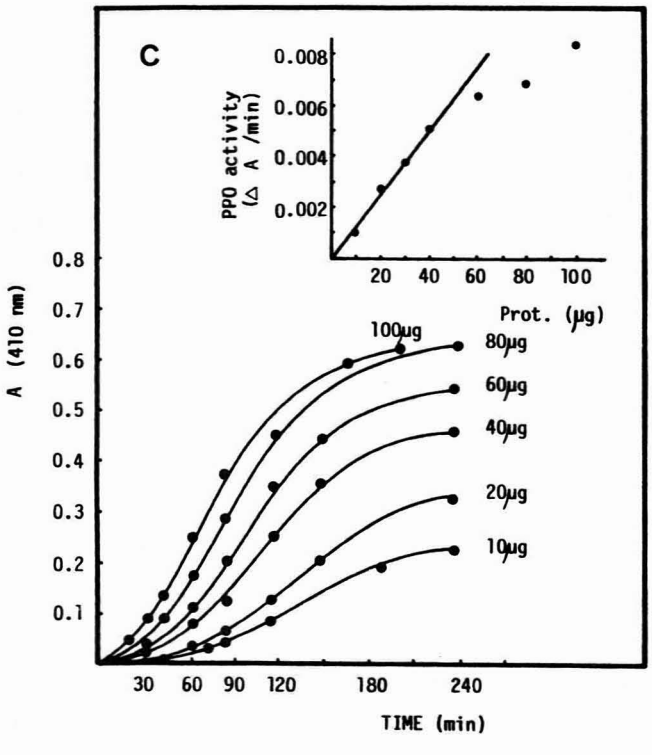


Fig. 1—The effect of enzyme concentration on the formation of product from L(−)tyrosine (A), tyramine (B), and p-cresol (C). The reaction mixture included, in a volume of 3 mL, 1.66 mM substrate, 50 mM sodium phosphate buffer (pH 6.5), and partially purified cherimoya PPO in the concentrations shown above (10 to 100 μg).

sugars or organic acids were assayed, 1.66 mM tyramine and catechol were used as monophenolic and o-diphenolic substrates; 11.66 μg/mL or 1 μg/mL of partially purified PPO were used, respectively.

RESULTS & DISCUSSION

Monohydroxyphenolase and dihydroxyphenolase activities of cherimoya epicarp PPO

Effect of enzyme concentration cherimoya epicarp PPO showed two different biochemical reactions: it hydroxylated monophenols like L(-)tyrosine, tyramine, and p-cresol to their corresponding diphenols and oxidized o-dihydroxyphenols, like catechol and L-DOPA, to o-quinone and dopachrome, respectively (Fig. 1 and 2). Monophenolase activity was lower than that of the dihydroxyphenolase; therefore, the former reaction was the limiting step of the monophenol oxidation catalyzed by cherimoya epicarp PPO.

Hydroxylation of the three assayed monophenols by cherimoya PPO was characterized by three consecutive phases: (1) an initial lag period, (clearly demonstrated with small quantities of the enzyme) that was inversely proportional to the enzyme concentration used; (2) a phase of relatively constant rate of hydroxylation, the rate of reaction being directly proportional to protein concentration up to at least 100 μg protein, probably due to the existence of greater reducing concentrations when there were greater enzyme concentrations; and (3)

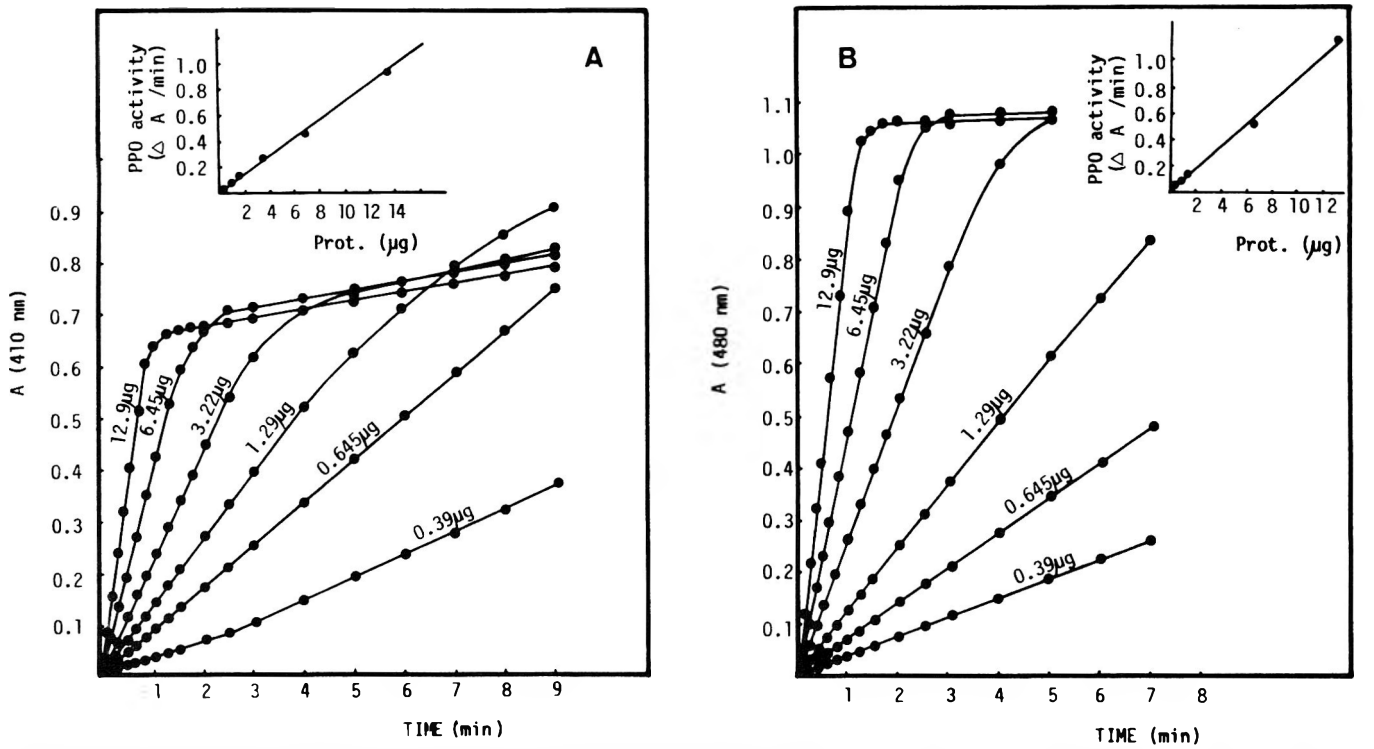


Fig. 2—The effect of enzyme concentration on the formation of product from catechol (A) and L-DOPA (B). The reaction mixture included, in a volume of 3 mL, 1.66 mM substrate, 50 mM sodium phosphate buffer (pH 6.5), and partially purified cherimoya PPO in the concentrations shown above (0.39 to 12.9 μg).

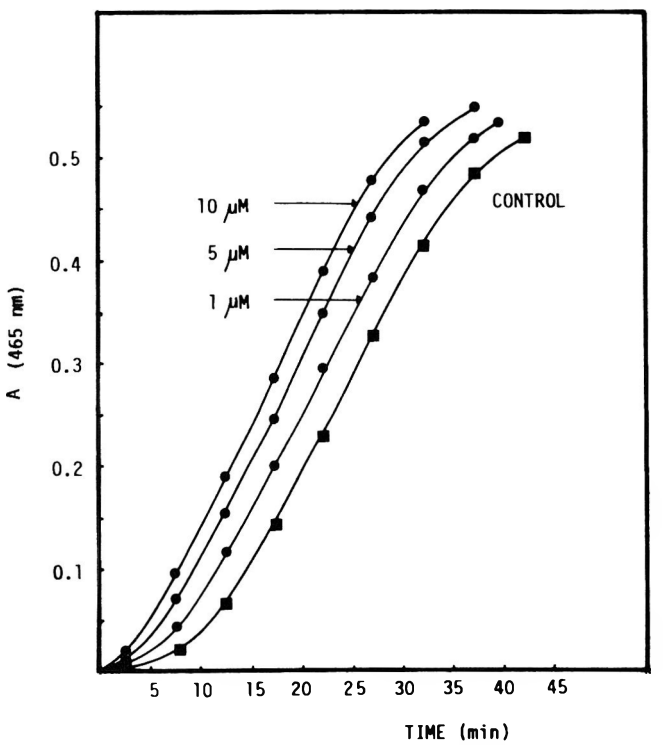


Fig. 3—Effects of different concentrations of catechol on the monophenol hydroxylase activity of PPO cherimoya epicarp. The reaction mixture included, in a total volume of 3 mL, 1.66 mM tyramine, 50 mM sodium phosphate buffer (pH 6.5), partially purified PPO (11.66 μg/mL), and catechol in the concentrations shown above (1 to 10 μM).

a decline of hydroxylation as the reaction ceased. The lag period was lower for tyramine than for either tyrosine or p-cresol (Fig. 1), but the rates of reaction were higher in the cases of tyramine and p-cresol.

Table 1—Oxidation constants and maximum rates of PPO cherimoya epicarp for different phenolic substrates

Substrates	Km (M)	VA_{max} (Δ/min/mg protein)
Catechol	6.25×10^{-3}	1.02
L-DOPA	6.66×10^{-3}	0.83
4-methyl-catechol	1.33×10^{-3}	0.47
Dopamine	0.55×10^{-3}	0.75
(−)epicatechin	1.18×10^{-3}	0.62

Oxidation of diphenols, catalyzed by PPO of cherimoya epicarp, only showed two phases: (1) a constant rate of oxidation phase proportional to enzyme concentrations and (2) an inactivation enzyme phase (Fig. 2A and 2B). No lag period was found. This behavior is similar to that shown by other PPO from different plant sources (Kahn, 1976; Mayer and Harel, 1979; Vaughn and Duke, 1984).

Km and V_{max} of cherimoya PPO for different diphenol substrates and shown in Table 1; in this particular case, the enzyme was only partially purified by ammonium sulfate precipitation. Plant PPO normally show low affinity for the phenolic substrates, except on rare occasions (Ashgar and Siddiqi, 1970); therefore, their Km are relatively high (Harel et al., 1973; Rivas and Whitaker, 1973; Tolbert, 1973). In our case the values of Km for PPO from cherimoya epicarp, obtained for several phenols assayed, also showed a low affinity of the enzyme for the substrate, but the Km were much higher than those reported in the literature for other sources (Golbeck and Cammarata, 1981; Mayer and Harel, 1979). This could be due to the spectrophotometric method used in those studies, since polarographic determinatives give a lower Km.

Effect of small amounts of diphenols on monophenolhydroxylase and PPO activity

The lag period, characteristic of the hydroxylation reaction, can be shortened by the addition of reducing agents which act

as co-substrates. Kahn and Pomerantz (1980) observed that DOPA and ascorbate shortened the lag period in the monophenol hydroxylation catalyzed by avocado PPO. Vaughan and Butt (1970) also demonstrated with PPO from spinach-beet leaves that the lag period could be shortened by NADH, dimethyltetrahydropterine, protocatechuic acid, 4-methyl-catechol, and DOPA. Likewise, the lag period of p-cresol and p-coumaric acid hydroxylation by mushroom PPO could be abolished in the presence of the corresponding dihydroxyphenols, 2-methylcatechol and caffeic acid, respectively (Kahn and Pomerantz, 1980).

The lag period of monophenol hydroxylation catalyzed by PPO of cherimoya epicarp can also be shortened by the presence of small amounts of diphenols such as catechol, 4-methylcatechol, L-DOPA, dopamine, (+)catechin and (−)epicatechin. The results obtained in the hydroxylation of tyramine, catalyzed by PPO of cherimoya epicarp, when different catechol concentrations were included in the reaction, are shown in Fig. 3. Higher concentrations of diphenol were not assayed because this compound was directly oxidized by the enzyme. The results obtained for the oxidation of the other diphenols were similar. Diphenols reduced lag period without modifying the corresponding reaction rates. Theoretically, the hydroxylation of a monophenol by PPO requires a certain amount of reducing agent. Diphenols, generated during hydroxylation, apparently, caused the reaction to be catalytic; thus, the hydroxylation rate of tyramine, in the absence of a reductant, was similar to that obtained with the addition of a diphenol electron donor.

Effect of different organic acids and sugars on cherimoya epicarp PPO activity

PPO from several plant sources are inhibited or activated by a substantial number of compounds (Mayer and Harel, 1979; Pifferi et al., 1974; Soler-Martinez et al., 1965). In the present work, the effects of some organic acids (acetic, oxalic, malonic, maleic, fumaric, succinic, glutaric, and glucuronic acids) and sugars (D(+)glucose, D(−)fructose, and sucrose) on cherimoya epicarp PPO were studied. Neither the organic acids nor sugars showed any effect on cherimoya PPO activity at 0.1 to 1 mM concentrations. These results could be contradictory to those obtained by other investigators (Pifferi et al., 1974) who demonstrated that acetic, maleic, fumaric, succinic, and oxalic acids slightly inhibited sweet cherry PPO; nevertheless, the inhibitor effect could be due to the high inhibitor concentrations (5 mM) assayed or to the low pH (pH = 4.2) used by these authors. It has been reported that non-ionized forms of aliphatic acids are the compounds responsible for the inhibition of PPO (Sinet and Garber, 1981). The pH was adjusted to 6.5 since a lower pH led to a decrease in cherimoya PPO activity.

Preincubation of PPO cherimoya epicarp with ascorbate, cysteine, and mercaptoethanol

The residual activities for tyramine hydroxylation and catechol oxidation catalized by PPO cherimoya epicarp previously incubated with different concentrations of ascorbate, cysteine and mercaptoethanol are shown in Table 2.

Preincubation of mushroom PPO with ascorbic acid, dithiothreitol or glutathione in the absence of a substrate causes a total loss of enzymatic activity in less than 4 hr (Golan-Goldhirs and Whitaker, 1984). Other authors, however, have suggested that ascorbic acid does not exert any effect on PPO activity (Varoquaux and Sarris, 1979). In this study, both cysteine and mercaptoethanol had a high protective effect on the stability of PPO cherimoya epicarp, the stability of monophenol hydroxylase being lower than that of o-diphenol oxidase; however, ascorbic acid did not show any protective action on the enzyme (Table 2). This suggested that the cherimoya PPO needed one or more thiol groups in a reduced form to maintain its activity. Thiol compounds did not appear to be suitable in preventing cherimoya browning.

Table 2—Effects of preincubation of PPO cherimoya epicarp with ascorbic acid, cysteine or mercaptoethanol on its monophenol hydroxylase and o-diphenoloxidase activities

		Remaining activity	
		Monophenol-hydroxylase	O-diphenol-oxidase
Enzyme extract control		12%	7%
Enzyme extract incubated	0.5 mM	8%	0%[a]
with ascorbic acid	0.2 mM	5%[a]	0%
Enzyme extract incubated	0.5 mM	62%[a]	43%[a]
with cysteine	0.2 mM	48%[a]	37%[a]
Enzyme extract incubated	0.5 mM	34%[b]	18%[b]
with mercaptoethanol	0.2 mM	29%[b]	9%

[a] $p < 0.01$, [b] $p < 0.05$ with respect to control extract.

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Pear Juice from Bartlett Pear Peels and Cores

T. BEVERIDGE, J.E. HARRISON, AND J.A. KITSON

ABSTRACT

Combination of high temperature-short time thermal treatment, enzymatic digestion, and decanter centrifugation allowed recovery of dilute juice in yields of 74.6–79.6% from Bartlett pear culls and peel and core material. Thermal treatment and/or enzymatic digestion of pear material resulted in increased concentrations of Fe, Ca, Mg, Na, epicatechin, chlorogenic acid, total acid and galacturonic acid in the clarified juices. Lack of heat and enzyme treatment resulted in dark brown off-flavored juice with an increase in pH, suspended solids, and amino nitrogen. No flavor differences were found between juices produced from 50% culls and peels and cores and 25% culls and 75% peels and cores. Both had a distinct pear flavor and were sweeter and more full-bodied than that produced from 100% peels and cores.

INTRODUCTION

WASTE PRODUCT utilization is an ongoing objective of fruit processors. In pear canning operations, discarded peels and cores have potential for recovery of economically important material. Pear juice is often used as a base for canning syrups and recovery of juice from peels and cores for this purpose represents a potential saving to the canning operation. Recently, a procedure was described (Beveridge and Harrison, 1986) for production of a clear, lightly colored juice from heated pear mashes. The application of this juice methodology to waste product recovery was explored in this study.

MATERIALS & METHODS

BARTLETT peel and core material and cull pears were obtained from a local cannery. The peel and core material was collected directly from the processing line into perforated asparagus baskets, allowed to drain for 5 min, then transferred into 10 kg pails. Sufficient ascorbic acid was then dusted on the surface of the peel and core material to allow transportation to the laboratory (~ 10 km) without enzymatic browning. On arrival, the peels and cores and cull pears were hammer-milled (9 mm holes) and thermally treated with scraped surface heat exchangers (Beveridge et al., 1986) to at least 90°C for at least 30 sec followed by rapid cooling to 35°C. Three batches (175–200 kg each) of heat-treated material were obtained containing, respectively, 100% peels and cores (PC,100), 75% peels and cores and 25% cull pears (PC:Culls, 75:25), and 50% peels and cores and 50% cull pears (PC:Culls, 50:50). The heat-treated mashes were treated with 0.02% (v/w) Pectinex Ultra SP (Swiss Ferment Co.) at 35°C for 4 hr. A fourth batch consisting of 100% peels and cores was prepared by hammer-milling but without either thermal or enzyme treatment (PC, 100-control). The four batches were frozen at −13°C for storage and later treatment.

Table 1—Juice yields form Bartlett pear culls and peel and core (PC) material using a decanter centrifuge

Samples	Thermal treatment[a]	Enzyme treatment[b]	Yield[c] (%)	Suspended solids (%)[d]
PC,100	YES	YES	74.6	1.60
PC:Culls, 75:25	YES	YES	74.7	3.02
PC:Culls 50:50	YES	YES	79.6	2.17
PC,100-control	NO	NO	67.1	23.1

[a] Thermal treatment: at least 90°C for at least 30 sec.
[b] Enzyme: 0.02% (v/w) Pectinex Ultra SP, 35°C, 4 hr.
[c] Calculated as 100 (juice weight recovered from decanter/weight of mash delivered to decanter).
[d] Determined as wt% material sedimentable at 16300g.

The authors are with Agriculture Canada Research Station, Summerland, B.C. VOH 1ZO, Canada.

Table 2—Color, soluble solids, pH, titratable acidity (TA) and amino nitrogen in clarified juices from Bartlett pear culls and peel and core (PC) material

Sample	°Brix	pH	TA (% Malic)	Amino nitrogen (mM glycine)	A_{420}[a]
PC,100	8.6	3.49	0.438	6.02	0.19
PC:Culls, 75:25	10.5	3.45	0.564	6.78	0.24
PC:Culls 50:50	11.2	3.51	0.553	7.32	0.21
PC,100-control	9.0	4.03	0.214	8.32	1.21

[a] Determined on clarified juice in 1 cm cells.

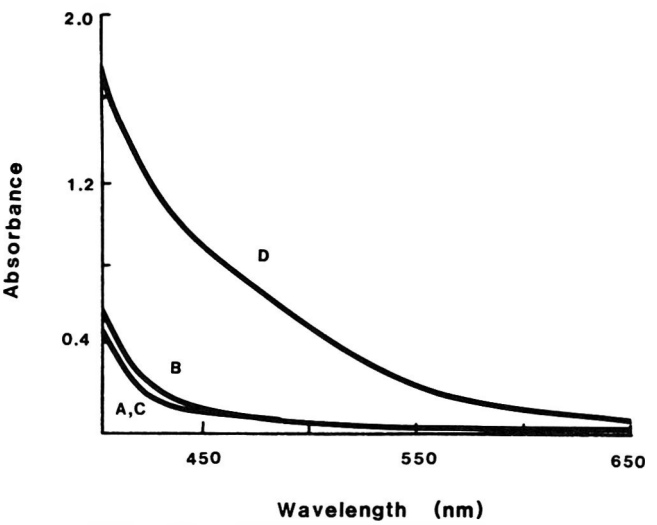

Fig. 1—Visible region scans of pear juices derived from Bartlett pear culls and peel and core material: A, PC,100; B, PC:Culls, 75:25; C, PC:Culls, 50:50; D, PC,100-control.

The frozen mashes were thawed at ambient temperature, then Moyno pumped cold into a decanter centrifuge (Dorr-Oliver Mercobowl, Hazleton, PA.) at 3-4 L/min for juice separation at 2700g. To the cloudy juice obtained from the three thermally treated batches was added 0.01% bentonite and the juice settled overnight. The clear or slightly hazy supernatant juice was mixed with 0.05% diatomaceous earth (prewashed with 1% malic acid, rinsed thoroughly with water and dried), then passed through a Buchner funnel using a pre-coated diatomaceous earth filter. Four liter samples of the three batches were collected for further analysis.

The juice obtained from the sample of PC,100-control material was very high in suspended solids with a consistency similar to apple sauce. Juice was obtained from this material by centrifugation at 16,300g for 10 min.

Carbohydrates

A Hewlett-Packard 1084B liquid chromatograph, fitted with a Brownlee Labs Inc. (Santa Clara, CA.) Polypore CA 10 μm (4.6 mm

Table 3—Mineral content of clarified juices obtained from Bartlett pear culls and peel and core (PC) material

	mg/100 mL				mg/L	
Sample	Na	K	Mg	Ca	Fe	Cu
PC,100	1.67	125	7.63	11.3	6.22	1.4
PC:Culls, 75:25	1.76	145	8.23	10.7	4.54	3.4
PC:Culls, 50:50	1.71	161	9.30	11.2	4.00	2.0
PC,100-control	1.20	124	5.86	5.64	1.45	1.2

× 22 cm) column, preceded by Polypore anion and CA guard columns (each 4.6 mm × 3 cm), was used for analysis. Injected samples (10 μL) of juice diluted 1/25 with water and containing 1 g/L mannitol as an internal standard were eluted at 65°C with double-distilled water flowing at 0.3 mL/min and detected refractometrically (Waters 410 Differential Refractometer) at 40°C.

Organic acids

Methodology was adapted from McCord et al. (1984). Samples were prepared by passing 1 mL pear juice through a 5 mm × 4 cm bed of Bio-Rad AG1-X8 100-200 mesh resin in the acetate form. Sugars were removed by washing the resin with 25 mL double-distilled water. Organic acids were then eluted with 1.5N sulfuric acid into a 25 mL volumetric flask containing 1 mL fumaric acid internal standard to give a final concentration of 200 mg/L of fumaric acid. Under these conditions recoveries of citric, galacturonic and malic acids were 95–100%. HPLC was performed using a Hewlett-Packard 1084B liquid chromatograph, equipped with a refractive index detector (Waters 410) and an Aminex HPX-87H organic acid column (Bio-Rad Laboratories), preceded by a Polypore cation (H^+) guard column (4.6 mm × 3 cm) Brownlee Labs Inc. Injected samples (30 μL) were eluted with 0.02N H_2SO_4 at 65°C flowing at 0.5mL/min.

Soluble solids, pH and titratable acidity

Soluble solids were measured as °Brix refractometrically at 20°C. Titratable acidity was determined by titrating 2.0 mL juice diluted to 10 mL with distilled water to pH 8.2 with 0.1N NaOH using a TTT80 automatic titrator operating through a PHM82 pH meter (Radiometer, Copenhagen) and reported as percent malic acid. The pH was measured using the same instrument.

Visible scans and amino acids

Absorbance spectrums of clarified juice, in 1 cm cells, were scanned between 400 and 700 nm using a Varian DMS 100 U.V.-Visible spectrophotometer equipped with a Varian DS 15 data station. Amino nitrogen values were determined according to Beveridge and Harrison (1985).

Minerals

Sodium, potassium, calcium and magnesium were determined by the method of standard additions. To 5 mL juice was added appropriate volumes of standard solutions and the mixture diluted to 50 mL with 0.5N HCl containing 6500 ppm lanthanum. Iron and copper were determined by diluting 10 mL of juice plus appropriate volumes of standard solutions to 50 mL with 0.5 HCl. Concentrations of both spiked and nonspiked samples were determined using a Varian AA575 atomic absorption spectrometer.

Sensory evaluation

Differences between heat-treated batches were determined by triangle testing (Larmond, 1977) using 20 panelists per comparison.

Phenols

Phenolic compounds were eluted with a methanol gradient on aqueous acetic acid (pH 2.8). A RP-18 Spheri-5 (C_{18} reverse phase, 5μ particle, 22 cm × 4.6 mm) column from Brownlee Labs, preceded by a 3 cm × 4.6 mm guard column of the same material, was used for separation. Pear juice phenolics were initially extracted using a C_{18} Sep-Pak (Waters Associates). Details of the method and recoveries of most of the phenolic materials have been described previously (Beveridge et al., 1986). Recovery of p-coumaric acid was 99.7%.

RESULTS & DISCUSSION

THE DECANTER-CENTRIFUGE extracted juice from peel and core material with yields varying between 74% and 80%, when the material was enzyme-treated (Table 1). Centrifugation of nonenzyme-treated material resulted in extraction of a very high insoluble solids juice in relatively poor yields. The high juice extraction rate for enzyme-treated pear waste is in agreement with data previously obtained for both Bartlett and d'Anjou pears (Beveridge et al., 1986). Similar differences between enzyme-treated and nonenzyme-treated, heated- or non-heated mashes, have been observed in McIntosh apples (Beveridge et al., 1987). Enzyme treatment is a necessary prerequisite for juice extraction with the decanter centrifuge.

Sensory evaluation of the three heat-treated samples (unoxidized) showed that clarified juice obtained from PC,100 could be distinguished from both PC:Culls, 75:25 and PC:Culls, 50:50 ($P \leq 0.05$) but PC:Culls, 75:25 were indistinguishable from PC:Culls, 50:50 ($P \leq 0.05$). PC,100 was considered a flat-tasting, bland, watery juice with a somewhat acidic, dilute pear flavor, with a distinct bitter note. This latter bitter note probably arose from the seeds and perhaps, the peel. The other two juices were more acceptable, being sweeter and more full bodied with a more distinct pear flavor. The presence of flavor and sugar from the cull pears appeared to have masked the peel and core-derived bitter notes since no bitter notes were recorded for either of the juices containing cull pears. However, none of the juices obtained from non-oxidized material were acceptable as juice since all were perceived as being dilute. The sample of oxidized (PC,100-control) juice was not included in the taste tests since it was dark brown in color, obviously different from the other three samples and unsuitable as a canning syrup without further refinement and color removal.

The soluble solids of the juices reflect the results obtained by sensory evaluation (Table 2). The soluble solids increased as increasing level of cull pears were added to the peels and cores. This was expected as some dilution or leaching of soluble solids from peels and cores undoubtedly occurred when they were washed from the production line. The juice pH values were as expected (Beveridge et al., 1986) with the higher value for the PC,100-control sample reflecting the fact that it was not enzyme-treated (Beveridge and Harrison, 1986). The titratable acidity values for the two cull-pear-containing juices are consistent with previous results (Beveridge et al., 1986), while the value for the PC,100 sample is lower but not unusual (Wallrauch et al., 1983). However, the titratable acidity value for the oxidized peels and cores is unusually low (Chen et al., 1982; Wallrauch et al., 1983). Part of the reason this value was lower than the others was due to the absence of enzyme-released galacturonic acid, but it was possible that skin ma-

Table 4—Soluble carbohydrates of clarified juices from Bartlett pear culls and peel and core (PC) material[a]

	g/100 mL			
Samples	Glucose	Fructose	Sucrose	Sorbitol
PC,100	1.11 ± 1.5	3.57 ± 1.4	0.205 ± 5.3	0.843 ± 1.2
PC:Culls, 75:25	1.36 ± 3.3	4.41 ± 4.1	0.190 ± 2.8	1.16 ± 4.4
PC:Culls, 50:50	1.65 ± 0.74	5.41 ± 1.1	0.290 ± 2.2	1.50 ± 2.1
PC,100-control	1.32 ± 1.8	4.75 ± 2.3	0.139 ± 3.2	1.13 ± 3.4

[a] ± values are coefficients of variation (%), n=9.

Table 5—Organic acid content of clarified juices from Bartlett pear culls and peel and core (PC) material[a]

Samples	g/100 mL Citric	Galacturonic	Malic	Quinic
PC,100	0.146 ± 8.5	0.359 ± 4.0	0.101 ± 10.9	0.035 ± 12
PC:Culls, 75:25	0.217 ± 3.4	0.248 ± 4.2	0.190 ± 16.8	0.042 ± 16.7
PC:Culls, 50:50	0.218 ± 2.2	0.410 ± 1.9	0.232 ± 2.1	0.043 ± 4.6
PC,100-control	0.125 ± 3.9	0.019 ± 7.3	0.143 ± 2.8	0.035 ± 8.6

[a] ± values are coefficients of variation (%), n = 9.

Table 6—Phenolic compounds in clarified juices from Bartlett pear culls and peel and core (PC) material[a]

Samples	µg/mL Catechin	Caffeic acid	Chlorogenic acid	Peak 4[b]	Epicatechin	P-coumaric acid	Peak 7[b]	Peak 8[b]	Peak 9[b]
PC,100	1.99 ± 42.2	5.35 ± 0.52	83.4 ± 3.24	7.36 ± 24.9	36.9 ± 22.3	4.21 ± 34.7	27.0 ± 1.62	30.0 ± 4.28	25.5 ± 2.29
PC:Culls, 75:25	3.18 ± 24.8	5.40 ± 0.35	97.8 ± 3.03	6.93 ± 17.0	43.9 ± 3.15	5.16 ± 26.0	28.1 ± 2.17	32.2 ± 1.64	25.7 ± 1.81
PC:Culls, 50:50	3.59 ± 56.2	6.52 ± 1.33	108.5 ± 10.7	6.79 ± 15.0	43.7 ± 5.12	6.36 ± 7.71	26.9 ± 1.67	30.3 ± 1.79	25.2 ± 2.27
PC,100-control	*	5.62 ± 1.13	0.681 ± 3.4	—	9.90 ± 50.1	1.86 ± 27.9	27.8 ± 6.80	28.7 ± 5.10	28.2 ± 4.69

[a] ± values represent % coefficient of variation (n = 9). — not present.
[b] Peaks 4, 7, 8, 9 quantitated as a chlorogenic acid.
* peak not consistently detected.

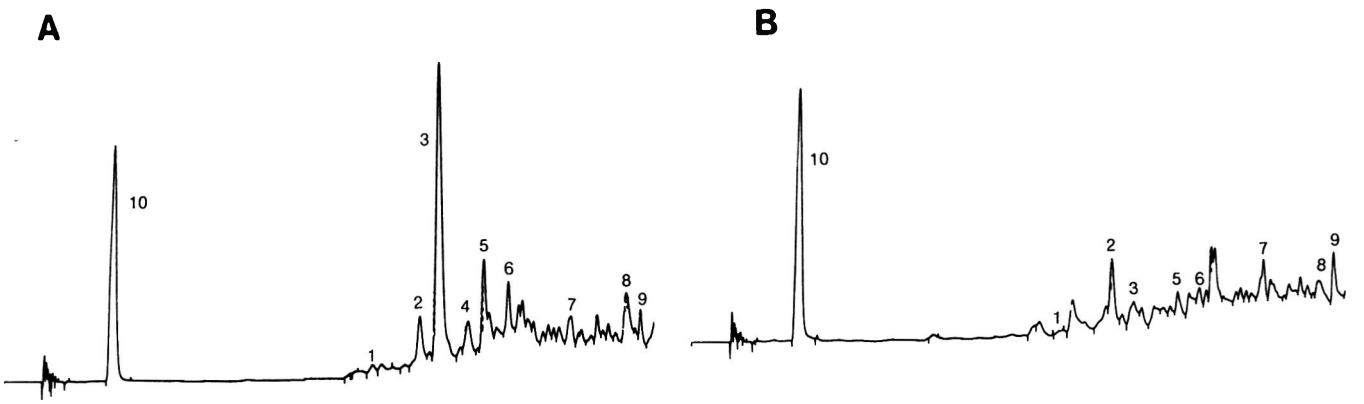

Fig. 2—High pressure liquid chromatography of phenolic material in A, juice prepared from thermized 100% peel and core material; B, juice prepared from 100% peel and core material allowed to oxidize freely prior to juice extraction. Peak identification is 1, (+)-catechin; 2, caffeic acid; 3, chlorogenic acid; 5, (−)-epicatechin; 6, p-coumaric acid; 4, 7, 8, and 9 are unknown; 10, gallic acid internal standard.

terial was lower in organic acids than was flesh and that this and/or dilution or leaching also contributed to the lower value. This explanation would also account for the reduced titratable acidity value obtained for the PC,100 sample.

Amino nitrogen, as measured by the juice reactivity with 2,4,6-trinitrobenzenesulfonic acid (TNBS), was in the expected range (Beveridge et al. 1986) but the PC,100 sample was in the lower portion of expected values while the PC,100-control sample was in the upper regions. No explanation of this difference was apparent although possibly the oxidative reactions generated compounds reactive with TNBS. The brown color of this oxidized juice relative to the other juices is clearly evident in Fig. 1, while the clarity and low visible region absorbance of the other juices is also evident. These differences are documented numerically in Table 2 as absorbance values at 420 nm. While these values are low, reflecting a light-colored juice, they are not as low as values previously reported (Beveridge et al., 1986) for whole pear juices, probably reflecting the increased proportion of peels in the mash and the resulting increase in color extracted from the skins.

The results of mineral analysis of the four juice samples are shown in Table 3. Generally, K and Mg increased with increased cull pear content while Na and Ca remained about constant. Iron decreased as cull pear content increased. These results suggest that the peel and core component of the pear contains more iron than the flesh but less Cu, Mg and K while Na and Ca are equally distributed; but, analyses of the skin, flesh and peel components of many pear varieties has shown that the peel and core portions of the pears contained more K, Ca, Cu and possibly Mg than the flesh of the pear (Faust et al., 1969; Brun et al., 1985). Therefore, the changes in the K and Mg contents of the peel and core and cull pear treatments must be due to leaching or dilution of the peel and core component prior to mash production. Possibly the reverse trend for iron is an artifact as the quantity present is approaching the lower detection threshold. Comparing the oxidized peels and cores sample with the unoxidized peels and cores sample provides same interesting contrasts. Clearly Na, Ca, Fe and perhaps, Mg were lower in the oxidized juice than in the unoxidized juice. This effect was probably due to the lack of both the pectinase and heat treatment in the PC,100-control sample. These minerals could have bound to the pomace, during oxidation, and to pectin galacturonic acid residues, which were not reduced by pectinase. The combination of enzyme and thermal treatment of the PC,100 mash apparently preserved at least two important nutrients, Ca and Fe, in the resulting juice. The increase in Fe content cannot be ignored since, as a known promotor of oxidation, it could seriously affect the storage stability of juices produced in this fashion.

The soluble carbohydrate content of the juices is shown in Table 4. The glucose, fructose, sucrose and sorbitol concentrations were about as expected since the values present here may be extrapolated to give sugar concentrations in good agreement with those previously reported by Beveridge et al. (1986) for whole pear juice. The juice obtained as a 50:50 mix of peels and cores and cull pears most closely approximated

whole pear juice and perhaps could substitute for this commodity. The other juices tended to be too low in carbohydrates to allow 1 for 1 substitution.

Generally, citric acid was higher, galacturonic acid lower and malic acid about the same as previously reported (Beveridge et al., 1986) for pear juice (Table 5). Both citric and malic acids increased with the introduction of cull pears while galacturonic acid gave mixed results. The galacturonic acid content of the PC,100-control sample was very low, as expected, since the mash was not enzyme-treated prior to juice separation. Quinic acid was a minor acid which increased slightly when cull pears were introduced into the juice mix.

High-pressure liquid chromatographic determination of C_{18} Sep-Pak retained phenolic material is shown in Fig. 2 and Table 6. Identification of the peaks as shown is tentative and based upon retention time equivalence with authentic standards (Beveridge et al., 1986). When compared to previous phenol values for Bartlett pear juice obtained by a similar process (Beveridge et al., 1986), (+)-catechin values are in reasonable agreement, (−)-epicatechin is slightly higher, chlorogenic acid a little lower and caffeic acid markedly lower than reported values. These differences may reflect seasonal or geographical variations since the juices were prepared in different years from pears grown in separate geographical locations. Also pear ripeness may be a factor since the juices analysed here were prepared from unripe pears whereas previous reports refer to fully ripe pears. A further difficulty with the analysis is reflected in Table 6 where large coefficients of variation are displayed for very small peaks such as (+)-catechin or other peaks such as (−)-epicatechin or p-coumaric acid which appear in crowded regions of the chromatogram.

Examination of Table 6 suggests that chlorogenic and p-coumaric acid concentrations increase as the cull pear content increases while the remaining compounds change only slightly at best. Comparison of the chromatographic profiles of peel and core material obtained before and after enzymatic oxidation (Fig. 2), by the polyphenol oxidase present in the pear fruit reveals marked changes. Recognized phenolic constituents of pears that are substrates for pear polyphenol oxidase include chlorogenic and caffeic acids, catechin and epicatechin (Walker, 1964; Rivas and Whitaker, 1973; Ranadive and Haard, 1971). However, cinnamic acids such as p-coumaric acid are known inhibitors of polyphenol oxidase (Walker, 1976; Rivas and Whitaker; 1973) Thus, the oxidation, which occurs in natural fruit material and which results in brown color development, is the net result of oxidation of substrates in the presence of natural inhibitors.

Examination of Fig. 2 and Table 6 suggests that catechin (peak 1) remains virtually unchanged or decreases during oxidation. This probably results from the generation of new compounds that elute near catechin during the oxidation since this peak was inconsistently detected in replicate chromatograms (Table 6). Chlorogenic acid and (−)-epicatechin were virtually eliminated from the phenolic spectrum as was peak 4. Elution of (presumed) caffeic acid in oxidized samples suggests this substrate is highly resistant to oxidation. Also, p-coumaric acid was markedly reduced during oxidation suggesting a role for this enzyme inhibitor in the oxidation of the natural substrate. The compounds comprising peaks 7, 8 and 9 do not appear to be involved in the reactions nor do the compounds comprising the two unresolved peaks eluting between peaks 5 and 7 in Fig. 1B.

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Starch/Solute Interaction in Water Sorption as Affected by Pretreatment

PEDRO J. CARRILLO, SEYMOUR G. GILBERT, and HENRYK DAUN

ABSTRACT

The effect of the freeze-drying process on the structure of starch and starch-sucrose mixtures was studied. The technique of inverse gas chromatography was used to study the interaction between organic compounds (probes) and starch (hylon 7) as well as starch-sucrose mixtures. The structural and morphological characteristics of the starch-solute mixtures from the thermodynamic parameters of these interactions was also examined. The porous freeze-dried samples interacted more with certain organic probes compared with untreated starch as indicated by higher values of specific retention volumes (Vg_o). Untreated starch apparently interacted only on the surface with organic probes. Freeze-dried starch reacted only with polar organic probes, particularly those with hydrogen bonding propensity. Cluster analysis of the water sorption isotherms correlated with data obtained from the injected probes.

INTRODUCTION

THE STRUCTURAL CONFIGURATION of starches and their interactions with other food constituents, such as proteins, sugars, and salts is of importance to the food industry. This knowledge can lead to more nutritive and palatable foods with longer shelf life stability. Water plays an important role before and after starch and different solutes are mixed to form various important food products. Water links to the active sites of the food ingredients, thereby changing the individual original structures by affecting the cohesive and adhesive forces and thus the physical properties of processed food.

The effects of sugars on the gelatinization properties of starch has been extensively reviewed (D'Appolonia, 1972; Osman, 1975; Bean et al., 1978; Bean and Yamazaki, 1978; Savage and Osman, 1978; Spies and Hoseney, 1982). In general, sugar type, chain length and concentration are major factors in starch/sugar interactions.

Hester et al. (1956) studied the effect of sucrose on the paste and gel properties of corn starch, wheat starch, wheat flours, and waxy-corn starches. The authors reported that the presence of sucrose decreased the hydration of starch granules and thus, the swelling and gel forming ability. Chinachoti and Steinberg (1984, 1986a, b, c) studied the water sorption characteristics of sucrose and starch mixtures before and after freeze-drying. They reported that freeze-dried mixtures interacted to produce changes in the sorption isotherms and that sucrose interacted more with gelatinized starch than with raw starch. Moreover, the hysteresis of a sucrose and waxy-maize starch mixture was attributed to the presence of amorphous sucrose which sorbed much more water than crystalline sucrose at Aw below 0.86. Furthermore, the crystallinity of sucrose in a mixture of sucrose and waxy-maize starch was linear with respect to weight ratios, increasing as Aw increased. The presence of sucrose had no effect on starch crystallinity during the absorption process as shown by X-ray diffraction.

The Zimm-Lundberg Theory (Zimm and Lundberg, 1956) for water cluster analysis can be used to study the water sorption behavior of the starch/solute mixtures. The tendency of water molecules to cluster is revealed by values of G_{11}/V_1 (cluster function) greater than -1. Negative cluster values indicate no formation of clusters and also that the molecules are widely dispersed in the system and clustering around specific sorption sites. If the cluster function is positive, the average solute molecule has more neighbors of its own nature than would be present in a random distribution. Moreover, average hydration site saturation is achieved and increases as relative humidity increases. As clusters form, the matrix expands and new binding sites may be exposed until all solutes or sites having enthalpy equal or greater than water-water bonds are occupied by clusters. Below saturation levels, water molecules occupy specific sites. If all sites have the same binding energy for water, only single water site occupancy will occur. But, if a range of sorption enthalpies exist, some sites already saturated with water will compete with weak enthalpies for the additional water even below saturation.

The inverse gas chromatographic (IGC) technique (Gilbert, 1984) involves the injection of known amounts of volatile probes into a column packed with the material to be studied. The probe has an initial linear velocity (uc) in the same direction of the flow of carrier gas. The molecular motion initiates collisions with the polymer surface. If there was no interaction, the initial linear velocity would be unaffected. However, if some interaction occurs, there could be a decrease in the velocity proportional to the strength and nature of this interaction.

The specific objectives of this study were: (1) to investigate water/starch/ solute interactions as functions of water content and temperature by the static (equilibrium relative humidity) method; (2) to determine the chemical and thermodynamic nature of the water interacting sites by using pulses of probe molecules of different polarity which sorb on sites not already occupied by water and/or other interacting solutes such as sucrose; (3) to examine the water cluster formation of water/starch/ solute systems.

MATERIALS & METHODS

Materials

Hylon 7 (high amylose corn starch, approx. 63%), hylon 5 (high amylose corn starch, approx. 55%), amioca (low amylose corn starch, less 5%) and potato starch (National Starch and Chemical Co., Bridgewater, NJ) were used because of their different amylose/amylopectin content. Sucrose was obtained from a local supermarket. The hylon 7 starch sample was used untreated and after being freeze-dried. Hylon 7 was also mechanically mixed with sucrose at different ratios (9:1, 1:1, 1:9 w/w), half of the mechanical mixture was dissolved in water and then freeze-dried in a Labconco Freeze Dry-12 instrument (Fisher Scientific, Springfield, NJ). The samples were frozen by placing them in an alcohol-ice bath mixture for 5 hr at -50°C; followed under vacuum (25 Torr) for 24 hr with the condenser at -75°C to constant weight.

Methods

Water sorption. Water sorption isotherms were determined by the equilibrium relativity method (Greenspan, 1977; Stokes and Robinson, 1949) at 25°C. Approximately 1.0g sample (200/300 mesh) was equilibrated in the head space of aluminum dishes in jars over different saturated salt solutions. Weight changes were monitored weekly until

The authors are with the Dept. of Food Science and the Center for Advanced Food Technology, Rutgers Univ., New Brunswick, NJ 08903.

equilibrium was reached. The weight gain or loss of a sample over the salt solution was used to calculate its equilibrium moisture content at the relative humidity of the particular salt solution. To determine the initial moisture content, approximately 1.0g sample was dried over anhydrous calcium sulfate ($Ca\ SO_4$). Hylon 7 untreated, hylon 7 freeze-dried, hylon 7-sucrose (1:1; 1:9; 9:1 w/w ratios) mechanical mixture and freeze-dried mixture were used for the sorption isotherms.

Water clusters. The sorption isotherms of these samples were studied by the Zimm-Lundberg Theory (Zimm and Lunberg, 1956) for water clustering on specific sites of water-polymer systems and were also used to determine the effects of the partial pressure and temperature on the system.

X-ray diffraction. X-ray diffraction patterns of hylon 7, hylon 5, potato starch and amioca were determined by using a ScinDag, PAD-V (Scientific Computers and Instruments, CA) X-ray diffractometer. The operating conditions were: 40 ma; 45 KV; diffracted angle was 2–40°; scanning rate was 2°/min., X-ray source was copper-k. Before X-ray determinations, the samples were sieved (200/300 mesh) and kept under 75% RH environmental conditions (NaCl, salt solution) for 2 days. The samples were analyzed untreated and after being freeze-dried.

Organic probe IGC analysis. Aluminum tubing columns (0.625 cm o.d., 30.5 cm long) were loaded with approximately 3.0g sample (200/300 mesh). In loading the column, 1.25 cm space was allowed at both ends of the column to accommodate the glass wool inserts. Five mL selected organic solvent (reagent grade) was introduced into a 60 mL glass vial, closed with rubber septa and placed in a −20°C refrigerated cold room. After sample vial had reached the environmental temperature (−20°C), different amounts of the vapor ranging from 1 to 25 µL, were introduced into the GC. Duplicates of each injection, and five different amounts of each organic probe, at each of four temperatures (25°, 30°, 35°, 40°C) were conducted for the organic probe IGC analyses. After obtaining at least 10 points (including duplicates), a "Curve Fit" program was used, and a least square fit was performed on the values (R^2 values better than 0.98). Using Lotus 1-2-3, the amount injected (x) vs retention time (y) was plotted to find the retention time at "0" infinite dilution as given by y intercept of the curve. After the "0" (zero) concentration value was obtained, it was fitted in Eq. (1)

$$V_r' = V_r - V_m \quad (1)$$

where V_m is the retention volume of methane, a noninteracting probe (Littlewood, 1970), V_r is the retention volume of injected probe at "0" concentration, and V_r' is the adjusted retention volume.

All the thermodynamic parameters were derived from the adjusted retention time (V_r'), the inlet (P_i) and outlet (P_o) column pressures and the net sample weight (w). From V_r', the specific retention volume (Vg_o) in moles of solute in 1 g of stationary phase/moles of solute in 1 mL of carrier gas, measured at 273.15 K., was obtained by Eq. (2):

$$Vg_o = (V_r'/w) \cdot j \cdot (273.15/T) \quad (2)$$

where w was the weight of the stationary phase (g), T was the column temperature (K), j was the velocity correction factor for pressure drop along the column (Littlewood, 1970) (Eq. 3).

$$j = (3/2) \cdot ((P_i/P_o)\text{^}2\text{-}1/((P_i/P_o)\text{^}3\text{-}1)) \quad (3)$$

After determining Vg_o for each organic solvent (at one temperature), ΔG_s was calculated (Littlewood, 1964) by using Eq. (4).

$$\Delta G_s = - RT \ln Vg_o \quad (4)$$

where R is the universal gas constant, and ΔG_s is the free energy change on the sorption of the probe.

The partial molar enthalpy of sorption (ΔH_s) is given by Eq. 5.

$$d(\ln Vg_o)/ d(1/T) = \Delta H_s/R \quad (5)$$

The entropy change (ΔS_s) is related to the above quantities by Eq. (6).

$$\Delta S_s = (\Delta H_s - \Delta G_s)/ T \quad (6)$$

The selection of the different organic probes (Table 1) was based on their molecular reactivity (Gilbert, 1984). The standard operating conditions for the untreated hylon 7, freeze-dried hylon 7 and hylon 7-sucrose mixture at 9:1 w/w ratio, mechanical mixture and freeze-dried mixture are shown on Table 2.

Table 1—Various organic solvents used for IGC analyses

Organic solvent	Reactivity
2-Propanol	Hydrophilic medium.
Toluene	Bond-aromatic
Methyl ethyl ketone	Carbonyl H-bonding
Ethyl acetate	As above, but more hydrophobic.
Heptane	Hydrophobic.

Table 2—Standard IGC conditions for organic probe analyses

	Product			
	Hylon 7		Hylon-7-Sucrose (9:1w/w)	
Conditions used	Untreated	Freeze-dried	Mechanical Mixture	Freeze-dried Mixture
Flow rate, mL/min	5	60	5	60
Back pressure, mm Hg	78	618	240	294
Length column, cm	30.5	30.5	30.5	30.5
Weight column, g	2.85	2.85	2.81	3.0
Factor "j"[a]	0.95	0.69	0.85	0.3
Range injections, µL	1-25	1-25	1-25	1-25

[a] Correction factor for pressure drop a ong the column.

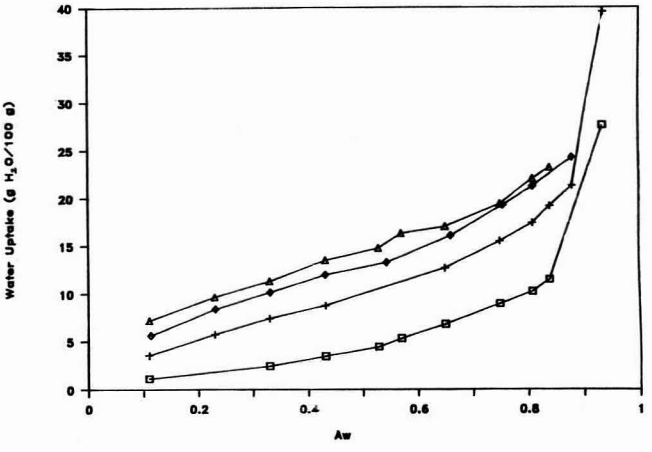

Fig. 1—Water sorption isotherms of hylon 7-sucrose (9:1 w/w ratio), determined at 25°C. Hylon 7-sucrose, mechanical mixture (□); freeze-dried hylon 7 (△); untreated hylon 7 (◇); hylon 7-sucrose, freeze-dried mixture (+).

RESULTS & DISCUSSION

Water cluster analysis

Figure 1 shows the data on the water sorption isotherm of hylon 7 mixture prepared mechanically and freeze-dried with sucrose at 9:1 w/w ratio. The water uptake of hylon 7 alone was higher than the mechanical and freeze-dried sugar/starch mixtures. The water uptake of the freeze-dried sample was always higher than the water uptake of mechanical mixtures. This probably was due to the opening of new sites upon the hydration-dehydration process to which freeze-dried samples were exposed or to the formation of more amorphous structures during the freeze-drying process. The water cluster formation (Fig. 2) for hylon 7 alone shows that water clusters start to form at lower Aw values with the mechanical mixture (0.44 Aw) compared with the freeze-dried sample (0.77 A_w). This indicated that when comparing mechanical and freeze-dried mixtures at the same partial pressure, water molecules on freeze-dried samples would form less water clusters because the water occupied or interacted with newly exposed binding sites in the more open structure. When analyzing the water sorption isotherms of hylon 7-sucrose mechanical mixture and freeze-dried mixture at 1:1, 1:9, 9:1 w/w ratios, both treatments showed the same water uptake behavior, i.e., as the sugar concentration increased, the sorption isotherm became more sugar-like.

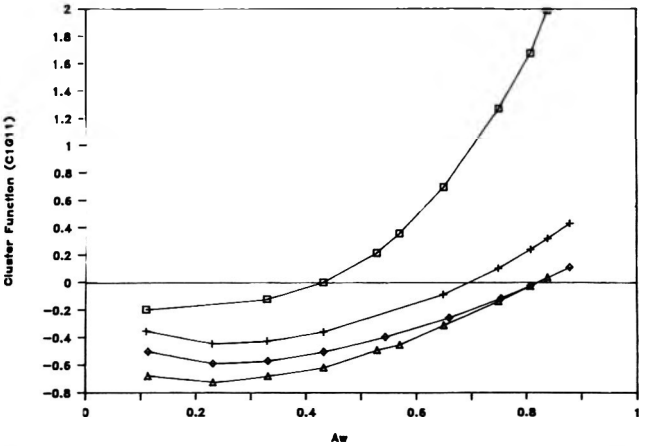

Fig. 2—Water cluster analysis of hylon 7-sucrose (9:1 w/w ratio), determined at 25°C. Hylon 7-sucrose, mechanical mixture (□); freeze-dried hylon 7 (△); untreated hylon 7 (◇); hylon 7-sucrose, freeze-dried mixture (+).

Water uptake was dependent upon the concentration in the mixture for both processes until the Aw reached 0.8, after which the moisture sorbed varied directly with the sugar concentration (osmotic effect). Water clusters started to form at almost the same Aw (mechanical mixture, 0.49, freeze-dried mixture, 0.47). However when the concentration of sugar (sucrose) was low (9:1 w/w ratio), the formation of a water cluster required higher water activities for freeze-dried samples (0.7 A_w) compared with mechanical mixtures. This was probably due to disruption of sucrose crystal structure by the solution step prior to freeze-drying with more sorption of water by amorphous sucrose, thus reducing the water available for clustering at starch sites.

X-ray diffraction analysis

Figure 3 shows the X-ray diffraction pattern of hylon 7 and potato starch, untreated and freeze-dried samples. The process of freeze-drying altered the starch structure with the development of more amorphous regions on the starch samples. Figure 4 shows the X-ray diffraction pattern of hylon 7, hylon 5, corn and amioca starches. It can be observed that high amylose starches (hylon 7, hylon 5) have a characteristic B-type crystalline structure, while high amylopectin starches (normal corn, amioca) have A-type crystalline structure.

Organic probe analysis

Specific retention volume. The specific retention volume (Vg_o) of various organic probes at 25°C when interacted with hylon 7 untreated, hylon 7 freeze-dried, and hylon 7-sucrose (9:1 w/w ratio), mechanical mixture and freeze-dried mixture is shown in Fig. 5. The specific retention volume of the organic probes was a function of column temperature and the reactivity of the probe. As the temperature increased, the interaction between organic probe molecules and hylon 7 samples diminished. This was probably due to the increase in volatility of the probe allowing less interaction and H-bonding in the column as the temperature increased.

The specific retention volume of hylon 7 freeze-dried starch was much higher than the untreated samples and hylon 7-sucrose samples. This was probably due to the opening of the starch structure producing more active sites for H-bonding interaction. Toluene had lower retention time when compared with methyl ethyl ketone (MEK) and ethyl acetate for hylon 7 untreated and hylon 7 freeze-dried. This was probably because the benzene ring structure of toluene had more spatial hinderance with less access to sites in the freeze-dried sample. Also

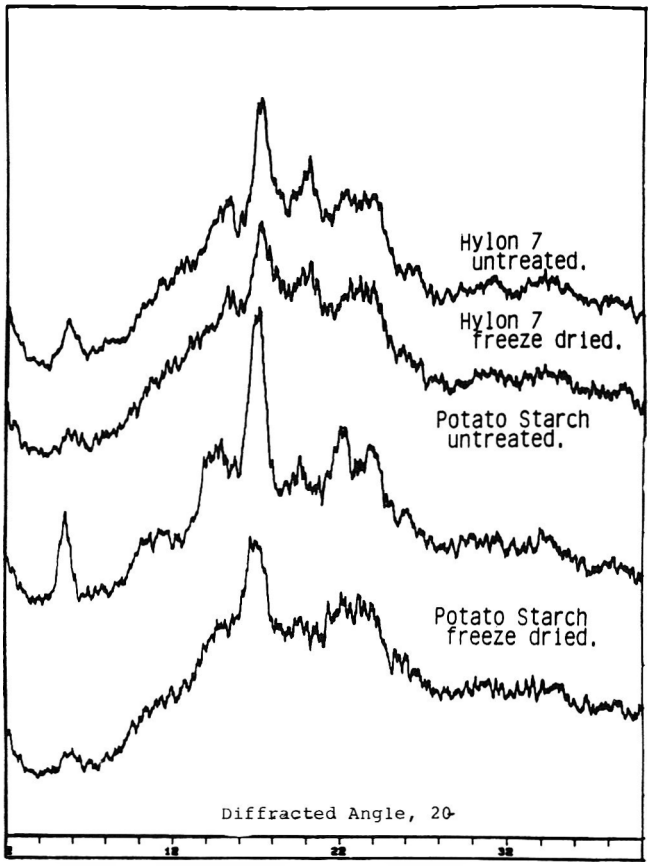

Fig. 3—Effect of freeze-drying on the X-ray diffraction pattern of hylon 7 and potato starch.

MEK and ethyl acetate have much more interaction with both hylon 7 untreated and hylon 7 freeze-dried due to their polar groups.

When comparing hylon 7-sucrose mixture (9:1 w/w ratio), mechanical mixture and freeze-dried mixture values were higher for organic probes interacting with the freeze-dried mixture compared with the mechanical mixture. However, the values found were not near the specific retention time values shown by hylon 7 freeze-dried. This was probably due to interactions between the starch and the sucrose, therefore, a lesser number of active sites remained free for interaction with the organic probes. The differences were more visible when using the more polar probes.

Free energy. Figure 6 shows the differential free energy of sorption at 25°C of hylon 7 untreated, hylon 7 freeze-dried and hylon 7-sucrose mixtures prepared mechanically and freeze-dried. At the same temperature, values for ΔG_s were more negative as the polarity of the probe increased, indicating that the polar probes were interacting more spontaneously with hylon 7. Also there was a higher sorption propensity for the organic polar probes with the polymer (hylon 7). The data for n-heptane at 35°C and 40°C showed more positive ΔG_s, indicating that at this level the low polarity probes were interacting less spontaneously with hylon 7. Freeze-dried samples also showed more negative free energy values when compared with the untreated ones.

Heat of sorption. The heats of sorption of hylon 7 untreated, hylon 7 freeze-dried and hylon 7-sucrose (9:1 w/w ratio), mechanical mixture and freeze-dried mixture and shown in Fig. 7. Enthalpy of sorption showed negative values for both polar and nonpolar organic probes. Negative values indicated an exothermic adsorption process for all hylon 7-solute combinations. The probes with groups participating in H-bond formation (2-propanol, MEK) showed the most negative ΔH_s in accordance to solvent polarity.

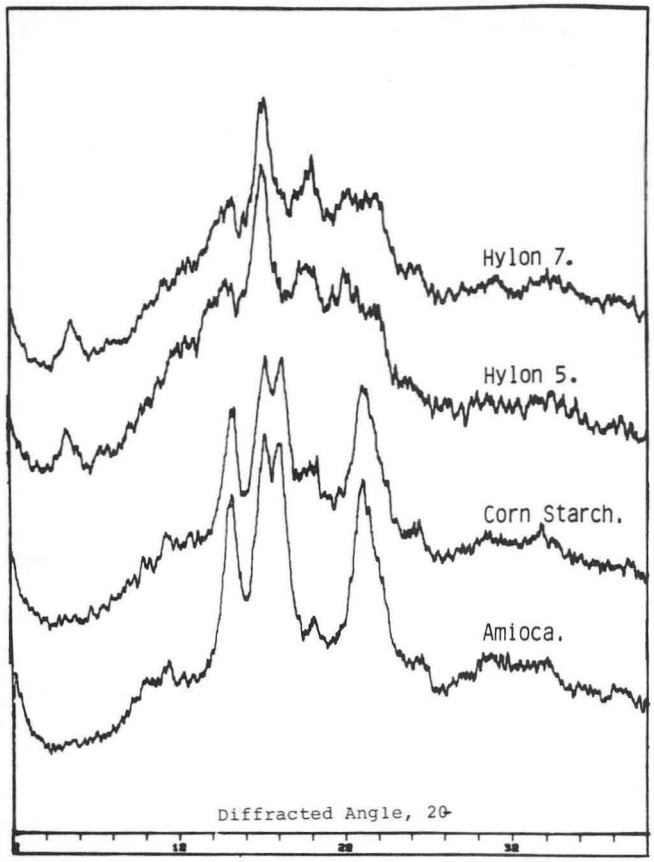

Fig. 4—X-ray diffraction pattern of hylon 7, hylon 5, corn and amioca sstarches.

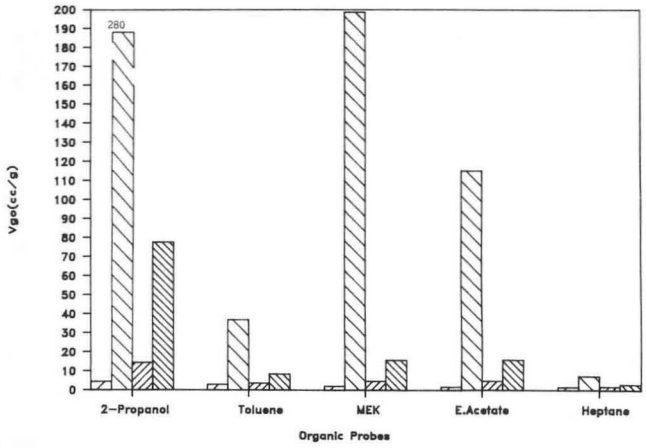

Fig. 5—Specific retention volume of hylon 7 untreated (▨), hylon 7 freeze-dried (▨), and hylon 7-sucrose (9:1 w/w ratio), mechanical mixture (▨), and freeze-dried mixture (▨) at 25°C. MEK-methyl ethyl ketone; E. acetate-ethyl acetate.

Entropy of sorption. Figure 8 shows the entropy of sorption for hylon 7 untreated, hylon 7 freeze-dried, and hylon 7-sucrose (9:1 w/w ratio), mechanical mixture and freeze-dried mixture. The entropy of adsorption for all probes remained approximately unchanged in the range 25°C to 40°C. The solute molecules having permanent dipole moments, (2-propanol, MEK) exhibited the greatest negative entropy and the least change with n-heptane.

CONCLUSIONS

FREEZE-DRIED HYLON 7 interacted much more with organic probes, compared with untreated hylon 7, reflecting the more amorphous structure and porosity created by freeze-drying. Upon freeze-drying, sucrose interacted with hylon 7 reducing the number of active sites available for the organic probes.

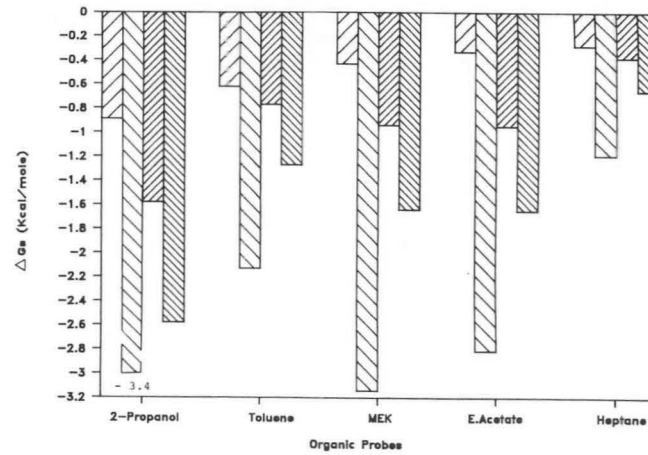

Fig. 6—Free energy of sorption of hylon 7 untreated (▨), hylon 7 freeze-dried (▨), and hylon 7-sucrose (9:1 w/w ratio), mechanical mixture (▨) and freeze-dried mixture (▨) at 25°C. MEK-methyl ethyl ketone; E. acetate-ethyl acetate.

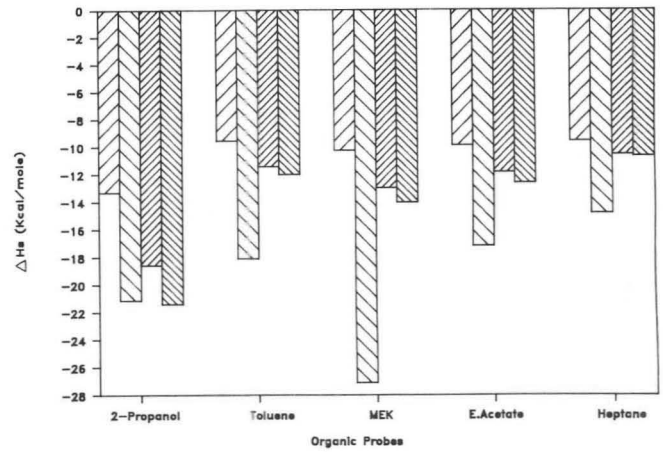

Fig. 7—Enthalpy of sorption of hylon 7 untreated (▨), hylon 7 freeze-dried (▨), and hylon 7-sucrose (9:1 w/w ratio), mechanical mixture (▨) and freeze-dried mixture (▨) at 25°C. MEK-methyl ethyl ketone; E. acetate-ethyl acetate.

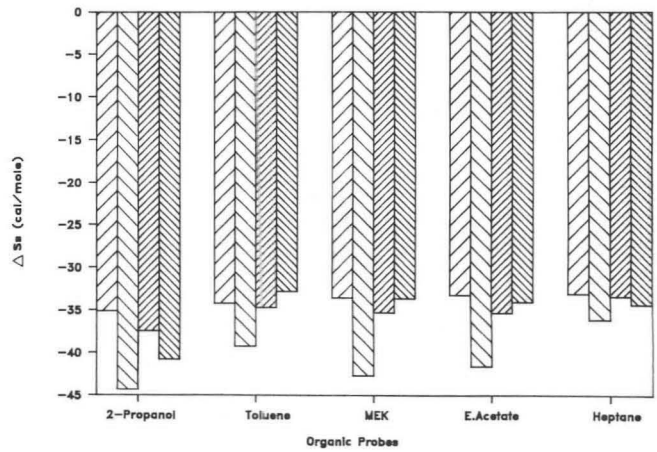

Fig. 8—Entropy of sorption of hylon 7 untreated (▨), hylon 7 freeze-dried (▨), and hylon 7-sucrose (9:1 w/w ratio), mechanical mixture (▨) and freeze-dried mixtured (▨) at 25°C. MEK-methyl ethyl ketone; E.acetate-ethyl acetate.

Water cluster analysis of the sorption isotherms confirmed the increase of adsorption observed for samples containing sucrose, since this sugar was water soluble. Organic probe IGC analysis was a very useful method to analyze food products for surface and bulk properties related to structure and diffusivity as well as thermodynamic properties.

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PECTIN AND DEHYDRATION OF PEACHES. . . From page 1190

higher TP and PP contents were observed in the 5 min blanched peaches, with a higher rehydration capacity (Fig. 2) and lower rehydration losses (Fig. 4). The decreased RW at room temperature after longer blanch (9, 15 min vs. 5 min) of the peaches (Fig. 3) is probably as a result of heat induced pectin degradation and configuration. The significant correlations found between the pectin content of the dehydrated peaches and their RW, as well as between the actual pectin content of the blanched peaches and their firmness, confirms the contribution of the pectins to the structural-textural characteristics of fruit products in general and dehydrated fruits in particular.

Sugars, acids, GA monomers and short oligomers, are both water- and alcohol-soluble. Therefore, they are partly removed during the blanching (condensed steam, cooling water), or AIS extraction. The relatively higher content of the complex insoluble PP after the blanching (0 min vs 5 min) is probably due to PP stabilization. One has to compare the 15 min blanch with the 5 min blanch, in order to assess the heat induced pectin degradation and its effects.

CONCLUSION

BLANCHING (as proposed by Mrak and Perry, 1948), is not a generally practiced method for stabilization of dehydrated fruits. This work indicates that adequate blanching stabilizes one of the CW and IC tissue components most susceptible to degradation-pectins—thereby affecting positively the rehydration capacity and reducing the rehydration losses of the dehydrated fruit. Optimum blanching, which depends on the tissue to be blanched, the size of the pieces, etc., can be recommended for stabilization of textural-structural quality characteristics, during dehydration and storage of dehydrated fruits.

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An *In Vitro* Digestibility Assay for Prediction of the Metabolizable Energy of Low-Calorie Dextrose Polymeric Bulking Agents

JOHN S. WHITE, CARL M. PARSONS, and DAVID H. BAKER

ABSTRACT

A method was developed to predict *in vivo* digestibility and metabolizable energy of dextrose polymers from *in vitro* enzyme digestibility measurements. A high correlation (r = 0.98) was established between *in vivo* true digestibility (TD) and *in vitro* percent dextrose yield (DY) for polymeric compounds representing a wide range of *in vivo* digestibility (22–96%). The linear regression equation describing this relationship was TD (%) = 1.11 DY + 10.68. The *in vivo* nitrogen-corrected true metabolizable energy (TMEn) of four test compounds was predicted from *in vitro* digestibility data with good accuracy using the linear regression equation TMEn (kcal/g) = 0.038 DY + 0.637 (r = 0.97). The *in vitro* method is most useful for predicting *in vivo* digestibility and metabolizable energy of dextrose polymers with *in vitro* dextrose yields less than 80% and *in vivo* metabolizable energy values less than 3.6 kcal/gram.

INTRODUCTION

FOOD SUPPLIES in affluent countries are abundant and lifestyles are often sedentary. A decade ago, up to 40% of the adult population in these societies was classified as obese (Bushkirk, 1974). In recent years, health-conscious individuals have become increasingly aware of the correlation between obesity and increased mortality from cardiovascular disease, hypertension, respiratory illness, and diabetes.

It is important that appealing and palatable low-calorie foods be formulated for individuals who experience difficulty in weight reduction and proper body-weight maintenance. Several classes of compounds have been proposed as low calorie bulking agents to augment the sensory and functional properties of synthetic sweeteners when sucrose is removed from dietary foods and beverages. Vegetable colloid, cellulose, pullulan, polyols (sorbitol, mannitol, xylitol, maltitol, and lactitol), gums, and polymerized dextrose (e.g. polydextrose) have been proposed as potential bulking agents (Beereboom, 1979). However, most of these products have not gained wide acceptance because of unacceptable flavor, functional properties, price or caloric value.

An accurate and rapid estimate of *in vivo* caloric value is critical in the assessment of new prototype bulking agents. Enzyme hydrolysis of carbohydrates *in vitro* with amylases has been used to estimate digestibility of native and modified starches (Leegwater, 1971; Conway and Hood, 1976; Bauer and Alexander, 1979; Wootten and Chaudhry, 1979; Batey, 1982) and nonstructural carbohydrates in forage plant tissues (El Faki et al., 1981; Khaleeluddin and Bradford, 1986). Methods for measuring caloric value of carbohydrates *in vivo* include the caloric assay of Rice et al. (1957) and the true metabolizable energy (TME) method of Sibbald (1976). Lowry et al. (1986) recently evaluated these two *in vivo* procedures for measuring the caloric value of polydextrose and concluded that the TME method was more suitable than growth assay methods such as the Rice assay. Several studies have measured *in vitro* digestibility of carbohydrates in conjunction with postprandial glucose and insulin responses in adult males and rabbits (O'Dea et al., 1981; Lee et al., 1985), hydrolysis by perfused enzymes in infants with acute diarrhea (Lebenthal, 1983), and *in vivo* digestibility in adult human ileostomists (Englyst and Cummings, 1985). In these studies, however, no attempt was made to measure carbohydrate caloric values.

The research presented herin had three objectives: (1) to develop an *in vitro* digestibility assay for dextrose polymers which reflects carbohydrase activities found in saliva as well as in the lumen and mucosa of the small intestine; (2) to evaluate the *in vivo* TME method for determining metabolizable energy of dextrose polymers with varying caloric values; (3) to establish quantitative relationships between *in vitro* digestibility and TME for future use in estimating the caloric value of experimental dextrose polymers.

Author White is with the Research Division, A. E. Staley Mfg. Co., 2200 E. Eldorado St., Decatur, IL 62525. Authors Parsons and Baker are affiliated with the Dept. of Animal Sciences, Univ. of Illinois at Urbana-Champaign. Address reprint requests to Dr. J. S. White.

MATERIALS & METHODS

Substrates

StarDri® 10 maltodextrin, Stadex® 82, 128, and 132 dextrins, and prototype low-calorie bulking agents A, B, and C are products of the A. E. Staley Mfg. Co. (Decatur, IL). Prototype low-caloric bulking agent D is a barley β-glucan kindly provided by NOVO Industri, A/S, Bagsvaerd, Denmark (Petersen, 1986). Anhydrous dextrose (Bacto-Dextrose) was purchased from Difco Laboratories (Detroit, MI). Polydextrose is a commercially available low-calorie bulking agent (Pfizer Chemical Division, New York, NY) manufactured by heating dextrose in the presence of organic acid (Rennhard, 1973).

Enzymes

α-Amylase (α-1,4-glucan-4-glycanohydrolase, E.C. 3.2.1.1) was purchased from Sigma Chemical Co. (St. Louis, MO) (Product No. A-6880, 12 U/mg, from porcine pancreas). Microbial enzymes with α-1,4 and α-1,6 bond hydrolyzing activities were substituted for commercially unavailable mammalian sucrase, maltase, and isomaltase. Amyloglucosidase (α-1,4-D-glucan glucanohydrolase, E.C. 3.2.1.3) was obtained from Sigma Chemical Co. (Product No. A-7420, 37 U/mg, from *Aspergillus niger*) or Enzyme Development Co., New York, NY (Enzeco, 26 U/mg, from *Aspergillus niger*). Pullulanase (Pullulan-6-glucanohydrolase, E.C. 3.2.1.41) came from Sigma Chemical Co. (Product No. P-2138, 175 U/ml, from *Enterobacter aerogenes*) or A. B. M. Chemicals, Ltd., England (Pulluzyme, 10 U/ml, from *Klebsiella aerogenes*).

In vitro enzyme digestibility assay

Five grams substrate (dry solids basis) were dissolved in 65 mL reaction buffer (0.15M sodium acetate, pH 5.5, supplemented with 1.5 mM calcium chloride) and equilibrated to 37°C in capped reaction bottles. Enzymes were dissolved individually in reaction buffer so the desired number of units would be contained in 10 mL. Hydrolysis was initiated with the addition of 10 mL each of amyloglucosidase (1600 units), α-amylase (15 units) and pullulanase (377 units) to each reaction bottle. Reaction mixtures were gently agitated at 37°C for 3 hr in a shaking water bath. Enzyme digestions were terminated by boiling aliquots of the reaction mixture for 10 min. Boiled aliquots were filtered through 0.45 μ Acrodiscs® (Gelman Sciences Inc., Ann Arbor, MI, Product No. 4217) and the percent dextrose in the aliquot

determined using the liquid chromatographic method of Scobell et al. (1977). An HPX-87C carbohydrate column (Product No. 125-0095, BioRad, Richmond, CA) was used.

Dextrose was reported as a percentage of total carbohydrate in the reaction mixture after 3 hr of enzymatic digestion. This time period was established as allowing essentially complete hydrolysis of the maltodextrin control compound. Reaction conditions of 37°C and pH 5.5 were chosen to accomodate optimum temperature and pH ranges of the three enzymes.

In vivo digestibility assay

True dry matter digestibility and TME were determined in adult male chickens as described by Sibbald (1976; 1986). The crop-intubated chicken is a good animal model for determining metabolizable energy, in that feed wastage and caprophagy are not confounding problems as they are in the rat. Adult Single-Comb White Leghorn cockerels averaging 10 mo in age were fasted for 24 hr to clear their digestive tracks of dietary residues. Four to six cockerels were then given 21.6 to 30 g of a test sample via crop intubation. The plastic intubation tube was 1.2 cm in diameter and 40 cm in length. All compounds were given as solutions in water, with concentrations varying from 30 to 60% (w/v) depending upon solubility of the compounds. The solutions were prepared just prior to intubation by very gradual addition of the test materials to a stirred volume of water (25°C) over a period of 4 to 6 hr. Cockerels given Prototype B, C, D, bacto-dextrose or polydextrose received one 50 mL dose of 60% (w/v) solution, whereas those given the other compounds received two equal 30 or 40 mL doses of a 36 or 30% solution, respectively, at 6-hr intervals. The latter regimen was used to ensure an adequate intake of low solubility compounds without causing regurgitation. Five additional cockerels remained on the fasting regimen for 48 hr to measure excretion of endogenous dry matter and energy. Excreta were collected quantitatively for 48 hr from all cockerels and then lyophilized, ground and analyzed for gross energy and nitrogen (AOAC, 1980). True dry matter digestibility and TME were calculated by the procedure of Parsons et al. (1982). The TME values were corrected to zero nitrogen retention (TMEn) using the value of 8.22 kcal/g for any nitrogen retained in the body or voided as products of tissue catabolism (Parsons et al., 1982).

After obtaining estimates of *in vitro* digestibility (i.e. dextrose yield), *in vivo* digestibility and TMEn for all substrates except prototype bulking agents, linear regression equations were calculated: TMEn (kcal/g) regressed on *in vitro* digestibility (%), *in vivo* digestibility (%) regressed on *in vitro* digestibility (%), and TMEn (kcal/g) regressed on *in vivo* digestibility (%). After establishing the veracity of linear fits, the regression equations were then used to predict the *in vivo* digestibility and TMEn of four additional experimental prototype low-calorie bulking agents from *in vitro* digestibility measurements. Predicted values were then compared with actual values of *in vivo* digestibility and TMEn determined in another series of bioassays with adult male chickens. Procedures were similar in this series of assays to those described above.

Statistical analyses

Data were analyzed by analysis of variance procedures. Treatment means were compared by the least significant difference procedure (Steel and Torrie, 1980). TMEn and *in vivo* digestibility were also regressed on *in vitro* digestibility as described in the previous section, and linear regression equations were calculated together with correlation coefficients. In the regression analyses, each *in vitro* digestibility value was regressed on all *in vivo* digestibility and TMEn values for that substrate because *in vitro* determinations were made in duplicate or triplicate, whereas *in vivo* determinations were performed on four to six birds each.

RESULTS

In vitro assay development

Enzyme ratios for optimum *in vitro* digestibility were established using a soluble starch hydrolysate, StarDri® 10 maltodextrin, as a model substrate. This substrate has an average of ten dextrose residues per polymer chain linked by α-1,4 and α-1,6 bonds. Proportions and levels of amyloglucosidase, α-amylase and pullulanase were varied until maximum hydrolysis of maltodextrin to dextrose in 3 hr was achieved. A high rate of hydrolysis of control maltodextrin to dextrose (96.7%) was achieved with 1600 units of amyloglucosidase, 15 units of α-amylase and 377 units of pullulanase. Maximum hydrolysis of control maltodextrin to dextrose was dependent on the enzyme sources used and varied approximately three percent in our assay system. Enzymatic hydrolyses of test substrates to dextrose were corrected by normalizing against dextrose yield from control maltodextrin (i.e., 96.7%) within each assay. The substrates listed in Table 1 displayed a wide range of digestibility in the *in vitro* assay system. The *in vitro* digestibility range of Stadex® dextrins reflects the extent of dextrinization, or heat and acid-catalyzed oligomer bond rearrangement, that these starch-derived dextrose polymers have undergone. Greater dextrinization results in greater bond rearrangement and lower enzymatic hydrolysis of substrate to dextrose. Polycextrose is a dextrose polymerization product claimed to possess low digestibility *in vivo* (Figdor and Rennhard, 1981) and it was found to have the lowest dextrose yield of the substrates in Table 1. The prototype bulking agents listed in Table 2 also displayed a wide range of digestibility in the *in vitro* assay system. Likewise, a wide range of *in vivo* digestibility and TMEn values were observed for the substrates and bulking agents listed in Tables 1 and 2.

Prediction equations

It is apparent that the readily digestible carbohydrate substrates (i.e. dextrose yields greater than 80%), while distinguishable in the enzymatic *in vitro* test, were indistinguishable in the avian *in vivo* tests. Thus, only substrates with dextrose yields less than 80% (i.e., samples 3 to 6 in Table 1) were used to construct regression lines relating *in vivo* results to *in vitro* dextrose yields. TMEn regressed on *in vitro* dextrose yields below 80% (Fig. 1) resulted in the equation TMEn (kcal/g) = 0.038 DY + 0.637 with a high degree of fit (r = 0.97). Likewise, the linear regression of *in vivo* digestibility on *in vitro* dextrose yield was TD (%) = 1.11 DY + 10.68 (Fig. 2), also providing a good fit (r = 0.98).

Substrates with the highest *in vivo* digestibilities also delivered the greatest *in vivo* TMEn. Regressing TMEn on *in vivo* digestibility for all six samples (Fig. 3) resulted in the equation: TMEn (kcal/g) = 0.034 TD + 0.266 (r = 0.99).

Table 1—In vitro and in vivo energy assessment of dextrose polymers with varying degrees of digestibility[a]

Sample no.	Carbohydrate substrate	In vitro assay (Dextrose yield, %)[b]	In vivo assay[c] Digestibility[d] (%)	In vivo assay[c] TMEn[e] (kcal/g)
1.	Maltodextrin Control	100 f	98.8 ± 1.71 f	3.69 ± 0.02 f
2.	Dextrose	100.81 ± 0.21 f	99.4 ± 0.54 f	3.60 ± 0.09 f
3.	STADEX® 82	78.84 ± 0.12 g	96.1 ± 2.05 f	3.60 ± 0.07 f
4.	STADEX® 132	53.22 ± 0.12 h	73.6 ± 2.57 g	2.79 ± 0.08 g
5.	STADEX® 128	32.11 ± 0.02 i	49.8 ± 4.31 h	1.94 ± 0.24 h
6.	Polydextrose	12.18 ± 0.15 j	22.2 ± 2.00 i	1.06 ± 0.09 i

[a] Means within a column not sharing a common letter differ significantly ($P<0.01$).

[b] Percent yield of monomer in an incubation medium containing a mixture of carbohydrase enzymes; results represent the mean ± SEM of duplicate determinations for samples 4 and 5 and triplicate determinations for samples 1, 2, 3, and 6 normalized against a maltodextrin control.

[c] Results represent the mean ± SEM of five adult male chickens for samples 1-5 and six adult male chickens for sample 6. Both digestibility and true metabolizable energy (corrected to nitrogen equilibrium) values were corrected for endogenous dry matter and gross energy excretion, respectively, using mean excreta values from five control roosters that had been fasted for 48 hr. Dextrose in vivo values were corrected for 9.1% moisture.

[d] Digestibility (%) =

$$\frac{\text{DM intubated} - (\text{DM excreted} - \text{DM excreted by fasted controls})}{\text{DM intubated}} \times 100.$$

[e] TMEn (kcal/g) = $\dfrac{\text{EI} - (\text{Ef} - 8.22\ \text{Nf}) + (\text{Efs} - 8.22\ \text{Nfs})}{\text{Amount of compound intubated}}$

where TMEn = nitrogen-corrected true metabolizable energy; DM = dry matter (g); EI = energy intubated/48 hr (kcal); Ef = energy excreted by intubated birds/48 hr (kcal); Nf = nitrogen excreted by intubated birds/48 hr (g); Efs = energy excreted by fasted birds/48 hr (kcal).

Table 2—Comparison of predicted and actual digestibility and TMEn values for prototype low-calorie dextrose polymeric bulking agents[a]

Prototype low-calorie bulking agent	In vitro dextrose yield (%)[b]	In vivo digestibility (%)[c]		In vivo TMEn (kcal/g)[c]	
		Predicted	Actual	Predicted	Actual
A	56.48 ± 0.13 d	73.37 m 0.15 d	77.26 ± 4.53 d	2.78 ± 0.006 d	2.94 ± 0.09 d
B	31.81 ± 0.69 e	45.99 ± 0.66 e	38.65 ± 6.23 e	1.85 ± 0.023 e	1.50 ± 0.04 e
C	12.46 ± 0.07 f	24.51 ± 0.08 f	29.44 ± 3.15 e	1.11 ± 0.003 f	1.07 ± 0.13 f
D	2.34 ± 0.08 g	13.29 ± 0.09 g	23.85 ± 4.39 e	0.73 ± 0.003 g	0.89 ± 0.19 f

[a] Means within a column not sharing a common letter differ significantly ($P<0.01$).

[b] Percent yield of monomer in an incubation medium containing a mixture of carbohydrase enzymes; results represent mean ± SEM of duplicate determinations for prototypes A and D and triplicate determinations for prototypes B and C, all normalized to a maltodextrin control.

[c] Results represent mean ± SEM of duplicate (prototypes A and D) or triplicate (prototypes B and C) predictions, and of four (samples B and D) or five (samples A and C) actual in vivo determinations. In vivo digestibility and TMEn were determined as described in Table 1; predicted TMEn and digestibility were calculated using equations shown in Fig. 1 and 2, respectively.

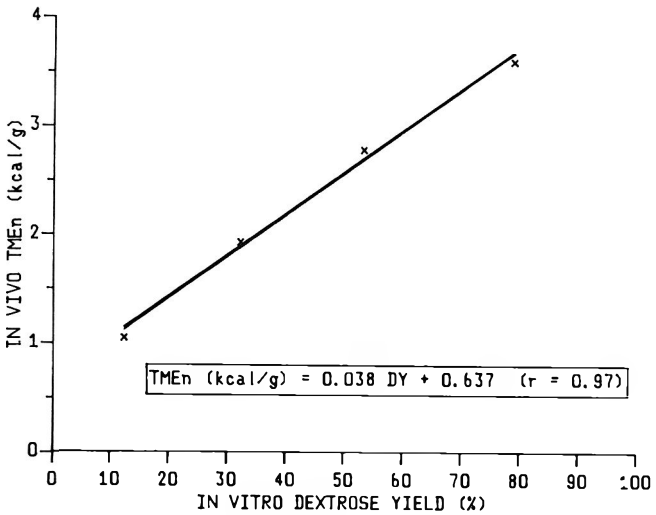

Fig. 1—Regression of in vivo true metabolizable energy (corrected to nitrogen equilibrium) on *in vitro* dextrose yield (samples 3-6, Table 1; n = 21).

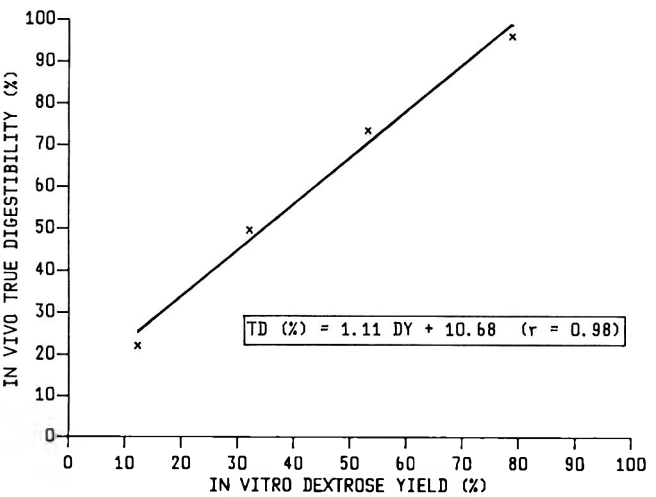

Fig. 2—Regression of in vivo true digestibility on *in vitro* dextrose yield (samples 3-6, Table 1; n = 21).

Prediction of TMEn from in vitro digestibility

Four low-calorie prototype bulking agents, all dextrose polymers, were measured for dextrose yield using the *in vitro* assay system. Dextrose yields ranged from 2 to 56% (Table 2). Using the regression equation described above (TMEn = 0.038 DY + 0.637), TMEn was predicted with values ranging from 0.73 kcal/g (prototype D) to 2.78 kcal/g (prototype A). To check the accuracy of the predicted TMEn values, TMEn was determined in the intact male chicken and resulting values ranged

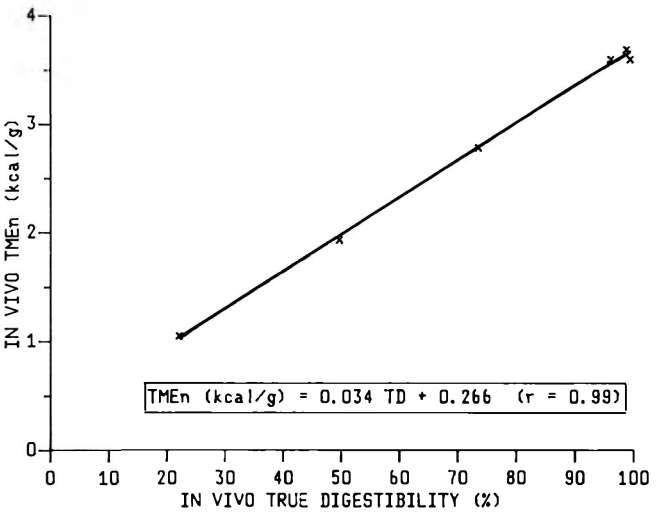

Fig. 3—Regression of in vivo true metabolizable energy (corrected to nitrogen equi ibrium) on *in vivo* true digestibility (samples 1-6, Table 1; n = 31).

from 0.89 kcal/g (prototype D) to 2.94 kcal/g (prototype A). Comparing determined TMEn values to predicted TMEn values indicated close agreement and high correlation (r = 0.91), with the possible exception of sample B whose SEM was indicative of the determined value being statistically lower ($P<0.05$) than the predicted value. All other prototype compounds had predicted TMEn values not statistically different ($P>0.05$) from determined values. Predicted and determined *in vivo* digestibility values were also highly correlated (r = 0.88).

DISCUSSION

HYDROLYSIS OF STARCH POLYMERS to maltose, maltotriose, and α-limit dextrins is begun by salivary amylase in the mouth and finished by pancreatic amylase in the lumen of the small intestine. In the mucosa of the small intestine, maltase and sucrase hydrolyze maltose and maltotriose to dextrose, and isomaltase hydrolyzes α-1,6-branched dextrins to dextrose and maltose (Roehrig, 1984). To construct an *in vitro* system which accurately simulates enzymatic digestion of carbohydrate polymers in the lumen and mucosa, it is necessary to have present α-1,4 and α-1,6 hydrolyzing activities. If commercially available carbohydrases of microbial origin (e.g., amyloglucosidase and pullulanase) are substituted for mammalian carbohydrases in developing the *in vitro* digestibility assay, enzyme assay costs can be substantially reduced.

In our *in vitro* digestibility system, enzymatic hydrolysis of substrates to dextrose is accomplished primarily by the exo-α-1,4 bond hydrolyzing activity of amyloglucosidase, supplemented with endo-α-1,4 hydrolysis by α-amylase and endo-α-1,6 hydrolysis by pullulanase. The addition of an excess of α-

amylase leads to a reduced dextrose yield in reaction products. This may be a result of too rapid conversion of maltodextrin by α-amylase to short polymer lengths which are unsuitable substrates for amyloglucosidase. Pullulanase aids in dextrose production by rapidly hydrolyzing the substrate α-1,6 bonds. Complete hydrolysis of control substrate to dextrose is limited by reversion reactions in which the *in vitro* enzymes catalyze dimer- and trimerization of dextrose product. Dextrose substrate underwent reversion during the assay that reduced its percent dextrose yield to levels comparable to the maltodextrin control.

The caloric value of dextrose polymers can be reduced by partially or fully reducing the proportion of enzymatically susceptible α-1,4 and α-1,6 bonds. Dextrins and polymerized dextrose are examples of compounds in which the proportion of susceptible bonds are reduced. The TMEn value of 1.06 kcal/g for polydextrose determined in our *in vivo* avian assay (Table 1) agrees with those assigned the compound in rat (Figdor and Rennhard, 1981) and human (Figdor and Bianchine, 1983) studies. Barley β-glucan (prototype compound D) is a β-1,3 and β-1,4-linked dextrose polymer which has no α-1,4 or α-1,6 bonds. As reported in Table 2, this compound was poorly digested in the *in vitro* test and it also delivered little metabolizable energy to the chicken in the *in vivo* test.

The *in vitro* enzyme system is less efficient than the *in vivo* avian system in hydrolyzing carbohydrate polymers (Table 1). The greater digestibility *in vivo* than *in vitro* is not readily explained solely by lower-gut fermentation and absorption of energy-furnishing fatty acids; the difference is too great. Thus, germ-free or cecectomized chickens would likely yield digestibility values only slightly lower than those obtained with the conventional birds used in our trials. Although lower, the *in vitro* digestibility values were highly correlated with *in vivo* values determined using a balance procedure with adult male chickens. This suggests that the *in vitro* assay developed herein is superior to the radiochemical procedures used by Figdor and Rennhard (1981) to estimate caloric value of polydextrose. Cooley and Livesey (1987) recently showed that these radiochemical procedures did not accurately predict the caloric value of polydextrose for rats as determined by a conventional energy-balance procedure.

In accordance with the findings of Lowry et al. (1986), the TME assay worked quite well for caloric assessment of carbohydrates varying widely in metabolizable energy. Moreover, *in vivo* digestibility accurately predicted TMEn over a broad range (Fig. 3), and the TME assay did not exhibit the problems associated with growth assay methods described by Lowry et al. (1986).

CONCLUSIONS

A PREDICTIVE linear regression equation was established between *in vitro* and *in vivo* digestibility of dextrose-based carbohydrate polymers for use in evaluating potential low-calorie bulking agents. The *in vitro* assay described in this paper has the greatest predictive utility for compounds with *in vitro* dextrose yields below 80%. Above this value the incremental increase in *in vivo* caloric energy compared with *in vitro* enzyme hydrolysis is greatly diminished. This does not limit the value of the assay, however, since only compounds with low dextrose yields and concomittant low *in vivo* digestibility are of interest as prototype low-calorie bulking agents. The high correlation between *in vitro* and *in vivo* measurements allows the linear regression equations developed herein to be useful in predicting the caloric value and dry matter digestibility of prototype low-calorie bulking agents. Screening can be done rapidly and without the high costs associated with *in vivo* caloric measurements.

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Immunogenicity and Allergenicity of Whey Protein Hydrolysates

J. ASSELIN, J. AMIOT, S.F. GAUTHIER, W. MOURAD, and J. HEBERT

ABSTRACT

Allergic reactions to cow's milk are common in children. The sera of allergic subjects have been found to contain specific IgE antibodies against several whey proteins such as α-lactalbumin (α-la) and β-lactoglobulin (β-lg). This work aims to study the effect of hydrolysis on the immunogenicity and the allergenicity of α-la and β-lg. Whey proteins were submitted to hydrolysis with immobilized α-chymotrypsin. High performance liquid chromatography analysis showed a decrease of native whey proteins and the appearance of low molecular weight peptides. The immunogenicity of these hydrolysates was not modified, but their allergenicity, as assessed by RAST inhibition, was decreased. The results may have important clinical implications.

INTRODUCTION

MILK being the first food for newborn babies, the nutrients in human milk are quantitatively and qualitatively adapted to their immature state and nutritive requirements. The proportions of cow's milk constituents used in infant food formulas are modified to correspond to the child's requirements but some allergies to bovine proteins can still develop.

Bahna (1978) estimated that 0.3 to 7.5% of babies, representing about 100,000 babies each year in the U.S.A., are allergic to cow's milk.

The allergenicity of cow's milk was related to heterogenicity of protein constituents and many authors considered β-lactoglobulin (β-lg) as being the major allergen because of its absence in human milk (Moneret-Vautrin et al., 1982; Bahna and Heiner, 1980); however the absence of β-lg in human milk is under controversy (Conti et al. 1980, Brignon et al. 1985). Gjesing et al. (1986) have shown that milk-allergic children had mainly IgE against α-lactalbumin, β-lactoglobulin, albumin, and immunoglobulin, the four major proteins of bovine whey, as well as IgE against the three casein components.

To reduce the allergenicity of milk proteins, the effect of heat treatment has been studied by many investigators. Ratner et al. (1958) showed that heating of whey proteins reduced antigenicity, but no change was observed with caseins. Bleumink and Young (1968) demonstrated that heat treatment of milk induced associations between β-lg and lactose by Maillard reaction and that the allergenic capacity of the product was a hundred times greater than that of native β-lg. The Maillard reaction was also associated with a loss of 24% of available lysine (Heppel et al., 1983). McLaughlan et al. (1981) observed that the milk with severe heat treatment during manufacture of commercial baby milk formula sensitized guinea-pigs less efficiently than mildly heated preparations. Kilshaw et al. (1982) established that severe heating applied to diafiltered whey completely abolished its sensitizing capacity for systemic anaphylaxis. Recently, Baldo (1984) demonstrated that even after severe heating (100°C for 3 h), the IgE antibodies in the serum of many patients still reacted with heated proteins and concluded that there was little possibility of developing a hypoallergenic milk by heat treatment.

Kaneko et al. (1985) suggested a method to eliminate selectively the β-lg from bovine milk whey with a maximal retention of Igs and α-la by a $FeCl_3$ precipitation. This hypoallergenic substitute is a new but expensive product and the α-la is also allergenic and thereby eliminated the usefulness of this product.

Commercial milk substitute formulas are made with goat milk or soy proteins, but those infant food formulas were also found to be responsible for allergic diseases and other nutritional and physiological disorders (Polonovski, 1979; Van der Horst, 1976). Goat milk proteins showed crossed immunologic reactions with bovine proteins, especially for caseins, β-lg and α-la (Bahna and Heiner, 1980). Dietary products made with casein hydrolysates are also available for children allergic to these proteins (Moneret-Vautrin et al., 1982).

The purpose of this study was to investigate the enzymatic hydrolysis of whey proteins as a means to eliminate the major milk allergens, β-lg and α-la, and thus find a solution for the allergies of infants towards cow's milk.

MATERIALS & METHODS

Materials

Enzymatic hydrolysis was performed on a commercial whey protein concentrate electrodialyzed and used for the fabrication of commercial infant food formulas (Wyeth Ltd., Toronto).

The following proteins were purchased from Sigma Chemical Company (St. Louis, Mo): β-lactoglobulin A from bovine milk, α-lactalbumin from bovine milk (Grade II), gelatin from swine skin and α-chymotrypsin from bovine pancreas (type II). The CNBr-activated Sepharose® 4B gel and the Radioallergosorbent test kits were obtained from Pharmacia Fine Chemicals (Sweden).

Sera of children with a history of reactions to cow's milk were obtained from the Centre Hospitalier de l'Université Laval. Among 14 sera analyzed, 7 sera were not used in the study because of no evidence of IgE-mediated cow's milk allergy.

Evaluation of specific IgE antibodies in human sera

Fourteen children (less than 2 years of age; 7 boys and 7 girls) with history of immediate reaction to cow's milk (gastrointestinal, cutaneous and/or respiratory symptoms) were evaluated by skin testing with cow's milk, casein, α-la and β-lg (Omega, Mtl) using the prick technique. The diameters of flare reactions were measured in millimeters. Serum specific IgE antibodies against cow's milk, α-la, and β-lg were measured by Pharmacia RAST, a commercial enzyme immunoassay manufactured by Pharmacia Diagnostics (Sweden). The test was performed as described in the manufacturer's procedure. Briefly, antigen-coated discs and human serum (50 μL) were incubated for 3 hr at 22°C. After three washes, enzyme-conjugated anti-human IgE (50 μL) were added and incubated overnight at 22°C. The reaction was then developed and read with an automatic spectrophotometer (Titertek) at 420 nm. The results were expressed in Phadezym RAST class on a scale from 0 to 4, where 4 represents highest levels of IgE antibodies and 0 undetectable levels.

The patients were also tested by opened milk challenge (including placebo) and the test was positive when elicitating immediate gastrointestinal, cutaneous and/or respiratory symptoms (Bédard and He-

Authors Asselin, Amiot, and Gauthier are with the Centre de recherche en nutrition, Département de sciences et technologie des aliments, Faculté des Sciences de l'agriculture et de l'alimentation, Univ Laval, Quebec, Canada, G1K 7P4. Author Gauthier is also affiliated with the Centre de recherches alimentaires de Saint-Hyacinthe (CRASH), Direction de la recherche, Agriculture Canada, 3600 Boul. Casavant Quest, Saint-Hyacinthe, Quebec, Canada, J2S 8E3. Authors Mourad and Hebert are with the Centre hospitalier de l'Université Laval, Unité de recherche en Inflammation et Immunologie-Rhumatologie, 2705, Boul. Laurier, Ste-Foy, G1V 4G2.

bert, 1987). Only the patients who were positive for skin tests and RAST and for whom the provocation was positive were included in the study.

Enzymatic hydrolysis of whey proteins

The α-chymotrypsin was immobilized on CNBr-activated Sepharose® 4B gel according to Pharmacia's procedure. The activity of fixed chymotrypsin, determined by pH-Stat at pH 7.0, using 0.02M N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate (10 mL) and 0.05M KCl (prepared in ethanol 5%) as described by Wilcox (1970), was 0.41 units/mg gel. One unit of esterase activity was defined as the amount of enzyme needed for the hydrolysis of 1 μmole substrate per minute at pH 7.0 at 22°C.

Solutions of whey proteins (200 mL) at 2.5% prepared in 0.1M sodium phosphate buffer, pH 7.5, were hydrolyzed under agitation for 0, 10, 30, or 60 min. in rotating bath (Lab-Line Instruments, Inc. Canlab, Quebec) at 150 rpm and 37°C. Enzymatic hydrolysis was started by the addition of 6g freeze-dried gel (immobolized α-chymotrypsin) and stopped by filtration through a millipore filter. The hydrolysis was performed in triplicate for each reaction time and the hydrolysates were freeze-dried.

Characterization of whey protein hydrolysates

The degree of hydrolysis (DH) is defined as the percentage of cleaved peptide bonds as assessed by the trinitrobenzenesulfonic acid (TNBS) method (Adler-Nissen, 1979). This method is a spectrophotometric assay of the chromophore formed by the reaction of TNBS with primary amines.

Residual α-chymotrypsin activity in each freeze-dried hydrolysate was measured with N-acetyl-L-tyrosine ethyl ester (ATEE) as substrate by the method of Schwert and Takenaka (1955). One chymotrypsin unit corresponds to the hydrolysis of 0.1 μmole ATEE per min at 23°C and pH 7.0. The residual activity of fixed enzyme was compared to that of free chymotrypsin activity previously measured to be 159 ATEE units/mg protein. The spectrophotometric method was more sensitive and allowed detection of traces of fixed enzymes in the gel.

The molecular weight distribution profile of hydrolysates were obtained by HPLC analysis, using a TSK-SW 2000 column and phosphate buffer 50 mM containing 0.1% TFA and 35% methanol as the mobile phase, according to the method developed in our laboratory by Vijayalakshmi et al. (1986).

Immunologic and allergenic analysis of hydrolysates

Four hydrolysates were tested for their antigenicity as assessed by their ability to induce antibody response in rabbit properly immunized. Each New Zealand white rabbit (adult male, 2–3 kg) was immunized with one concentration (25, 50, or 75 mg/mL) of one of the four hydrolysates by intramuscular injections of 1 mL of antigen emulsified with an equal volume of Freund's adjuvant. Two subsequent injections were made at intervals of three weeks with the same antigen emulsified with incomplete Freund's adjuvant. Before the first injection, blood (20 mL) was collected as pre-immune sera and the negative control. One week after the last injection, blood was collected and the serum obtained.

To determine the rabbit immune response, Ouchterlony double immunodiffusion (Hébert et al., 1985) was carried out with whey proteins.

Specific IgG antibodies were also measured by the immunodot method. Ten μL antigen (whey, β-lg or α-la) were fixed to nitrocellulose discs and after saturation of the nonbinding sites with 0.2% gelatin solution and three washes, 100 μL rabbit antisera were added and incubated overnight in a moist chamber at room temperature. After extensive washing with PBS, 100 μL of ^{125}I-labeled goat anti-rabbit IgG were added for 12 hr in the same conditions. Finally, bound radioactivity was counted in a LKB Gamma Counter after three washes with PBS.

The allergenicity of the various hydrolysates was analyzed by their ability to inhibit the binding of human serum specific IgE antibodies to β-lg or α-la coated disc (radioallergosorbent test inhibition). Briefly, allergen-coated discs (β-lg or α-la) were first incubated with a pool of serum from subjects allergic to milk (as defined above) in the presence or absence of various concentrations of unhydrolyzed whey proteins (T_0) in order to determine the concentrations capable of inhibiting 50% of the reaction. This concentration (10 mg/mL) was used when hydrolyzed proteins were tested as inhibitors in subsequent experiments. Results were expressed as the percentage of inhibition for each hydrolysate.

RESULTS & DISCUSSION

Incidence of allergies to β-lactoglobulin and α-lactalbumin for patients allergic to cow's milk

Skin tests and RAST results are presented in Table 1. Sera originating from fourteen young patients who showed a history of reactions to cow's milk were analyzed. These children were allergic to cow's milk at some previous time and all had positive provocation, but 7 of 14 children showed no evidence of IgE-mediated cow's milk allergy (Bock, 1982). In some cases, RAST lacked the sensitivity to detect the presence of specific IgE antibodies to milk allergens so, the skin test had to be done to confirm the allergy. Skin tests and RAST are complementary, specific, and very useful for diagnosis of allergic reaction (Bédard and Hébert, 1987).

Among the fourteen patients under study, seven were allergic to whey proteins. All patients produced IgE antibodies against both β-lg and α-la. Therefore, the children were considered to be allergic to both or neither whey proteins. These results could suggest an antigenic cross reactivity between these two determinants responsible, in part, for allergy symptoms to cow's milk. Using purified protein preparations, Saperstein (1960) also suggested an immunologic similarity between these two proteins.

Since β-lg and α-la proteins seem to be major allergenic determinants and no hypoallergenic substitute made with cow's milk is commercially available, whey appears to be a good substrate for proteolysis treatment.

Characterization of the hydrolysates

The percentage of peptide bonds cleaved after 0, 10, 30, and 60 min. of hydrolysis were 0, 3.95, 5.85, and 7.46, respectively, as measured by the TNBS method (Table 2). The enzymatic activity of the chymotrypsin was more extensive in the first ten minutes and gradually decreased thereafter.

Residual enzymatic activity (REA) in the hydrolysates (Table 2) indicate no chymotrypsin activity up to 30 minutes of reaction. After 60 minutes of hydrolysis, a small quantity of enzyme was found in the hydrolysate (0.03 U/mg) but represented less than 0.02% (w/w) of the final product. Axen (1974) observed that the conjugates ribonuclease A attached to cyanogen-bromide activated agarose must be washed extremely carefully to release any noncovalently adsorbed enzyme. He also observed that all stirring and shaking procedures

Table 1—Immunological evaluation of patients with history of reaction to cow's milk

Patients	Skin tests CM[a] (mm)	Specific IgE antibodies levels[b] CM	α -la	β-lg	Opened milk challenge
MG	ND[c]	0	0	0	–
FT	6	0	0	0	–
LDM	4	3	3	2	+
BLV	3	2	2	1	+
LS	8	4	3	4	+
HM	0	0	0	0	–
LD	0	1	0	0	–
RA	2	2	2	2	+
MGF	4	2	3	2	+
DN	10	4	3	3	+
SHK	ND	0	2	1	–
FJK	2	1	0	0	+
DJ	ND	0	0	0	–
MRK	0	0	0	0	–

[a] Skin test by prick technique with cow's milk (CM): diameter of flare reaction in millimeter.

[b] Phadezym RAST class: 4 = very high level of allergen specific IgE antibody; 0 = Absent or undetectable

[c] Not determined

Table 2—Degree of hydrolysis (DH) and residual enzymatic activity (REA) measured for each hydrolysate of whey proteins

Hydrolysate	Reaction time (min.)	Degree of hydrolysis[a] (%)	Residual enzymatic activity[b] (U/mg hydrolysate)
T_0	0	0	0
T_{10}	10	3.95	0
T_{30}	30	5.85	0
T_{60}	60	7.46	0.03

[a] Measured by trinitrobenzenesulfonic acid (TNBS) method
[b] Free enzyme activity was 159 U ATEE/mg protein

must be gentle to avoid mechanical disintegration, which may cause mechanical leakage during the use of the immobilized enzymes, and to avoid the passage of enzymatically active microbeads through filter discs.

The molecular weight distribution profiles of whey protein hydrolysates (T_{10}, T_{30}, and T_{60}) in comparison to whey proteins (T_0) are shown in Fig 1. Even after 60 minutes of reaction, there was a small amount of unhydrolyzed proteins. The major peak on the left represents aggregates of intact proteins with molecular weight range of 44000 daltons. These proteins gradually disappeared with increase in time of hydrolysis and, correspondingly, new peaks of molecular weight averaging around 3500 to 125 daltons appeared. Although their amounts increased with the length of hydrolysis, their molecular distribution remained about the same with extent of hydrolysis, indicating α-chymotrypsin's specific and limited activity on the proteins. The α-chymotrypsin was chosen for the enzymatic hydrolysis of the whey proteins because of its specific activity and expected hydrolysate composition. It seemed however that the α-chymotrypsin cleaved more than the bonds involving the aromatic amino acids since the peak on the right side is composed of unexpected low molecular weight peptides and free amino acids: this fact was observed by Pélissier (1984) with the hydrolysis of caseins by α-chymotrypsin. In this regard, the sensory properties should be evaluated on the final product considering that Pregestimil® (a formula based on casein hydrolysate) is less palatable than other formulas (Brady et al., 1986).

Antigenicity of the hydrolysates

The presence of antibodies specific to whey (T_0), α-la, and β-lg in rabbit antisera was determined by qualitative double immunodiffusion (Table 3) and semi-quantitative immunodot (Table 4). Results of both methods indicated that the immune response was comparable for all rabbits immunized against whey or against hydrolysates, independently of the degree of hydrolysis. The antigenic determinants present in whey and responsible for antibody production by rabbit were conserved in the hydrolysates. The antibodies produced by animals sensitized to hydrolysates reacted with whey, α-la, and β-lg. These results were expected since some of the two native proteins remained in the hydrolysate as demonstrated by HPLC analysis.

Allergenicity of the hydrolysates

The results of the RAST inhibition indicated a modification of the allergenicity of α-la and β-lg by hydrolysis (Table 5). However, when the degree of hydrolysis increased, its ability to inhibit the reaction between antigen and serum IgE antibodies decreased. The allergenicity associated with whey proteins was not completely eliminated by the experimental conditions applied in this study as shown by the inhibition seen with higher degree of hydrolysis (T_{60}). These results, however, constitute a good indication that the elimination of the allergenicity of whey proteins could be achieved; however, further work must be done to refine the procedure so that all the allergenicity of the native proteins can be eliminated.

CONCLUSION

THE RESULTS herein suggest the existence of a relationship between the major whey proteins, α-la and β-lg, and the allergic reactions in newborn babies. Furthermore, this study could suggest cross reactivity between these two whey proteins, since the children were found to be allergic for both or neither proteins.

The enzymatic hydrolysis of whey proteins modified the allergenic properties of the proteins to some extent under conditions used in this study but did not completely eliminate the allergenicity associated with β-lg and α-la.

The immobilized enzyme system was used to avoid all contamination with exogenic substances (catalyst or inhibitor), up to 60 minutes of reaction time and to obtain an interesting peptide profile with significant decrease of allergenicity of α-la and β-lg. This system was also selected for the following reasons: no significative residual enzymatic activity in the hydrolysates, control of the extent of hydrolytic activity and industrial feasibility. The molecular weight distribution profile of peptides obtained seems to be less than the theoretical pro-

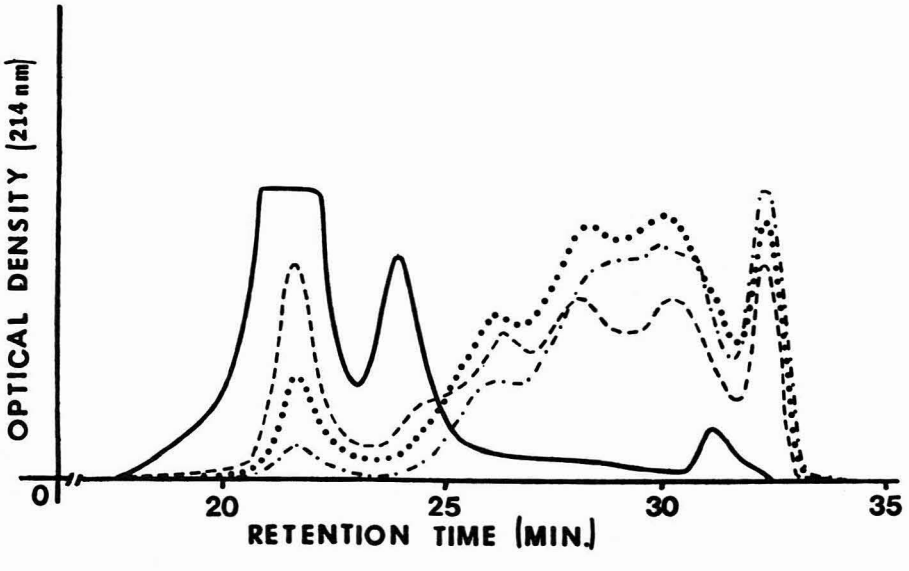

Fig. 1—Chromatographic analysis on HPLC-TSK-2000 SW column of the whey proteins hydrolyzed by α-chymotrypsin after 0 (—), 10 (--), 30 (...), and 60 (—·—·) min.

Table 3—Antibody production in rabbits immunized with the different hydrolysates, as assessed by double immunodiffusion[a]

Ag injected	Dilution of serum 1:1	1:2	1:4	1:8	1:16	1:32
T_0 (25 mg/mL)	+	+	+	−	−	−
T_0 (50 mg/mL)	+	+	+	+	−	−
T_0 (75 mg/mL)	+	+	+	+	−	−
T_{10} (25 mg/mL)	+	+	−	−	−	−
T_{10} (50 mg/mL)	+	+	+	−	−	−
T_{10} (75 mg/mL)	+	+	+	−	−	−
T_{30} (25 mg/mL)	+	+	−	−	−	−
T_{30} (50 mg/mL)	+	+	+	−	−	−
T_{30} (75 mg/mL)	+	+	+	+	−	−
T_{60} (25 mg/mL)	+	−	−	−	−	−
T_{60} (50 mg/mL)	+	+	+	+	−	−
T_{60} (75 mg/mL)	+	+	+	−	−	−

[a] Central well (Ag): Hydrolysate T_0 (12.8 mg/mL); Circumferential wells (Ab): Antiserum diluted; Reaction +: Presence of a precipitation line; Reaction −: No reaction.

Table 4—Antibody production in rabbits immunized with the different hydrolysates, as assessed by immunodot

Ag injected	Fixation (%)[a] T_0	α-la	β-lg
T_0 (25 mg/mL)	40.2	21.9	30.0
T_0 (50 mg/mL)	48.3	33.9	29.6
T_0 (75 mg/mL)	40.6	31.0	32.8
T_{10} (25 mg/mL)	39.3	24.2	23.6
T_{10} (50 mg/mL)	40.1	33.8	34.3
T_{10} (75 mg/mL)	41.0	20.9	21.1
T_{30} (25 mg/mL)	42.6	31.7	35.8
T_{30} (50 mg/mL)	45.7	39.7	41.0
T_{30} (75 mg/mL)	48.8	37.2	43.5
T_{60} (25 mg/mL)	40.1	30.3	29.3
T_{60} (50 mg/mL)	47.6	31.7	26.9
T_{60} (75 mg/mL)	37.2	21.8	13.6

[a] Calculation:

$$\% \text{ Fixation} = \left(\frac{\text{CPM OF } ^{125}\text{I-labeled anti-IgG* fixed}}{\text{CPM of } ^{125}\text{I-labeled anti-IgG* added}}\right) \times 100$$

file expected and can be explained by chymotrypsin cleaving more than peptide bonds involving aromatic amino acids.

In view of industrial applications, the intact whey proteins remaining in the hydrolysates should be eliminated. This could be achieved by modifying the enzymatic system (change of the enzyme or the solid support or lengthening the time of hydrolysis) and/or by removing α-la and β-lg from the hydrolysate by ultrafiltration.

Enzymatic hydrolysis seems to be an efficient method for the production of hypoallergenic infant food formula and a good alternative for children allergic to whey proteins.

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Table 5—Percentage of inhibition of IgE binding to α-lactalbumin or β-lactoglobulin by various hydrolysates

Hydrolsates (10 mg/ml)	α-la	β-lg
Control[a]	0	0
T_0	46.9*	58.0
T_{10}	38.7	54.5
T_{30}	36.4	37.8
T_{60}	24.9	11.1

[a] Phosphate-buffered saline (PBS) was used for control. Calculation:

$$* \% \text{ inhibition} = \left[1 - \left(\frac{\text{O.D. obtained in presence of hydrolysate}}{\text{O.D. obtained in absence of hydrolysate}}\right)\right] \times 100$$

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Effect of Carbon Dioxide on the Thermodynamic State of Water in Collagen

J.J. SPECCHIO, E. KARMAS, H. DAUN, S. PAIK, and S.G. GILBERT

ABSTRACT

Thermodynamic activities of polar sites of collagen in the presence of CO_2 were observed by inverse gas chromatographic techniques using water as a probe. The interactions between collagen and the water probe were evaluated by determining the specific retention volume (Vg°) and partition coefficient (Kp) at 25°C, 30°C, and 35°C. Thermodynamic parameters were determined from these data. CO_2 exhibited a significant effect on the water binding of collagen as shown by increased Vg° and Kp values as compared to N_2- and He-treated collagen. The thermodynamic parameters of partial molar Gibb's free energy ($\Delta\bar{G}_T^\circ$), partial molar enthalpy ($\Delta\bar{H}_T^\circ$) and partial molar entropy ($\Delta\bar{S}_T^\circ$) indicated CO_2 significantly increased the average energy of water binding by collagen.

INTRODUCTION

THE EXACT ROLE of water in the structure and function of biopolymers in food systems still remains unclear. The importance of water as a food constituent is recognized, however, the physical chemistry of these systems is poorly understood (Gilbert et al., 1983).

Protein-water interaction is of great importance from a theoretical and practical point of view. At the lower vapor pressure region, specific binding sites are believed to be responsible for this interaction (Coelho et al., 1979). These binding sites are believed to be polar amino acid side chains (Wolfenden et al., 1981). Recently, studies have been undertaken to evaluate changes in thermodynamic parameters such as enthalpy of absorption which indeed is related to binding energy of polar amino acids.

Coelho et al. (1979) reported that the heat of sorption at lower vapor pressure regions was not linearally related to that at a higher vapor pressure region. This indicated that the heterogenic amino acid composition in natural proteins was responsible with the polar amino acids in minor concentrations being most active.

Understanding and determining the effect of CO_2 gas on dry protein-water interaction during the hydration process would provide useful information on bacterial spoilage as well as sensory properties. Therefore, the understanding of the behavior of protein-water interactions in a CO_2 atmosphere could become an indispensable tool for studies of the determination of shelf life and the use of modified atmospheric packaging for low to intermediate moisture products.

Recently, inverse gas chromatography (IGC) has been employed as an effective technique in moisture sorption studies of food systems such as proteins, starches, bakery products and freeze-dried coffee (Helen, 1983; Paik, 1984; Tanaka, 1984; Smith et al., 1982). Due to the high sensitivity of the detector system, more rapid and accurate information regarding specific binding sites at the lower vapor pressure regions is facilitated. The observation and evaluation of a parameter such as specific retention volume (Vg°) enable an in-depth investigation of the thermodynamics of specific binding sites (Coelho et al., 1979).

The objective of this study was to determine the effects of CO_2 and N_2 atmospheres on the thermodynamic state of water in collagen by studying their interactions during inverse gas chromatography. Specific objectives included the calculation of the thermodynamic parameters of CO_2-collagen and H_2O interactions which govern sorption mechanisms and to determine the protein-water interaction at lower moisture where the effect of specific binding sites would be dominant.

Author Specchio is with the Dept. of Food & Nutrition, Montclair State College, Upper Montclair, NJ 07043. Authors Karmas, Daun, Paik, and Gilbert are with the Dept. of Food Science, Rutgers Univ., New Brunswick, NJ.

THEORY

Inverse gas chromatography

Inverse gas chromatography (IGC) involves the assay of the changes of a stationary phase by determining the interaction with a mobile phase that is known (Guillet, 1973; Guillet and Galin, 1973; Nakajima and Gocho, 1981; Gilbert, 1984).

Retention values. In gas chromatography, the net retention volume (Vn) is presented by Eq. (1)

$$Vn = Vr - Vo \tag{1}$$

where, Vr = retention volume of probe and Vo = retention volume of an unabsorbed peak (air).

The net retention time can also be presented by Eq. (2)

$$Vn = tr \cdot w \cdot j \frac{Tc}{Ta} \cdot \left(\frac{Po - Pa}{Po}\right) \tag{2}$$

where, tr = empirical column pressure gradient correction factor, w = carrier gas flow rate, Tc = column temperature, Ta = ambient temperature, Po = atmospheric pressure at ambient temperature and Pa = vapor pressure at ambient temperature.

The j factor can be calculated from Eq. (3) (James and Martin, 1952)

$$j = \frac{3}{2}\frac{(Pi - Po)^2 - 1}{(Pi - Po)^3 - 1} \tag{3}$$

where, Pi = column insert pressure.

The specific retention volume (Vg°) which is the net retention volume per unit weight, corrected to 0°C, is expressed by Eq. (4)

$$Vg^\circ = \frac{273}{Tc} \cdot Vn \cdot \frac{1}{w_s} \tag{4}$$

where, w_s = weight of polymeric material in a stationary phase.

The retention volume of the probe (Vr) can also be determined by Eq. (5)

$$Vr = Vo + Kp \cdot Vs \tag{5}$$

where, Vs = volume of polymeric material and Kp = partition coefficient, defined as the ratio between the probe concentration in the polymeric material and in the mobile phase. The net retention volume can, therefore, be expressed by combining Eq. (1) and (5).

$$Vn = Kp \cdot Vs \tag{6}$$

Kp can be expressed as

$$K_p = \frac{Vg^\circ \cdot \lambda s \cdot Tc}{273^\circ} \tag{7}$$

where, λ = density of polymeric material.

Thermodynamic parameters from Vg°

Theoretical work on liquid-gas interactions of polymer-solute thermodynamics has been extensively studied using retention values (Varsano, 1971). Khalil (1976) and Orr et al. (1981) expanded this work using a gas-solid mode. Thermodynamic parameters can be calculated for a gas-liquid system by employing Vg°C. The Vg° calculation is based on the assumption that Vg° is related to the partition coefficient (Kp) which is not affected by probe concentration. Vg°, however, generally shows a probe concentration dependence in a gas-solid system, therefore, Vg° must be defined at an infinite dilution to calculate thermodynamic parameters. When the probe concentration is at an infinite dilution, and the vapor pressure of the probe is extremely low, Kp is governed by Henry's Law because probe-probe interaction is negligible.

Orr et al. (1981) based the following discussion on thermodynamic parameters using Vg°. $\Delta\bar{G}_T°$, which constitutes the total standard partial molar Gibb's free energy as given using the following equation:

$$\Delta\bar{G}_T° = -RT \ln Kp \quad (8)$$

where R and T are gas constant and column temperature, respectively. Kp is obtained by Vg° by Eq. (5), as previously cited.

$\Delta H_T°$, which is the total standard partial molar enthalpy is calculated using the following equation:

$$\Delta\bar{H}_T° = R \cdot \frac{d(\ln Vg°)}{d(1/T)} \quad (9)$$

The total standard partial molar entropy, $\Delta S_T°$, is calculated from the Gibb's-Helmholtz equation:

$$\Delta\bar{S}_T° = \frac{\Delta H_T° - \Delta G_T°}{T} \quad (10)$$

MATERIALS & METHODS

Sample preparation

Fresh microcut calf-hide collagen (supplied by Devro, Inc., Somerville, NJ) was used in this study. A collagen dispersion in water was prepared by dropping the pH to 2.5 using dilute acetic acid. A known weight of this dispersion together with a known weight of inert support (Supelcoport 60/70 mesh) and water at 0°C was blended for 5 min. Care was taken to ensure that the temperature of the blend did not exceed 25°C. After freeze-drying for 24 hr, the mixture was further dried by heating in a forced circulation oven at 45°C for 24 hr (Coelho et al., 1979).

The dry stationary phase was passed through a 70 mesh sieve and packed into a 100 × 0.64 cm (o.d.) aluminum column using a vacuum pump and a vibrating device. The amount of stationary phase packed into the column was determined by weighing the column before and after the packing, and also by the difference in the weight of the container holding the stationary phase before and after packing.

To remove most of the moisture (without affecting the structural properties of the collagen) that might have been absorbed during the process of packing, the column was conditioned for 48 hr by passing dry helium (50 cc/min) through it. A separate column for CO_2, N_2, and He was employed to assure the direct affect of each gas on the collagen/H_2O relations.

Inverse gas chromatography (IGC) techniques

A Varian 1700 dual column gas chromatograph with thermal conductivity detector was used for the IGC analysis. Column temperature: 25°C, 30°C, and 35°C; detector temperature: 160°C; injection port temperatures: 150°C; carrier gases: CO_2, N_2, He; carrier gas flow rate: 60 cc/min - pulse, 40 cc/min - frontal; and filament current: 150 mA. High purity grade helium, CO_2 and N_2, manufactured by Union Carbide Company (Linden, NJ) was further purified by moisture and oxygen traps (R&D Separation, N. Highlands, CA) after the respective tanks.

The column temperature was monitored by a thermocouple attached on the surface of the midpoint of the column. The constant temperature was maintained by a circulating water bath around the column in the GC oven. The columns were separated from the heated injection ports and detector oven by spacer columns (5 cm long) filled with siliconized glass wool. Detector signals were recorded by a Hewlett-Packard 7127B stripchart recorder using a chart speed of 1.27 cm/min. The retention time of polar probes and peak area were monitored by an integrator (model C-EIB, Shimazu Company, Kyoto, Japan). Room temperature was monitored by a calibrated mercury thermometer. Carrier gas flow rate was measured at the detector outlet at room temperature by a soap bubble flow meter manufactured by Supelco, Inc. The column inlet pressure was measured by a mercury manometer. An outlet pressure of one atmosphere was used based on prior work.

Calculation of thermodynamic parameters

The specific retention volume Vg° was calculated from Equation 4 and thermodynamic parameters of sorption calculated from Vg° included partition coefficient (Kp), total standard partial molar Gibb's free energy ($\Delta\bar{G}_T°$), total standard partial molar enthalpy ($\Delta\bar{H}_T°$), and total standard partial molar entropy ($\Delta\bar{S}_T°$).

Statistical Analysis

An analysis of variance (ANOVA, confidence level of 95%) was used to determine the correlation between the thermodynamic parameters and the gaseous atmospheres.

RESULTS & DISCUSSION

Effect of gas on the sorption thermodynamics of Supelcoport

Partition coefficient (Kp°), specific retention volume (Vg°) and partial molar Gibb's free energy ($\Delta\bar{G}_T°$) were calculated at an infinite dilution to observe the interaction of the gas with sorption themodynamics of Supelcoport. Vg° values for CO_2, N_2 and He at 25°C, 30°C and 35°C were calculated by the relative retention time (Vn) by using Eq. (2) and (4).

The relationship between probe size, gas and Vg° is presented in Table 1. For all three gases, the relationship between probe size and specific retention volume Vg° indicated independence. The relationship was the same for three gases at a constant temperature. By obtaining Vg° at the infinite dilution, thermodynamic parameters of Kp° and $\Delta\bar{G}_T°$ were calculated. From the above data is was concluded that thermodynamics of the absorption of water on Supelcoport was gas independent.

Retention values

Retention time was calculated as in previous work which utilized the peak front for the observation of retention time for Vg° values (Varsano and Gilbert, 1973; Orr et al., 1981). The relative retention time (Vn) was graphically determined from the chromatograms generated by IGC using air as an unabsorbed peak.

Significant differences in the presence of CO_2, N_2, and He

Table 1—Specific retention volume (Vg°) of Supelcoport at 25°C, 30°C, and 35°C[a]

Amount Injected (μL)H_2O	Vg° 25°C	Vg° 30°C	Vg° 35°C
He			
1.0	2.56	2.98	2.97
2.0	2.57	2.96	2.98
3.0	2.57	2.98	2.98
4.0	2.56	2.98	2.96
5.0	2.55	2.97	2.98
CO_2			
1.0	2.97	2.57	2.55
2.0	2.96	2.57	2.55
3.0	2.97	2.55	2.57
4.0	2.97	2.56	2.57
5.0	2.97	2.57	2.57
6.0	2.96	2.57	2.57
N_2			
1.0	2.95	2.55	2.57
2.0	2.97	2.57	2.56
3.0	2.97	2.57	2.57
4.0	2.97	2.56	2.56
5.0	2.97	2.57	2.56
6.0	2.96	2.57	2.56

[a] Data are means of three determinations.

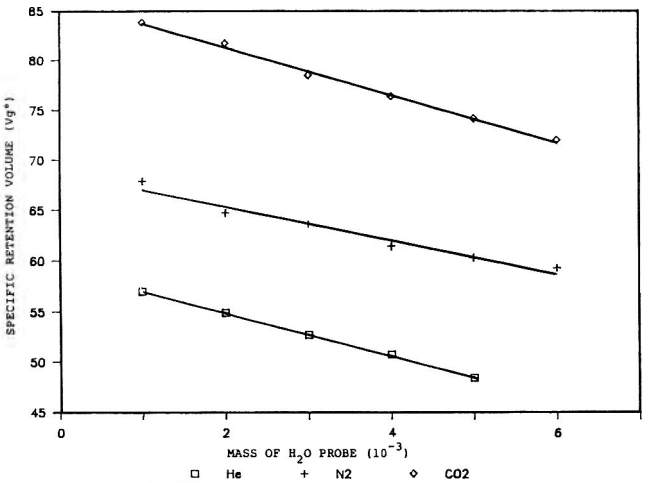

Fig. 1.—Effect of CO_2, N_2, and He on the specific retention volume (Vg°) in collagen at 25°C.

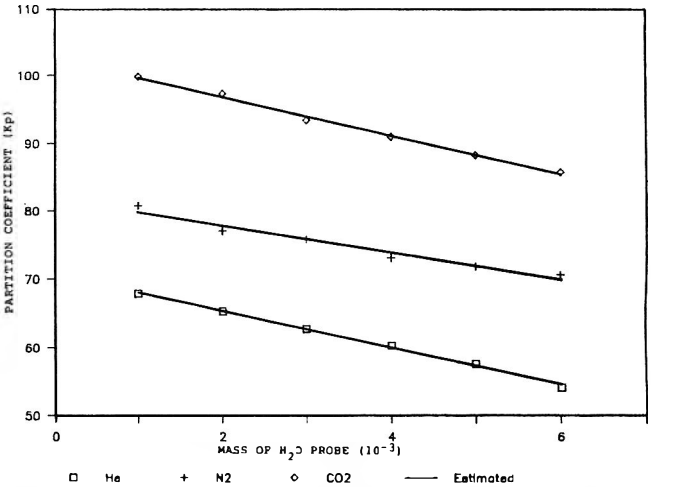

Fig. 2.—Effect of CO_2, N_2, and He on the partition coefficient (Kp) in collagen at 25°C from (Vg°) values.

Table 2—Effect of CO_2, N_2, and He on the specific retention volume (Vg°) of collagen at 25°C, 30°C, and 35°C[a]

Amount injected (μL) H_2O	Vg°		
	25°C	30°C	35°C
He			
1.0	57.04*	50.54	43.17
2.0	54.88	48.49	37.69
3.0	52.71*	47.36	34.51
4.0	50.67*	45.19	33.37
5.0	48.39*	43.14	32.35
CO_2			
1.0	83.84*	67.80*	50.91*
2.0	81.71*	64.67*	48.89*
3.0	78.46*	63.66*	47.77*
4.0	76.44*	61.53*	45.64*
5.0	74.20*	59.29*	43.51*
6.0	72.07*	58.28*	42.50*
N_2			
1.0	67.86*	52.82	40.89
2.0	64.67*	51.68	38.84
3.0	63.64*	49.63	36.67
4.0	61.37*	46.33	35.65
5.0	60.34*	44.17	33.37
6.0	59.32*	43.03	32.46

[a] Data are means of three determinations.
*Asterisk indicates that means for a comparison between gaseous atmospheres within a specific temperature are significantly different at $p < 0.05$ by ANOVA.

Table 3—Effect of CO_2, N_2, and He on the partition coefficient (Kp) of collagen at 25°C, 30°C, and 35°C[a]

Amount injected (μL) H_2O	(Kp)		
	25°	30°C	35°
He			
1.0	67.93*	61.20	53.14
2.0	65.36*	58.72	46.40
3.0	62.77*	57.35	42.48
4.0	60.34*	54.72	41.07
5.0	57.63*	52.24	39.82
CO_2			
1.0	99.85*	82.10*	62.66
2.0	97.31*	78.31*	60.18*
3.0	93.44*	77.09*	58.80*
4.0	91.04*	74.51*	56.18*
5.0	88.37*	71.79*	53.56*
6.0	85.83*	70.57*	52.31*
N_2			
1.0	80.82*	63.96	50.33
2.0	77.02*	62.58	47.81
3.0	75.79	60.10	45.14
4.0	73.09*	56.10	43.88
5.0	71.86*	53.49	41.07
6.0	70.64*	52.10	39.95

[a] Data are means of three determinations.
* Asterisk indicates that means for a comparison between gaseous atmospheres within a specific temperature are significantly at $p < 0.05$ by ANOVA.

Table 4—Effect of CO_2, N_2, and He on the total partial molar Gibb's free energy ($-\Delta\bar{G}_T$) of collagen at 25°C, 30°C, and 35°C[a]

Amount injected (μL) H_2O	$-\Delta\bar{G}°_T$		
	25°	30°C	35°
He			
1.0	2.50*	2.48	2.44
2.0	2.48*	2.46	2.35
3.0	2.45*	2.44	2.30
4.0	2.43*	2.41	2.28
5.0	2.40*	2.38	2.26
CO_2			
1.0	2.73*	2.66*	2.54*
2.0	2.71*	2.63*	2.51*
3.0	2.69*	2.62*	2.50*
4.0	2.68*	2.60*	2.47*
5.0	2.66*	2.58*	2.44*
6.0	2.64*	2.57*	2.43*
N_2			
1.0	2.60*	2.51	2.40
2.0	2.58*	2.49	2.37
3.0	2.57*	2.47	2.33
4.0	2.55*	2.43	2.32
5.0	2.53*	2.40	2.28
6.0	2.52*	2.38	2.26

[a] Data are means of three determinations.
* Asterisk indicates that means for a comparison between gaseous atmospheres within a specific temperature are significantly different at $p < 0.05$ by ANOVA.

Table 5—Effect of CO_2, N_2, and He on the total partial molar entropy ($-\Delta\bar{S}°_T$) of collagen at 25°C, 30°C, and 35°C[a]

Amount injected (10^{-3})	$-\Delta\bar{S}°_T$		
	25°C	30°C	35°C
He			
1.0	7.99	7.92*	7.92*
2.0	7.72*	7.67*	7.83*
3.0	5.81*	5.74*	5.94*
4.0	5.54*	5.51*	5.84*
5.0	5.30*	5.28	5.55*
CO_2			
1.0	10.87*	10.92*	11.13*
2.0	10.60*	10.69*	10.91*
3.0	9.32*	9.41*	9.64*
4.0	9.13*	9.41*	9.60*
5.0	9.09*	9.21*	9.51*
6.0	8.83*	8.91*	9.22*
N_2			
1.0	8.28	8.44*	8.67*
2.0	8.28*	8.44*	8.60*
3.0	8.05*	8.28*	8.59*
4.0	7.79*	8.05*	8.24*
5.0	7.25*	7.55*	7.82*
6.0	6.98*	7.33*	7.59*

[a] Data are means of three determinations.
* Asterisk indicates that means for a comparison between gaseous atmospheres within a specific temperature are significantly different at $p < 0.05$ by ANOVA.

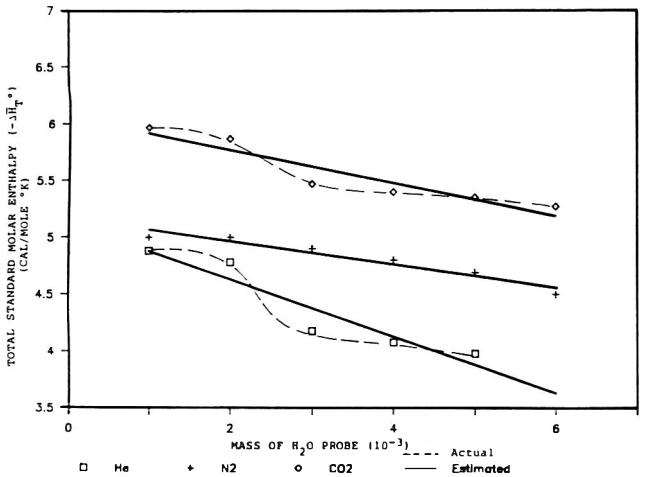

Fig. 3.—Effect of CO_2, N_2 and He on the total standard molar enthalpy ($-\Delta\bar{H}_T°$) in collagen from 25°C (Vg°) values.

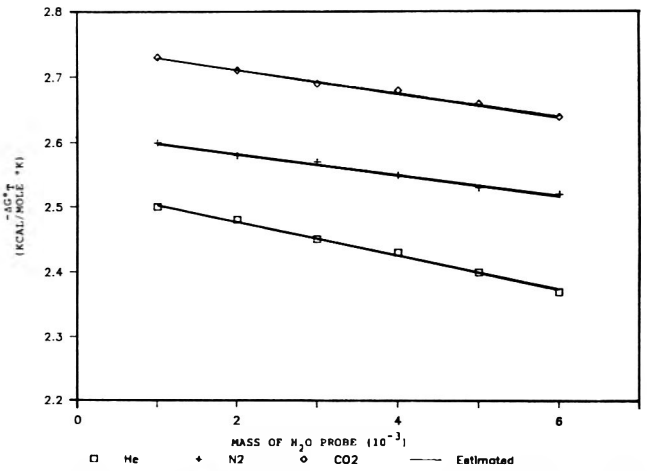

Fig. 4—Effect of CO_2, N_2, and He on the standard partial molar Gibb's free energy ($\Delta\bar{G}_T°$) in collagen at 25°C.

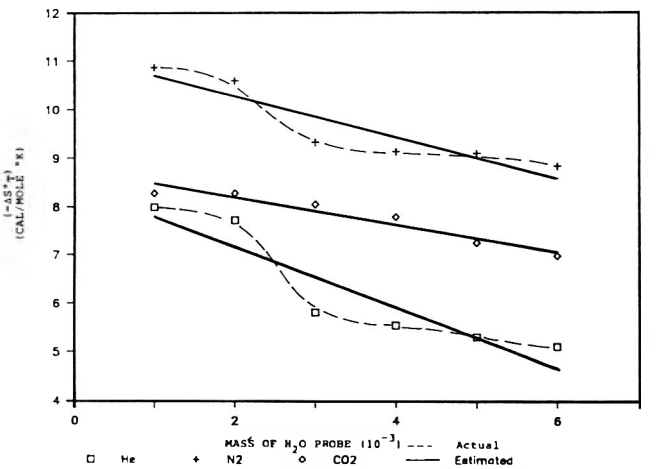

Fig. 5.—Effect of CO_2, N_2, and He on the standard partial molar entropy ($\Delta_T°$) in collagen at 25°C.

were observed in the Vg° values for collagen at 25°C, 30°C and 35°C as shown in Table 2. The CO_2-treated collagen exhibited larger retention volume followed by N_2 and He the lowest (Table 2). This effect appeared to be greatest at 25°C (Fig. 1). The relationship showed that Vg° decreased with a temperature increase. The effect of probe concentration on Vg° among the treatments in shown in Table 2. Vg° volumes increased as the probe concentration decreased. The increased Vg° with CO_2, compared to N_2 and He, indicated an increase in the availability of the polar binding sites in CO_2-treated collagen.

The effect of CO_2, N_2 and He on the partition coefficient (Kp) of collagen at 25°C is presented in Fig. 2. Significant differences were observed between the CO_2-treated collagen and the N_2- and He-treated collagen at 25°C, 30°C, and 35°C (Table 3). Since Kp is defined as the concentration of probe in the stationary phase versus concentration of probe in the mobile phase, it is directly related to Vg°. The Kp of the CO_2-treated collagen was significantly larger over the entire range of sorption than the N_2- and He-treated collagen, indicating a higher binding energy, i.e., transport velocity was low, therefore, more mobile phase was required to achieve elution.

Thermodynamic parameters from Vg°

Calculations of the thermodynamic parameters for observing the effect of CO_2, N_2, and He on the probe protein interactions at 25°C, 30°C, and 35°C were achieved by defining Vg° at the infinite dilution for each atmosphere. The density of collagen, obtained from Devro, Inc., agreed with literature value of 0.091. The assumption was made that there would be no changes in the density due to the gas atmospheres for the calculations of $\Delta G_T°$.

The $\Delta\bar{H}_T°$ values were most negative in CO_2-treated collagen at 25°C, 30°C and 35°C followed by N_2 and He (Fig. 3). This indicated that the sorption process of the water probe was most favored in the CO_2 atmosphere directly followed by N_2 and He atmospheres. The $\Delta\bar{S}_T°$ values followed a similar trend as $\Delta\bar{G}_T°$ (Tables 4 and 5). These data indicated that the probe-CO_2 system was relatively more ordered than the probe-N_2 and probe-He system at 25°C, 30°C, and 35°C. The greatest effect of CO_2 on $\Delta\bar{G}_T°$ and $\Delta\bar{S}_T°$ was observed at 25°C (Fig. 4 and 5). Probe-He exhibited the least negative $\Delta\bar{G}_T°$ and $\Delta\bar{S}_T°$ values (Tables 4 and 5). These results indicated that CO_2 significantly increased energy of water binding by collagen at 25°C, 30°C and 35°C. The significant difference in $\Delta\bar{H}_T°$ values between CO_2 and N_2 indicated a change of protein-water interaction with CO_2 having a stronger affinity of water and involving binding to the less polar specific sites.

CONCLUSIONS

CO_2 APPEARED to have a significant effect on the H_2O binding of collagen as shown by increased Vg° values as compared to N_2- and He-treated collagen.

The evaluation of the thermodynamic parameters of $\Delta\bar{G}_T°$, and $\Delta\bar{H}_T°$, and $\Delta\bar{S}_T°$ from Vg° data at different temperatures indicated that CO_2 significantly increased average energy of water binding by collagen.

Significant differences in moisture sorption in the CO_2-, N_2-, and He-treated collagen were observed at both high and low vapor pressure regions. CO_2-treated collagen appeared to significantly increase water sorption by collagen at 25°C, 30°C, and 35°C. This observation suggested that with the CO_2-treated collagen, more or higher activity sites became available for sorption at the low and intermediate vapor pressure regions.

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A Research Note

An Empirical Model for the Description of Moisture Sorption Curves

M. PELEG

ABSTRACT

Published sorption curves in the form of moisture vs time relationships of milk powder and rice, exposed to moist atmosphere or soaked in water, were fitted by a two parameter, nonexponential empirical model. The model enabled prediction of moisture contents after long exposure from experimental data obtained in relatively short time, i.e., well before the moisture level appeared to reach a plateau. The model implied that the moisture equilibrium was somewhat higher than that determined on the assumption that the sample reached a constant weight, but there was no conclusive evidence that this was really the case.

INTRODUCTION

MOST EQUILIBRIUM moisture sorption isotherms of foods are determined by allowing a dry sample to absorb moisture in an atmosphere of controlled humidity. The equilibrium moisture is usually determined by weighing the sample periodically until a constant weight is reached. In evacuated desiccators with saturated salt solutions, at ambient temperature, the time to reach constant weight is usually on the order of 24–48 hr.

Since moisture is absorbed at different rates by different materials or by the same material under different moisture and humidity conditions, there has been interest in characterizing the whole sorption process and not only the equilibrium conditions. Thus, sorption data, in the form of moisture vs time relationships, have been fitted and interpreted by different kinds of mathematical models originating from diffusion and other theories (e.g., Hayakawa 1974; Engels et al., 1987). Similar models were also applied to water absorption by soaking on the basis that such processes are also regulated by water diffusion within the food (Hendrickx et al., 1987).

The objective of this communication was to describe and demonstrate the applicability of a single simple mathematical model that fitted published sorption curves and to explore some of its potential implications in the analysis of sorption data.

DEVELOPMENT OF MODEL

A NOTABLE FEATURE of most published sorption curves is their characteristic shape shown schematically at the top of Fig. 1. Similar shapes are observed in a large variety of physical processes from powder's compaction by tapping (Sone, 1972) to electrostatic charge accumulation (Malave and Peleg, 1985) or mechanical creep (Peleg, 1980). In all these cases, the curves could be described by the same two parameter models, that if applied to sorption would assume the form:

$$M(t) = M_0 + t/(k_1 + k_2 t) \quad (1)$$

where M(t) is the moisture after time t, M_0 the initial moisture, and k_1 are constants. The units of k_1 and k_2 will correspond to those of the moisture units. Thus, if M(t) and M_0 are given in % weight on dry basis, and the time units are in hours, then the unit of k_1 will be hour per % weight and that of k_2 in the reciprocal of % on dry weight basis. It is easy to show that the equilibrium moisture, M_E, according to this model, i.e., when $t \to \infty$, is given by:

$$M_E = M_0 + 1/k_2 \quad (2)$$

Similarly, the momentary sorption rate dM(t)/dt is given by:

$$dM(t)/dt = k_1/(k_1 + k_2 t)^2 \quad (3)$$

and the initial rate, i.e., at $t = 0$ by $1/k_1$. One of the characteristics of Eq. 1 is that it can be transformed to a linear relationship in the form:

$$t/[M(t) - M_0] = k_1 + k_2 t \quad (4)$$

The latter, as shown in Fig. 1, offers a simple way to test the applicability of the model to sorption data and to calculate its parameters by linear regression.

RESULTS & DISCUSSION

THE FIT OF EQ. (4) to sorption data reported in three different publications is shown in Fig. 1. The figure demonstrates that despite the differences in the materials and conditions the degree of fit, as judged by the regression coefficient, was about the same. It should be noted that the data shown in the figure were intentionally taken from the clearly curved part of the sorption curves, i.e., far enough from the region where the weight gains are extremely small. (In that region, the fit of Eq. (1) or Eq. (4) will be largely a tautology because $M(t) - M_0 \approx$ constant).

The predictive capability of the model is demonstrated in Table 1 where the constants k_1 and k_2, which were derived from the linear fit, were used to calculate the moisture content at times well beyond the range of the regression data. Similar agreement was observed with practically all the points within the reported range. It should be added though that the moisture content, after 24 hr in the first two cases and after 1 hr in the third, remained practically unchanged and consequently, they could be treated as representing equilibrium conditions. According to the discussed model, however, the value of the equilibrium moisture was significantly higher. If the model truly describes the sorption pattern, then the discrepancy can easily be explained. As can be seen from Table 1, the sorption rates fall dramatically when these moisture contents are reached and, therefore, the net weight gain can fall below the accuracy of the moisture determination method. With respect to the test duration, this can also be formulated as follows. Let R be the ratio

$$R = [M(t) - M_0]/(M_E - M_0) \quad (5)$$

It follows from the mathematical structure of Eq. (1) that if, for example, $t_{1/2}$ is the time needed to reach $(M_E - M_0)/2$, i.e.

Author Peleg is with the Dept. of Food Engineering, Univ. of Massachusetts, Amherst, MA 01003.

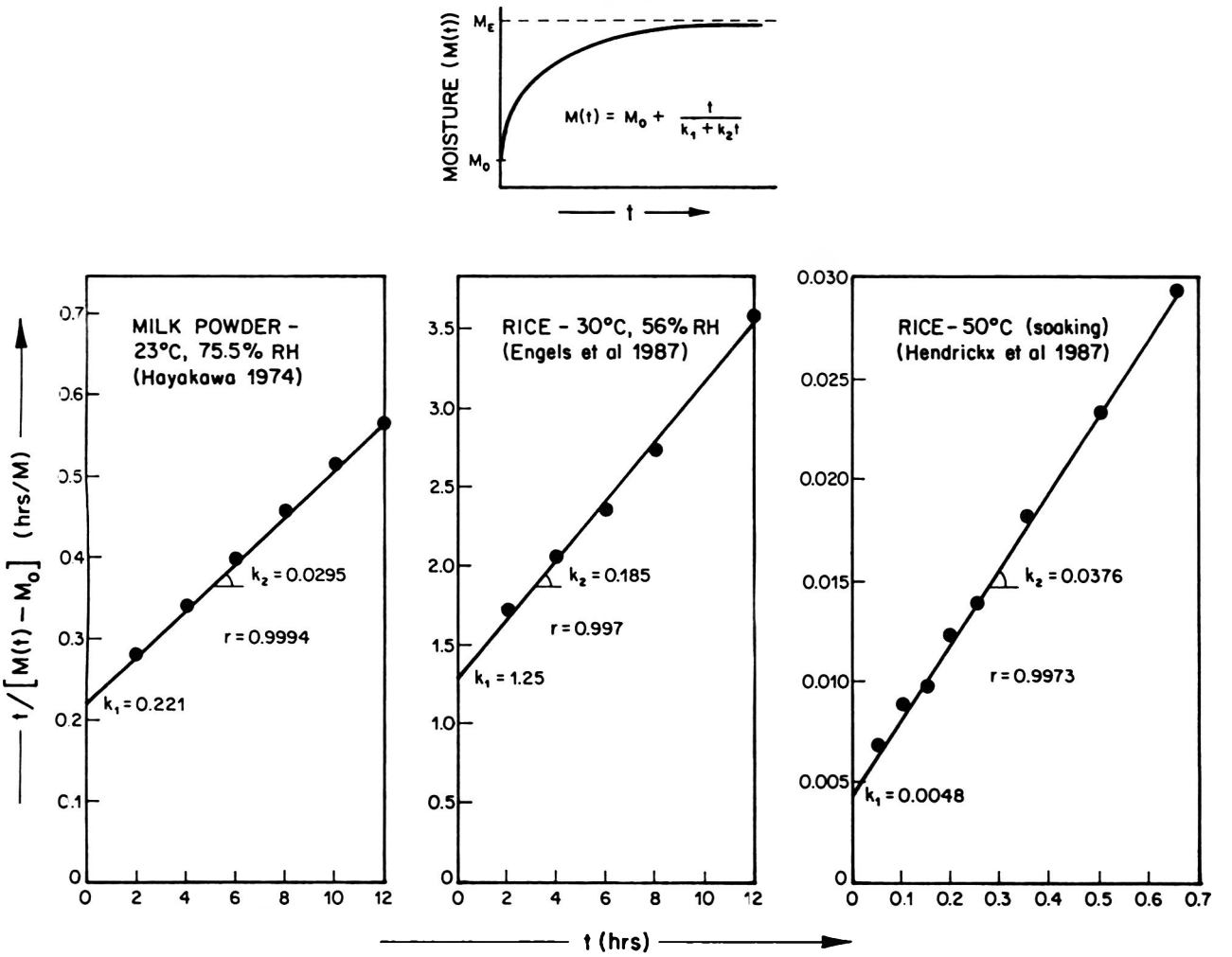

Fig. 1—Linearization of moisture sorption curves, top, and three examples of the fit of the linear model to published sorption data, bottom. M(t) is the moisture content at time, t, and M_o and M_E the initial and equilibrium moisture contents, respectively; k_1 and k_2 are constants. The relation of k_1 and k_2 to equilibrium conditions and rate is expressed by Eq. (2) and (3).

Table 1—Water sorption parameters calculated from published data.

Material, Conditions & data source	Initial moisture (% db)	Moisture (%db) After time (hr)	Calc.[1]	Observed	Calc.[b] M_E (%db)	Calc. sorp. rate (%/hr)[c] Init al	After 24 hr	After 48 hr	$t_{1/2}$[d] (hr)	t_R(hr)[d] R = 0.95	R = 0.99
Nonfat milk powder, 23°C 75.5% FH (Hayakawa, 1974)	2.29	24	28.1	28.5	36.2	4.5	0.26	0.083	7.5	142	742
Rice, 30°C, 56% RH (Engels et al. 1987)	8.5	24	12.7	12.4	13.9	0.80	0.038	0.012	6.7	128	668
Rice, 50°C soaking (Hendrickx, 1987)	12.5	1.45	36.9	34.5	39.1	208	after 3 hr 0.35	after 6 hr 0.090	0.13	2.45	12.9

[a] Calculated by Eq. (1) with k_1 and k_2 determined from data during the first 12 hr (first and second rows) and 0.65 hr (third row).
[b] Calculated by Eq. (2) with k_2 determined as described above.
[c] Calculated by Eq. (3) with k_1 and k_2 determined as described above.
[d] See Eq. (5) and (6).

half the added moisture to reach equilibrium, then the time to reach any given level of R, namely t_R, is given by:

$$t_R/t_{1/2} = R/(1-R) \quad (6)$$

As can be seen from Table 1, the times to reach R = 0.95, let alone 0.99, are longer than what was considered practical. The reported experiments were of a duration of 30 min, 50 min and 1.5 hr, respectively, and in at least the last third of these periods the moisture data appear, at least superficially, to be at the equilibrium or very close to the equilibrium level.

Model advantages and limitations

The model can be employed to predict successfully, or at least estimate, long range moisture gains from experimental data obtained in tests of relatively short duration. The predictions, it ought to be added, hardly depend on the test duration

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A Research Note

Moisture Sorption Method for Hygroscopic Samples Using a Modified Proximity Equilibration Cell

P. B. KANADE and J. S. PAI

ABSTRACT

The study was undertaken to develop a suitable design to accommodate liquefying hygroscopic samples in the Proximity Equilibrium Cell (PEC) for equilibration to desired water activity. The system suggested has overcome the limitation of the earlier reported methods using PEC and is found to be rapid compared to other gravimetric methods used for hygroscopic materials.

INTRODUCTION

WATER VAPOR SORPTION measurements are very important in food analysis. Considering the frequency with which these measurements are made, it is necessary that they be made precisely and economically. Different methods of studying the sorption phenomenon have been published. Audu et al. (1978) used a dynamic system in which air was circulated as a carrier for the transfer of water vapor to and from the sample. Problems may arise in this method with respect to the maintenance of exact humidity values in the circulating air. Smith et al. (1981) described a method for sugar using Inverse Gas Chromatography which required sophisticated instruments. Most of the methods require precise calibration and expensive instrumentation. Gal (1975) has reviewed various methods used.

The use of a Proximity Equilibration Cell (PEC) to rapidly equilibrate a sample to an atmosphere of known relative humidity was developed by Lang et al. (1981). This method has shown advantages over methods suggested by Young and Nelson (1967), Bosin and Easthous (1970) and others because it is rapid, precise and simple. Design of the sample holder suggested by Lang et al. (1981) was found not to be suitable for sugar and hygroscopic samples as these samples dissolved and dripped in the saturated salt solution. This study was undertaken to devise a simple and efficient system requiring a balance and a simple equilibration chamber to accomodate hygroscopic samples in PEC and to evaluate it in comparison with other static gravimetric methods.

MATERIALS & METHODS

Materials

The barium chloride used was reagent grade. Maltodextrin used for sorption study was a commercial grade 19 DE sample obtained from Laxmi Starch Ltd., Bombay.

Sample preparation

The sample to be equilibrated was dried in vacuum oven at 50°C and 29 in Hg vacuum for 48 hr over phosphorus pentoxide. This sample was kept in a petri dish at an approximately 2 mm thick layer in a partially evacuated desiccator for 48 hr over phosphorus pentoxide.

Authors Kanade and Pai are with the Dept. of Chemical Technology, Univ. of Bombay, Matunga, Bombay-400019, India.

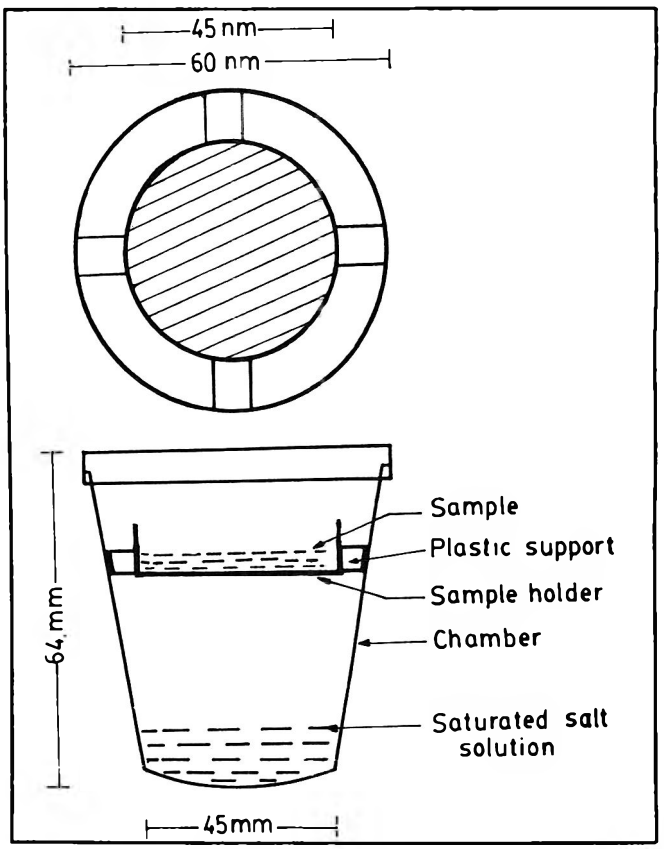

Fig. 1—Proximity equilibration cell with modified sample holder for sugars and hygroscopic samples.

Equilibration to known relative humidity

Method I. This method is a modification of that of Lang et al. (1981). The sample holder was a plastic weighing dish, 45 mm diameter and 15 mm deep, to which were stuck four 5 sq. mm size rectangular supports on the outer diameter as shown in Fig. 1. A clean, dry sample holder was weighed and placed in the PEC containing saturated salt solution for 24 hr at 20°C. The sample holder was weighed again after 24 hr and a 1g sample was placed in the holder in an approximately 2 mm thick layer. The sample holder was then inserted in the PEC for equilibration.

Method II. This method was used by Chinachoti and Steinberg (1984). A clean and dry weighing bottle of 5 mL capacity was placed in the PEC with the saturated salt solution outside the bottle. After 24 hr, a 100 mg sample was weighed in the weighing bottle and kept for equilibration.

Method III. This is the modified conventional method carried out by partially evacuating the desiccator. A glass vacuum desiccator 150 mm diameter and 160 mm deep, was used with saturated salt solution in the base. A 1g sample was weighed in a plastic weighing dish, 45 mm diameter and 15 mm deep, and placed on the solid support in the desiccator under partial vacuum.

All samples were equilibrated at 20°C temperature to relative humidity of 91.03 using a saturated solution of $BaCl_2$ (Rockland, 1960). Every experiment was carried out in triplicate. Moisture content was determined on weight gain basis.

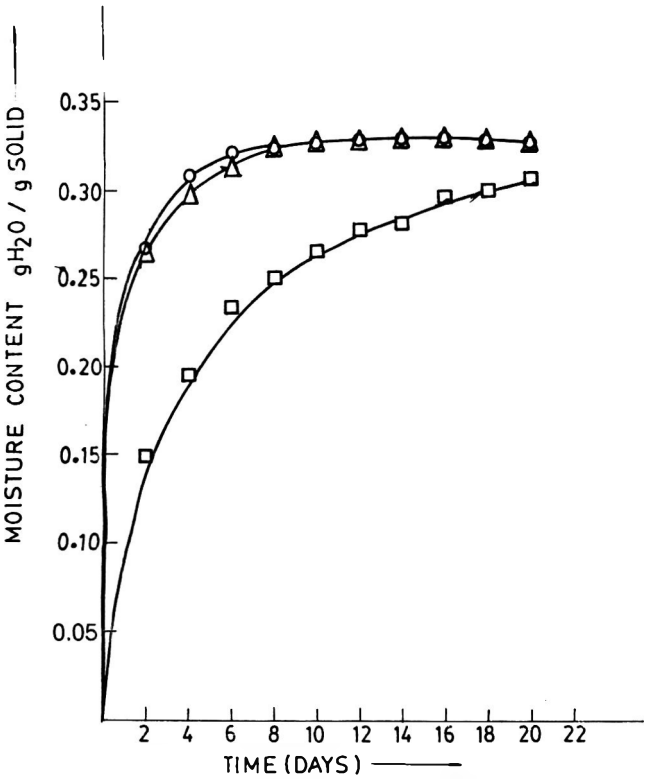

Fig. 2—Water vapor sorption by maltodextrin against a saturated salt solution at0.9103 a_w and 20°C in ○—○ modified equilibration cell, □—□ method used by Chinachoti and Steinberg (1984), and △—△ partially evacuated desiccator.

RESULTS & DISCUSSION

THE PEC METHOD of Lang et al. (1981) has the advantage of having an increased surface area of the saturated salt solution per unit vapor volume as well as shorter mean free path for the water vapor. This results in decrease in the equilibration time from more than 30 days to about 7 days. However, this method can not be used for sugars and hygroscopic samples. The results of the modifications used are shown in Fig. 2. The PEC method with the weighing bottle (Chinachoti and Steinberg, 1984) employs a weighing bottle instead of the aluminum cut-out weighing dish. Although this solves the problem of hygroscopic samples dissolving in absorbed moisture and dripping into the salt solution, it reduces the surface area of sample and increases the reverse path for water vapor to travel towards the sample surface. This greatly increases the equilibration time. In the PEC method using the modified holder, although water vapor has to travel longer distance than original method of Lang et al. (1981), the additional distance is small. Moreover a large surface area of sample, much larger than with weighing bottle, facilitates faster equilibration as can be seen from Fig. 2. The desiccator method also gives a fairly rapid equilibration, however, cost of materials and labor is higher. Thus, the PEC method with modified sample holder has advantages of being rapid, accurate and simple for water vapor sorption measurements.

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EMPIRICAL MODEL FOR MOISTURE SORPTION CURVE DESCRIPTION. . . From page 1217

itself, and the values arrived at in regression of data obtained during the first 6 hrs in the first two experiments, and 0.3 hr in the third, do not differ by much from what is reported in Table 1. Naturally, the predictions would improve if more data were included but not by more than about 1–2% moisture. It ought to be remembered, however, that the model as expressed in Eq. (1) or (4), is only an empirical model that was not derived from any set of physical laws or diffusion theories. Despite its demonstrated success, therefore, its general applicability cannot be taken for granted and the latter needs to be established independently for any system where its use is considered. This does not appear to be a serious drawback though since the model, particularly in its linear form, offers a simple and convenient way to test its own applicability by linear regression. As to the equilibrium moisture contents, the model predictions, as previously stated, are significantly higher than those based on the assumption that true equilibrium is reached after 24–48 hr. Because the reported food materials become biologically and/or physically unstable after long exposure to moist environment, there are no data on their sorption behavior in long term experiments, i.e., on the order of weeks in the first two cases and several hours in the third. Consequently, the question of whether true equilibrium can be reached at all under such circumstances, and if so, at what moisture levels, cannot be settled on the basis of the available data.

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A Research Note

Comparison of Four Methods for the Dimethylacetal-free Formation of Fatty Acid Methyl Esters from Phospholipids of Animal Origin

R.L. CRACKEL, D.J. BUCKLEY, A. ASGHAR, J.I. GRAY, and A.M. BOOREN

ABSTRACT

Fatty acid methyl esters (FAMEs) were prepared from phospholipid extracts of beef, pork, lamb, chicken and turkey using four methylating procedures: BF_3/methanol, KOH/BF_3, direct transesterification with KOH, and a commercial preparation, Meth Prep II. Gas chromatographic analysis of the FAMEs revealed the presence of dimethyl acetals (DMAs) in samples methylated with BF_3/methanol or KOH/BF_3. These DMAs were evident when samples were analyzed on both polar and non-polar columns. DMAs were not found in samples derivatized by direct alkali transesterification or by Meth Prep II.

INTRODUCTION

PLASMALOGENS are natural constitutents of animal lipids (Peng and Dugan, 1965; Grigor et al., 1972) and contain long chain aldehydes, commonly C_{16} and C_{18}, bound as enol ethers. While these alkyl and alk-1-enyl glyceryl ethers comprise a small portion of the neutral lipids, they are present in greater concentrations in the phospholipid fraction (Grigor et al., 1982; Maxwell and Marmer, 1983).

Plasmalogens are aldehydogenic lipids under acidic conditions. Acid hydrolysis coupled with methanolysis yields a stable dimethylacetal (DMA) derivative of the aldehyde. DMAs are readily formed under conditions commonly used to produce fatty acid methyl esters (FAMEs) for gas chromatographic (GC) analysis. The use of HCl/methanol or boron trifluoride (BF_3)/methanol produces DMAs (Morrison and Smith, 1964; Mahadevan et al., 1966; Sun and Horrocks, 1968) which, under certain conditions, can interfere with the analysis of FAMEs. In the analysis of neutral or total lipid fractions from animal tissues, DMAs would appear in only trace amounts; however, they would be significant components of the GC profile of polar lipid FAMEs (Maxwell and Marmer, 1983).

It appears that in several studies DMA derivatives may have been mistaken for FAMEs because the two classes of compounds have very similar GC retention times when analyzed using a polar stationary phase (Marion and Woodroof, 1965; Marion et al., 1967; Igene et al., 1981). In these cases, FAMEs were prepared under acidic conditions using either H_2SO_4/methanol or BF_3/methanol. However, it has been reported that the formation of DMAs can be minimized if methanolysis is carried out under basic conditions (Maxwell and Marmer, 1983).

The objective of this study was to compare several procedures for preparing FAMES of phospholipids from various meat samples and to demonstrate the potential for misidentifying DMAs as FAMES in the GC profiles of animal phospholipid fatty acids.

Authors Crackel, Asghar, Gray, and Booren are with the Dept. of Food Science Human Nutrition, Michigan State Univ. East Lansing, MI 48824. Author Buckley is with the Dept. of Dairy & Food Technology, University College Cork, Ireland.

MATERIALS & METHODS

BEEF ROUND STEAK, pork loin chops, lamb chops, and chicken and turkey breast meat were purchased at a local supermarket. Portions from each meat sample were ground twice through a 3/16 in. plate. Skin was removed from the poultry samples before grinding. Neutral and phospholipid fractions were extracted from duplicate meat samples using the dry column procedure of Marmer and Maxwell (1981). FAMEs were prepared from the phospholipid fractions of each sample using the following methylating procedures: BF_3/methanol (Morrison and Smith, 1964); KOH/methanol (Eichhorn et al., 1985); direct transesterification (Maxwell and Marmer, 1983); and a commercial preparation, Meth Prep II (Alltech, Deerfield, IL) which is a methanolic solution of m-trifluoromethylphenyl trimethylammonium hydroxide. FAMEs were separated on a glass column (3m × 2mm i.d.) packed with 10% SP-2330 on 100/120 Supelcoport (Supelco, Bellefonte, PA). FAMEs from beef and chicken were also separated on a 10% DEGS (Anspec Co., Ann Arbor, MI) column of similar dimensions. The gas chromagraph (Hewlett Packard 5880A) was operated isothermally at 190°C. Component fatty acids were tentatively identified by comparing retention times to those of standard FAMEs assayed under identical conditions. The identities of FAMEs and DMAs were confirmed by GC-mass spectrometry (Hewlett Packard 5985A) using the same S-2330 column as described above. Operating conditions included: electron impact voltage, 70eV; electron multiplier voltage, 240eV; threshold, 0.6; source temperature, 200°C; analog/digital measurements, 3/sec and ion source detection in the positive mode.

RESULTS & DISCUSSION

GC PROFILES OF FAMEs which were prepared with either BF_3/methanol or KOH/BF_3 consistently contained three peaks with retention times which did not match those of fatty acid standards when analyzed on the nonpolar (SP-2330) column. These peaks were found in FAMEs from all meat samples (Table 1). One compound eluted near $C_{16:0}$ while the other two eluted between $C_{16:0}$ and $C_{18:0}$ (Fig. 1A). The peaks comprised approximately 10% of the total peak area. When separated on the more polar DEGS column, the unknown compounds again did not correspond to any of the FAME standards. These peaks were not evident in samples derivatized by direct transesterification (Fig. 1B) or by Meth Prep II. GC/MS analysis of two of the peaks confirmed the presence of DMA derivatives of hexadecanal and octadecanal, while the third unknown compound was not identified.

It is possible that several researchers have mistakenly identified DMAs as FAMEs because of their very similar retention times on packed columns using polar stationary phases such as DEGS. Significant amounts of $C_{14:1}$, $C_{14:2}$, $C_{15:1}$, and $C_{15:2}$ have been reported in chicken and beef phospholipids (Marion and Woodroof, 1965; Marion et al., 1967; Igene et al., 1981; Igene and Tukura, 1986). However, studies by Gardner et al. (1972) and Moerck and Ball (1973) revealed no significant amounts of $C_{14:2}$, $C_{15:0}$ or $C_{15:2}$ in chicken phospholipids but rather the presence of compounds having GC retention times and mass spectra characteristics of C_{16} and C_{18} DMAs. Wood (1983) did not detect any $C_{14:2}$ or $C_{16:2}$ in beef phospholipids but did report 11 to 14% DMAs in the FAMEs prepared by BF_3/methanol. DMA values reported in Table 1 generally agree

Table 1—Percent areas of 16:0 and 18:) dimethylacetals and unknown peak in fatty acid methyl ester profiles of meat phospholipids, as influenced by method of methylation[a,b]

Method of methylation	BEEF				PORK				LAMB				CHICKEN				TURKEY			
	16:0	18:0	Unk	Total	16:0	18:0	Unk	Total	16:0	18:0	Unk	Total	16:0	18:0	Unk	Total	16:0	18:0	Unk	Total
BF_3/methanol[c]	7.1	3.6	2.1	12.8	6.9	2.6	1.9	11.4	5.9	4.1	1.9	11.9	4.3	1.9	0.9	6.9	8.1	3.4	1.4	12.9
Saponification[d] + BF_3	7.8	3.4	1.8	13.0	5.5	2.5	1.9	9.9	6.4	3.9	1.6	11.9	5.3	1.3	0.7	7.3	9.4	2.4	1.1	12.9
Direct[e] transerterification	–	–	1.1	1.1	–	–	–	–	–	–	1.2	1.2	–	–	–	–	–	–	–	
Meth-Prep II[f]	–	–	tr[g]	tr	–	–	tr	tr	–	–	tr	tr	–	–	tr	tr	–	–		

[a] Figures in table represent percentages of total area.
[b] Average of duplicate meat samples, two analyses per sample.
[c] Morrison and Smith (1964).
[d] Eichhorn et al. (1985)
[e] Maxwell and Marmer (1983);
[f] Alltech, Deerfield, MI.
[g] <0.1% of total peak area.

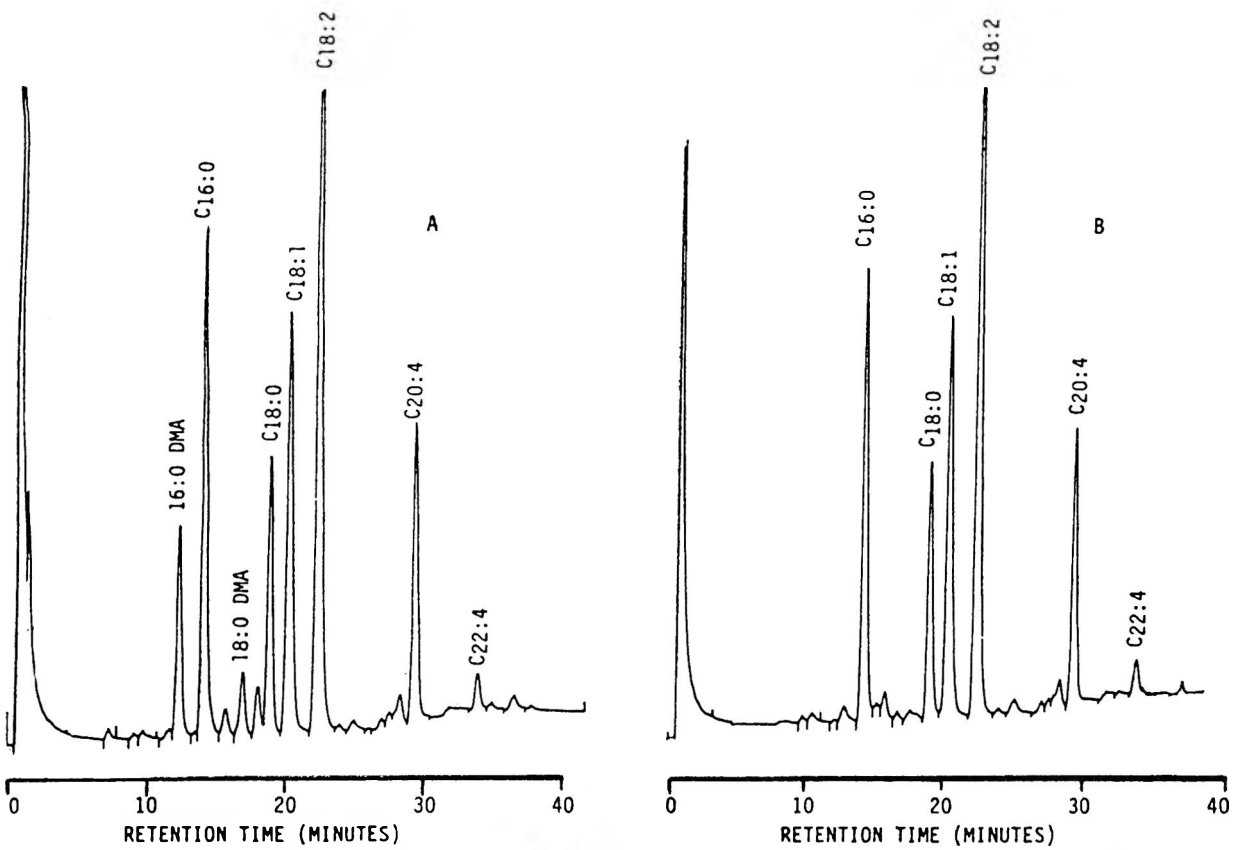

Fig. 1—Gas chromatographic profiles of fatty acid methyl esters of pork phospholipids prepared by (A) BF_3/methanol and (B) direct transesterification methylating procedures.

with those reported in the literature when acidic methanolysis conditions were used (Gardner et al., 1972; Wood, 1983).

It has been reported that the formation of DMAs can be minimized if methanolysis is carried out under basic conditions. Several researchers have used a saponification/reesterification method involving KOH and BF_3/methanol for the preparation of FAMEs (Metcalfe et al., 1966; Eichhorn et al., 1985). However, this procedure can promote the formation of DMAs as evidenced by the current study (Table 1) and by the findings of Moerck and Ball (1973) who identified DMAs in FAMEs prepared using the method of Metcalfe et al. (1966). Maxwell and Marmer (1983) developed a direct transesterification procedure for the preparation of FAMEs from phospholipid concentrates. Results of the current study confirm their findings that room temperature transesterification with KOH (2N) produced FAMEs that are free from contaminating DMA derivatives (Table 1). Similar results were obtained with Meth Prep II, although many extraneous peaks with retention times greater than that of $C_{20:4}$ were observed.

This study has confirmed many previous observations that acid methanolysis of fatty acids of animal phospholipids will lead to the formation of DMA, which can be, and have been, misidentified as FAMEs. In addition, the presence of relatively high percentages of DMAs can lead to inaccurate information on the percentages of component fatty acids present in phospholipids. The transesterification procedure of Maxwell and Marmer (1983) is fairly rapid and simple, and greatly decreases the possibility of misidentifying DMAs as component fatty acids. These advantages, coupled with the low cost per sample analysis, makes the transesterification procedure a recommended technique for preparing FAMEs from phospholipid fractions. Meth Prep II is another possible alterative, however, it is more expensive and can generate additional peaks, the identities of which were not addressed in this study.

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—*Continued on page 1233*

A Research Note

Effect of High Altitude on Copper and Zinc Content of Beef

L. C. MEDEIROS, R. A. FIELD, and D. M. MEDEIROS

ABSTRACT

Copper and zinc content of longissimus (LD) and semimembranosus (SM) muscle from concentrate-fed steers raised at 549 or 2145m above sea level were compared. Data on younger range-grazed steers raised at 2145m were also obtained. Copper and zinc content of muscles did not differ by altitude but copper and zinc in muscles from concentrate-fed steers was greater than that from younger range-grazed steers ($P < 0.05$).

INTRODUCTION

COPPER, ZINC, AND IRON are essential nutrients for humans and animals and interactions between these minerals may influence health. The copper-containing compound ceruloplasmin, contains the ferroxidase enzyme activity responsible for iron ionic form conversions (Bezkorovainy, 1980). Zinc is essential for growth and development (Gibson, 1985) but excessive zinc may interfere with iron absorption (Evans, 1971) and iron storage (Macara et al., 1973) and may result in decreased muscle iron content (Field et al., 1985). Skeletal muscle zinc and copper may be affected by diet (Standish et al., 1969; Standish and Ammerman, 1971) and by age of animal (Doornenbal and Murray, 1981; Kotula and Lusby, 1982). The most pronounced differences in mineral content occur among species (Doyle, 1980) and among different muscles (Zenoble and Bowers, 1977; Doornenbal and Murray, 1981; Kotula and Lusby, 1982; Zarkadas et al., 1987).

A previous study found that the ratio of heme to nonheme iron in longissimus (LD) and semimembranosus (SM) muscles was altered by high altitude hypoxia (Medeiros et al., 1988). A greater proportion of heme iron in the muscles of animals reared at high altitude when compared to those reared at low altitude was found. Because there are a number of metabolic interrelationships between zinc, copper and iron, it would seem reasonable that a change in iron content of skeletal muscle could also result in a concurrent change in the zinc and copper content of muscle. Therefore, the purpose of this study was to determine whether altitude differences that influence heme iron also influences zinc and copper content of skeletal muscle.

METHODS

A DESCRIPTION of the steers and methods used to prepare muscle samples have been previously reported (Medeiros et al., 1988). Steers were allotted to three treatments: (1.) 24 mo old steers raised at 2145m above sea level and fed concentrate (HIFD); (2) 24 mo old steers raised at 549m above sea level and fed concentrate (LOFD); and (3.) 17 mo old steers raised at 2145m above sea level and pasture-grazed (HIGR). Forages consumed by the HIGR steers ranged from 4.9 to 14.2 ppm Cu and 2.9 and 31.3 ppm Zn with the lower levels being found in mature plants that the animals were consuming at time of slaughter (Hamilton and Gilbert, 1972). Concentrates consumed by the HIFD and LOFD steers ranged from 12 to 20 ppm zinc and from 4 to 10 ppm copper (National Research Council, 1982). However, predicting muscle mineral composition based on dietary intake in ruminate animals is difficult because the concentration of mineral absorbed is influenced by a number of factors including age of animal and the bacterial action of the rumen (National Research Council, 1984).

Copper was determined in raw muscle samples and zinc was determined in raw and cooked muscle samples. Steaks from the LD and SM muscles were cooked on a conventional gas oven boiler to 71°C internal temperature.

Zinc and copper contents were determined by atomic absorption spectrophotometry. The procedure used for wet ashing the samples has been previously reported (Mustafa and Medeiros, 1985; Medeiros et al., 1988). A bovine liver sample purchased from the National Bureau of Standards (Reference No. 1577a) was used as an external standard. Liver samples were processed in a manner identical to the muscle samples. Zinc and copper concentrations were determined with a Perkin Elmer model 2280 flame atomic absorption spectrophotometer (Perkin-Elmer Corporation, Norwalk, CT). Bovine liver zinc and copper values were within ± 1 standard deviation of the mean, confirming the accuracy of the analytical methods. Values for zinc content were analyzed by analysis of variance (ANOVA) as a 3 × 2 × 2 factorial design using the BMDP-4V software program (Dixon, 1983). Factors were groups (HIFD, LOFD and HIGR), muscle (LD and SM) and cooking treatment (raw or cooked). Group means within the two cooking treatments were separated by Fisher's protected least significant difference test (Steel and Torrie, 1980). Copper values were analyzed by a similar procedure except that cooking treatment was eliminated from the design. Group means within each muscle were separated by Fisher's protected least significant difference test (Steel and Torrie, 1980).

RESULTS & DISCUSSION

COPPER CONTENT of muscle from HIFD and LOFD steers of the same age was similar (Table 1). Steaks from the LOFD and HIFD groups generally contained more copper than those from the HIGR group. Muscle iron content is known to increase with age (Kotula and Lusby, 1982), but muscle copper

Table 1—Means for copper content (ppm) of raw longissimus and semimembranosus muscle

Variable	n	Longissimus	n	Semimembrancsus
Copper (fresh weight)[a]				
HIFD[b]	23	1.29[cd]	23	1.85[c]
LOFD	18	1.49[c]	20	1.64[c]
HIGR	20	1.08[d]	20	1.07[d]
SEM		.05		.07
Copper (moisture-fat free)[a]				
HIFD	23	5.45[cd]	23	8.15[c]
LOFD	18	6.39[c]	20	7.07[c]
HIGR	20	4.58[d]	20	4.58[d]
SEM		.21		.34

[a] Muscle × group interaction ($P < 0.05$).

[b] HIFD-feedlot beef from high altitude, LOFD = feedlot beef from low altitude, HIGR = range-grazed beef from high altitude, SEM = standard error of mean.

[c,d] Variable means within columns followed by different superscripts differ ($P < 0.05$).

Authors L. C. Medeiros and D. M. Medeiros are affiliated with the Dept. of Home Economics and Author R. A. Field is affiliated with the Dept of Animal Science, Univ. of Wyoming, Laramie, WY 82071.

content of 14 to 16 mo animals when compared to 3 to 12 yr old animals was not different (Doornenbal and Murray, 1981). For the HIGR group, copper content of the two muscles was not different, whereas copper content of the SM muscle was higher than the LD muscle for the HIFD and LOFD groups ($P<0.05$). Differences in copper content among muscles has been previously reported (Zenoble and Bowers, 1977; Doornenbal and Murray, 1981).

Unlike muscle heme-iron content (Medeiros et al., 1988) where an altitude effect existed, a clear altitude effect on copper was not evident. Dietary copper requirements of cattle are relatively low so none of the animals in the present study were on a copper deficient diet (National Research Council, 1984). In addition, dairy calves and veal calves are able to maintain hemoglobin values without copper supplementation (Matrone et al., 1957; Bremner and Dalgarno, 1973).

There was no difference in the zinc content of raw steaks between HIFD and LOFD steers of the same age (Table 2). Zinc content of steaks from the HIGR group was lower than that of the other two groups ($P<0.05$). However, these animals were younger than those in the HIFD and LOFD groups. Kotula and Lusby (1982) reported a trend toward increased zinc content in muscles from steers 12 to 72 mo, but the difference between zinc content of animals 18 to 24 mo of age was not significant ($P>0.05$). In the present study, age differences between the HIGR group and the HIFD group may partly explain observed differences in zinc content but differences in the management practices and diets could also influence zinc content (Doornenbal and Murray, 1981; Kotula and Lusby, 1982).

Zinc in moisture/fat free muscle decreased during cooking and this was probably a result of drip loss. An interaction was observed between muscles and cooking treatment ($P<0.05$), and between group and cooking treatment ($P<0.05$) as the amount of zinc loss was not uniform between group and cooking treatment ($P<0.05$) as the amount of zinc loss was not uniform between muscles or groups.

In summary, the zinc and copper content of skeletal muscle is a function of many interacting factors but exposure to high altitude hypoxia does not appear to alter their content in muscle. Observed variations in mineral content underline the problem with reporting nutritional data on retail packages of meat.

Table 2—Means for zinc content (ppm) of raw and cooked longissimus and semimembranosus muscle

		Longissimus			Semimembranosus	
Variable	n	Raw	Cooked	n	Raw	Cooked
Zinc (fresh weight)[a,b]						
HIFD[d]	23	37.9[e]	49.4[e]	23	35.8[e]	42.3[e]
LOFD	18	37.3[e]	49.0[e]	20	37.0[e]	43.2[e]
HIGR	20	31.5[f]	45.2[f]	20	30.9[f]	37.5[f]
SEM		.57	.77		.55	.64
Zinc (moisture-fat free)[a,b,c]						
HIFD	23	160.8[e]	139.7[f]	23	157.5[e]	122.2[e]
LOFD	18	160.2[e]	155.3[e]	20	159.0[e]	128.9[e]
HIGR	20	133.3[f]	121.4[g]	20	131.6[f]	106.5[f]
SEM		2.52	2.83		2.49	2.02

[a] Cooking treatment significant ($P < 0.05$).

[b] Muscle × cooking treatment interaction ($P < 0.05$).

[c] Group × cooking treatment interaction ($P < 0.05$).

[d] HIFD = feedlot beef from high altitude, LOFD = feedlot beef from low altitude, HIGR = range-grazed beef from high altitude, SEM = standard error of mean.

[e,f,g] Variable means within each column followed by different superscripts differ ($P < 0.05$).

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A Research Note

Effect of Temperature on Collagen Extractability and Kramer Shear Force of Restructured Beef

ELIZABETH D. STRANGE and RICHARD C. WHITING

ABSTRACT

Collagen extractability and Kramer shear force were measured for raw and heated restructured beef products made with trimmed (epimysium removed) and untrimmed clods (triceps brachii, infraspinatus, and supraspinatus). Collagen extractability was significantly ($p<0.001$) higher for untrimmed samples heated at 50°C than for trimmed samples. Kramer shear forces were significantly higher ($p<0.01$) for untrimmed than trimmed when raw or heated at 35°C, 45°C, 50°C, and 55°C. Collagen extractabilities showed no distinct changes with temperature for trimmed samples. Collagen extractability of untrimmed samples increased then decreased as heating temperature increased. Kramer shear forces decreased between 55°C and 60°C for both trimmed and untrimmed samples.

INTRODUCTION

TEXTURE of restructured beef (RSB) meat products is dependent on connective tissue content (epimysial and intramuscular) and myofibrillar structure of meat as well as binding between meat pieces. Muscles with most intramuscular collagen were reported to be toughest (Dransfield, 1977); muscles with higher rates of intramuscular and/or epimysial collagen solubilization were reported to have lower shear forces and better sensory scores (Goll et al., 1964a; Moller, 1980-81; Light et al., 1985). Others reported that total intramuscular collagen was unrelated to Warner-Bratzler measurements or that soluble intramuscular collagen had no major influence on sensory or Instron texture characteristics of muscle (Goll et al., 1963; Fogle et al., 1982; Naewbanij et al., 1983). The lack of standardization in cooking and analytical procedures in various studies make comparisons difficult.

The objective of this study was to relate Kramer Shear force measurements to collagen extractability in RSB made with different epimysial collagen contents and heated at different temperatures.

MATERIALS & METHODS

THE TRICEPS BRACHII, infraspinatus and supraspinatus from three clods ('A' maturity, Choice grade, Yield grade 2, NAMP #114) were divided into two portions containing equal parts of each muscle. One portion was trimmed of visible epimysial tissue, while the other portion was not. Meat was crust-frozen and diced (0.62 cm on the side). RSB products were made by gently mixing diced meat with 0.75% sodium chloride and 0.125% sodium tripolyphosphate for 2 min in a Hobart mixer, stuffing into 5 cm diameter cellulose casings and holding at +11°C for 4 hr to enhance binding before freezing at −20°C. After initial freezing, products were tempered, sliced 2.5 cm thick and individually vacuum-packaged and stored at −20°C.

Slices of trimmed and untrimmed RSB (one slice from each clod for each temperature) were defrosted overnight at +4°C and heated in water baths at 35°, 45°, 50°, 55°, 60°, 65°, 70°, 75°, or 80°C for 1 hr in vacuum packages. After the slices had cooled to 20°C, each was divided into three pie-shaped wedges (mean wt = 19.3 ± 2.7g, N = 180) and peak force (Newtons/g product) was determined with a Kramer multiblade shear cell at a cross head speed of 50 mm/min.

Nine slices of trimmed and untrimmed RSB (three slices from each clod) were ground in a food processor. The pH of both ground products was 5.8 (combination electrode). Three five gram samples of the ground RSB products were heated in 50 mL round bottom tubes for 1 hr at the same temperatures as above. Ten grams water were added to each tube and to three raw samples of each (60 samples) and extracted overnight at +4°C before grinding with a Brinkman Polytron for 1 min (power setting of 5). The polytron was rinsed 4X with 3 mL water and the ground meat and rinses combined and centrifuged at 10,000 x *g* for 1 hr. The supernates were divided into three aliquots, freeze-dried, hydrolyzed with 2 mL 6N HCl at 120°C overnight and the hydroxyproline (Hyp) of the supernate determined (Woessner, 1961). The Hyp of each residue was determined in triplicate. Hyp values multiplied by 7.14 yield collagen content (Dransfield, 1977). Percent extractable collagen was calculated by dividing the total extracted collagen (supernates) by the sum of total insoluble collagen (residues) and total extracted collagen (supernates) times 100.

Heating temperature effects were tested for trimmed or untrimmed RSB by Duncan's New Multiple Range Test and differences between the means of trimmed and untrimmed RSB at the same temperature were tested using Student's t (Steel and Torrie, 1980).

RESULTS & DISCUSSION

UNTRIMMED RSB had higher shear forces when raw or heated below 55°C than for higher temperatures (Fig. 1). Trimmed RSB was significantly tougher ($p<0.05$) when heated at 50°C and 55°C than at other temperatures. Differences in shear forces of trimmed and untrimmed RSB were significant ($p<0.05$) at temperatures ≤ 60°C.

The epimysial connective tissue in untrimmed RSB resulted in higher shear forces when heated ≤ 60°C compared to trimmed RSB. Trimmed RSB averaged 1.86% collagen while the untrimmed RSB averaged 3.52% collagen ($t = 7.25$, $p<0.01$, $N = 40$). Shear forces changed similarly in both trimmed and untrimmed RSB. Shear force increased before 55°C for both trimmed and untrimmed RSB due to coagulation and shrinkage of myofibrillar proteins. Heat denaturation of connective tissue usually results in decreased shear force while heat denaturation of myofibrillar protein results in increased shear force (Schmidt and Parrish, 1971).

At temperatures of 60°C, the contribution of the collagen to the overall texture decreased, and there were no significant differences in shear force values between trimmed and untrimmed RSB. Penfield and Meyer (1975) showed decreases in shear values after cooking beef semitendinosus to 60°C, and Laakkonen et al. (1970) and Laakkonen (1973) reported several studies which showed decreases in shear values of various beef muscles heated between 50°-60°C, due to the shrinkage of intramuscular collagen. In other studies (Paul et al., 1973; Moller, 1980-81; Brady and Penfield, 1981) heating above 60°C showed no effect of ultimate temperature on shear values.

Authors Strange and Whiting are with the USDA-ARS, Eastern Regional Research Center, 600 East Mermaid Lane, Wyndmoor, PA 19118.

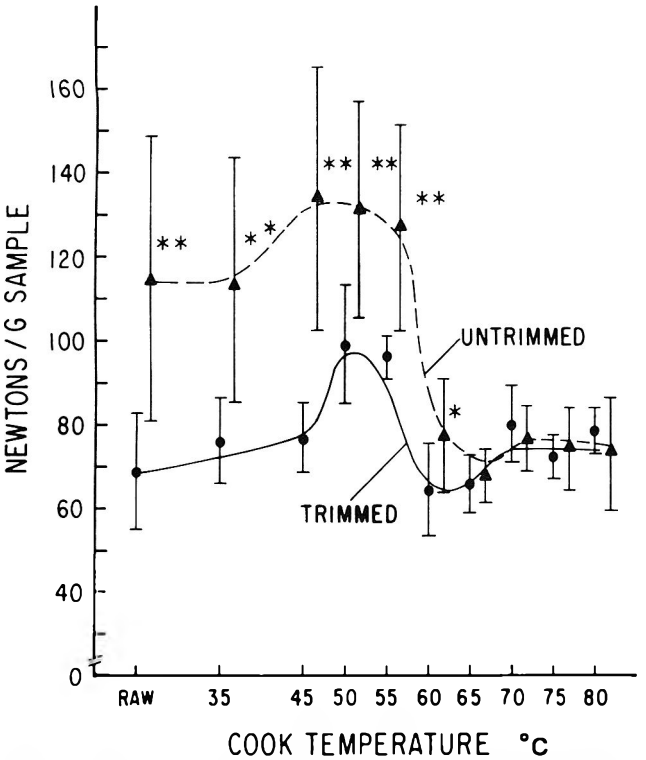

*Fig. 1—Relationship between Kramer multiblade shear forces and cooking temperature (°C) of restructured meat products made from trimmed and untrimmed beef clods: ● — ● trimmed samples; △ — △ untrimmed samples; bars on graph are standard deviation; **p<0.01 and *p<0.05 for Student's t of Kramer shear force between trimmed and untrimmed samples cooked at temperature shown.*

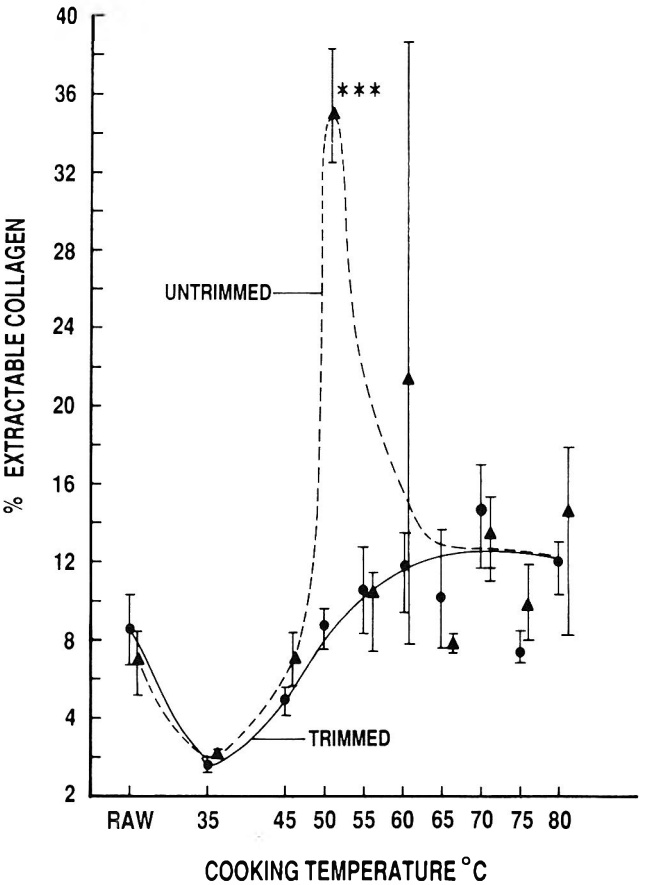

*Fig. 2—Relationship between % extractable collagen and cooking temperature of restructured meat products made from trimmed and untrimmed beef clods. ● — ● trimmed samples; △ — △ untrimmed samples; bars on graph are range of extractabilities; ***p<0.001 for Student's t of % extractable collagen between trimmed and untrimmed samples cooked at temperature shown.*

For trimmed RSB, the percent extractable collagen showed no distinct variation with temperature except for a decrease at 35°C (Fig. 2). Goll et al. (1964b) and Penfield and Meyer (1975) showed a minimum intramuscular collagen solubility at 40°C. The extractable collagen of untrimmed RSB, which contained more epimysial connective tissue than trimmed RSB, behaved similarly to the extractable collagen of trimmed RSB except for a peak between 50°C and 60°C. The variation in extractable collagen between 50°C and 60°C in RSB containing epimysial collagen and the subsequent decrease may possibly be due to interaction between localized aggregation and resolubilization of Type I collagen (Engel, 1987). Differences in the extractabilities of trimmed and untrimmed RSB were significant at 50°C. Strange and Whiting (1987) also noted greater collagen extractability in cooked (60°C) RSB samples with added epimysium compared to those made with trimmed meat. Mohr and Bendall (1969) showed that tendon was more soluble than intramuscular connective tissue. Field et al. (1970) showed that epimysial collagen from young pigs was more soluble than intramuscular collagen. Recently, Burson and Hunt (1986) demonstrated that Type I collagen was more heat-labile than Type III. Light et al. (1985) found that epimysial connective tissue contained more Type I collagen than did the intramuscular connective tissues.

Collagen extractability is a function of type of collagen present and temperature of heating. Shear force is unrelated to total collagen extractability in RSB if several types of connective tissue are present. Increasing solubility of specific collagens by using collagenases or chemical treatments may be effective for tenderizing restructured meats by lowering the temperature where collagen solubilizes during heating, thereby reducing the contribution of connective tissue to shear force of RSB.

In conclusion, collagen content was related to shear force only if the shear force was measured on restructured samples cooked to less than 60°C; collagen extractability was a poor predictor of texture for restructured meat.

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—Continued on page 1233

A Research Note

Effects of Phage Concentration, Bacterial Density, and Temperature on Phage Control of Beef Spoilage

G. GORDON GREER

ABSTRACT

Retail beef steaks, inoculated with graded amounts of a *Pseudomonas* spp. and homologous phage, were subjected to a range of retail case temperatures to assess the significance of any interactive effects upon retail case life. The potential of phage to increase the case life of beef was unaffected by temperature within the range 1–10°C but was significantly influenced by the interactive effects of initial bacterial density and phage concentration. A maximum increase in the retail case life of beef, from 3.4 to 6.4 days, required initial bacterial densities of 4.6×10^3 CFU/cm² and phage concentration of 9.7×10^7 PFU/cm².

INTRODUCTION

VIRULENT, psychrotrophic bacteriophages for *Pseudomonas* spp. (Greer, 1982) and *Brocothrix thermosphacta* (Greer, 1983) have been isolated from naturally spoiling retail beef cuts. The potential of these phages as a unique biocontrol system is supported by recent evidence that homologous phage could inhibit *Pseudomonas* growth and double the retail case life of artificially inoculated beef (Greer, 1986a,b). However, studies to date have been restricted to a single temperature and the limited research on the effects of the concentration of phage and contaminating bacteria has not permitted a study of possible interactive effects (Greer, 1986a,b). There is, however, some limited evidence that a specific threshold density of host bacteria is required if phages are to interact with bacteria in natural ecosystems (Ellis et al., 1972; Wiggins and Alexander, 1985).

The present study was designed to evaluate the effects of bacterial density, phage concentration and temperature upon the ability of homologous phage to extend the case life of *Pseudomonas*-inoculated beef.

MATERIALS & METHODS

BEEF RIB-EYE STEAKS were cut from Longissimus muscle after 6 days of carcass aging at 1°C. At each of five retail temperature treatments (1, 4, 6, 8, and 10°C), 25 steaks were randomly assigned to each of five inoculation treatments. The preparation of the *Pseudomonas* D23 and phage C35 inocula and the surface inoculation of steaks has been described previously (Greer, 1986a). Following inoculation, steaks were wrapped in an oxygen permeable, polyvinyl film (Vitafilm Choice Wrap) and placed in a commercial, horizontal retail cabinet under simulated conditions of retail display (Greer and Jeremiah, 1981). At daily intervals, for 10 days, each steak was evaluated by an experienced, five-member sensory panel using previously described subjective scales to determine case life on the basis of appearance (Greer and Jeremiah, 1981).

Data were analyzed by analysis of variance according to the General Linear Models procedure of the Statistical Analysis System (SAS Institute, Cary, NC). The models included the main effects of phage and bacterial concentration, temperature and appropriate interactions.

Author Greer is with Agriculture Canada, Research Station, Lacombe, Alberta, Canada TOC 1S0.

RESULTS & DISCUSSION

THE DATA in Table 1 show that neither the three-way interaction (temperature × bacteria × phage) nor the two-way interaction (temperature × phage) were significant ($p > 0.1$). Thus, homologous phage control of spoilage by a *Pseudomonas* sp. was unaffected by beef temperature within the range 1 to 10°C. However, beef case life (Table 1) was influenced by a significant two-way interaction of bacteria × phage and of temperature × bacteria ($p < 0.001$).

That the variation in beef case life is dependent upon the interrelationship of bacterial numbers and temperature is well documented (Greer and Jeremiah, 1981; Gill, 1986). In keeping with the objectives of the current study, the bacteria × phage interaction is the critical issue. The nature of this interaction is shown in Table 2. In general, at higher levels of bacterial contamination, less phage were required to produce a significant increase in case life (Table 2). However, phage did not affect beef case life until initial bacterial densities reached 46 colony forming units/cm² (CFU/cm²). At bacterial densities from 46 CFU/cm² to 4.6×10^5 CFU/cm², increases in the concentration of added phage produced a progressive increase in the case life of beef.

Minimum requirements for phage to significantly ($p < 0.05$) increase case life were initial *Pseudomonas* loads of 46 CFU/cm² and phage concentrations of at least 9.7×10^5 plaque forming units/cm² (PFU/cm²). Under these conditions, beef case life was increased from about 6.3 to 7.1 days and up to 7.7 days at the highest phage concentration (9.7×10^7 PFU/cm²). This threshold level of bacterial contamination is con-

Table 1—Effects of phage and bacterial concentrations and temperature on the retail case life of beef

Source	DF[a]	Mean Square	F-value
Temperature	4	845.72	451.14***
Bacteria	4	459.18	244.94***
Temperature × Bacteria	16	10.62	5.67***
Phage	4	55.26	29.48***
Temperature × Phage	16	1.01	0.54[ns]
Bacteria × Phage	16	5.21	2.78***
Temperature; × Bacteria × Phage	64	1.88	1.01[ns]
Error	500	1.87	

[a] Degrees of freedom.
*** Significant at 0.1% level.
[ns] Not significant at 10% level.

Table 2—Effect of phage and bacterial concentrations on beef case life

Phage C35 (PFU/cm²)	Case life (Days) *Pseudomonas* D23 (CFU/cm²)				
	0	0.46	46	4.6×10^3	4.6×10^5
0	6.70	6.94	6.27	3.44	1.70
97	6.18	6.87	6.55	4.58[a]	1.75
9.7×10^3	6.96	7.32	6.81	5.23[a]	2.62[b]
9.7×10^5	7.16	7.66	7.14[a]	5.49[a]	2.91[b]
9.7×10^7	7.16	7.56	7.65[a]	6.39[a]	4.45[b]

[a] Least squares means in the same column with a superscript differ significantly from the control (0 phage) at the 5% level. Standard error of all means was 0.27.

siderably lower than that reported by Wiggins and Alexander (1985) who found that phages could not effect the growth or metabolic activities of bacteria in water or sewage at a density of the host species below 10^4 CFU/mL. Furthermore, Ellis et al. (1972) found that *P. fragi* phage was unable to control bacterial numbers in milk unless initial bacterial loads exceeded 10^3 CFU/mL.

In the present study, however, the limited improvement in the case life of beef (1.4 days), mediated by phage at low bacterial densities, is of dubious practical relevance. For phage to promote an increase in case life of any practical magnitude, initial loads of *Pseudomonas* of 4.6×10^3 CFU/cm^2 were necessary. At these initial bacterial levels all phage concentrations within the range of 97 PFU/cm^2 to 9.7×10^7 PFU/cm^2 produced a progressive and significant increase in case life ($p < 0.05$). Maximum shelf life extension was found at phage concentrations of 9.7×10^7 PFU/cm^2 where the retail case life of beef was increased from about 3.4 to 6.4 days.

Since high titer lysates (ca. 10^{10} PFU/mL) of *Pseudomonas* phages can readily be prepared in the laboratory, the limiting factor would be the initial levels of bacterial contamination. In this regard, Ellis et al. (1972) argued that the requirement for unrealistically high levels of bacteria would reduce the likelihood of phage influencing the shelf life of milk. In accordance with these views and the knowledge that *Pseudomonas* constitutes only 1% of the initial contamination of beef (Gill, 1986), the potential of phage as an effective biological control agent for beef spoilage is questionable.

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A Research Note

Identification of Geosmin as the Major Muddy Off-flavor of Louisiana Brackish Water Clam (*Rangia cuneata*)

T. C.-Y. HSIEH, U. TANCHOTIKUL, and J.E. MATIELLA

ABSTRACT

The major compound responsible for the objectionable muddy or musty/earthy off-flavor in Louisiana brackish water clams (*Rangia cuneata*) was positively identified as geosmin (trans-1,10-dimethyl-trans-9-decalol), based on gas chromatographic retention times, electron ionization mass spectra, and odor characteristics. Confirmation was based on analysis of authentic geosmin under experimental conditions comparable to those of the clam samples.

INTRODUCTION

RANGIA CLAM (*Rangia cuneata*) is found mainly in brackish waters along the Gulf of Mexico coast (LaSalle and de la Cruz, 1985). The largest concentration of living clams has been found in the Louisiana coastal area with a standing crop of 50 billion clams. However, a strong and objectionable muddy off-flavor limits their commercialization for food consumption. The objective of this study was to identify the chemical structure of the major compound(s) responsible for the muddy off-flavor.

The authors are affiliated with the Dept. of Food Science, Louisiana Agricultural Experiment Station, LSU Agricultural Center and College of Agriculture and Louisiana Sea Grant College Program, Louisiana State Univ., Baton Rouge, LA 70803–4200

MATERIALS & METHODS

LOUISIANA RANGIA CLAMS were collected by dredging from the marshes in St. Bernard Parish area. The clams were placed in plastic bags and transported on ice within 4 hr to the LSU Food Science Department in Baton Rouge, LA. After overnight storage on ice, the clams were washed with water and steamed for about 45 min until the shells were open. Samples were purged in a dynamic headspace concentration system (Tekmar Co., Cincinnati, OH) at 60°C purge temperature and 40 mL/min helium (99.999% purity) purge flow rate to trap volatile compounds in the headspace onto a Tenax TA (Chrompack, Raritan, NJ) sorbent cartridge. Each sample of 70g clam tissue was distributed into 10 purge tubes (7g per tube), and each tube was purged, in sequence, for 30 min followed by 40 min of dry-purge. Additional dry-purge to remove trapped moisture in the Tenax TA trap was also carried out. The trapped volatiles were then flash-heated at 185°C and transferred to a GC column with simultaneous cryogenic focusing. Separation of components was achieved by high resolution gas chromatography on a fused silica capillary column Supelcowax 10 (60m length × 0.25 mm i.d. × 0.25 μm film thickness; Supelco, Inc. Bellefonte, PA) in a Hewlett-Packard (HP) 5792 GC with the oven temperature programmed from 60°C to 175°C at 2°C/min and set at 175°C for 15 min. For GC-coupled sniffing, the column effluent was split into two streams at a ratio of 1:1 with one stream going to a hydrogen flame-ionization detector and the other stream to a heated (200°C) sniffing port with a make-up flow (30 mL/min) of moistened breathing air. Descriptive sensory evaluation at the sniffing port was performed by two expert panelists familiar with the sniffing techniques

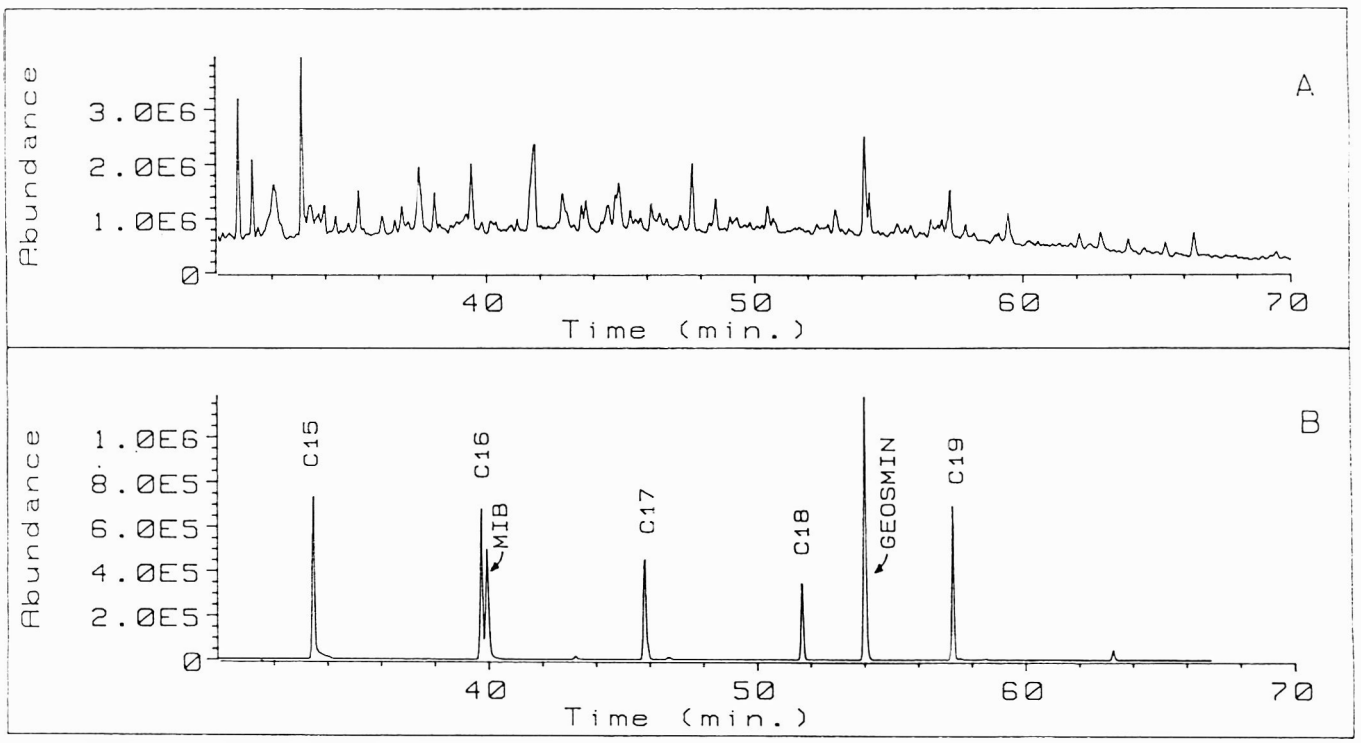

Fig. 1—(A) Total ion chromatogram of volatile components in steamed Rangia clam. (B) Total ion chromatogram of a standard mixture of MIB, geosmin, and hydrocarbons.

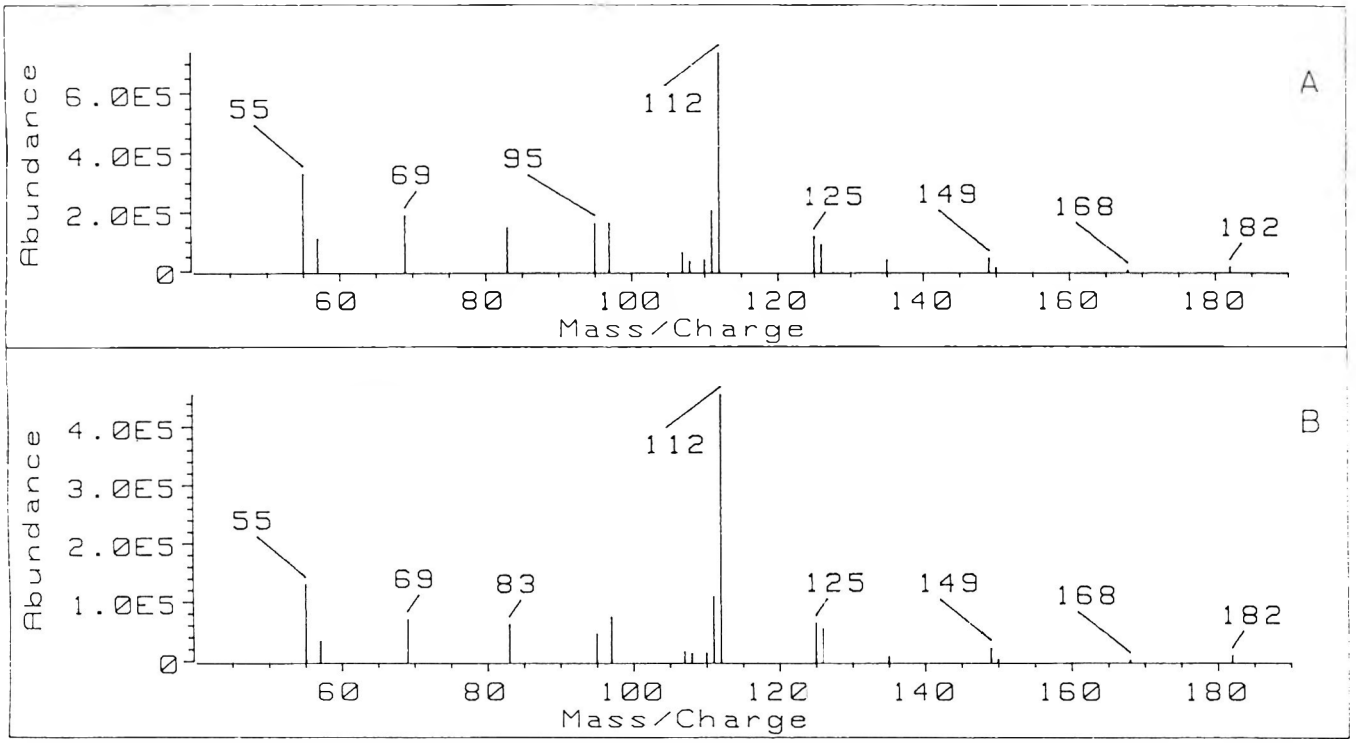

Fig. 2—(A) Mass spectrum at retention time 54.129 min of sample in Fig. 1(A,. (B) Mass spectrum at retention time 54.025 min in Fig. 1(B)

and the muddy off-flavor characteristics of the Louisiana Rangia clams. In GC/mass spectrometry (GC/MS) experiments, the GC was coupled to an HP 5970B mass selective detector operating in the electron ionization (EI) mode with the ion source temperature set at 200°C and the electron multiplier voltage at 1800 V. Initial experiments were carried out in full MS scan with a mass range of m/z (mass/charge) 40–290. Better MS sensitivity was achieved by selective ion monitoring for the following ions: m/z 55, 57, 69, 83, 95, 97, 107, 108, 110, 111, 112, 125, 126, 135, 149, 150, 168, and 182. Authentic standards of geosmin (trans-1,10-dimethyl-trans-9-decalol) and 2-methylisoborneol (MIB) were a generous gift from Dr. R. T. Lovell of Auburn University.

The standard geosmin was originally obtained from A. R. Hochstetler (Givaudin Corp., Clifton, NJ) and was synthesized by the procedure described by Marshall and Hochstetler (1968), and the standard MIB was synthesized at the USDA Southern Regional Research Center, New Orleans, LA by the procedure of Wood and Snoeyink (1977). Standard hydrocarbons were purchased from PolyScience Corp., Niles, IL.

RESULTS & DISCUSSION

POSITIVE IDENTIFICATION of geosmin as the major muddy off-flavor compound in Louisiana Rangia clam has been achieved. A total ion chromatogram of a clam sample and that of a standard mixture from GC/MS analysis in EI mode are shown in Fig. 1. The mass spectrum of the clam sample at a retention time of 54.129 min (Fig. 2A) and that of the standard geosmin at retention time of 54.025 min (Fig. 2B) are essentially identical. The two retention times were considered matched within experimental error.

The odor characteristics of the GC effluent also were carefully examined by descriptive sensory evaluation at the GC sniffing port. Within the entire chromatographic period of the volatiles from each sample, the only retention time segment during which the muddy off-flavor of the clams was detected and announced reproducibly by the two expert sniffers was found to coincide with the retention time segment of the geosmin peak in separate sniffing analyses for the standard. In addition, the muddy odor characteristics of the specific area in the clam chromatogram were found indistinguishable from those of the standard geosmin and the muddy taint of the clam samples. Geosmin has been found to cause muddy odor in water and fish (Tabachek and Yurkowski, 1976; Persson, 1980), and in pond-cultured penaeid shrimp (Lovell and Broce, 1985). Another muddy flavor compound, 2-methylisoborneol, has also been found in fish (Persson, 1980). However, no evidence using chromatography, mass spectrometry, and sensory evaluation has suggested the presence of 2-methyl-isoborneol in these clam samples. Samples collected over a period of one year had similar results. These data suggested that geosmin was the major compound contributing to the muddy off-flavor of the clam samples studied.

The procedure developed and information obtained from this study will be used to monitor concentrations of geosmin in various clam samples and to evaluate the effectiveness of various off-flavor reduction procedures. Successful reduction of the muddy off-flavor eventually could lead to commercialization of this abundant shellfish resource.

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A Research Note

Effect of Lipids on the Properties of Extruded Products

M. BHATTACHARYA and M.A. HANNA

ABSTRACT

Corn gluten having 3.9, 1.8 and 0.2%, lipid (dry basis, db) was blended with defatted soy protein concentrate in the ratio of 25:75 w/w. The blends were made up of 20, 30, and 40% moisture (db) and then extruded through 4.24, 3.0, and 2.12 mm diameter dies at screw speeds of 60, 120, and 180 rpm. The dependent variables measured were puff ratio, bulk density, shear strength, and water-holding capacity. Shear strength increased when lipids decreased from 3.9% to 1.8% but then decreased when lipids were reduced to 0.2%. Water-holding capacity and puff ratio increased with decreasing lipids. Bulk density was unaffected by lipid concentration.

INTRODUCTION

THE USE OF high-temperature short time (HTST) extrusion cooking has led to the production of fabricated foods consisting primarily of cereals, starches and vegetable proteins. This process has almost limitless applications in the processing of cereal-based foods and other materials including various blends. Literature reviews on protein-texturization via extrusion can be found in Harper (1979), Kinsella (1978), Clark (1978) and Stanley and de Man (1978). However, very little has been reported on the possible interactions between lipids and proteins during extrusion. Even though the nutritional value of lipids may decrease at high temperature because of an increased oxidation rate and the formation of toxic compounds (Linko et al., 1981), it is possible that temperatures during extrusion cooking are rarely high and residence time long enough to cause thermal destruction of lipids (Nielsen, 1976).

The effect of lipids on extrudate properties is complex and has been shown to vary with the type, the amount and the hydrophilic-lipophilic balance of the lipid and the material being extruded (Faubion et al., 1982). Faubion and Hoseney (1982) observed that the diameter of lipid-extracted, low-protein hard wheat flour extrudate increased by 28% over nonextracted flour. Textural strength was also found to increase upon removal of free lipids. In a study by Bhattacharya et al. (1986), different levels of lipids in the product, as obtained by blending corn gluten meal (CGM) and soy concentrate (SC) in varying proportions, resulted in different puff ratios, bulk densities, water holding capacity and shear strengths after extrusion. However, the differences in textural properties could also have been due to varying levels of the two protein types (corn and soy), since protein types have been known to affect properties (Breshnahan et al., 1982). This study was, therefore, undertaken to separate the effects of lipids from proteins and to observe the effect of lipid concentration on textural properties.

MATERIALS & METHODS

DEFATTED SOY PROTEIN concentrate was obtained from Central Soya (Fort Wayne, IN); wet corn gluten meal was obtained from ADM (Clinton, IA). The details of sample preparation can be found in Bhattacharya et al. (1986).

Lipids from CGM samples were extracted using solvent Skellysolve B in a modified Soxhlet extraction apparatus having an extraction volume of 2200 mL. Samples of flour were extracted for 48 hr to reduce the average lipid content of CGM from 3.91% to 1.8% and for another 48 hr to further reduce the lipid content to 0.2%. The defatted materials were air-dried at room temperature (23°C) until solvent odor was no longer noticeable.

A Brabender laboratory food extruder with a 1.90 cm diameter barrel and a 20:1 barrel length to diameter ratio was used. Screws rotated at speeds of 60, 120, and 180 rpm. The screw compression ratio was 3:1. The detailed barrel and screw dimensions can be found in Bhattacharya et al. (1986). The temperature of the first (heating) zone was kept at 80°C, whereas those of the second and die zone were held at 145 ± 1°C. The nozzle diameters were 4.24, 3.00 and 2.12 mm having length to radius (L/R) ratios of 6.73, 10.73, and 14.05, respectively. The melt temperature at the die entrance was within ± 2-3°C of the reported barrel temperature.

Before extruding the blends, the extruder was brought to equilibrium with corn meal. Care was taken to ensure that flights at the feeding port were kept full throughout the extrusion runs. The extruded samples were dried at 45°C for 16–24 hr to bring the final products to approximately the same moisture (2–3%). The products were sealed in polyethylene bags and stored in a freezer until further analyzed.

Eleven samples from each blend were extruded at different moistures with different die and screw speed combinations. The detailed experimental design can be found in Walker and Parkhurst (1984).

Textural properties (puff ratio, bulk density, shear strength and water-holding capacity) were determined as outlined in Bhattacharya et al. (1986). Die diameter and screw speed were expressed as shear rate, the detailed calculation of which can also be found in an earlier paper (Bhattacharya et al., 1986).

Duncan's multiple range test (Barr et al., 1979) was used to determine mean differences between lipid concentrations. General Linear Model (GLM) procedure was used to conduct analysis of variance.

RESULTS & DISCUSSION

EXPERIMENTAL DATA can be found in Table 1. Statistical analysis of linear quadratic and linear cross product effects of independent variable on puff ratio, bulk density, shear strength and water-holding capacity is shown in Table 2. Puff ratio was affected by linear and quadratic effects of shear rate as well as interactions of shear rate with moisture content and lipid concentration. Increased shear rate caused product to puff more. Lower lipid concentrations also increased the puff ratio of the products. Higher moisture caused products to puff less.

Bulk density was affected by shear rate and moisture but not by concentration of lipid in the product. Increasing shear rate decreased bulk density whereas increased moisture increased bulk density.

Shear strength was affected by moisture and lipid concentrations. Shear strength was highest at 1.8% lipids but dropped off at the 3.9% and 0.2% lipids. At 20% and 30% product moisture, the shear strength at 3.9% lipids was higher than at 0.2% lipids. At 40% product moisture, the reverse was observed. Duncan's Multiple Range Test (DMRT) showed that mean shear strength at 1.8% lipids was higher than at 0.2% lipids (5% level of significance). No statistical differences were observed between means at 3.9% and 1.9% or 3.9% and 0.2%.

Author Bhattacharya is Assistant Professor, Dept. of Agricultural Engineering, Univ. of Minnesota, St. Paul, MN. Author Hanna is Professor, Depts. of Agricultural Engineering and Food Science & Technology, Univ. of Nebraska, Lincoln, NE.

Table 1—Effect of lipid concentration on textural properties of extruded corn gluten meal/soy concentrate blend[a]

MC	rpm	Diam	Puff ratio			Shear strength (kPa)			Bulk density (kg/m^3)			Water-holding capacity[b]		
			LL1	LL2	LL3	LL1	LL2	LL3	LL1	LL2	LL3	LL1	LL2	LL3
20	60	4.24	1.24	1.43	1.78	3530	4596	2584	514.5	508.9	523.9	3.37	3.63	3.60
20	120	2.25	2.54	3.99	4.85	1410	3000	820	272.5	241.7	165.9	3.56	3.59	3.81
20	180	3.00	2.46	3.34	6.93	1500	2665	859	414.5	313.7	239.0	3.48	3.64	3.65
30	60	2.25	1.76	2.11	2.53	2200	3744	2594	486.5	434.8	475.7	2.83	3.43	3.44
30	120	4.24	1.35	1.41	3.17	2210	3252	1066	506.5	530.3	384.2	3.33	3.24	3.25
30	120	3.00	1.75	2.28	3.88	2380	2716	1034	484.0	457.2	325.0	3.07	3.31	3.26
30	120	2.25	1.41	2.68	3.67	1940	2247	1238	387.0	367.0	304.9	2.93	3.27	3.27
30	180	4.24	1.56	1.83	3.58	1890	2117	841	487.5	479.1	317.0	3.25	3.42	3.40
40	60	3.00	1.28	1.86	1.43	5040	5520	6913	551.5	498.8	612.3	2.99	3.14	3.11
40	120	4.24	1.24	1.31	1.58	1760	4294	2708	557.5	571.0	644.2	3.09	3.22	3.23
40	180	2.25	1.74	1.93	2.27	1580	3960	3109	477.5	451.7	512.2	2.88	3.37	3.44

[a] LL1–Unextracted CGM (3.9% lipid); LL2–CGM extracted once (1.8% lipid); LL3–CGM extracted twice (0.2% lipid); MC–Moisture content (dry basis); rpm–Screw speed (rev/min); Diam–Die diameter (mm).

[b] Water-holding capacity has units of (g H_2O/g substrate).

Table 2—Analysis of variance of dependent variables as affected by independent variables[a]

	PR>F			
	Puff ratio	Bulk density	Shear strength	Water-holding capacity
MCDB	NS	S	S	S
Shear	HS*	HS*	NS	NS
LL	NS	NS	NS	S
MCDB*MCDB	NS	NS	S	S
Shear*Shear	HS*	S	NS	NS
LL*LL	NS	NS	HS	S
MCDB*Shear	HS*	S	NS	NS
MCDB*LL	S	NS	NS	NS
Shear*LL	HS	NS	NS	NS

[a] MCDB–Moisture Content (Dry basis); Shear–Wall Shear Rate (Sec^{-1}); LL–Lipid concentration; NS–Not significant at 5% level; S–Significant at 5% level; HS–Significant at 1% level; HS*–Significant at 0.1% level.

Water-holding capacity was affected by both linear and quadratic effects of lipid level and moisture. Increasing moisture decreased water-holding capacity whereas decreased lipids increased water-holding capacity. DMRT showed that water-holding capacity at 3.9% lipid concentration was significantly lower than at 1.8 and 0.2% lipids.

Extrudate properties have been known to vary with the amount and type of lipids (Faubion et al., 1982). An analysis of the lipids showed that the lipid profiles of all three blends were the same. Very little is known regarding lipid-protein interaction. Protein interaction which is responsible for texturization (Holay and Harper, 1982) is probably inhibited by the presence of lipids leading to a less puffed and weaker product. As lipid concentration decreases, the protein may have the freedom to form disulfide bonds thus increasing the shear strength. However, the data suggested that some lipid was necessary. Since molecular interactions are complex and their measurement quite difficult, more work needs to be done on a molecular level to explain these interactions.

CONCLUSIONS

DECREASING the lipid concent in products resulted in a puffier extrudate that had higher water-holding capacity. The shear strength of the extrudates increased with a decrease in lipid concentration and then decreased with further reductions in lipid content. This might indicate that a certain percent of lipid was necessary to impart strength in extrudates. The combination of lipid concentration and extrusion conditions made it possible to produce extruded products with a wide range of textural properties.

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A Research Note

Effect of Centrifugation on Hemagglutinin Activity Assessment in Common Beans

O. PAREDES-LOPEZ, M. L. SCHEVENIN, and F. GUEVARA-LARA

ABSTRACT

The effect of centrifugation in the determination of hemagglutinin (lectin) activity was assessed. Seeds of two bean cultivars (*Phaseolus vulgaris*) were used for this study. The saline extracts of lectins from soaked and/or cooked samples showed significant decreases in hemagglutinin activity after centrifugation, whereas the activity of the same extracts from raw beans (control) did not change after this separation step. These results suggest that the use of centrifugation affects the hemagglutinin activity assessment.

INTRODUCTION

COMMON BEANS are an important source of protein, calories, B-complex vitamins, dietary fiber, and mineral in Latin America. The problems associated with bean consumption are well known, particularly those related to the presence of some anti-nutritional factors and production of gastrointestinal distress (Paredes-López et al., 1986). Raw beans may be toxic when fed to animals and the principle toxicant is lectin or phyto-hemagglutinin proteins (Liener, 1976). The action of lectins is believed to be due to binding to intestinal mucosal cells, causing malfunction and consequently interference with the absorption of nutrients (Thompson et al., 1986). This antinutritional effect of lectins may be eliminated by moist heat treatment applied during cooking (Thompson et al., 1983).

The method most commonly used to measure lectin activity is based on the agglutination capacity of red blood cell suspensions (Lis and Sharon, 1972). It was noticed that the use of centrifugation of raw lectin extracts from bean flours to assess hemagglutinin activity (HA) was involved in the procedure followed by some workers (Grant et al., 1983; Thompson et al., 1986; Kadam and Smithard, 1987), while others did not include this separation step (Tan et al., 1983; Thompson et al., 1983). In view of the remarkable binding properties of lectins, it was assumed that the use of centrifugation might affect the HA results provided by such procedure. Thus the objective underlying this study was to evaluate the effect of centrifugation in the HA assessment of common bean extracts.

MATERIALS & METHODS

Bean samples

The bean seeds from the varieties FM-C and FM-RMC (*Phaseolus vulgaris* L.) used for this study were sown in the local experimental farm in 1986/87. Mature seeds were harvested, cleaned and stored in tightly sealed containers at 4°C until used.

Sample preparation

Samples for determination of HA were soaked for 16 hr in distilled water (1:4, w/v) and drained. Another portion was soaked as described and cooked in fresh water for 86 min at 95°C. Cooking time was determined using a modified Mattson bean cooker (Juárez et al., 1988). Beans were also soaked and cooked as before but in soaking water. A final lot was cooked without presoaking. All samples were freeze-dried and milled to pass a 100-US mesh screen.

Lectin extraction and analysis

The PBS (phosphate buffer saline, 0.136M NaCl-2.7 mM KCl-8.1 mM Na_2HPO_4-1.5 mM KH_2PO_4)-soluble protein was extracted from the seed flour (1:25, w/v), pH 7.4, in an Ultra-turrax homogenizer at maximum speed for 5 min at room temperature. The homogenate was magnetically stirred for 16 hr at 4°C and, unless otherwise stated, centrifuged at 40,000 x g for 30 min at 4°C. The supernatant contained the raw extracts of lectins (Felsted et al., 1975; Guevara-Lara, 1987). Starting with 50 µL of the extracts, serial twofold dilutions were made with PBS in Cooke microtiter V plates. Trypsinized rabbit red blood cell suspension (50 µL), prepared according to Lis and Sharon (1972), was then added. The plates were shaken for 10 min and after a 2-hr rest at room temperature the agglutination patterns were observed. HA was expressed as the reciprocal of the highest dilution giving positive agglutination (Thompson et al., 1983). Reported values represent means of four determinations, each obtained as duplicate analyses of two replicate experiments.

RESULTS & DISCUSSION

THE RAW SEED SAMPLES of the FM-C cultivar showed the same HA without and with the use of centrifugation (Table 1). A similar pattern was exhibited by the raw beans of the genetically improved cultivar FM-RMC. It is interesting to note that a different effect of centrifugation on the HA level occurred for soaked and/or cooked samples. The lectin extracts that were centrifuged had much lower values of HA than those that did not go through this separation step. The HA of some of the centrifuged samples from both cultivars decreased to below detectable levels. Also, the use of low-speed centrifugation (e.g., 2000 x g) produced the same effect as described. The derived cultivar FM-RMC tended to show higher lectin activity in relation to its progenitor (FM-C). The HA of the control beans FM-RMC (noncentrifuged sample) declined after soaking, while no significant changes were found for the equivalent sample of the FM-C cultivar. Some workers (Noah et al., 1980) have observed lectin losses during soaking but others (Thompson et al., 1983) reported nonsignificant changes after this treatment.

The results of this study suggest that the saline extracts contain suspended particles with lectin activity; therefore, centrifuged samples exhibit lower HA.

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Authors Paredes-Lopez and Schevenin are with Unidad Irapuato, CIEA-Inst. Politécnico Nal., Apdo. Postal 629, 36500 Irapuato, Gto., México. Author Guevara-Lara's present address is Dept. of Crop Sciences & Plant Ecology, Univ. of Saskatchewan, Saskatoon, Saskatchewan, S7N 0W0, Canada.

Table 1—Effect of centrifugation on the hemagglutinin activity level of saline extracts from seeds of two bean cultivars[a]

Sample	FM—C		FM—RMC	
	A[b]	B[b]	A	B
Raw bean (control)	1.15×10^6	1.15×10^6	2.32×10^6	2.32×10^6
Soaked bean	1.10×10^6	0.55×10^6	1.09×10^6	0.54×10^6
Soaked and cooked	7,500	500	16,200	200
Soaked and cooked bean in soaking water	4,000	0	15,900	0
Cooked bean without presoaking	8,600	500	21,000	0

[a] Results in hemagglutinin units/g sample (dry basis).

[b] A, B = Lectin extracts without and with centrifugation, respectively.

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A Research Note

Haemagglutinating and Trypsin inhibitor Activities of Lupin Seed (*Lupinus angustifolius*)

CHAN SHICK KIM AND K.T. MADHUSUDHAN

ABSTRACT

The phosphate buffer saline (PBS) extracts of defatted lupin seed flour showed haemagglutin activity only with trypsinized rabbit erythrocytes. The lectin tested in crude extracts showed specificity towards galactose and lactose. Heat stability studies of PBS extracts on incubation for 10 min showed no reduction of haemagglutinin activity in the temperature range 40°C to 80°C while no haemagglutination was observed when incubated at 90°C for 8 min. The trypsin inhibitor activity of flour extracts was 14.4 TUI/mg protein.

INTRODUCTION

LUPIN SEED is an important legume with promising potential for use in foods in addition to its present use in livestock and poultry feeds (Fontain, 1973; Anon, 1974). The composition and nutritive value of lupin seed has been studied by various workers (Gabriel and Marcos, 1976; Schoeneberger et al., 1982; Buendia, 1983; Hill, 1983). Debittering of lupin seed flour has been accomplished by the use of organic solvents (Blaicher et al., 1981; Oritz and Mukherjee, 1982; Lee and Kim, 1984). The possibility of substituting lupin seed flour in place of soy flour in some products was suggested by both its high protein content and yellow color (Morad et al., 1980; Boundy, 1982; Hill, 1983). Furthermore, it has been reported that lupin seed is free from anti-nutritional factors, such as trypsin inhibitors, phytohaemagglutinins, and from flavor problems that are present in soybean (Gladstones, 1977; Valdebouze et al., 1982; Gross, 1983; Hill, 1983; Rahma and Narasinga Rao, 1984). The present study was initiated to reinvestigate the possible presence of haemagglutinating and trypsin inhibitor activities in a particular cultivar of lupin seed.

MATERIALS & METHODS

LUPIN SEED (*(Lupinus angustifolius)* cultivar Sweet White was obtained from Australia. Soybean, cultivar Bragg, was purchased form Nagpur, India. D (+) - glucosamine, N-acetylglucosamine, 1-0-methyl-α-D-glucopyranoside and N α-benzoyl-DL-arginine p-nitroanilide (BAPNA) HCl were obtained from Sigma Chemical Co. St. Louis, MO). D(+)-mannose was from E. Merck, West Germany. Lupin or soybean seeds were dehulled, flaked, and defatted with food grade hexane at ambient temperature until the fat content was less than 1%. The defatted flakes were pulverized and passed through a 60 mesh (BSS) sieve and the resultant flour was used for all experiments.

Haemagglutinating activity

Defatted lupin seed or soy flour was extracted with 0.01 M sodium phosphate buffer, pH 7.0, containing 0.9% NaCl (PBS), and haemagglutinating activity was determined in the extracts, using 2% trypsinized rabbit erythrocytes by the method of Paulova et al. (1971). Erythrocytes of cow, sheep, rabbit, guinea pig and humans (A, B, and O groups) were prepared in the same way as rabbit erythrocytes. Haemagglutination was observed visually after incubating the protein and erythrocytes mixture at 37°C for 1 hr. One haemagglutination unit (HU) is defined as the minimum concentration of protein necessary for agglutination under the experimental conditions. Sugar specificity was determined by incubation of a phosphate buffer saline (PBS) extract of flour with various amounts of sugar at 37°C for 1 hr, followed by incubation of the sugar-protein mixture with the rabbit erythrocytes at 37°C for 1 hr.

Trypsin inhibitor activity

This was determined by the method of Kakade et al. (1969) using a synthetic substrate, BAPNA-HCl. PBS extracts of lupin or soybean flour were extensively dialyzed against water and the water-soluble portion was used for the assay. Nitrogen content of the flour extracts was determined by the micro-Kjeldahl method and protein calculated (N × 6.25).

RESULTS & DISCUSSIONS

HAEMAGGLUTINATING ACTIVITY of phosphate buffer saline extracts of lupin seed was observed only with the trypsinized rabbit erythrocytes. These values were 12.5 and 32 haemagglutination units (HU) per mg protein for the PBS extract and PBS extract dialyzed against the same buffer, respectively. However, the absence of haemagglutination activity in lupin seed extracts has been reported (Valdebouze et al., 1982; Gross, 1983; Hill, 1983; Rahma and Narasinga Rao, 1984). Sugar specificity tests with the crude extracts indicated that the agglutination was inhibited by galactose and lactose at a concentration of 180 and 342 μg, respectively. Glucose, mannose, glucosamine-HCl, N-acetylglucosamine and methyl glucopyranoside had no effect on agglutination. This showed that the lectin was specific to galactose. The heat stability of the haemagglutin was studied by incubation of the PBS extract of lupin seed flour for 10 min in the temperature range of 40°C to 100°C. There was no reduction in the haemagglutination activity up to 80°C, beyond which there was a complete loss of agglutination activity (Fig. 1A). The reduction of haemagglutinating activity of PBS extracts at 90°C was in the order of 50, 75, and 100% during an incubation period 4, 6 and 8 min, respectively (Fib. 1B).

The trypsin inhibitor activity of lupin seed flour was 14.4 TUI/mg protein and this activity was completely lost on incubation of the extract at 90°C for 8 min. The trypsin activity (76 TUI/mg protein) of soybean flour, dialyzed against distilled water and haemagglutinating activity (104 HU/mg protein) of soybean flour, dialyzed against PBS, were much higher than in trypsin seed flour (14.4 mg protein and 104 HU/mg protein, respectively.

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Author Madhusudhan is with the Dept. of Biochemistry & Molecular Biology, Univ. of Oklahoma Health Sciences Center, P.O. Box 26901, Oklahoma City, OK 73190. Author Kim's present address is The Research Institute for Food Science, Kyoto Univ., Uji, Kyoto 611, Japan.

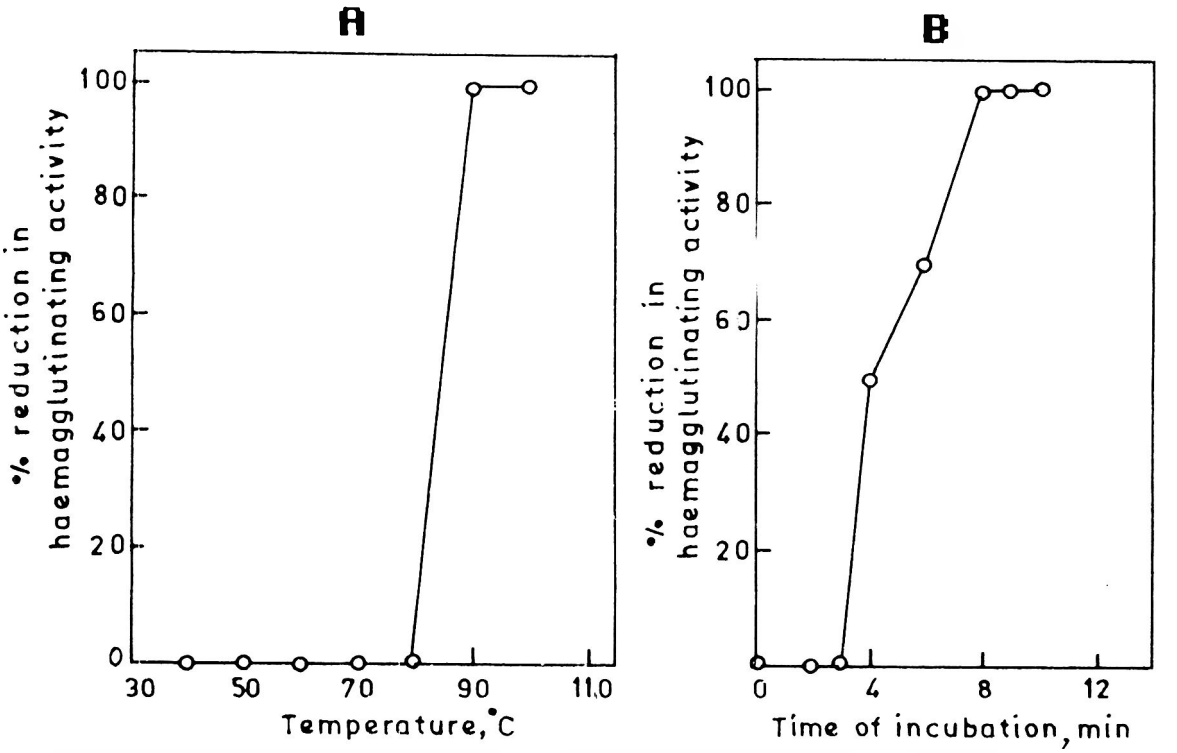

Fig. 1—Effect of time and temperature on the loss of haemagglutinating activity of PBS extracts of lupin seed flour: (A) Effect of temperature, time of incubation, 10 min; (B) Effect of time, temperature of incubation, 90°C.

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A Research Note

Utilization of Endo-Polygalacturonase from *Kluyveromyces fragilis* in the Clarification of Apple Juice

L. GOMEZ-RUIZ, M. GARCIA-GARIBAY, and E. BARZANA

ABSTRACT

Endo-polygalacturonase (endo-PG) from *Kluyveromyces fragilis* produced on whey was used for the clarification of apple juice and its efficacy was compared with that of a commercial pectinase. Endo-PG was recovered from the fermentation broth with a yield of 60.7% and a purification factor of 3.85. Clarification experiments were conducted at 30°C and 50°C for different times. No significant statistical differences in the final turbidity were observed at the 1% level for samples treated with either enzyme. It was concluded that endo-PG could be successfully used in the process of clarification of apple juice.

INTRODUCTION

PECTIC ENZYMES or pectinases are generic terms for those enzymes which modify in any way the structure of the pectic substances present in plant tissues. Fungal pectinases are of commercial importance representing approximately 3% of the total enzyme market (Godfrey and Reichelt, 1983) with a wide range of applications in the fruit and wine processing industries.

Pectinases may be divided into pectinesterases and depolymerizing enzymes. The latter group includes the galacturonases, which operate through a hydrolytic mechanism, and the lyases, which act by transeliminative cleavage (Kilara, 1982). Further classification depends on the preference for pectate or pectin as substrate and whether the enzyme acts through an endo- or exo-mode of attack. Esterases de-esterify pectin producing methanol and pectate. Endo-polygalacturonase (endo-PG, E.C. 3.2.1.15) acts on pectate and is widely produced by plants and molds. Endo-PG is the only pectic enzyme produced by the yeast *Kluyveromyces fragilis* (Luh and Paff, 1951) and consists of multiple forms of different molecular weights. Lim et al. (1980) have reported three, while Call and Emeis (1983) have reported twenty-one and Sakai et al. (1984) four. Therefore, it seems that the pectinolytic enzyme system depends on the strain.

No practical application for endo-PG from yeasts is yet available. Yeast endo-PG has a higher cost of production and lacks the multiple activity of commercial mixed enzyme preparations of fungal origin. For instance, Ultrazym 100, the commercial preparation used in this study contains high levels of polymethylgalacturonases and lyases and negligible esterase activity. However, we have recently demonstrated that endo-PG from *K. fragilis* can be produced inexpensively as a by-product in the processing of industrial single cell protein from cheese whey (Garcia-Garibay et al., 1987a,b). Preliminary studies on the potential use of endo-PG are scarce. Most of them deal with the use of the enzyme as a macerating agent (Kobayashi and Matsuo, 1979; Lim et al., 1980; Ozawa et al., 1959; Voragen et al., 1980). Sakai et al. (1984) have reported protopectinase activity besides the endo-PG activity. Regarding its flocculating abilitiy, Luh and Phaff (1951) reported that a 2% pectin solution could be clarified using this enzyme. Pollard and Kieser (1959) found that an endo-PG produced by an unidentified yeast during fermentation of apple juice was able to clarify the resulting cider by acting in conjunction with pectin methylesterase derived from the fruit.

The objective of this study was to assess the potential industrial use of *K. fragilis* endo-PG produced from whey in the clarification of apple juice by comparing its clarifying ability to that of a commercial pectinase of fungal origin.

Author Gómez-Ruiz is with Departamento de Alimentos, Facultad de Química, Universidad Nacional Autónoma de México, México, D.F., 04510, México. Author García-Garibay's current address: Centro de Investigación sobre Ingeniería Genética y Biotecnología, UNAM A.P. 70-479, México D.F., 04510, México. Author Bárzana (to whom inquiries should be addressed) is on leave at the Dept. of Applied Biological Sciences, Room 56-109, Massachusetts Institute of Technology, Cambridge, MA 02139.

MATERIALS & METHODS

KLUYVEROMYCES FRAGILIS CDBB L-278 was obtained from the Culture Collection of the Department of Biotechnology and Bioengineering, National Polytechnic Institute, Mexico City. The enzyme was produced in whey as described previously (Garcia-Garibay et al., 1987b). At the end of the fermentation the culture broth (0.76 uPG/mL, 0.13 uPG/mg protein, where uPG indicates units of endo-PG activity) was centrifuged to remove cells and endo-PG was recovered in the fraction precipitated between 35% and 65% (w/v)$(NH_4)_2SO_4$, as described by Phaff and Demain (1956), with an activity yield of 60.7%. No pectinolytic activity was detected in the precipitate below 35% or in the supernatant within this range. The precipitate was centrifuged, resuspended in acetate buffer (0.1M, pH 5) and dialyzed against the same buffer for 12 Hr. The final specific activity was 0.5 uPG/mg protein indicating a purification factor of 3.85. The enzymatic solution obtained was subsequently used in the clarification studies.

Pectinolytic activity was determined by the increase in reducing groups released from 0.% (w/v) pectic acid solution as described previously (Garcia-Garibay et al., 1987a). The concentration of reducing groups was established according to the method of Nelson (1944). One unit of endo-PG activity is defined as the amount of enzyme which liberates 1 umol of reducing groups per minute at 30°C, pH 5.

Apple juice was prepared from ripe Golden Delicious apples with a home juice extractor; gross materials were removed by filtration through a cheese cloth. The product obtained was subjected to clarification tests using the enzymatic solution described above. For comparison, juice samples were also treated with Ultrazym 100, a commercial pectinase suitable for apple juice clarification. This enzyme is an *Aspergillus niger* preparation commercialized as Irgazyme 100 by Ciba-Geigy AG, Basel, Switzerland. Both endo-PG and Ultrazym 100 were diluted in acetate buffer (0.1M, pH 5) to obtain a solution with 2.17 uPG/mL. An amount equivalent to 75 ppm of the commercial enzyme (as recommended by the supplier) or its equivalent in activity of endo-PG was added to the apple juice (53.3 uPG/L juice). For each assay a control without enzyme was conducted in parallel. The clarification experiments were conducted at 30°C or 50°C for various intervals. Controls and the enzyme treated samples were incubated at corresponding temperatures and times, filtered through filter paper (Whatman No 1) and the turbidity values measured at 650 nm in a spectrophotometer (Pye Unicam SP 30). Each assay was performed in triplicate and results were compared by analysis of variance (Snedecor and Cochran, 1971). The turbidity at 650 nm of a

commercial clear apple juice purchased from a local supermarket was also determined.

RESULTS & DISCUSSION

TURBIDITY VALUES obtained for each treated sample of apple juice are shown in Table 1. The results for both enzymes were independent of temperature and time of treatment, as revealed by analysis of variance ($P<0.01$). There were no significant differences in the clarification power of endo-PG from *K. fragilis* compared to the commercial enzyme ($P>0.01$), although they were significantly different from controls ($P<0.01$) for all treatment conditions. For instance, 98.6% of the turbidity was removed by endo-PG compared to 99% for the commercial enzyme after only 0.5 hr at 50°C. From the visual appearance of the samples treated for 2 hr at 50°C (Fig. 1) there was no subjective difference in clarity of samples treated with either endo-PG or the commercial enzyme. The industrial process of clarification is normally conducted at 50°C for 3 to 6 hr (Birch et. al., 1981). The results obtained suggest that endo-PG is thermostable enough to act efficiently at this temperature. The turbidity obtained with endo-PG under the conditions tested was not significantly different ($P<0.01$) from the turbidity of the commercial clear apple juice (Table 1).

Table 1—Turbidity values obtained from different treatments of apple juice[a]

Incubation time (hr)	Temp (°C)	Absorbance at 650 nm		
		Endo-PG	Commercial pectinase	Control (no enzyme)
0.5	50	0.026	0.013	1.689
1	50	0.023	0.013	1.580
2	50	0.029	0.009	1.699
2	30	0.035	0.014	1.303
4	30	0.022	0.011	—
Turbidity of commercial apple juice:		0.058		

[a] Data represent the mean value of triplicate experiments. Turbidity measurements of samples treated as indicated were conducted after filtering through filter paper (Whatman No 1).

Endo-PG is specific for oligo- and polygalacturonic acids, with preference for longer polymers. However, it can hydrolyze pectin in a limited way and at a rate inversely proportional to the degree of methylation (Luh and Phaff, 1954). It is very probable that the clarification takes place by the combined activities of endo-PG and the pectinesterases present in the apple, as reported by Pollard and Kieser (1959) and Yamasaki et. al. (1967). The control experiment (Table 1) indicates that in the absence of endo-PG no substantial clarification was obtained at the conditions used suggesting that native pectinesterase (Hulme, 1971) by itself is unable to promote clarification. Normally, the clarification step in industrial operations is conducted before pasteurizing the juice (Nelson and Tressler, 1980), when the pectinesterase is still active.

In conclusion, endo-PG from *K. fragilis* produced economically from cheese whey could be used in the clarification of apple juice. The enzyme was easily recovered from the fermentation culture with high yield. Previous reports on the ability of this enzyme to reduce the viscosity of pectin solutions (Garcia-Garibay et al., 1987a; Luh and Phaff, 1954), its macerating power (Lim et al., 1980; Ozawa et al., 1959; Voragen et al., 1980), and its protopectin-solubilizing activity (Sakai et al., 1984) suggest that this enzyme has potential uses in the industrial preparation of fruit concentrates and purees and in

—*Continued on page 1240*

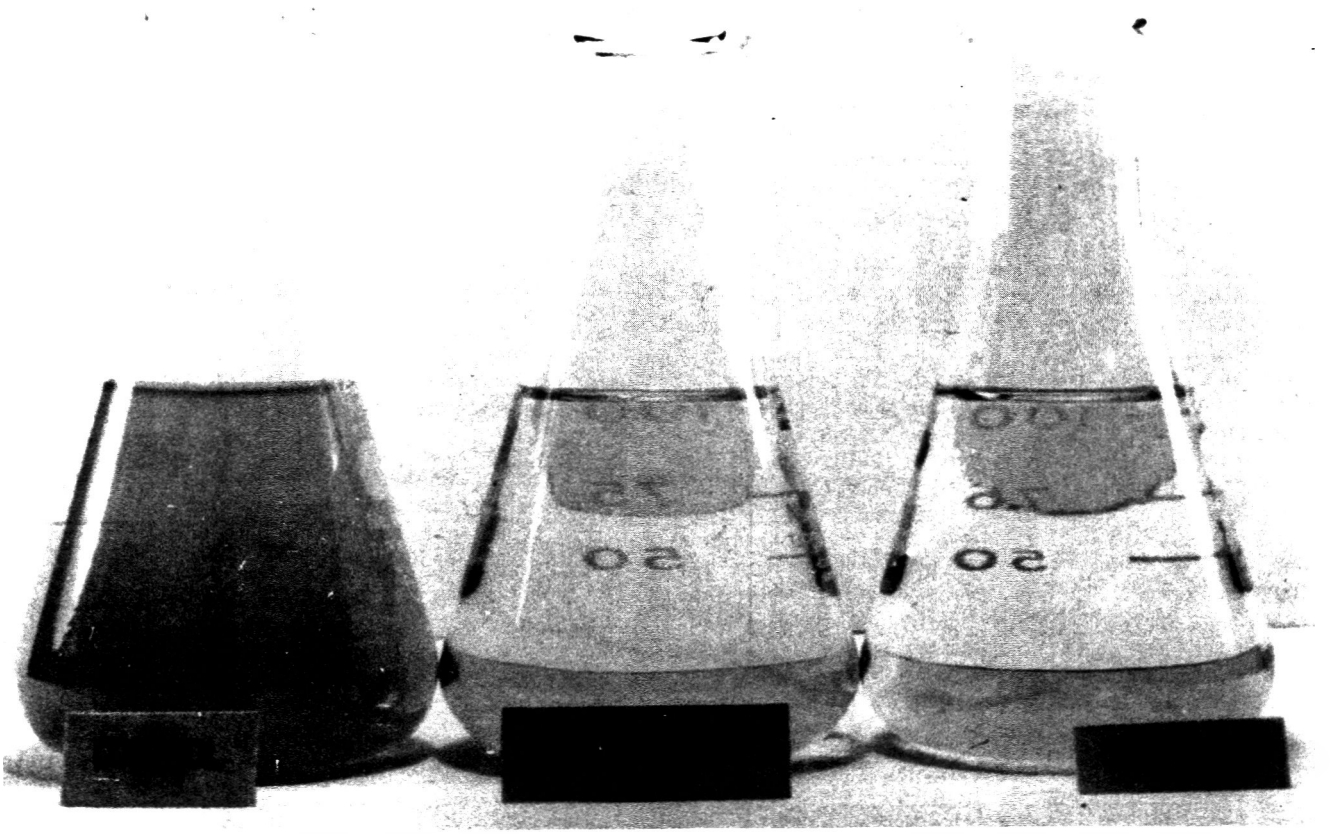

Fig. 1—Visual appearance of filtered apple juice after treatment with endo-PG (K. fragilis), commercial pectinase (commercial), and without enzyme (control) for 2 hr at 50°C.

A Research Note

Incidence of *Listeria monocytogenes* in Market Samples of Fresh and Frozen Vegetables

R. L. PETRAN, *E. A. ZOTTOLA*, and R. B. GRAVANI

ABSTRACT

Samples of the outer portions of fresh beets, broccoli, cabbage, carrots, cauliflower, corn, head lettuce, leaf lettuce, mushrooms, and potatoes, as well as frozen green beans, green peas, pea pods, and spinach were examined for the presence of *Listeria monocytogenes* using a 7-day enrichment procedure at 30°C. Samples were streaked on modified McBrides agar, and isolates were tested for characterization reactions. Fresh carrots, lettuce, and mushrooms, and frozen spinach were also examined using a cold enrichment procedure at 4°C. Samples were taken weekly, streaked onto modified McBrides agar, and were characterized. It was determined that no *L. monocytogenes* was detectable in any of these samples.

INTRODUCTION

LISTERIA MONOCYTOGENES is prevalent in nature and has been isolated from soil, animal feed, sewage, and animals (Armstrong, 1985). Additional evidence suggests that *L. monocytogenes* may be a saprophyte. The organism is able to multiply at low temperatures, will survive for an extended time in soil, and has been recovered from dead vegetation (Welshimer and Donker-Voet, 1971). *Listeria* has been isolated from the feces of animals (Weis and Seeliger, 1975) which may serve as a source of contamination of agricultural crops (Schlech et al., 1983).

Welshimer (1968) reported that multiplication of *L. monocytogenes* may be favored by decay of organic plant material and *Listeria* may be isolated more frequently from portions of the plant near the ground. *L. monocytogenes* has been isolated from samples of ground corn and soybeans (Welshimer, 1968), from old, faded, or moldy plants (Weis and Seeliger, 1975) and from still green or recently dead vegetation (Welshimer and Donker-Voet, 1971).

Recovery of *L. monocytogenes* from vegetation or soil samples is enhanced by holding the sample at 4°C (Welshimer and Donker-Voet, 1971) for a period of at least 20 days (Welshimer, 1968). *L. monocytogenes* was isolated from refrigerated coleslaw in a listeriosis outbreak in Canada. The same serotype (4b) was isolated from a patient's blood. It is likely that this contamination came from cabbage from a farm with ovine listeriosis (Schlech et al., 1983). *Listeria* will grow in 1.5% NaCl and low pH. Consequently, proliferation can occur on vegetables and in brines used in fermentation procedures (Conner et al., 1986).

Foodborne outbreaks of listeriosis have been attributed to milk (Fleming et al., 1985), cheese (James et al., 1985), and coleslaw (Schlech et al., 1983). Consumption of raw vegetables, especially raw celery, tomatoes and lettuce, was implicated in an outbreak of listeriosis in Boston in 1979 (Ho et al., 1986). The purpose of this study was to survey fresh and frozen vegetables for the presence of *L. monocytogenes*.

Authors Petran and Zottola are with the Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108. Author Gravani is with the Dept. of Food Science, Cornell Univ., Stocking Hall, Ithaca, NY 14853.

MATERIALS & METHODS

Sample preparation

Fresh lettuce, potato peels, corn husks, broccoli stems, cabbage outer leaves, carrot peels, cauliflower stems, mushroom stems, spinach, and beet peels, and frozen green beans, pea pods, green peas, and spinach were obtained from a local supermarket, stored at 4°C or −20°C until analysis, and examined by the method of Lovett et al. (1987). Twenty-five grams of each vegetable were homogenized in a stomacher for 1 min in the enrichment broth (Lovett et al., 1987) and incubated as recommended by this procedure.

Microbiological analysis was started within 48 hr of purchase. The positive control was 1 mL of a 24-hr culture of *L. monocytogenes* added to the enrichment broth, and the negative control was an uninoculated broth. All control cultures were obtained from the FDA laboratory in Minneapolis, Minn. One sample each of the fresh vegetables was examined in the fall of 1986 and the spring of 1987. Frozen vegetables were analyzed in the spring of 1987.

Isolation procedure

After 24 hr and 7 days incubation, the enrichment broths were streaked onto modified McBride agar (MMA) (Lovett et al., 1987). Following 48 hr incubation at 35°C, five blue-gray colonies were picked from each MMA plate for characterization. The most common isolate reactions are reported in this paper.

Cold enrichment

Carrot peels, lettuce leaves, mushroom stems, and frozen spinach were examined in a cold enrichment procedure (Doyle et al., 1985). Twenty-five grams of vegetable were placed in 225 mL tryptose broth (20 grams tryptose [Difco], 5g NaCl, and 1g dextrose in 1 L distilled water), homogenized in a stomacher for 1 min, and incubated at 4°C. The same positive and negative controls were used. The broths were sampled weekly by making spread plates on MMA using samples both undiluted and diluted 1:10 in 0.5% KOH. Plates were incubated at 35°C for 48 hr. The blue-gray colonies were examined as described previously.

RESULTS & DISCUSSION

Fresh vegetable isolates

Fall. Colonies isolated from all the vegetable samples except cauliflower, mushroom, and beet appeared blue-gray on both MMA and trypticase soy agar with yeast extract (TSA-YE). All microbes were isolated on TSA-YE, and all were catalase-positive except for the organism from the potato. The organisms isolated from the beet were gram variable cocci. No further tests were performed on these samples. Biochemical tests were done on the remaining isolates, and a summary of their reactions is found in Table 1.

Motility was examined in wet mounts and in SIM media (Difco). All isolates showed the presence of motile rods except the isolate from the mushroom, which did not produce the characteristic "inverted pine tree" growth in SIM. All of the isolates were Vogues Proskauer (VP) negative and the microorganisms from the cabbage and cauliflower were negative in the Methyl Red (MR) test. Only the isolates from the lettuce and carrot produced the characteristic Triple Sugar Iron (TSI) reaction.

Table 1—Cellular morphology and biochemical reactions of microorganisms isolated from fresh vegetables, fall, 1986

Sample	Colony appearance of isolates on MMA	Catalase reaction	Wet mount	Gram reaction	SIM	MR	VP	TSI	Dextrose	Esculin	Maltose	Rhamnose	Mannitol	Xylose
Positive control	Blue-gray	+	Short rod, tumbling	+	+	+	+	Acid slant and butt, no H_2S	+	+	+	+	−	−
Beet	Blue-gray	+	Cocci	Variable										
Broccoli	Blue-gray	+	Short rods, tumbling	+	+	+	−	Acid butt only	+	−	+	−	+	−
Cabbage	Blue-gray	+	Coccoic rods, tumbling	+	+	−	−	Acid butt only	+	−	+	−	+	−
Carrot	Barely blue	+	Small rods, tumbling	+	+	+	−	Acid slant and butt	+	−	+	−	+	+
Cauli-flower	Creamy	+	Small rods, tumbling	+	+	−	−	Acid butt only	+	−	+	−	+	−
Corn	Blue-gray	+	Long rods, tumbling	+	+	ND*	ND	Acid butt only	ND	ND	ND	ND	ND	ND
Leaf lettuce	Blue-gray	+	Small rods, no tumbling	+	+	+	−	Acid slant and butt	+	+	+	−	+	−
Mush-room	Creamy	+	Small rods, tumbling	+	−	+	−	Acid butt only	+	−	+	−	+	+
Potato	Creamy	−												

* ND = Not done.

Table 2—Cellular morphology and biochemical reactions of microorganisms isolated from fresh vegetables, spring, 1987.

Sample	Colony appearance on MMA	Catalase reaction	Wet mount	Gram reaction	Urea hydrolysis	NO_3 reaction	SIM	MR	VP	TSI	Dextrose	Esculin	Maltose	Rhamnose	Mannitol	Xylose	Hemolysis zone
Positive control	Blue-gray	+	Short rod, tumbling	+	−	−	+	+	+	Acid slant and butt no H_2S	+	+	+	+	−	−	1 mm
Beet	Creamy color	+	Cocci in chains	+													
Broccoli	Irri-descent	−															
Cabbage	Yellow/ brown	+	Cocci in chains	+													
Carrot	Irri-descent	−															
Cauli-flower	Irri-descent blue	+	Small coccoic rods	+	−	−	+	+	+	Acid slant and butt	+	−	+	+	+	−	1 mm
Head lettuce	Irri-descent	+	Cocci	+													
Leaf lettuce	Creamy color	+	Long and short rods	+	+	−	−	+	+	Acid slant and butt	+	−	+	+	+	−	17 mm
Mush-room	Fluor-escent yellow green	+	Cocci and rods	−													
Potato	Irri-descent creamy	−															
Spinach	Creamy color	+	Cocci	+													

In the carbohydrate tests typical acid without gas reactions were observed for all isolates in dextrose and maltose. Only the isolate from the lettuce produced acid without gas in esculin broth. In the rhamnose broth, none of the isolates resulted in the characteristic acid without gas reaction. In atypical reactions, all isolates produced acid from mannitol; the isolates

from the lettuce, carrot, and mushroom produced acid from xylose.

Spring. The reactions of isolates from the fresh vegetables examined in the spring are listed in Table 2. These reactions were noncharacteristic for *L. monocytogenes*. All microbes were initially isolated on TSA-YE plates and all appeared blue-gray except for the cabbage and mushroom isolates. All were catalase-positive except for those isolates from broccoli, carrot, and potato. Isolates from the beet, spinach, cabbage, head lettuce, and mushroom were all cocci. Further biochemical tests were performed only on the isolates from the leaf lettuce and cauliflower.

The isolate from the leaf lettuce gave non-listeria reactions in the urea, SIM, esculin, mannitol, and xylose reactions. The hemolysis zone on blood agar was too large; the usual hemolysis zone for *L. monocytogenes* is approximately 1 mm. The isolate from the cauliflower showed characteristic biochemical reactions in all tests except for the esculin and mannitol fermentations. However, serological testing performed on the isolate using commercially available antisera showed no agglutination.

Frozen vegetable isolates

The isolates from the pea pod and green pea were both catalase-negative, and those from the green beans and spinach were gram-positive cocci. No further biochemical or serological tests were performed on any of these isolates. None appeared blue-gray on MMA or TSA-YE.

Cold enriched samples

After 8 wk of sampling from the samples incubated at 4°C, no *L. monocytogenes* was isolated from any of the vegetables. All microorganisms that were isolated were catalase-negative, gram-positive cocci or rods, and were not motile.

CONCLUSIONS

NONE of these vegetable samples were determined to harbor detectable levels of *L. monocytogenes*. No further identification of any of these non-*Listeria* organisms was performed. The results obtained in this study show that there are a variety of organisms present on fresh and frozen vegetables. However, based on these limited tests, it appears that the microflora of both types of vegetables is diverse and would require a much more exhaustive study to completely identify all the microbes present.

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the extraction of essential oils. Further research is needed to evaluate those alternatives in detail.

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A Research Note

Anthocyanins in Fruits of *Aronia Melanocarpa* (Chokeberry)

JAN OSZMIANSKI and JEAN C. SAPIS

ABSTRACT

Anthocyanins from fruits of *Aronia melanocarpa* (chokeberry) were extracted with acidified ethanol and methanol and fractionated by high performance liquid chromatography. The pigment composition was very simple as there were only four compounds. Semi-preparative HPLC, thin-layer chromatography and spectral techniques indicated cyanidin as a single aglycone and glucose, galactose, arabinose, and xylose as associated sugars. The relative proportions of anthocyanins were determined. The major components were cyanidin 3-galactoside and cyanidin 3-arabinoside.

INTRODUCTION

ARONIA MELANOCARPA (chokeberry) is a widely distributed plant in Eastern Europe, the Soviet Union, and North America. Its berries are dark red, almost black since skins and pulp are very rich in phenolics and particularly anthocyanins. The berries are useful as raw material for natural colorings and for pharmaceutical preparations or for production of red fruit wines (Wilska-Jeszka et al., 1986; Rosa and Krugty, 1987; Plocharski and Smolarz, 1987). Crop yield is high, about six tons per ha. No work dealing with qualitative and quantitative composition of *A. melanocarpa* anthocyanins has previously been reported. The purpose of this study was to investigate the composition of the anthocyanins.

MATERIALS & METHODS

Extraction from fruit

A sample of 5g of *A. melanocarpa* fruits picked from the University of Wroclaw (Poland) botanical garden was crushed under liquid nitrogen in a ball grinder to obtain very fine powder. Anthocyanins were extracted twice with 80% ethanol (50 mL) containing 0.1% HCl. Extraction was carried out a third time with 80% methanol and HCl as before. After filtration (Millipore, 0.45 μm), the extract was concentrated under vacuum at 35°C and extracted again with petroleum ether (25 mL) to remove inconvenient nonpolar compounds (Romeyer, 1984), and then with ethyl acetate (3 × 25 mL) for extraction of flavonoids (Kärppä et al., 1984). Solvents were evaporated with a Rotavapor. Anthocyanins present in the aqueous phase were diluted with distilled water adjusted at pH 2 by addition of HCOOH and filtered. A 20 μL aliquot of this solution was injected into the liquid chromatograph.

Analytical HPLC of anthocyanins

A Varian 5500 liquid chromatograph fitted with a Varian UV-200 detector set at 546 nm and connected to a Varian Vista 401 computer was used. Fractionation was carried out using an analytical RP-18 (5 μm particles) 250 × 4 mm ID column, protected with a guard cartridge of the same packing (Brownlee Labs. Inc., Santa Clara, CA). Elution was carried out using a mixture of two solvents (Albersheim et al., 1967; Bishop and Nagel, 1984). Solvent A consisted of 10% HCOOH in water; solvent B was 10% HCOOH, 60% H_2O, 30% CH_3CN. The increasing gradient was run as follows: 0–10 min, 20–40% B in A; 10–25 min, 40–50% B in A; 25–26 min, 50–100% B in A; 26–30 min, 100% B. The flow rate was 1 mL/min.

Author Oszmianski is affiliated with the Dept. of Fruit & Vegetable Processing, Academy of Agriculture, 50375 Wroclaw, Poland. Author Sapis is with Institut des Produits de la Vigne, INRA, 34060 Montpellier Cedex, France.

Semi-preparative fractionation of anthocyanins

Semi-preparative fractionation was carried out using HPLC with a larger RP-18 column (250 × 7 mm ID) and a flow rate of 2 mL/min. Each peak was collected at column outlet; acetonitrile was evaporated under vacuum and the extract, dissolved in 1 mL distilled water, was passed through Sep-Pak C18 (Waters Associates, Bedford, MA). Anthocyanins were eluted with 0.1% HCOOH in methanol. After evaporation of this solvent, hydrolysis was carried out with 2N trifluoroacetic acid for 60 min at 100°C. The solution was then passed through Sep-Pak. Sugars, not retained by C18 column, were completely removed by rinsing with 0.1N trifluoroacetic acid. Anthocyanidins were then eluted with 0.1% HCOOH in methanol.

Thin-layer chromatography of sugars

Sugars were chromatographed on silica gel plates (Andary, 1975). The mobile phase was ethyl acetate:isopropanol:water (65:30:10). Elution time was 3 hr. Chromatograms were sprayed with 2% naphthoresorcinol in absolute ethanol with 5% concentrated sulfuric acid followed by heating to 100°C for 5 min.

Identification of anthocyanidins and sugars

Anthocyanidins and sugars were identified by reference to authentic samples (Fluka) of anthocyanidins (cyanidin) and sugars (glucose, galactose, arabinose, xylose).

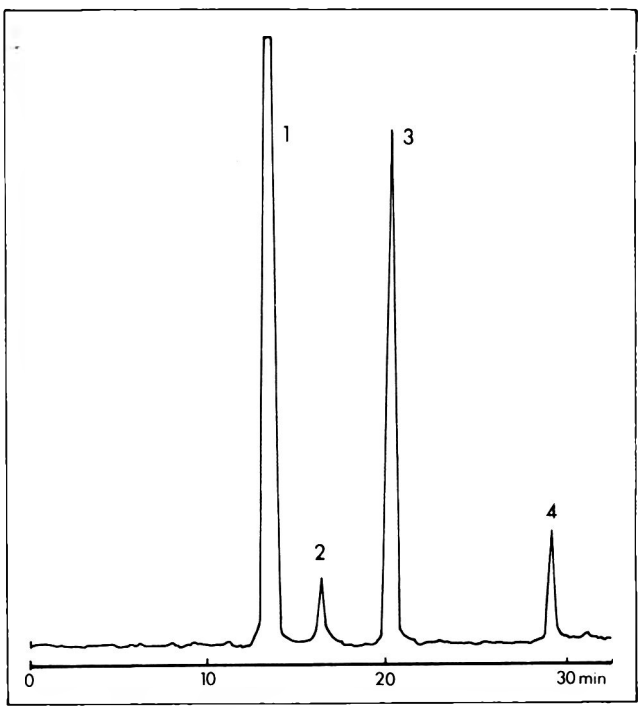

Fig. 1 – HPLC separation of A. melanocarpa *anthocyanins. (1) cyanidin 3-0-galactoside; (2) cyanidin 3-0-glucoside; (3) cyanidin 3-0-arabinoside; (4) cyanidin 3-0-xyloside.*

Table 1—Anthocyanin composition of fruits of Aronia melanocarpa

Peak	Anthocyanin	Rt[a]	K[b]	α[c]	Area %
1	cyanidin 3-O galactoside	16.06	5.80	—	64.5 ± 0.1
2	cyanidin 3-O glucoside	17.63	6.53	1.11	2.4 ± 0.1
3	cyanidin 3-O arabinoside	19.97	7.53	1.15	28.9 ± 0.1
4	cyanidin 3-O xyloside	27.09	10.58	1.40	4.2 ± 0.2

[a] Rt: retention time (min).
[b] K: capacity factor (t_r − to)/to (to = 2.34 min).
[c] α: separation factor K_2/K_1, i.e. the capacity factor of the peak divided by the capacity factor of the preceding peak.

Quantitation of anthocyanins

Quantitative evaluation of each *Aronia melanocarpa* anthocyanin was also carried out. Standard deviations were calculated by successive determination on four batches of 5g of fruits, picked at random at the same moment in the same area.

RESULTS & DISCUSSION

CHROMATOGRAPHIC PATTERNS of *A. melanocarpa* extracts displayed profiles with four peaks as shown in Fig. 1. Absorption spectra of solutions from each peak collected showed that the maxima were identical (517 nm), indicating the presence of the same anthocyanidin. This was confirmed by cellulose thin-layer chromatography and by HPLC which revealed the presence of cyanidin alone.

Sugars associated with anthocyanidins were identified using thin-layer chromatography as previously described: galactose, glucose, arabinose, xylose. In addition, the absorbance ratio between 440 nm and λ max (the value of which was 25) confirmed that glycosides were located in position 3 (Zapsalis and Francis, 1965; Francis, 1982). The anthocyanin composition is given in Table 1. Qualitative, anthocyanin was composed of four compounds. Quantitatively, the most plentiful were cyanidin 3-0 galactoside and cyanidin 3–0 arabinoside.

Recent identification, using HPLC techniques, of some similar anthocyanins was made in many fruits such as grapes (Wulf and Nagel, 1978; Hrazdina and Moskowitz, 1980; Mc Closkey and Yengoyan, 1980; Estrella et al., 1984; Fuleki and Somerville, 1984; Roggero et al., 1984a and b, 1986; Bakker and Timberlake, 1985, 1986; Hrazdina, 1986; Kampis et al., 1986); fruits of *Vaccinium* species (Ballinger et al., 1979, 1982; Baj et al., 1983; Andersen, 1985, 1987; Fuleki, 1986); *Sambucus nigra* (Bronnum-Hansen and Hansen, 1983); *Empetrum nigrum* (Kärppä et al., 1984); *Amelanchier alnifolia* (Mazza, 1986). From a qualitative point of view, the anthocyanin composition of fruits of *A. melanocarpa* is, however, one of the most simple as they contain only four compounds with the same anthocyanidin in each case and no diglucosides. The occurence of cyanidin 3-0 xyloside must also be noted as it seldom occurs in anthocyanin composition of fruits. Quantitatively only two compounds (cyanidin 3-0 galactoside and cyanidin 3-0 arabinoside) account for 93% of the total anthocyanins.

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A Research Note

Quantitative Analysis of γ-Nonalactone in Wines and Its Threshold Determination

S. NAKAMURA, E.A. CROWELL, C.S. OUGH, and A. TOTSUKA

ABSTRACT

Thirty-eight California and French wines were analyzed for γ-nonalactone by Freon extraction and gas chromatography. The California white wines had from 0 to 16 μg/L (average for 6 samples was 7 μg/L and the red wines from 12 to 43 μg/L (average for 20 samples was 24 μg/L). French red wines obtained similar amounts, 14 to 41 μg/L (average for 12 samples was 21 μg/L). The sensory threshold for γ-nonalaconte was determined to be 30 μg/L.

INTRODUCTION

AMONG THE MANY volatile constituents of wine, the lactones, some of which might be responsible for the pleasant aroma of wine, have been reviewed by Muller et al. (1973). However, most of the studies have been aimed at the isolation and identification of the constituents (Kepner et al., 1972; Muller et al., 1969, 1972; Augustyn et al., 1971) and not their quantification because of the difficulties associated with their isolation and low concentration in wine.

Several studies (Otsuka et al., 1974; Onishi et al., 1977) have looked at β-methyl-γ-octalactones from wood sources found in brandies and liquors. Simpson and Miller (1984) quantitated them in Chardonnay wine that had been barrel-aged. They were below sensory threshold levels.

γ-Nonalactone (γ-NL) which has an odor reminiscent of coconut, is one of the flavor constituents in apricots, peaches, pineapples, and coconuts (Allen, 1965; Tang and Jennings, 1968). Recently, it also has been found in distilled beverages and wines (Kahn et al., 1969; Brander et al., 1980; Nakamura et al., 1985). Although the amount of γ-NL found in wines is a negligible quantity, its odor threshold is supposed to be extremely low and, hence, very small amounts might be sufficient to affect the total flavor.

The purpose of this paper is to report results of the quantitative analysis of γ-NL in California varietal wins and bordeaux red wines and to determine its threshold sensory level.

MATERIALS & METHODS

Wines

Twenty-six commercial California wines and 12 Bordeaux wines were analyzed in this study. Details of the wines are provided in Table 1.

Preparation of wine extract

Seven hundred milliliters of the wine were extracted with 100 mL of Freon-11 in continuous extraction equipment (Rapp and Knipser, 1980). After 40 hr extraction, the Freon-11 was evaporated in a water bath at 30°C to leave a concentrated extract (3 to 4 mL). Further concentration to final volume of 1 mL was accomplished under a gentle stream of nitrogen.

Table 1 – Wines analyzed and the amounts of γ-nonalactone

Variety	Vintage	Winery	Origin	γ-NL (μg/L)
		California		
Chardonnay	1984	Cha. Montelena	Sonoma	3
Sauvignon blanc	1985	Iron Horse	Sonoma	3
Chenin blanc	1985	Husch	Mendocino	–
J. Riesling	1985	Clos du Bois	Sonoma	7
Moscato d'Oro	1983	Robert Mondavi	Napa	16
Gewürztraminer	1985	Joseph Phelps	Napa	12
Zinfandel	1983	Burgess	Napa	31
Zinfandel	1985	Gundlach Bundschu	Sonoma	25
Zinfandel	1985	Shenandoah	Amador	43
Pinot Noir	1983	Louis M. Martini	Napa	19
Pinot Noir	1981	Spring Mountain	Napa	27
Pinot Noir	1983	Buena Vista	Carneros	16
Merlot	1984	Markham	Napa	14
Merlot	1984	Boeger	El Dorado	24
Merlot	1983	Franciscan	Napa	16
Cabernet Sauvignon	1983	St. Clement	Napa	30
Cabernet Sauvignon	1984	Ridge	Santa Cruz	27
Cabernet Sauvignon	1983	Clos du Val	Napa	12
Cabernet Sauvignon	1982	Dehlinger	Sonoma	27
Cabernet Sauvignon	1983	Stag's Leap	Napa	20
Cabernet Sauvignon	1983	Alexander Valley	Sonoma	20
Cabernet Sauvignon	1978	Spring Mountain	Napa	31
Cabernet Sauvignon	1983	Carmenet	Sonoma	28
Cabernet Sauvignon	1984	Buena Vista	Carneros	16
Cabernet Sauvignon	1982	Heitz Cellar	Napa	28
Cabernet Sauvignon	1983	Beaulieu	Napa	26
		Bordeaux		
Chateau du Tertre		1983	Margaux	14
Chateau Rausan Segla		1981	Margaux	16
Chateau Beychevelle		1979	St. Julien	20
Cha. Mouton Baronne Philippe		1983	Pauillac	17
Chateau Lafon Rochet		1976	St-Estéphe	20
Chateau Haut Bailly		1983	Grave	22
Chateau Pavie		1983	St-Emilion	22
Chateau L'Eglise Clinet		1983	Pomerol	22
Chateau Lanessan		1983	Haut-Médoc	31
Chateau Le Boscq		1983	Médoc	41
Chateau Potensac		1982	Médoc	14
Chateau Plagnac		1984	Médoc	16

Analytical gas chromatography

A Hewlett Packard 5721A gas chromatograph equipped with a flame ionization detector was used. A fused silica capillary column, Supelcowax 10, (0.25 mm ID × 60 m, essentially a stabilized carbowax 20 M) supplied by Supelco, Inc., was employed. Nitrogen flow to column was 1 mL/min (40:1 split) and makeup N_2 30 mL/min was used as carrier gas. The injector and detector temperatures were maintained at 250°C. The programmed oven temperature was raised from 105°C to 135°C at the rate of 0.1°C/min.

Two μL of the concentrate were injected. Typically, isobutyl benzoate, used as internal standard, eluted at 59 min and γ-NL at 212 min. A standard curve was prepared by plotting area ratios of γ-NL/isobutyl benzoate. The concentration of the internal standard was 0.3 mg/mL. Samples containing increasing amounts of γ-NL were prepared in the white wine. Each standard was extracted and concentrated as described before.

Author Crowell and Ough are with the dept. of Viticulture & Enology, Univ. of California, Davis, CA 95616. Author Nakamura's present address: Sanraku Incorp. 15-1,Kyobashi 1-Chome, Chuo Ku, Tokyo 104. Author Totsuka's present address: National Institute Brewing of Japan, 2-6-30 Takinogawa Kitu ku, Tokyo 114.

Odor threshold test in a wine extract concentration solution

A Freon-11 concentrate of Chenin blanc (No. 3 in Table 1), in which no γ-NL was found by GC, was diluted with 10% ethanol solution to its original wine volume (700 mL), and this solution served as a basic wine control for the odor threshold tests. Six concentrations of γ-NL in the wine solution were evaluated. Each concentration was served as 3-glass set known to contain the same wine solution, and the different concentrations were lined up in the order of increasing strength. Ten judges were asked to select the odd sample in each set. Correct responses were plotted against concentration, and the concentration at which 50% of the judges answered correctly was designated the odor threshold.

RESULTS & DISCUSSION

TABLE 1 presents the analyses of California and Bordeaux wines and their concentrations of γ-NL. γ-NL was found in all wines with one exception. The ranges for six white varieties were from 0 to 16 μg/L and red varities contained from 12 to 43 μg/L.

The first eight Bordeaux wines were ranked as Grands Crus Classe and the last four wines were so-called Crus Bourgeois in Médoc. The concentration of lactone for all eight Crus Classe wines ranged from 14 to 22 μg/L. These levels should have little influence on the total flavor. Only two wines in Crus Bourgeois contained exceptionally high amounts of γ-NL. Generally, it is considered that the quality of Crus Classes is superior to that of Crus Bourgeois. Therefore, if there is any relationship between γ-NL and the quality of Bordeaux wines, the analytical result seems to indicate that γ-NL acts as a negative factor to the quality; although the data are not sufficient for a more positive statement.

In comparing Crus Classe wines in Médoc with California Cabernet Sauvignon, the latter, as a whole, had a tendency to contain higher levels of γ-NL Exceptions of this were Clos du Val and Buena Vista.

Figure 1 illustrates the results for threshold determination of γ-NL. This procedure gives a practical threshold which can be considered to effect the actual influence to the total flavor of the wine.

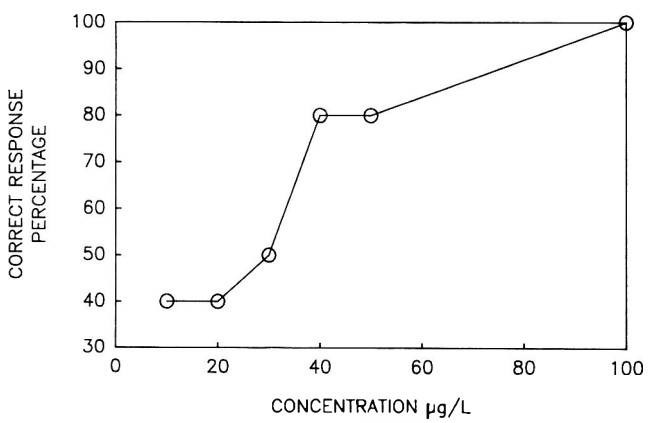

Fig. 1—Relationship between % correct responses and concentration of pure compound (γ-NL) in the wine concentrate solution.

Judging from the threshold obtained above (30 μg/L), the analytical values of the white varieties were so far below this threshold that the concentration of γ-NL found in white wines not be expected to affect their total flavor. On the other hand, some of the red wines produced from Zinfandel, Pinot noir, Merlot and Cabernet Sauvignon contained γ-NL close to or exceeding the threshold. Among red varieties, the average values for the Zinfandel and Cabernet Sauvignon were higher than those for the Pinot noir and Merlot. However, more data are necessary to confirm the relationship between γ-NL contents and variety.

It has been said that California cabernets are fruitier and more palatable in their youth than Bordeaux red wines. In the course of the threshold test, several judges pointed out that the first sensation of the wines in which γ-NL had been added had somewhat sweeter, more pleasant and fruitier aroma than that of original basic wine solution. They also pointed out that the subtle and delicate aroma of the original basic wine solution seemed to be covered by the addition of γ-NL. However, comparing statistically the mean values of the measured red wines of California to those of France, there was no statistical difference (student t = 0.995, 30 D.F.). Comparing only the 14 cabernet type California wines to the 12 from France, the mean values were even closer and not significantly different.

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A Research Note

Influence of pH on Egg Yolk Lipid Oxidation

O. A. PIKE and I. C. PENG

ABSTRACT

The influence of pH on lipid oxidation of liquid egg yolk was studied. Liquid yolk samples were prepared to contain 60% yolk, 40% distilled water, 0.012% sodium azide, and pH was adjusted from the normal pH of 6.0 to 2.0, 3.0, 4.0, and 5.0 with 1N HCl. Samples were agitated at room temperature (ca 23°C) for 20 days and lipid oxidation measured using the distillation thiobarbituric acid test. No increase in thiobarbituric acid reactive substances occurred in the control (pH 6) or pH 5 yolk samples. The degree of oxidation significantly increased ($P<0.01$) in the more acidic samples in the following order: pH 3 >> pH 2 > pH 4. Addition of 1.2% EDTA significantly lowered ($P<0.01$) the degree of oxidation but did not inhibit oxidation completely.

INTRODUCTION

THE RATE of lipid oxidation in food and model systems is markedly affected by pH (Richardson and Korycka-Dahl, 1983). Using a model system of linoleic acid emulsion at 50°C, Mabrouk and Dugan (1960) found that as pH increased from 4.5 to 5.5 the rate of oxygen uptake increased, then gradually decreased as pH increased to 8.0. Wills (1965) found that pH greatly affected metal-catalyzed oxidation of linoleic acid emulsions. Oxidation was inhibited at pH 4 but then increased rapidly and peaked at pH 5.5 for Fe^{3+}, and at pH 6.5 for Co^{2+}, then declined as pH increased to 9.0. Several studies investigating lipid oxidation in meat have measured the influence of pH. In ground pork, pH values below approximately 6.1 resulted in rapid increases in lipid oxidation, and it was postulated that histidine ionization ($pK=6.0$) may reduce the chelation of metal catalysts by protein (Yasosky et al., 1984). Work with ground raw poultry meat indicated that the lower the pH value is, as low as pH 3, the higher is the rate of oxidation (Chen and Waimaleongora-Ek, 1981). Fischer and Deng (1977) reported increases in oxidation with increasing acidity, to pH 4, of a fish homogenate in a linoleic acid emulsion. Thus, the pH optimum seen in linoleic acid emulsion model systems was not observed in meat systems.

The influence of pH on lipid oxidation in egg yolk has not been investigated. An understanding of the effect of pH on the oxidative stability of yolk may have implications for the stability of yolk-containing foods. The purpose of this study was to investigate the influence of pH on lipid oxidation of liquid egg yolk.

MATERIALS & METHODS

EGGS were collected from a flock of White Leghorns and used within 24 hr. Yolks of approximately 6 dozen eggs were separated and adhering albumen removed by rolling on a moist paper towel; the yolk sac was broken and the contents pooled. Erlenmeyer flasks (250 mL) containing 60 grams of yolk were diluted with 10 mL water to decrease yolk viscosity and provide rapid dispersion of acidulant. Sodium azide (0.012%, w/w) was added to prevent microbial growth. The pH was adjusted using 1N HCl and water added to adjust total liquid added to 40 mL. Duplicate flasks were prepared at pH 1, 2, 3, 4, and 5, and a control at pH 6. To investigate the influence of a metal chelator, additional flasks were prepared at pH 2 and 4 containing EDTA (1.2%, w/w). Flasks were covered with paraffin to retain moisture yet not exclude oxygen. To insure adequate and even exposure of flask contents to air, all flasks were agitated simultaneously using a wrist action shaker, with approximately 200 strokes per minute, at room temperature (ca 23°C).

At stated intervals, 10-g samples were removed from each flask and measured for thiobarbituric acid reactive substances (TBARS) using the distillation method of Tarladgis et al. (1960). To prevent oxidation during the assay procedure, butylated hydroxytoluene (0.3%, w/w) was added to each sample before distillation (Pikul et al., 1983). Results are reported as absorbance at 532 nm. Data were analyzed using analysis of variance and significant differences among treatments were established using the Newman-Keuls test (SAS, 1985).

RESULTS & DISCUSSION

YOLK ADJUSTED to pH 1 underwent gelation during agitation, inhibiting uniform exposure to air and, therefore, results are not included. The control (native yolk, pH 6) and yolk adjusted to pH 5 did not result in the formation of TBARS (Fig. 1). However, the level of oxidation in the more acidic samples was significantly affected: pH 3 >> pH 2 > pH 4 ($P<0.01$). At pH 3, values exceeded 2.0 absorbance units. To determine the possible involvement of metal ions, the effect of 1.2% EDTA on pH 4 and pH 2 adjusted yolk was simultaneously measured. The presence of EDTA resulted in significantly lower TBARS formation ($P<0.01$), although oxidation was not entirely eliminated. Thus, it appears that acidified yolk undergoes oxidation that can be partially inhibited by the inclusion of a metal chelator.

The ability of the yolk protein phosvitin to chelate metal ions suggested that the results of this study might be caused by denaturation of phosvitin by acid. However, the experiment was repeated using yolk plasma which was void of phosvitin; the effect of EDTA at pH 3 was also studied. Yolk plasma was prepared by centrifuging diluted yolk at 78,000 × g for 1 hr at 4°C. A pattern (data not shown) similar to Fig. 1 was seen with pH-adjusted yolk plasma. Again, a very rapid rate of oxidation was observed at pH 3 and the addition of EDTA partially inhibited the oxidation at pH 2, 3, and 4, indicating a metal-catalyzed reaction.

The results of this study agree with those of Chen and Waimalcongora-Ek (1981) and Fischer and Deng (1977), indicating that an increase in acidity, as low as pH 3, causes an increase in lipid oxidation. Because of the complexity of egg yolk, it is difficult to pinpoint a single cause for the rapid rate of oxidation seen in yolk at pH 3. It is possible that denaturation of the protein surrounding low density lipoprotein (LDL) occurred which may result in disruption of its structure, and subsequent exposure of lipids to pro-oxidants in the surrounding environment (Pike and Peng, 1988).

Since EDTA did not completely inhibit the formation of TBARS in yolk, other factors besides metal ions (e.g., light) may be partially responsible for causing the oxidation.

Finally, results of this experiment suggested that consideration should be given to the acidity of foods containing yolk.

Author Peng is with the Dept. of Food Science, Purdue Univ., West Lafayette, IN 47907. Author Pike's current address is: Dept. of Food Science & Nutrition, Brigham Young Univ., Provo, UT 84602.

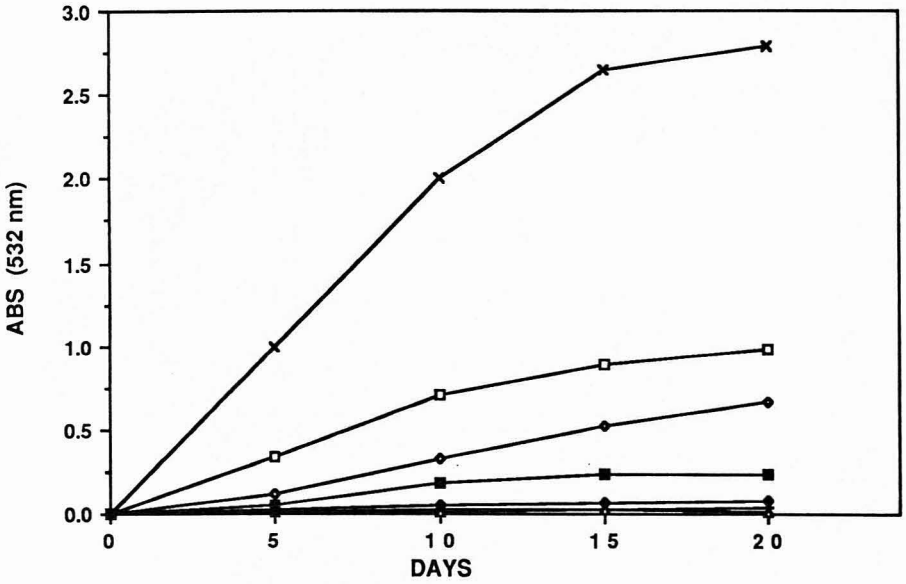

Fig. 1—Effect of pH and 1.2% EDTA on the formation of TBARS in yolk. Yolk concentration: 60% (w/w). Control, pH 6 (△), pH 5 (+), pH 4 (◇), pH 3 (×), pH 2 (□), pH 4/EDTA (◆), pH 2/EDTA (■).

In an acidic environment, it appeared that yolk was less stable to lipid oxidation than at its normal pH of 6.

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INSTITUTE OF FOOD TECHNOLOGISTS
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