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AUTHOR INDEX
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Letters

Storage Stability of Low-fat Ground Beef

By now this is ancient history, but this is a note regarding the paper in the *Journal of Food Science*, 59(1): 6–9 (1994), Storage Stability of Low-fat Ground Beef Made with Lower Value Cuts of Beef, by Bullock, Huffman, Egbert, Mikel, Bradford, and Jones.

On page 8, in the right hand column, the authors refer to work of Kraft and Ayres [*Food Technology* 6(1): 8, 1952] for frozen steaks. We did not work with frozen steaks, but with meat refrigerated at about 4.4°C, never frozen. Since their work dealt with frozen steaks, the two projects are not directly comparable with regard to storage temperature, although the results were similar.

I doubt if too many people would be concerned about these details, but they should be clarified here. I'm glad that the results were similar for both types of storage temperatures, since undoubtedly similar phenomena occurred (oxidation and dehydration).

Regardless of the discrepancy mentioned in the literature review, the paper is appreciated. I wish the authors continued success in their research.

—Allen A. Kraft, Professor Emeritus, Food Science & Nutrition, 3624 Ross Road, Ames, IA 50014

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Oxidative Changes of Heat-Sterilized Meat in Trays

BRUNO GÜNTENSPERGER and FELIX E. ESCHER

ABSTRACT

We studied oxidative changes of ground pork meat filled into plastic or aluminum trays, sterilized at 121 or 131°C and stored up to 56 days at 20 or 37°C in the dark or exposed to light, respectively. Sterilization temperature did not influence either thiobarbituric acid reactive substances (TBARS) of the samples or ethane concentrations in headspace of trays. An increase of storage temperature from 20 to 37°C increased TBARS and ethane concentrations regardless of filling method and packaging material. No changes in double-bond indices were observed. Nitrogen flushing in combination with light protection reduced lipid oxidation up to 85–95%

Key Words: lipid oxidation, ground pork packaging, ethane, TBARS

INTRODUCTION

CONSUMER DEMAND has been increasing for partially or fully cooked meat in small and medium-size portions as part of convenience-type menus (Cross et al., 1987; Graf and Panter, 1991). Heat sterilization is a main method of preservation, in particular when such products are subjected to extended storage. Lipid peroxidation appears to be the primary mechanism of quality loss in such foods (Kanner, 1992). It affects appearance, texture, flavor and nutritional value (Vercellotti et al., 1992). Product quality is limited mainly by off-flavors developing via autoxidation of polyunsaturated fatty acids, PUFA (Gray and Pearson, 1987), or oxidative effects on proteins, peptides and amino acids (Spanier et al., 1992a).

Modern sterilization techniques for meat products are designed to minimize heat-induced changes in nutritional and sensory properties. Nevertheless, overall quality is impaired during processing and storage. Canned vegetable and potato products are very stable over extended storage, when they are packed in hermetically sealed cans or aluminum pouches and oxygen is removed during filling (Bloeck et al., 1986, 1988 a,b; Margadant, 1991). Menu components containing meat are usually packed in trays in order to reduce heat damage (due to more favorable geometry than that of cans) and to increase convenience and product appeal. Therefore, in addition to being more susceptible to lipid oxidation than vegetables or potatoes, meat products are frequently less protected by packaging. This is especially true when nonaluminum package material is used and no precautions are taken to expel residual oxygen during filling.

Our objective was to investigate the effects of process temperature and nitrogen flushing during filling on oxidative stability of heat-sterilized meat products, using aluminum trays. We also measured and compared oxidative changes in products on high-barrier plastic trays as affected by storage temperature, light exposure, and oxygen permeability.

MATERIALS & METHODS

Preparation and heat sterilization of samples

Pork from animals of known genetic background and constant feeding regime was used. One day after slaughtering, the shoulder part was deboned and the depot fat, as well as the rind, was cut off in a local slaughter house (Schlachthof Chur). Fat was added to provide a constant fat content of 450 g/kg and the mixture was ground in an industrial grinder. The ground meat (90g) and 180g deionized water were filled into aluminum trays (Type 73300, 138 × 138 × 28 mm; Wilhelm Wagner GmbH, Esslingen, Germany) and 102g ground meat and 204 g deionized water were filled into plastic trays (material: PP/EVOH/PP, Type BC 138, 138 × 138 × 34 mm; ONO, Auneau, France). The trays were sealed with a peelable aluminum lid foil (Flexalpeel 45/1000; Alusingen, Singen, Germany) with and without prior removal of air by evacuating to 200 mbar and flushing back with N_2 to atmospheric pressure (CPM H175 VG; Packaging Machinery, Cornwall, England). The trays were then sterilized in hot water in a batch retort (Pilot Rotor 400; Hermann Stock GmbH, Neumünster, Germany) without agitation at 121 and 131°C, respectively, to a F_0-value of 8–9 min for aluminum trays and 10–11 min for plastic trays. The F_0-value denotes the heating time at 121.1°C to which a sterilization process with a z-value of 10°C (*Clostridium botulinum*) is equivalent. (The z-value is the temperature change which results in a tenfold change in the decimal reduction time). The internal temperature was recorded by thermocouples (Ellab, Roedovre, Denmark).

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Storage of products

Aluminum trays were stored up to 62 days at 20°C. Plastic trays were stored alternatively (a) up to 62 days in the dark at 20 and 37°C, (b) up to 62 days exposed to light at 20°C, and (c) exposed to light for 1 day and then up to 17 days in the dark at 20°C. Samples for light exposure were placed on a shelf at a distance of 36 cm under five 40 W fluorescent lamps and 1270–3500 lux (warm-white LF 31, percentage of UV radiation less than 0.1%). Nonsterilized control samples were removed immediately after sealing, placed into ice-cooled boxes and stored overnight at 2–4°C.

Headspace analysis

Oxidative changes in headspace were monitored by a headspace sampler developed by Bertoli (1989) and a gas chromatographic (GC) method according to Bertoli (1989) and Margadant (1991). With the sampling device headspace gas was transferred from trays directly into the GC. Gas samples were analyzed for concentration of low-carbon short chain alkanes. Although other alkanes (methane, propane, butane and pentane) were present as well, ethane was the most prevailing so that it served best as an indicator for lipid oxidation. In our study, only ethane concentration was used as an indicator for oxidative changes. Therefore, results were reported as ng ethane/mL headspace.

2-Thiobarbituric acid (TBA) test

2-Thiobarbituric acid reactive substances (TBARS) were determined by the distillation method of Tarladgis et al. (1960) with some modifications: The meat product was transferred quantitatively into a liquid nitrogen cooler and after 2 min transferred quantitatively to a mixer (Cut-o-mat H4R; Kneubühler Co. AG, Luzern, Switzerland). The sample was mixed for 10 sec at speed level 1 and 20 sec at speed level 2. About 20g of the homogenized sample was blended with 97.5 mL deionized water in a 500-mL flat-bottom flask. Prior to distillation, two boiling chips, 2.5 mL HCl (4 mol/L) and 10 drops of antifoam (Fluka AG, Buchs SG, Switzerland) were added. The sample was distilled for about 10 min at the highest heating rate and 50 mL distillate was collected. Five mL of distillate and 5 mL of TBA reagent (2-thiobarbituric acid; Fluka AG, Buchs SG, Switzerland; 0.02 mol/L in 90% acetic acid) were pipetted into a 20-mL test tube with screw-cap and mixed in a Vortex mixer. The test tube was held in a boiling water bath for 35 min and then cooled in tap water for 10 min. Absorption was measured at 532 nm in a UV spectrophotometer (UVIKON 940; Kontron Instruments, Milano, Italy). Preparation of standard curve: 0, 1, 2, 4, 6 and 8 mL of a standard solution (1,1,3,3 tetraethoxypropane; Fluka AG, Buchs SG, Switzerland; 0.1 mMol/L in 40% ethanol) were pipetted into a 500-mL flat-bottom flask and deionized as described. TBARS were expressed as mg malondialdehyde (MDA)/kg product.

Fatty acid analysis

Lipid extraction. The extraction method of Winter (1963) was modified as follows: About 20g of homogenized sample was weighed in a 250-mL beaker. Solvent (chloroform/methanol, 2:1 v/v, 100 mL) was added and the mixture was blended in a homogenizer (Polytron PT 45–80; Kinematica GmbH, Littau, Switzerland). The homogenizer rod was washed with ca 50 mL solvent and remaining particles were transferred with tweezers into the beaker. Two large spatulas of celite high-flow (Super Cel L; Schneider Dämmtechnik, Winterthur, Switzerland) were filled into a D3 filter funnel and 20 mL of a $MgCl_2$ solution (0.074 mol/L) and ca 120 mL deionized water were placed in a separatory funnel. After standing 1 hr at room temperature (≈23°C) the sample was quantitatively transferred over the D3 filter funnel into the separatory funnel. The D3 filter funnel was washed with 2 × 50 mL solvent, while the funnel was dried between washes by a Weiss suction ring. Afterwards the funnel was filled with pure chloroform and kept for 15 hr. The lower phase was transferred to a 200-mL volumetric flask which was then filled to the mark with chloroform.

Fractionation into neutral and complex lipids. The fractionating procedure of Dittmer and Wells (1969) was modified as follows: for recovery of neutral lipids one small spatula of Celite and three small spatulas of Silica gel 60 (0.063–0.200 mm; Fluka AG, Buchs SG, Switzerland) were filled into a D2 Allihn filter tube. Three mL of standard 1 (C13:0 methyl ester/chloroform, 10 mg/mL) and 25 mL of the extract were pipetted onto the column and collected in a flat-bottom flask. Then the column was washed with 2 × 25 mL chloroform and the flat-bottom flask was removed. Chloroform was evaporated by a rotary evaporator. Hexane (8 mL) was added and about 2 mL solution was transferred to a centrifuge tube with two boiling chips.

For recovery of complex lipids, 3 × 25 mL of extract was pipetted onto the same column. It was washed with 2 × 25 mL chloroform and neutral lipids were collected in another flat-bottom flask. A new flask was placed under the column, 2 mL standard 2 (C13:0 methyl ester/chloroform, 1 mg/mL) and 2 × 25 mL methanol were pipetted onto the column and collected. Methanol was evaporated and 4 mL hexane was added to the residue.

Fatty acid esterification and GC. Both fractions were converted to fatty acid methyl esters (FAME) by adding 2 mL methanolic NaOH (0.5 mol/L) boiling for 2 min in a sandbath and adding 3 mL boron trifluoride/methanol (100 mL/L). Seven mL of NaCl/H_2O (20 g/L) and 2 mL hexane were added. FAME were analyzed with a GC (Model 5890A; Hewlett Packard, Palo Alto, CA) equipped with a flame ionization detector. A 30m × 0.25 mm i.d. fused silica capillary column (Supelcowax™ 10; Supelco, Inc., Bellefonte, PA) was operated with nitrogen carrier gas. The oven was initially held at 175°C for 0.1 min, then increased at 3°C/min to 230°C and held for 13 min at 230°C. The injector and detector were held at 250°C. Identification of FAME was by comparison of retention times with FAME standards. Fatty acid distribution for each fraction was based on peak areas and reported as weight percent of total methyl esters. Results were expressed by calculating the double-bond index (DBI) (number of double-bonds per 100 fatty acids) according to Vogg (1989).

RESULTS & DISCUSSION

TBARS and ethane concentration

Heterogeneity of raw material can never be fully excluded when working with meat. Nevertheless, our experiments with several batches of raw material showed very similar results. This was mostly due to rigorous control of factors expected to affect oxidative deterioration, such as genetics, animal feed, sample preparation and analytical procedures. TBARS of control samples before heat processing were near 0 mg MDA/kg product. Also, no ethane was detected in the headspace indicating no lipid peroxidation occurred during sample preparation and 1-day storage at 0–4°C.

Sterilization temperature at equal F_0-values did not influence TBARS (Fig. 1). Likewise, ethane concentrations, (Fig. 2) were not significantly lower when the processing temperature was raised from 121 to 131°C. Usually, an increase in process temperature at equal F_0 results in a decrease of quality changes because cook values C (heating time at reference temperature to which a heating process is equal in quality change) do not increase at the same rate as F values. This principle is exploited basically in the high-temperature short-time sterilization (HTST) concept. For meat products in trays the higher process temperatures do not appear to contribute to a notable quality improvement.

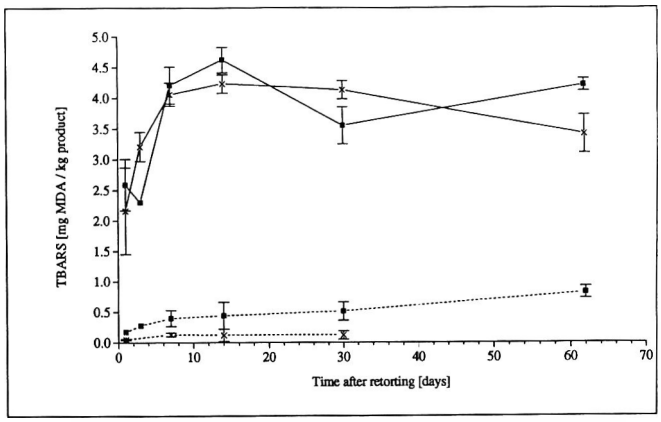

Fig. 1—Effect of retort temperature and filling conditions on the TBARS of heat-sterilized meat products in aluminum trays during storage at 20°C. F_0 = 8-9 min. Analytical values = mean of four samples ± standard deviation. TBARS value before retorting = 0.06 mg MDA/kg product. ■—■ 121°C, no flushing; ■---■ 121°C, N_2 flushing; x—x 131°C, no flushing; x---x 131°C, N_2 flushing.

In contrast, nitrogen flushing greatly inhibited lipid oxidation as judged by both TBARS (Fig. 1) and ethane concentration (Fig. 2). In nitrogen-flushed products stored for 62 days at 20°C, TBARS were reduced by 80% (Fig. 1) and ethane concentration by >95% (Fig. 2). Similar results were obtained when ground beef patties were cooked and stored under vacuum in a refrigerator for 4 days (Spanier et al., 1992b,c). TBARS slightly increased with increase in storage temperature from 20 to 37°C, but dropped after 14 days storage, regardless of filling conditions (Fig. 3). Ethane concentrations were higher when storage temperature was increased from 20 to 37°C (Fig. 4).

MDA reacts with amino acids, nucleic acids, proteins, glycogen and other food components to form products in which MDA exists in bound form (Kwon et al., 1965; Buttkus, 1967; Karel, 1973; Schauenstein et al., 1977; Gardner, 1979). However, those bonds are broken during the acid/heat treatment of the distillation step during the TBA test (Kwon et al., 1965). Nevertheless, in our study TBARS could only be used as a measure for oxidative deterioration during the first 14 days storage. Thereafter, TBARS decreased, probably due to the increased reaction rate of MDA decomposition by secondary oxidation or polymerization (Hoyland and Taylor, 1991).

Storage-induced changes in non-N_2-flushed meat products in both aluminum and plastic trays were more pronounced than heat-induced changes during sterilization. This might be due to the high oxygen content prior to sterilization. Oxygen removal in canned vegetable and potato products is usually achieved by hot-filling leading to high oxidative stability during long-time storage of 540 days at 20°C (Margadant, 1991). In contrast, trays are cold-filled and, therefore, oxygen removal by evacuating and flushing of trays is absolutely necessary to prevent or lessen oxidative deterioration.

Lipid peroxidation can be induced by oxygen or by light. The effects of light on food quality can be explained by both photolytic autoxidation or, in presence of photosensitizers such as hemoproteins, bound or free iron by photosensitized oxidation (Bradley and Min, 1992; Spanier et al., 1992b). Effects of light exposure of plastic trays on ethane concentrations in headspace (Fig. 5) showed at 20°C ethane concentrations increased greatly and, after 29 days, reached the level of samples stored in the dark at 37 °C. When samples that had been flushed with nitrogen where exposed to light, a higher oxidative change was found than in nonflushed samples that had

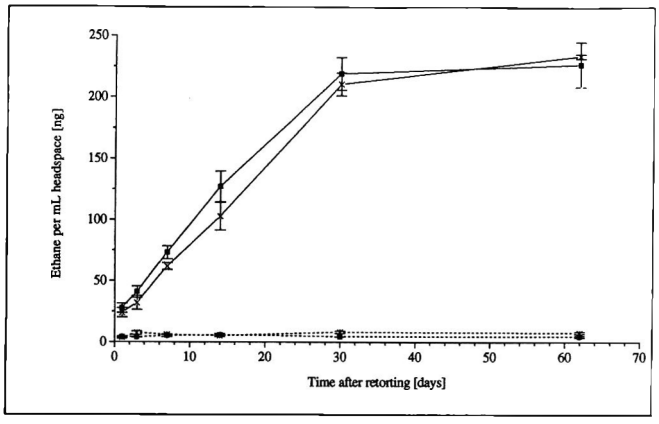

Fig. 2—Effect of retort temperature and filling conditions on the ethane concentration of heat-sterilized meat products in aluminum trays during storage at 20°C. F_0 = 8-9 min; Analytical values = mean of four samples ± standard deviation; Ethane concentration before retorting < 1.0 ng/mL headspace. Symbols same as Fig. 1.

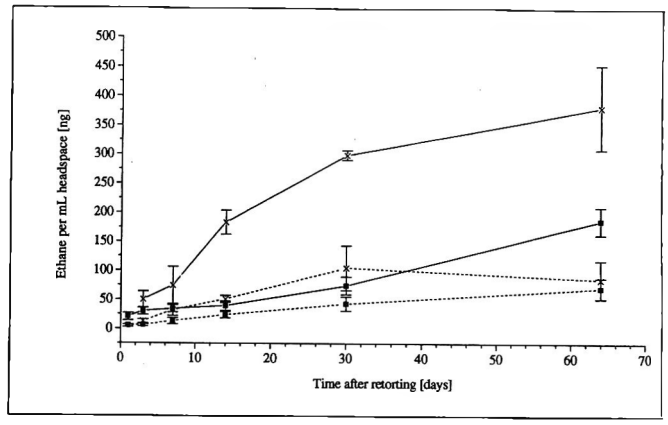

Fig. 4—Effect of storage temperature and filling conditions on the ethane concentration of heat-sterilized meat products in plastic trays. Process temperature: 131°C. F_0 = 10-11 min. Analytical values = mean of six samples ± standard deviation. Ethane concentration before retorting < 1.0 ng/mL headspace. Symbols same as Fig. 3.

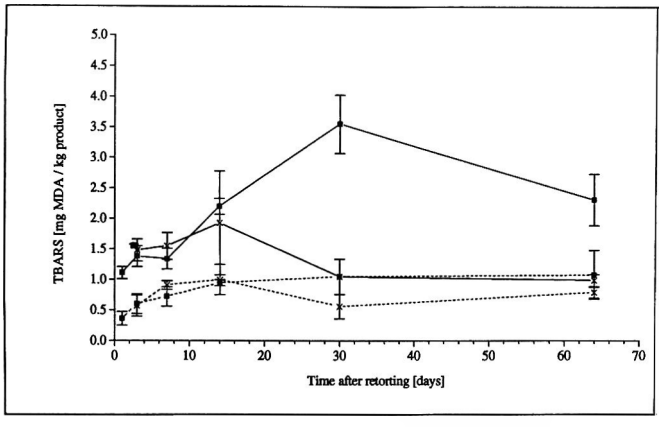

Fig. 3—Effect of storage temperature and filling conditions on the TBARS of heat-sterilized meat products in plastic trays. Process temperature: 131°C, F_0 = 10-11 min. Analytical values = mean of six samples ± standard deviation. TBARS value before retorting = 0.01 mg MDA/kg product. ■—■ 20°C, no flushing; ■---■ 20°C, N_2 flushing; x—x 37°C, no flushing; x---x 37°C, N_2 flushing.

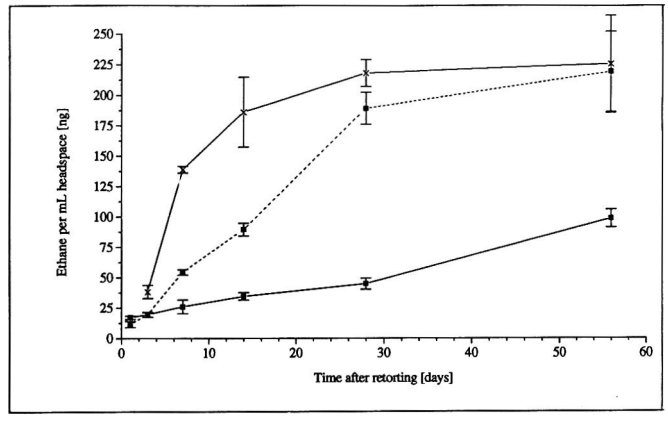

Fig. 5—Effect of storage temperature and light exposure on the ethane concentration in the headspace of heat-sterilized meat products in plastic trays. Process temperature: 131°C. F_0 = 10-11 min. Analytical values = mean of six samples ± standard deviation. Ethane concentration before retorting < 1.0 ng/mL headspace. ■—■ 20°C; ■---■ 20°C, light; x—x 37°C.

been stored in the dark at the same temperature (Table 1). The same relationship was found for flushed samples that had been exposed to light for only one day and then stored in the dark. Therefore, while nitrogen flushing was effective for preventing excessive oxidative changes, light protection from the very beginning of storage was equally important for quality retention in sterilized meat products.

Double-bond index

Only peroxides which have unsaturation α, γ to the peroxide group can undergo cyclization with ultimate formation of malonaldehyde. Such peroxides can only be formed from fatty acids with three or more double bonds. Therefore, it becomes imperative to know the fatty acid profile (Gray, 1978). The development of double-bond indices (DBI) of both neutral and complex lipid fractions of heat-processed meat in plastic trays with and without nitrogen flushing were compared (Fig. 6). Although some differences were found and a very slight decrease of DBI values during storage was observed, the method did not appear to be sensitive enough to detect heat and oxidation-induced changes of the lipids. This was also the case for complex lipids where the concentration of polyunsaturated fatty acids was much higher (36–42 Mol-% vs 9–11 Mol-% in neutral lipids). Similar results were obtained in a study on pork meat patties heated up to 80–100°C for 15–120 min, where also no changes occurred in the complex fatty acid profile (Häuser, 1992).

CONCLUSION

In preparing shelf-stable meat products in trays, an increase of process temperature from 121 to 131°C and a reduction of cook value C at equal F values did not improve oxidative stability. Higher storage temperatures (37 vs 20°C) enhanced oxidative deterioration. Removal of air by nitrogen flushing during filling led to increased storage stability in both aluminum and plastic trays. In plastic protection of packages against light was critical at any storage whether filling occurred with or without nitrogen flushing. There were definite indications that residual oxygen permeability of the high-barrier plastic trays we used had little effect on controlling storage stability.

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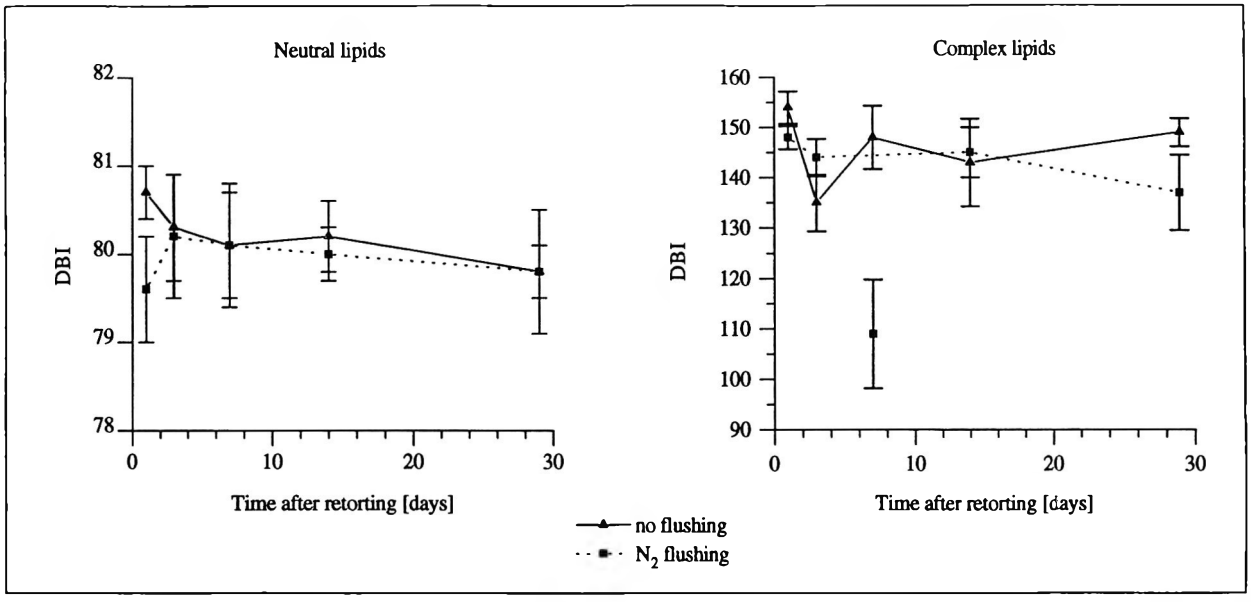

Fig. 6—Effect of filling conditions on the double-bond index (DBI: number of double bonds/100 fatty acids) in the neutral and complex lipid fraction of heat-sterilized meat products in plastic trays during storage at 20°C. Process temperature: 131°C. F_0 = 10–11 min. Analytical values = mean of six samples ± standard deviation. DBI of neutral lipids before retorting = 80.5. DBI of complex lipids before retorting = 148.5.

Table 1—Effect of light and time of exposure on ethane concentration in headspace of conventionally packed (no flushing) and N_2-flushed heat-sterilized meat products in plastic trays. (Retort temperature 131°C, F_0 = 10–11 min)

Time after retorting [days]	Ethane concentration[a] [ng/mL headspace, mean ± standard dev.]				
	A	B	C	D	E
1	11.6±2.5	n.m.[b]	n.m.	17.2±1.4	5.0±2.1
3	19.5±1.8	n.m.	n.m.	19.6±2.2	5.9±2.0
7 (8)	54.6±2.2	(47.8±4.7)	27.7±3.0	26.0±5.6	13.1±5.4
14 (17)	89.6±5.4	(52.6±5.3)	(44.5±5.5)	34.6±3.2	23.7±5.6

[a] A—no flushing, permanent light exposure during storage (n=3); B—N_2 flushing, permanent light exposure during storage (n=3); C—N_2 flushing, light exposure for 1 day with subsequent storage in darkness (n=3); D—no flushing, stored in darkness (n=3); E—N_2 flushing, stored in darkness (n=6); Product preparation in 3 individual batches A/D, B/C and E, respectively.

[b] n.m.—not measured because batch A and D did not significantly differ after 1 and 3 days of storage.

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Thermal Processing Canned Meat with Sub-Freezing Initial Temperatures: Thermal and Microbial Validation

G.M.M. SANDBERG, T.D. DURANCE, P. RICHARD, and P.H. LEUNG

ABSTRACT

Retorting from the frozen state is attractive with respect to product quality and economy and was investigated in canned bentonite and flaked ham. A two dimensional finite difference model was written in which thermal diffusivity varied with temperature. Heat penetrations were completed with initial temperatures of 0, −5°, −10°, −15° and −20°C. In ham, decreasing initial temperatures resulted in increased lag factors (j_h) but did not affect heating rate index (f_h). In bentonite, f_h but not j_h increased. D and Z of *Clostridium sporogenes* PA 3679 spores were not affected by previous freezing. Sub-freezing initial temperatures were judged to be consistent with safe processes if validated with heat penetration studies.

Key Words: meat, ham, bentonite, model system, thermal process

INTRODUCTION

GENERALLY IN FOOD CANNING INDUSTRIES products with initial temperatures < 0°C are not processed without prior thawing. A survey of Canadian government registered processes and various published processes data indicated very few scheduled thermal process for retorting food products with initial temperatures below the freezing point of water. The possibility of ice in the product and the extra heat required for the phase change causes concern for spore-forming organisms escaping the full sterilization heat treatment.

The possibility of processing food products with initial temperatures below freezing is interesting due mainly to rising material costs in the meat processing industry. In an effort to reduce such costs, the comminuting of meat and other ingredients to create meat emulsions with structures analogous to intact muscle protein is common. In this process, protein extractability is of prime importance (Schmidt, 1987). Maximum extraction of myosin protein occurs in the range −5°C to +2°C (Bard, 1965). At the lower of this range the possibility of ice in canned products must be considered. Furthermore, the thawing of meat results in loss of some soluble protein through drip-loss (Miller et al., 1980). The use of frozen ingredients could result in notable cost-savings. Prior thawing also increases the potential for microbial deterioration of highly perishable components before thermal processing. Some preliminary work in the laboratories of American National Can Company (Fairbrother, 1989) during the early 1960s, involved canned luncheon meat with initial temperature −28.9°C. The experiments suggested that while the heating rate index (f_h) and heating lag factor (j_h) were increased somewhat, the process time (P_t) was not substantially lengthened. Subfreezing initial temperatures and their effects require further study and may provide potential advantages.

Bentonite-water dispersions have been used to simulate foods in many studies of thermal processes. Their use as a model food, has been primarily due to low cost and chemical stability compared to food systems (Unklesbay, 1982). Researchers concluded that bentonite-water dispersions were effective models of food systems for food service energy research, and that the bentonite component of such models simulated the solids content of foods (Niekamp et al., 1984). The water component simulated moisture and fat.

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Table 1—Composition of chunk, emulsion and blended portions of flaked ham and bentonite dispersion

Material	Percent composition			
	Fat	Moisture	Ash	Protein
Chunk	5.0	73.5	1.0	22.0
Emulsion	10.0	69.0	7.5	13.5
Blend	6.7	71.0	1.0	21.3
Bentonite	0.0	63.8	36.2	0.0

Computer simulations of heating can be useful, as a means of testing hypotheses regarding a particular system, and as a convenient vehicle for prediction of heat penetration profiles (Richard et al., 1991). In our study, a two-dimensional finite difference model of thermal processing with frozen initial temperature was compared to true heat penetration data of bentonite and flaked ham in tinplate steel cans.

Ultimately, the safety of a thermal process depends on its potential to destroy/prevent microorganisms. In low-acid foods, *Clostridium sporogenes* (PA 3679), a close but nontoxic relative of *Clostridium botulinum*, has often been employed to measure adequacy of heat processes. Heat shocking increases thermal resistance of PA 3679 but no published results of the effects of freezing on thermal resistance was found. The impact of freezing on D and z values of PA 3679 was therefore examined, to test the hypothesis that frozen low-acid foods might require greater lethalities than unfrozen product.

Our objectives were thus, to evaluate thermal processes of canned ham and bentonite with various freezing initial temperatures, to look for anomalies in temperature histories by comparison with zero degree initial temperature heat penetrations and computer simulations of heating, and finally to test the hypothesis that previous freezing would not affect the thermal death rates of a clostridium spore former, PA 3679.

MATERIALS & METHODS

Materials

The flaked ham product consisted of a 1:1 blend of 95% lean ham, ground through a 19 mm plate and a comminuted meat emulsion of lean ham trim, water, lean ham shanks, salt, smoke flavor, sodium phosphate, sodium erythorbate and sodium nitrite. The finished product was allowed to cure for 36 hr prior to canning. Proximate composition was determined (Table 1). The bentonite model food was a 36.2% solids dispersion of bentonite (X-tra Gel, American Colloid Co., Arlington Heights, IL) in distilled water.

Thermal diffusivity (α)

Measurements were performed by the method of Dickerson (1965). A cylindrical diffusivity cell (5 cm × 25 cm) was fabricated from chrome plated brass tubing with a 5 cm thick Teflon end caps (Fig. 1). The bath (Coliform Incubator Bath, GCA Precision Scientific, Chicago, IL), which had a submersible pump to provide agitation and a Model 70 D5X thermostat control (United Electric Controls (Canada) Ltd., Mississauga, ON) to provide a linear heating rate, was filled with a 1:1 mix of a commercial ethylene glycol (Prestone II) and distilled water, and was pre-cooled to −25°C in a commercial freezer. Thermocouples were positioned as shown (Fig. 1) and connected to a datalogger. Temperatures at the center (T_c) and surface (T_s) of material packed in the diffusivity cell were recorded at 1 min intervals as the

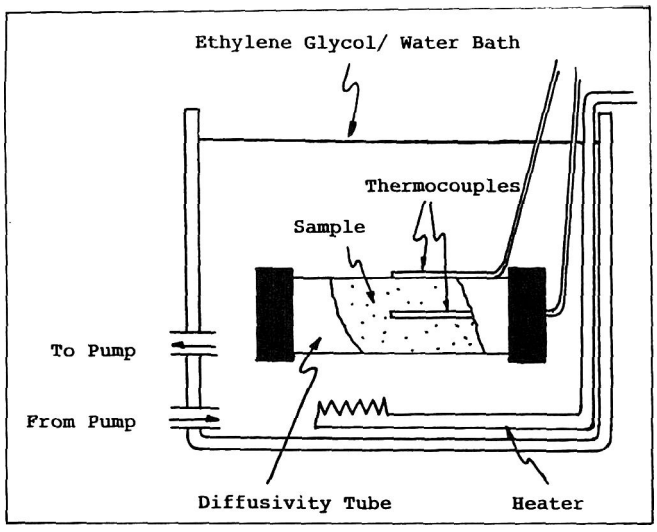

Fig. 1—Apparatus for measurement of thermal diffusivity of bentonite and flaked ham at various temperatures.

Table 2—Apparent thermal diffusivity of flaked ham and bentonite at various temperatures

Temperature (°C)	Thermal diffusivity[a] ($\times 10^{-7}$ m²/sec) Flaked ham	Bentonite
−20	11.00	5.88
−15	6.14	4.33
−10	1.22	2.79
−5	0.52	1.67
0	0.40	0.45
5	0.42	0.48
10	0.46	0.53
15	0.50	0.60
20	0.54	0.66
25	0.58	0.74
30	0.64	0.84
35	0.70	0.95
40	0.76	1.08
45	0.83	1.22
50	0.91	1.39
55	0.97	1.57
60	1.10	1.71
65	1.24	2.00
70	1.43	2.20
75	1.68	2.45
80	1.99	2.60
85	2.51	3.10
90	3.00	4.07
95	3.61	4.15
100	4.10	4.65
105	4.50	5.03
110	5.20	5.57
115	5.84	5.98
120	6.15	6.50
125	6.74	6.92

[a] *Values were influenced by phase changes at the extremes of the temperature range*

cell was heated at a constant rate (B) of about 1°C/min. Thermal diffusivity was assumed constant over narrow temperature ranges (e.g., 4C) and was computed as follows:

$$\alpha = \frac{B\,R^2}{4\,(T_s - T_c)} \qquad (3)$$

R was the radius of the diffusivity cell. Diffusivities calculated at temperatures of phase transition were influenced by latent heats of fusion and condensation and were therefore apparent rather than true thermal diffusivities.

Heat penetration

Thermocouples were located at the geometric center of 307 × 111 two-piece tinplate cans. They were filled with a minimal head space (fill weight 190g) and hermetically sealed on an Angeles 61H steamflow closing machine (Angeles Corp, Anaheim, CA). The cans were

Table 3—Mean time to reach 0°C from various initial product temperatures

	Initial temperature (°C)	Time to reach 0°C (min) Actual	Simulation[b]
Ham	0	0.0	0
	−5	6.4 (1.3)[a]	7
	−10	10.1 (1.0)	8
	−15	11.0 (1.6)	9
	−20	11.3 (1.3)	10
Bentonite	0	0.0	0
	−5	5.6 (1.6)	6
	−10	6.2 (1.5)	6
	−15	7.0 (1.5)	7
	−20	7.8 (1.6)	8

[a] Standard deviation.
[b] *Computer simulation recorded temperatures at 1 minute intervals.*

Table 4—Mean values of f_h, and j_h for bentonite and ham at various initial temperatures

	Initial temperature (°C)	f_h (min)	j_h
Ham	0	25.91 (2.59)[a]	1.64 (0.47)[a]
	−5	25.37 (2.49)	1.97 (0.56)
	−10	25.08 (1.45)	2.44 (0.57)
	−15	23.81 (1.65)	2.51 (0.59)
	−20	24.44 (0.77)	2.78 (0.28)
Bentonite	0	25.84 (2.63)	1.01 (0.10)
	−5	26.09 (2.31)	1.02 (0.20)
	−10	26.81 (2.71)	1.04 (0.24)
	−15	28.44 (2.55)	0.90 (0.16)
	−20	27.95 (2.06)	1.00 (0.28)

[a] Standard deviation.

then placed in a freezer set to the desired initial temperature or IT (−20°C, −15°C, −10°C, −5°C, or 0°C) and allowed to equilibrate over a 24 hr period. Cans were placed into a vertical, saturated steam, nonagitating retort. Center point thermocouples, calibrated vs flowing steam, were attached to a Calwest model 32 datalogger (Calwest Technologies, Canyon Country, CA), connected to a Toshiba T1000 computer equipped with Calsoft software program for acquisition and analysis of heat penetration data (Technical Inc., Metairie, LA). Only cans with actual initial temperature ± 1C° of the nominal IT were used in data analysis.

At least 20 containers for each initial temperature, were processed at 125°C until a lethality (F_o) of ≥6.0 min was achieved in the heating phase, as calculated by the CalSoft program using the general method. Come up time was 10 min. Cans were water cooled (T_w = 13°C) and cooling data were also collected. Temperature data were corrected for effects of thermocouple fitting (Ecklund, 1955). Data were then plotted and analyzed using the general method (Patashnik, 1952) and Ball's formula (Ball and Olson, 1957). Computer simulation center point temperature histories were analyzed by the same methods. In all cases, Ball's process times were calculated for an F_o of 6 min.

Spore suspension

A culture of *Clostridium sporogenes* PA 3579 (American Type Culture Collection, Rockville, MD) was propagated in Oxoid Reinforced Clostridium medium (RCM) and sporulated in liver infusion broth (Stumbo, 1973). Spores were harvested aseptically in a centrifuge (10000 × *g*, 10 min) and washed two times with sterile buffer, before being resuspended in 0.067M potassium phosphate, pH 6.6 (K-P).

Thermal resistance

Spores (1.1 × 10^6/mL K-P) were sealed into 100 µL borosilicate glass capillary tubes and heat treated in an agitated oil bath for 0–60 sec at 121°C, 0–210 sec at 116°C, or 0–16 min at 110°C, then immediately cooled in ice slush. The ends of the capillary tubes were aseptically broken and spores flushed into a tube of fresh RCM broth with 0.9 mL sterile peptone water. Serial dilutions were made in RCM to produce 5-tube MPN series. The medium surface of inoculated tubes was sealed with 2 mL of warm 1:1:4 vaseline:paraffin:mineral oil and tubes were incubated at 30°C for 10 days. Positive tubes displayed turbidity and gas production.

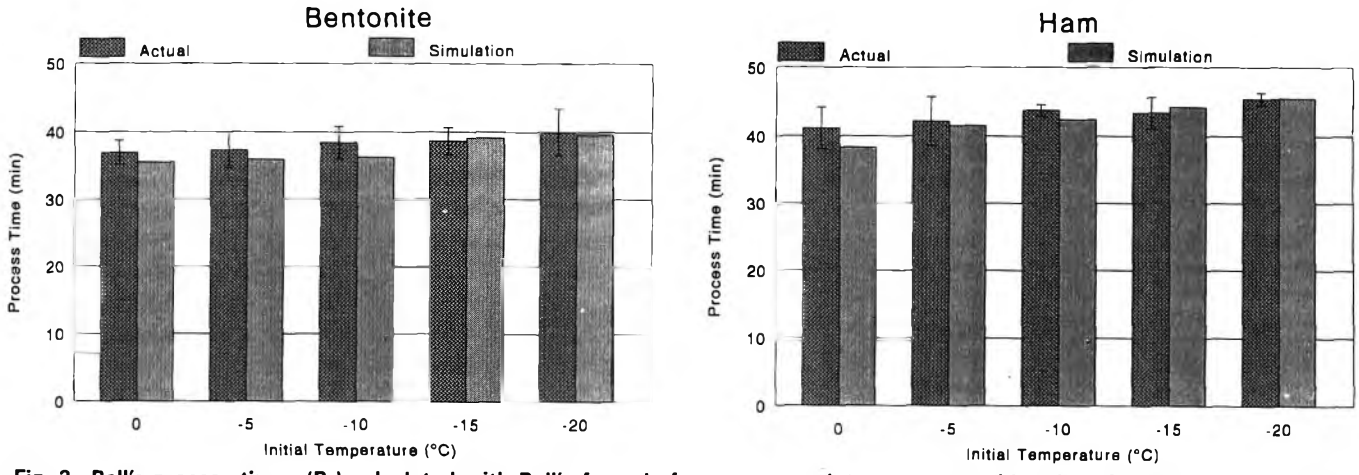

Fig. 2—Ball's process times (B_B) calculated with Ball's formula from center point temperature histories of cans (n=20) and from computer simulations at five different initial temperatures: (a) bentonite; (b) flaked ham.

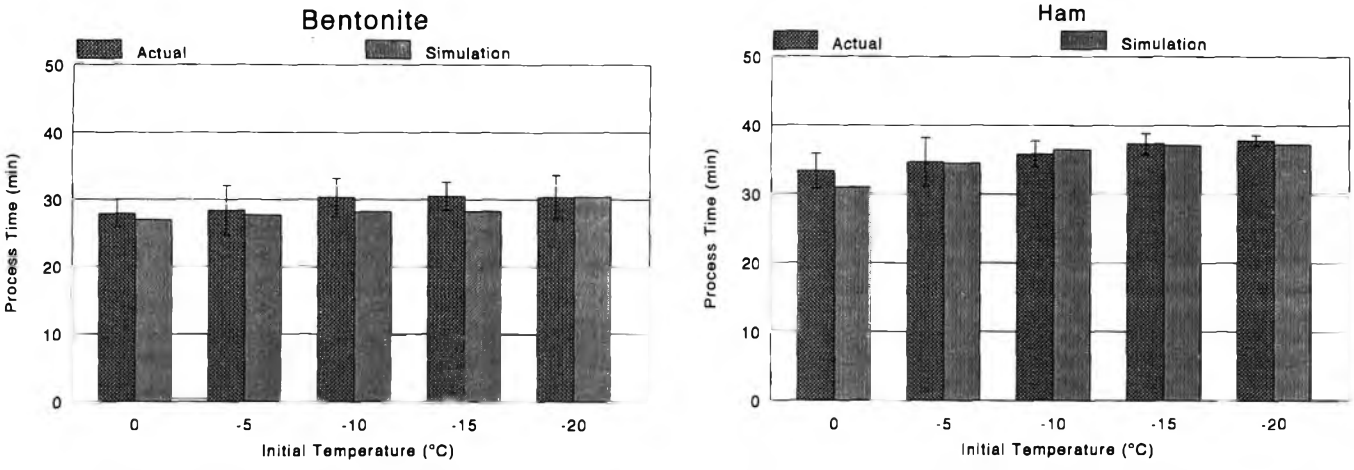

Fig. 3—Ball's process times (B_B) calculated by the general method from center point temperature histories (n=20) and from computer simulations at five different initial temperatures: (a) bentonite; (b) flaked ham.

Computer simulation of retort process

The objective of any heat-transfer analysis is to predict heat flow or the temperature which results from a specified heat flow (Holman, 1976). In our two dimensional model of unsteady heat conduction in a cylinder, x and r denoted increments in the height and radial position within a cross-section of a can.

$$\frac{\partial^2 T}{\partial x^2} + \frac{\partial^2 T}{\partial r^2} + \frac{1}{r}\left(\frac{\partial T}{\partial r}\right) = \frac{1}{\alpha}\frac{\partial T}{\partial t} \qquad (1)$$

Thus, x and r coordinates denote a "node" for which a temperature (T) can be calculated at any point in time (t). If the initial temperature and the temperatures of surrounding nodes are known at any time, the temperature of a node after a time increment may be calculated with a finite difference Eq. (2), so as to obtain temperatures (T2) at t+1. For a detailed review see Teixeira et al. (1969).

$$T2^{t+1} = \left[\Delta t\alpha\left(\frac{1}{\Delta r^2} + \frac{1}{2r_m\Delta r}\right) T2\ A^t\right] + \left[\Delta T\alpha\left(\frac{1}{\Delta r^2} - \frac{1}{2rm\Delta r}\right) TB^t\right] + \left[\left(\frac{\Delta t\alpha}{x^2}\right) T3^t\right] + \left[1 - \left(\Delta t\alpha\left(\frac{2}{\Delta r^2} + \frac{2}{\Delta x^2}\right)\right)\right] \qquad (2)$$

By choosing the appropriate radial and linear increments (in this study, 0.00218m and 0.0010715m, respectively) and a suitable time increment (0.01 sec), the unsteady-state heat transfer was simulated. The modeled system was a 0.0873m by 0.027m steel can undergoing thermal processing in a static-cooking, vertical saturated steam retort. An array of α at 5°C temperature intervals was used in our model rather than a single average α for the entire temperature range as is common practice. Temperature calculations for a particular node were based upon the apparent thermal diffusivity of that node one time increment in the past.

The product temperature was assumed to be uniform throughout the can at the beginning of the cook. Heat penetration measurements were used for comparison with the model only if the measured center point initial temperature was <1°C from the nominal initial temperature. A perfect thermal contact at the surfaces of the container was also assumed, in an attempt to simplify the model. Lastly, due to the large temperature difference between the interior of the container and the saturated steam environment of the retort, the convective boundary condition was ignored and the first node of the finite difference grid was located at the container surface and was set at retort temperature at the beginning of the process.

RESULTS & DISCUSSION

APPARENT THERMAL DIFFUSIVITIES of ham and bentonite at −20°C to 125°C were determined (Table 2). Phase transitions had a substantial influence at the extremes of the measured temperature range and thus those values were not true diffusivities. However, given that the same transitions occurred within cans in the retort, the measurements were appropriate for our purpose.

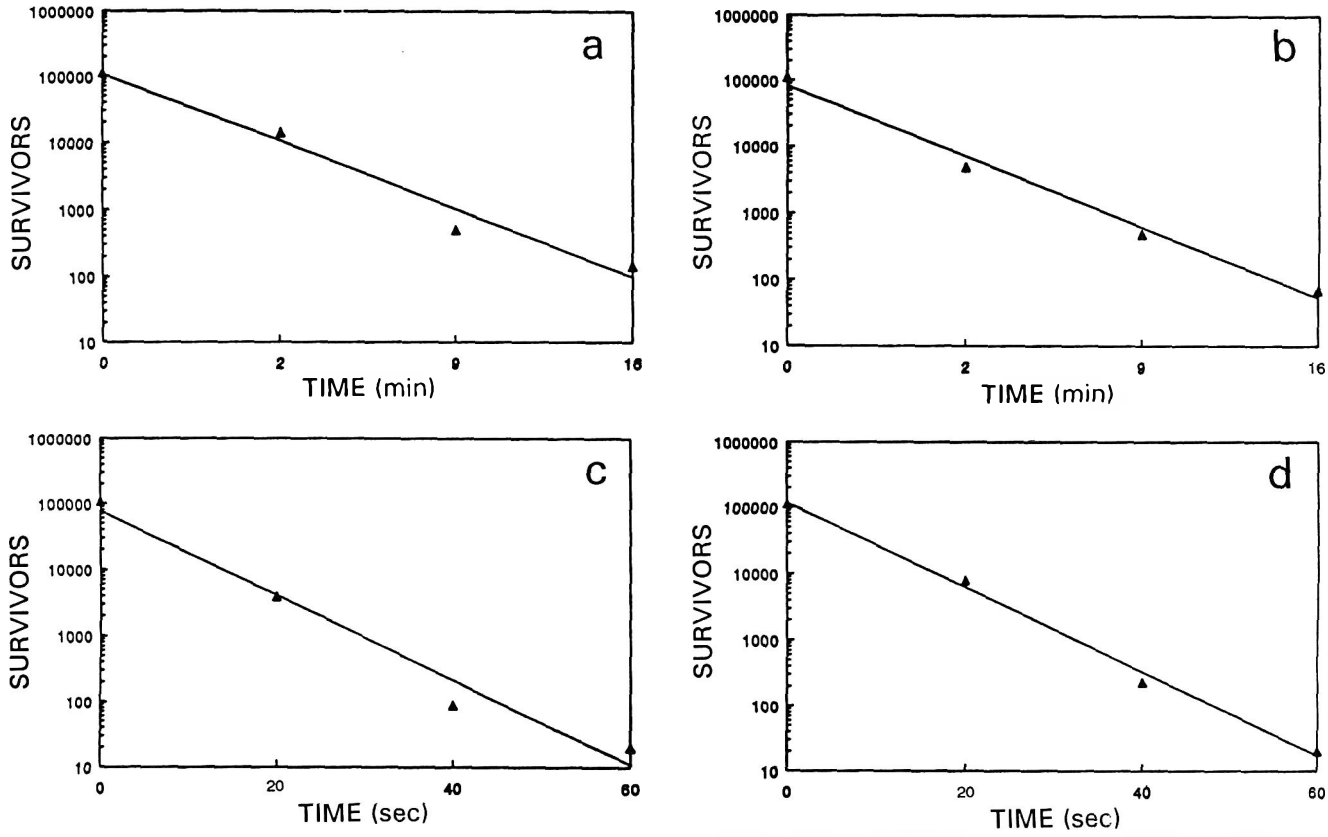

Fig. 4—Log survivor curves of PA 3679 spores in potassium phosphate buffer: (a) control spores at 110°C, r^2= 0.92; (b) previously frozen spores at 110°C, r^2= 0.91; (c) control spores at 121°C, r^2= 0.97; (d) previously frozen spores at 121°C, r^2= 0.99.

Times for cans of ham and bentonite to thaw in the retort (i.e. reach 0°C) were determined (Table 3). Most thawing time was associated with the −10°C to 0°C temperature change. The slight increase in thawing times of lower initial temperature samples may be associated with freezing of more stongly bound water or eutectic points of dissolved salts (Fennema et al., 1973). Differential Scanning Calorimetry (DSC) studies of similar samples, also indicated that most thawing occurred between −10° and 0°C (Sandberg and Durance, unpublished data).

The net result of lower initial temperatures on heat penetration pattern of the ham was an increase in j_h (Table 4). The value for f_h was virtually unaffected. Analysis of variance indicated no significant effect of IT on f_h ($p>0.2$) while the efffect on j_h was significant ($p<0.001$). This was expected because freezing was not thought to notably alter the structure of the ham product. The meat had been frozen prior to curing and canning. Bentonite did not behave in the same manner; j_h was not significantly affected by IT but f_h was slightly increased ($p<0.001$). This suggested that the freezing treatment altered the structure of the bentonite suspension such that its heating rate decreased.

Using the center point temperature histories from both heat penetrations and computer simulations, we calculated the required process time to achieve a lethality (F_o) of 6.0 min with Ball's formula method and Patashnik's general method. Results of these calculations (Figs. 2 and 3) showed the process times calculated from computer simulations differed from experimental averages by up to a few minutes in some cases. However, they were generally within one standard deviation of empirical values. Usually the model underestimated the experimental process time, possibly due to assumptions in the model that the outside node of the can was at retort temperature and that retort temperatures were achieved instantaneously.

Thermal death behavior of PA 3679 suspended in pH 6.6 buffer was estimated in 100 μL capillary tubes. Spores previously frozen and stored 48 hr at −20°C exhibited similar phantom thermal death time curves at 121°C and 110°C (Fig. 4). Estimates of D and z over this range were also very similar (Table 5).

Table 5—Decimal reduction times and z values of PA 3679 spores[a] with and without previous freezing

	Temperature (°C)	D (min)	Z (C°)
Control	110	5.70	
	116	1.16	
	121	0.27	8.6
Frozen[b]	110	5.2	
	121	0.27	8.2

[a] Suspended in 0.067M potassium phosphate, pH 6.6.
[b] Stored 48 hr at −20°C.

Several conclusions can be drawn from our results. Processing directly from the frozen state initially slowed heating of the center of the can of ham but, once thawed, the heating curve resembled that of initially thawed product. The lag factor (j_h) was increased but f_h was not affected. In the bentonite, initial freezing slightly increased the f_h but did not effect j_h. The process time had to be increased up to 5 min for ham and up to 3 min for bentonite, when cans were placed in the retort at −20°C. Bentonite was therefore not a good model for flaked ham. We postulate, that at least for muscle foods, bentonite may not be a suitable model for studies involving freezing and thawing.

The thermal resistance of *Clostridium sporogenes* PA 3679 was not affected by previous freezing; neither $D_{121°C}$ nor z appeared to be altered. This combined with the conclusion above, indicates that there is no safety impediment to subfreezing initial temperatures, provided a proper heat penetration study of the product is completed.

Use of an array of different α values, indexed to temperature, allowed a good fit between measured center point temperatures and those predicted by computer model. However,

—Continued on page 719

Ultrasonic Spectral Analysis for Beef Sensory Attributes

B. PARK, A.D. WHITTAKER, R.K. MILLER, and D.S. HALE

ABSTRACT

Ultrasonic spectral feature analysis was conducted for measuring beef sensory attributes noninvasively. Spectral features were compared with instrumental texture, chemical and sensory evaluation measures. The most significant ($P < 0.05$) ultrasonic parameter was the number of local maxima for juiciness ($\rho = 0.49$), connective tissue amount ($\rho = 0.52$), flavor intensity ($\rho = 0.39$), percent total collagen ($\rho = 0.34$), and shear force ($\rho = 0.51$). However, the central (resonant) frequency was the most dominant parameter for tenderness ($\rho = 0.45$; $P < 0.05$). Multivariate linear regression models were developed for predicting each palatability attribute. Standard errors of calibration for models were 0.253 for juiciness, 0.745 for muscle fiber tenderness, 0.244 for connective tissue amount, 0.754 for overall tenderness, and 0.224 for flavor intensity. Accuracy of prediction models was not adequate for use as a tool but this approach has potential for nondestructive sensory attribute measurement.

Key Words: beef, juiciness, tenderness, ultrasound, palatability

INTRODUCTION

THE UNITED STATES BEEF INDUSTRY is shifting from commodity-oriented to consumer-oriented industry (Cross et al., 1989). Changing consumer preferences in beef and a health-conscious public, are causing beef producers to place more emphasis on carcass merit. The National Consumer Retail Beef Study (Savell et al., 1987, 1989) revealed that tenderness or meat texture was the most important factor affecting consumer perception of taste. Measurement of beef tenderness in an automated grading system to predict carcass merit would be applicable in such a consumer driven industry.

The method for tenderness measurement uses human sensory panelists. Instrumental measurement of tenderness, however, has been considered important for quantitative evaluations. The earliest instruments designed for objective tenderness measurement have been evaluated by many investigators (Kulwitch et al., 1963; Sharrah et al., 1965; Voisey and Hansen, 1967; Ockerman et al., 1981). The Warner-Bratzler Shear Device is a standard measurement used primarily for intact muscle tissues. Most instruments used for tenderness evaluation are destructive of the sample. However, destructive methods are not efficient in an industrial setting because of time to prepare and process samples and the local nature of information from the sample. The National Beef Tenderness Survey (Morgan et al., 1992) showed beef cuts were being merchandized at the retail level that would be rated tough by consumers. The quality grade system for identifying less tender beef does not sufficiently segment cuts based on tenderness. A more direct measure of tenderness is needed.

Park and Whittaker (1991) introduced an ultrasonic, noninvasive shear force measurement for predicting meat tenderness through ultrasonic velocity measurement. A real-time ultrasonic imaging analysis method was investigated for measuring beef sensory attributes using a textural analysis method (Park and Whittaker, 1992). Specific objectives of our research were to (1) determine the feasibility of ultrasonic spectral analysis for predicting beef sensory attributes, (2) select ultrasonic spectral parameters for tenderness measurement, and (3) develop multivariate linear regression models to predict beef tenderness and other palatability attributes.

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MATERIALS & METHODS

Ultrasonic signal processing

Frequency spectra may be obtained by invoking the Fourier transform relationship from the radio frequency (RF) wave form. Mathematically, the Fourier spectrum is obtained by transforming time-domain signal to frequency-domain spectrum. The Fourier transform $F(\omega)$ is defined in Eq. (1):

$$F(\omega) = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{+\infty} f(t) \exp(-j\omega t)\, dt \quad (1)$$

and the inverse transform as Eq. (2):

$$f(t) = (2\pi) \int_{-\infty}^{+\infty} F(\omega) \exp(j\omega t)\, d\omega \quad (2)$$

In typical digital frequency transformation algorithm, Eq. (1) may be written as Eq. (3):

$$\mathcal{F}[f(t)] = F(\omega) = \int_{-T}^{+T} f(t) \exp(-j\omega t)\, dt \quad (3)$$

If the Fourier transform of $f(t)$ is $F(\omega)$, then the transform of $f(t-t_o)$ can be expected as equation (4) by the shift property of the Fourier transform.

$$\mathcal{F}[f(t-t_o)] = \exp(-j\omega t_o)\, F(\omega) \quad (4)$$

The spectra can be determined from the magnitude of the Fourier transform of $f(t)$. If a second identical pulse were added and the total delay between the two pulses was $2t_o$, these pulses could represent the two signals from the extreme boundary points of a muscle tissue. In that specific case, the spectrum could be determined from Eq. (5).

$$|\mathcal{F}[f(t+t_o) + f(t-t_o)]| = |2\cos\omega t_o||F(\omega)| \quad (5)$$

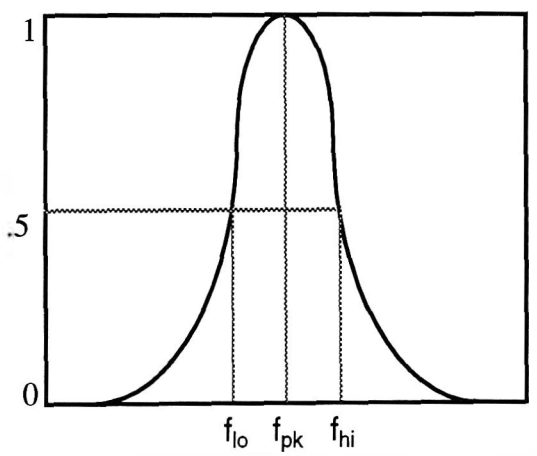

Fig. 1—Ideal spectrum power curve for ultrasonic transducer: f_{lo} = lower frequency; f_{pk} = peak frequency, f_{hi} = higher frequency.

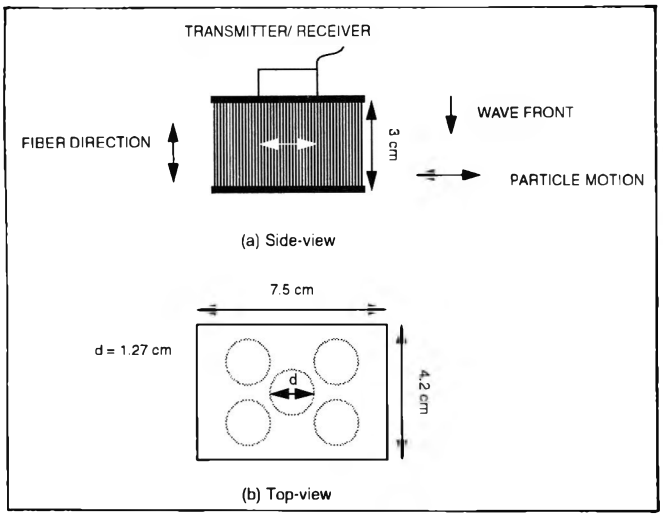

Fig. 2—Ultrasonic shear wave propagation through meat sample and the five scanning locations of each probe.

The envelope of the resulting spectrum would be the same as that obtained for a single pulse, but the spectrum maxima and minima would depend on t_o.

Ultrasonic spectral analysis

Spectral analysis methods reveal a variety of characteristics in biological materials. Normally, in frequency analysis experiments, broadband, highly damped transducers are used to send a wide range of frequencies into a material. Spectral analysis then may indicate which frequencies are most affected by inhomogeneities within the material such as connective tissue amount or muscle fiber tenderness. A typical spectral curve for a homogeneous ultrasonic signal is a Gaussian or normal shape (Fig. 1). The spectrum is symmetric about the peak frequency (f_{pk}). The frequencies at half-power points (f_{lo} and f_{hi}), in this case, occurred equidistance to either side of the peak. The central frequency (f_{cen}) is defined and can be calculated by lower half-power frequency and upper half-power frequency as Eq. (6):

$$f_{cen} = \frac{1}{2}(f_{lo} + f_{hi}) \quad (6)$$

For symmetric spectrum, the peak-power frequency clearly equals and central frequency. The percentage bandwidth (B_{ndw}), expresses the breadth of the curve and can be calculated by Eq. (7):

$$B_{ndw} = \frac{(f_{hi} - f_{li})}{f_{cen}} \times 100 \quad (7)$$

and skewness (F_{sk}), may be represented by Eq. (8):

$$f_{sk} = \frac{(f_{pk} - f_{lo})}{(f_{hi} - f_{pk})} \quad (8)$$

From the spectrum of each meat specimen, changes in lower or upper half-power points on the spectrum (f_{lo} and f_{hi}) may reflect differences in muscle fiber tenderness. High frequency components are normally attenuated in muscle tissue and should be reflected by connective tissue and other components of beef muscle which would cause the spectrum to shift toward lower frequencies. This could be indicated by upper frequency component values as well as shifts in peak and central frequencies, bandwidth and skewness. In addition, local maxima, (discontinuity of the Fourier spectrum) were used as another parameter for measuring beef tenderness and other sensory attributes. Because the ultrasonic spectrum is affected by the elasticity and density of muscle tissue, tenderness may be predicted by measuring spectral parameters. Thus, the central (resonance) frequency could be used as an indicator of tenderness. Also, because attenuation and scattering of ultrasonic waves could occur in muscle tissue, bandwidth and skewness of spectra could change as a function of muscle fiber tenderness.

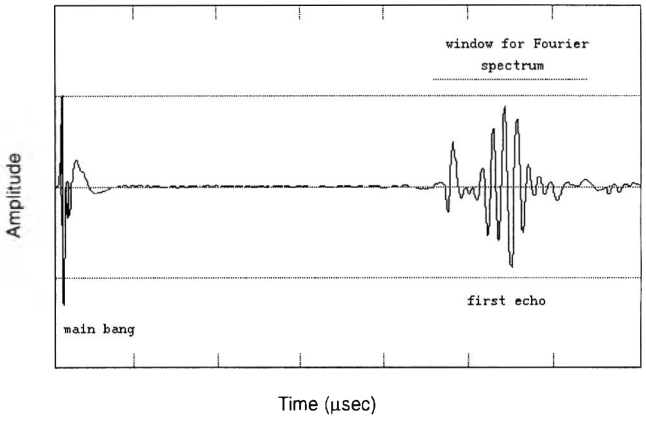

Fig. 3—A typical reflected ultrasonic signal through a meat sample (longissimus muscle; 2.298% fat, 72.57 moisture content, 30 mm thickness) scanned by 2.25-MHz shear probe. Time base = 3.8 μsec/div.

Materials

Beef carcasses (n=72) were obtained from Simmental-Angus crossbred steers and Angus-Hereford crossbred heifers that had been slaughtered at Rosenthal Meat Science and Technology Center at Texas A&M University. At 24 hr postmortem, beef carcasses were ribbed between the 12–13th rib interface by a cross-sectional cut to the longissimus muscle. Marbling score and carcass maturity were determined by a trained evaluator. Thirty minutes after ribbing, the marbling score was determined as described by USDA (1989). Carcasses were 9 to 30 mo of age (A maturity; USDA, 1989) and ranged in marbling score from Traces to Abundant (USDA, 1989). Loin steaks 3 cm thick were excised anterior to posterior from the longissimus muscle beginning at the 13th rib from the right side of each carcass. The first steak was used to determine ultrasonic spectral features (higher and lower frequency, central frequency, peak frequency, bandwidth, skewness, and number of local maxima) and the second steak was used for sensory panel evaluation. The steak identified for ultrasonic evaluation was used for establishing ultrasonic Fourier spectra.

Instrumentation and procedure

Panametrics (Waltham, MA) contact transducers with 1.27 cm diameter, 1-, 2.25-, and 5-MHz longitudinal and shear waves, were used for ultrasonic A-scan. An ultrasonic analyzer (Model 5052UA, Panametrics), which incorporates a broadband pulser/receiver and a stepless gated peak detector, was used to collect ultrasonic radio frequency (RF) signals. The signal from the ultrasonic analyzer was acquired through a PCTR-160 A/D board and processed by PCDAS software. The single board Personal Computer Transient Recorder (PCTR-160) combined an 8-bit, 20-MHz analog-to-digital converter, 4K bytes of high-speed buffer memory, and digital control logic. PCDAS is a high speed data acquisition and processing system designed for the IBM-PC compatible computer.

Ultrasonic measurements were performed using the normal incidence pulse-echo method. Each meat sample was scanned at 5 locations parallel and perpendicular to muscle fiber orientation (Fig. 2). Five locations were used to minimize effects of uneven marbling. Received RF signals throughout longitudinal and shear probes (Fig. 3) were recorded for spectral analysis.

Chemical analysis

Sarcomere length was determined using a Timbrell/Coulter Shearicon particle counter/size analyzer as described by Cross et al. (1981). Total collagen and its solubility were analyzed and calculated utilizing a hydroxyproline procedure described by Hill (1966) and Berman and Loxley (1963). Myofibrillar fragmentation index (MFI) was calculated according to the methods of Culler et al. (1978).

Sensory evaluation

A descriptive attribute panel technique (Cross et al., 1978) was used for meat sensory evaluation. The meat descriptive panel consisted of four steps—personal interview, screening, training, and performance

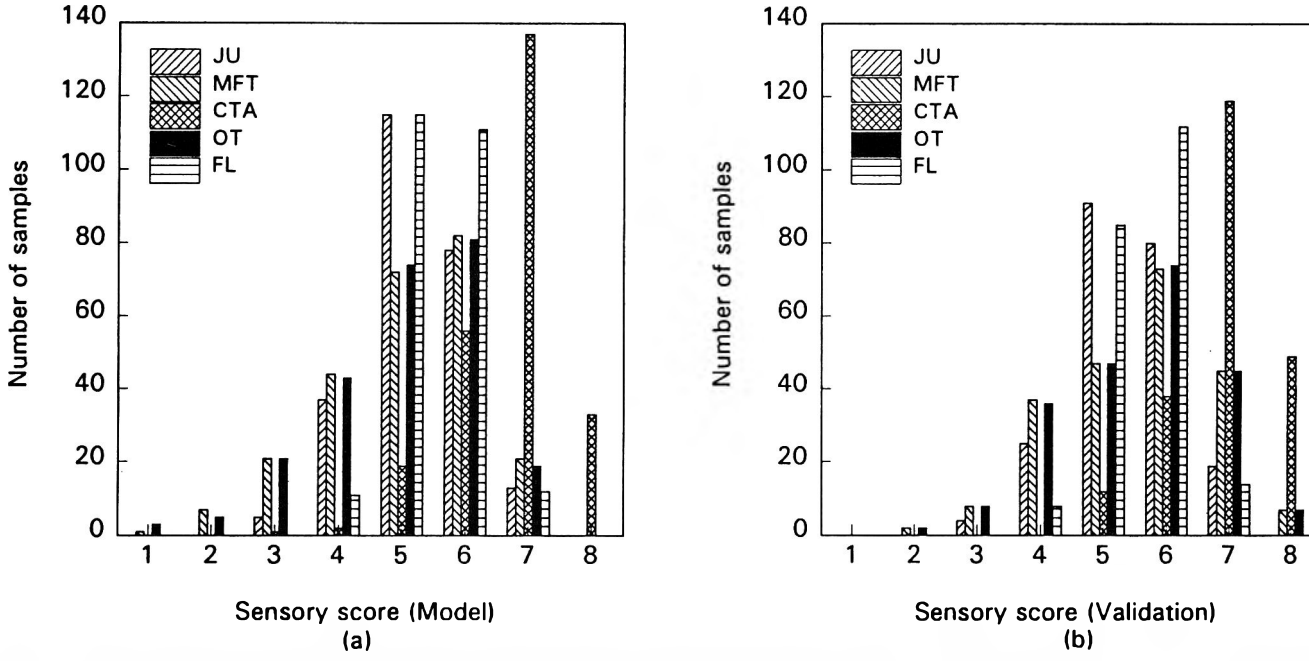

Fig. 4—Histogram of sample distribution based upon sensory scores: (a) Calibration (N=223) (b) Validation (N=222). (JU=juiciness; MFT=muscle fiber tenderness; CTA=connective tissue amount; OT=overall tenderness; FL=flavor intensity.

Table 1—Pearson's correlation coefficients of the most significant parameters to the sensory attributes (N=223)

Sensory attribute[a]	Frequency (Mhz)	Probe type	Correlation coefficient	Spectral feature
Juiciness	2.25	Shear	0.49	Local maxima
Muscle fiber tenderness	5.0	Shear	0.45	Central frequency
Connective tissue amount	1.0	Longitudinal	0.52	Local maxima
Overall tenderness	5.0	Shear	0.44	Central frequency
Flavor intensity	1.0	Longitudinal	0.39	Local maxima
Shear force mean[b]	1.0	Longitudinal	0.51	Local maxima
Percent total collagen	1.0	Shear	0.34	Local maxima
Percent soluble collagen	2.25	Shear	0.35	Bandwidth

[a] 8=extremely juicy, extremely tender, no connective tissue, extremely tender and extremely flavorable; 1=extremely dry, extremely tough, abundant amount of connective tissue, extremely tough and extremely unflavorable.
[b] Measured by Warner-Bratzler shear device.

evaluation. Panelists were trained for 4 mo and tested four times. Eight panelists were used. Steaks selected for sensory evaluation (25% of steaks selected) were cooked on a Farberware Open-Hearth Electric Grill (Model 350A, Kidde, Inc., Bronx, NY) to internal temperature 70°C. Copper-constantan thermocouples were placed in the geometric center of each steak and temperature was monitored (Omega Digital Thermometer, Model 871A, Omega Engineering, Inc., Stamford, CT). After cooking, exterior edges were removed and steaks were cut. The amount of sample a panelist placed in the mouth was standardized (1.0 cm × 1.0 cm × 1.0 cm sections per sample). Each steak was evaluated for juiciness, muscle fiber tenderness, overall tenderness, flavor intensity, and connective tissue amount using 8-point scales (8=extremely juicy, tender, flavorful or none; 1=extremely dry, tough, unflavorable or abundant, respectively).

Warner-Bratzler shear force measurement

Steaks for shear force determination were cooked as described and cooled for 2 hr at room temperature (≈23°C). Ten 1.3 cm diameter cores were taken from each steak parallel to the muscle fibers. The shear force for each steak was measured by Warner-Bratzler Shear Tester (Ockerman et al., 1981) and averaged.

Statistical analysis

A cross-validation method was used on regression models. This method split the data into two sets: one for calibration (n=223) and the other for validation (n=222). Splits of the data were made at random. The interactions between sensory attributes such as juiciness, muscle fiber tenderness, connective tissue amount, overall tenderness, flavor intensity and ultrasonic spectral parameters were evaluated from the Pearson's correlation coefficient using SAS (Statistical Analysis System, Inc., 1990). Comparison was considered significant at the $P < .05$ level. Multivariate linear regression analysis was used for modeling. The most appropriate regression model was selected by stepwise forward regression. Calibration performance of models was calculated in terms of the R^2 value and standard error of calibration (SEC), defined as

$$SEC = \sqrt{\frac{1}{N-1-t}\sum_{i=1}^{N}(Y_i-R_i)^2} \quad (9)$$

where Y_i and R_i are predicted and observed sensory attribute values, respectively. N is the number of samples and t is the number of parameters in the model. When applied to the palatability validation data set, the performance of the model was evaluated by standard error of prediction (SEP) defined as

$$SEP = \sqrt{\frac{1}{N-1}\sum_{i=1}^{N}[(Y_i-R_i)-\bar{D}]^2} \quad (10)$$

where $\bar{D}$ is the average of (Y_i-R_i), also referred to as bias between calibration data set and validation data set.

RESULTS & DISCUSSION

Sample distribution

Determining the exact number of animals needed for an experimental study of this type of research was practically impossible, because values for beef sensory attributes were

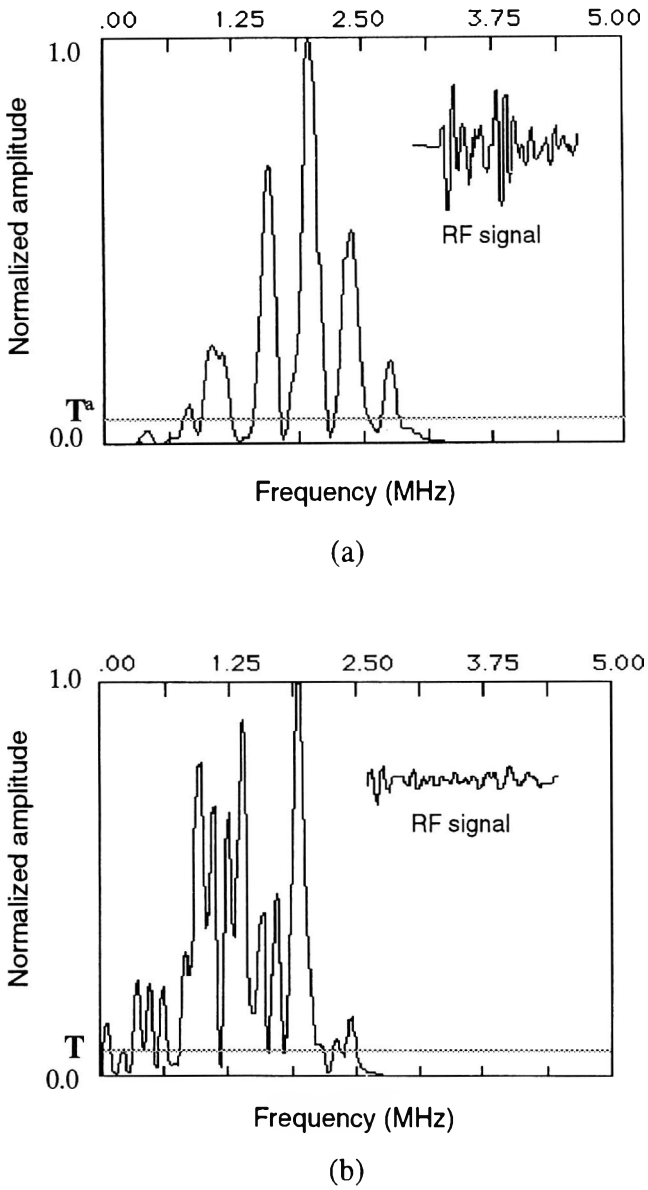

Fig. 5—Ultrasonic frequency spectra and their RF signal of meat samples (a) Slightly tender (Overall tenderness = 5; Shear force = 8.79kg) and (b) Very tender (Overall tenderness = 7; Shear force = 5.77kg). [a]T = Threshold for counting the number of local maxima.

assigned after each sample had been measured ultrasonically. The distribution of test animals was not uniform. A representative distribution for this study (Fig. 4) seems bell-shaped, skewed to the left, for both samples for models and for validation. This indicated the models developed for predicting palatability attributes may be more appropriate for meat with panel scores between 4 (slightly tough) and 7 (very tender) than for scores of <4 or >7. Sample distributions, both calibration and validation, were useful for this study because young (A maturity) animals represent the majority of beef consumed.

Ultrasonic spectral features for sensory attributes

Seven sensory attributes (juiciness, muscle fiber tenderness, connective tissue amount, overall tenderness, flavor intensity, percent total collagen, and percent soluble collagen) and instrumental shear force were compared initially through Pearson's correlation coefficients (Table 1). Among seven ultrasonic spectral features (lower frequency, upper frequency, central frequency, peak frequency, bandwidth, skewness, and number of local maxima), the most significant parameter in the frequency domain was the number of local maxima. Correlation coefficients were not high but the number of local maxima correlated most ($P < 0.05$) with juiciness ($\rho = 0.49$), connective tissue amount ($\rho = 0.52$), flavor intensity ($\rho = 0.39$), and percent total collagen ($\rho = 0.34$). The number of local maxima also correlated more with shear force ($\rho = 0.51$) than other spectral features. Whereas the central frequency, which represents a resonance frequency, correlated most ($P < 0.05$) with muscle fiber tenderness ($\rho = 0.45$) and with overall tenderness ($\rho = 0.44$). Additionally, bandwidth correlated ($P < 0.05$) with percent soluble collagen ($\rho = 0.35$).

The longitudinal type transducer was useful for connective tissue amount, flavor intensity, and shear force prediction, whereas the shear transducer provided information more consistently related to juiciness, muscle fiber tenderness, overall tenderness, and collagen. A shear probe with a 5-MHz frequency was useful for tenderness measurement. Because the higher frequency produced better resolutions, it could detect muscle fiber texture precisely. However, a 2.25-MHz shear probe was useful for both juiciness and percent soluble collagen measurement and a 1-MHz probe was useful for connective tissue amount, flavor intensity, and shear force measurement. High frequency could scan muscle texture with high resolution but, because normally high frequency components were attenuated in the muscle, the central (resonant) frequency of muscle was shifted to the lower frequency. As a result the connective tissue amount and shear force measurement correlated more with ultrasonic parameters at low frequency (1-MHz).

Local maxima

In biological tissues, ultrasonic wave propagation may be delayed due to heterogeneities. This time delay may be caused by acoustical impedance and/or density of muscle tissues, which could be responsible for meat tenderness. As described previously (Park and Whittaker, 1990), the number of local maxima seemed to be a function of not only marbling, but also texture of the muscle fiber, which has been related to tenderness (Joseph and Connolly, 1979). We found that overall tenderness increased and Warner-Bartzler shear force decreased with increasing numbers of local maxima (Fig. 5).

Parameter selection

Several spectral parameters correlated with palatability attributes but no single parameter was useful for prediction models with high accuracy. Because the interaction of ultrasonic characterization with biological tissue materials is complex, multivariate regression analysis was conducted to determine sensory attributes using spectral parameters. Multivariate linear regression analysis showed that partial linear regression models were useful for palatability prediction models because some spectral parameters have colinearity with each other that made the full model unstable. In development of the partial linear model, the number of local maxima and lower frequency were dominant parameters for predicting connective tissue amount. The standard error of calibration was 0.244, whereas three parameters were useful for juiciness (peak frequency, central frequency, and skewness of the ultrasonic spectrum) and for flavor intensity (number of local maxima, lower frequency, and skewness of the spectrum). In that case, the standard errors of calibration were 0.253 for juiciness and 0.224 for flavor intensity prediction. Tenderness prediction models including four spectral parameters were useful, i.e., the lower frequency, peak frequency, bandwidth, and skewness of the spectrum were optimum parameters for the tenderness prediction model.

Table 2—Determinant coefficients and corresponding variables for calibration (N=223)

Palatability attributes	R^2	SEC[a]	Parameters[b]	Probe type	Frequency (Mhz)
Juiciness	0.49	0.253	f_{pk}, f_{cen}, f_{sk}	Longitudinal	5.0
Muscle fiber tenderness	0.43	0.745	$f_{lo}, f_{pk}, B_{ndw}, f_{sk}$	Longitudinal	5.0
Connective tissue amount	0.64	0.245	Lm, f_{lo}	Longitudinal	1.0
Overall tenderness	0.43	0.754	$f_{lo}, f_{pk}, B_{ndw}, f_{sk}$	Longitudinal	5.0
Flavor intensity	0.49	0.224	Lm, f_{lo}, f_{sk}	Longitudinal	1.0

[a] Standard error for calibration.

[b] f_{pk}=peak pulse frequency of power spectrum (MHz); f_{cen}=central pulse frequency of power spectrum (MHz); f_{sk}=skewness of power spectrum; f_{lo}=frequency at low half power point on power spectrum (MHz); B_{ndw}=bandwidth, Lm=number of local maxima.

Table 3—Multivariate linear regression models for predicting palatability attributes (N=223)

MODEL: $PA = \beta_0 + \beta_1(Lm) + \beta_2(f_{lo}) + \beta_4(f_{pk}) + \beta_5(f_{cen}) + \beta_6(B_{ndw}) + \beta_7(f_{sk})$[a]

PA[b]	β_0	β_1	β_2	β_4	β_5	β_6	β_7	R^2
JU	3.6750	N/A	N/A	−8.1129	8.4466	N/A	0.8389	0.49
MFT	0.6109	N/A	13.9946	−12.1716	N/A	0.1053	0.3338	0.43
CTA	9.4982	−0.2957	−3.1626	N/A	N/A	N/A	N/A	0.64
OT	0.6168	N/A	14.4279	−12.5848	N/A	0.1067	0.3587	0.43
FL	4.8476	−0.2563	1.2951	N/A	N/A	N/A	0.2649	0.49

[a] Lm=number of local maxima; f_{lo}=frequency at low half power point on power spectrum; f_{hi}=frequency at upper half power point on power spectrum; f_{pk}=peak pulse frequency of power spectrum; f_{cen}=central pulse frequency of power spectrum; B_{ndw}=bandwidth; fskw=skewness of power spectrum.

[b] Palatability attributes; JU=juiciness; MFT=muscle fiber tenderness; CTA=connective tsissue amount; OT=overall tenderness; FL=flavor intensity.

Table 4—Validation of models for predicting palatability attributes (N=222)

Palatability attribute	SEP[a]	RMSD[b]	Bias[c]
Juiciness	0.5380	0.7173	0.0412
Muscle fiber tenderness	1.1317	1.2810	−0.5939
Connective tissue amount	0.4600	0.5085	−0.2138
Overall tenderness	1.1448	1.3157	−0.6394
Flavor intensity	0.3082	0.3126	0.0868

[a] Standard error for prediction.

[b] Root mean square error of difference between predicted and observed values.

[c] Mean of difference.

Standard errors of calibration for tenderness were 0.745 for muscle fiber tenderness and 0.754 for overall tenderness.

Determinant coefficients of each predictable sensory attribute were compared with their corresponding standard error of calibration (SEC) and dominant parameters for multivariate regression models (Table 2). Longitudinal probes were useful for all regression models for predicting sensory attributes. The ultrasonic spectral parameters of the higher frequency probe (5-MHz) were useful for predicting juiciness, muscle fiber tenderness, and overall tenderness. Spectral parameters from the 1-MHz probe were more useful for connective tissue amount and flavor intensity prediction.

Multivariate linear regression models

Through stepwise linear regression, the multivariate models for predicting sensory attributes were developed. As illustrated (Table 3) the highest determinant coefficient was .64 for connective tissue amount prediction. The juiciness was predictable with R^2=0.49. The determinant coefficients of models for tenderness prediction were .43 for predicting muscle fiber tenderness and 0.43 for overall tenderness. Flavor intensity was predictable with R^2=0.49.

Model validation

To validate the models, predicted palatability attribute values were calculated through the multilinear models developed, and those values were compared to measured values. To show the accuracy of each model, the standard error of prediction (SEP) was calculated and the standard error of difference between true values and predicted values was determined. The standard errors of prediction for each sensory attribute were 0.538 for juiciness, 0.460 for connective tissue amount, 1.131 for muscle fiber tenderness, 1.144 for overall tenderness, and 0.308 for flavor intensity.

To validate the true difference of prediction values of each sensory attribute from observed values numerically, the root mean square (RMS) errors of difference between observed and predicted values from the models were calculated from validation data (Table 4). The RMS error of the model for predicting flavor intensity was 0.312, the lowest of the models developed. The RMS errors of other attributes were 0.508 for connective tissue amount prediction model and 0.717 for juiciness prediction model. However, the RMS errors of tenderness prediction models were higher than other attributes 1.281 for muscle fiber tenderness and 1.316 for overall tenderness. The large RMS errors of tenderness prediction models could have been caused by the variability of spectral parameters. Since the ultrasonic beam propagation is sensitive to muscle fiber orientation, more consistent acoustical beam propagation measurement in the muscle should be considered. Another possible reason for high error for tenderness prediction is that no ultrasonic spectral parameter correlated highly with percent total collagen. The correlation coefficient was only 0.34. It may be that crosslinking of collagen is more important than total collagen. The meat samples were from longissimus muscle, so other major subprimals need to be measured for developing complete models.

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Soy Protein Isolate Antioxidant Effect on Lipid Peroxidation of Ground Beef and Microsomal Lipids

S. Y. WU and M. S. BREWER

ABSTRACT

Soy protein isolate antioxidant (SPIA) was separated from soy protein, fractionated, and purified. SPIA (300 or 900 ppm) was added to a ground beef model system; oxidation was initiated by adding Fe^{+2}/Fe^{+3}. Sealed vials were stored at 4°C for 24 hr. Oxidation products were assessed using the 2-thiobarbituric acid (TBA) test, sensory analysis, gas chromatographic separation, and mass spectrometric identification of headspace volatiles. SPIA (900 ppm) was also added to a beef microsomal protein/linoleic acid model system. Beef containing 900 ppm SPIA had lower TBA numbers, less rancid odor, hexanal, and total volatiles after 16 and 24 hr than did samples containing 300 ppm SPIA and controls.

Key Words: beef, microsomes, lipids, antioxidants, soy protein

INTRODUCTION

LIPID OXIDATION IS A PROBLEM in muscle foods when microbial spoilage is controlled by frozen storage of fresh meat and during refrigerated storage of cooked meat. Rate and degree of lipid oxidation is related to the tissues involved, postmortem carcass handling, and degree of cellular disruption (grinding). Dupuy et al. (1987) reported that subcutaneous fat produced about 50 volatile compounds during meat flavor deterioration (MFD), while intramuscular fat produced more than 200 volatiles. Lipid oxidation is more extensive when polyunsaturated glycerides are in bilayers such as cellular membranes (St. Angelo et al., 1987). In the presence of ADP-chelated iron, oxygen radicals are generated in the sarcoplasmic reticulum of muscle foods (Decker and Hultin, 1990). Muscles also contain notable amounts of iron, a known prooxidant (Hazell, 1982). Soluble chelates of low-molecular-weight (LMW) iron can activate oxygen and initiate lipid peroxidation, probably through production of hydroxyl radicals (HO•) from hydrogen peroxide (Decker and Hultin, 1992; Kanner, 1992; Spanier et al., 1992). Oxidizing lipids form hydroperoxides which are susceptible to further oxidation or decomposition to secondary reaction products, including aldehydes, ketones, acids, and alcohols. Aldehydes are major contributors to off-odor and off-flavor, and MFD because of their high reactivity, and their low flavor thresholds (Ullrich and Grosch, 1987; Simic et al., 1992). Ketones and alcohols have high flavor thresholds and are lesser causes of off-flavors (Min et al., 1979).

Exogenous antioxidants have been added to beef products to preserve quality (Faustman et al., 1989; Wheeler et al., 1990). Free radical scavengers appear to be most effective inhibitors of MFD; however, different substrates and systems have responded differently (St. Angelo et al., 1990). Phenolic antioxidants, butylated hydroxyanisole (BHA) and *tertiary*-butylhydroxyquinone (TBHQ), added to restructured beef-pork steaks, protected color and inhibited lipid oxidation (Chastain et al., 1982). Treating ground beef patties with metal chelators, free radical scavengers, rosemary, and sodium alginate has retarded lipid oxidation as assessed by chemical methods (Caldwell et al., 1959; Shahidi et al., 1987). Antioxidants extracted from soybean flour, concentrate, and isolate added to beef have decreased lipid oxidation (Pratt, 1972; Sato et al., 1973; Hammerschmidt and Pratt, 1978; Rhee et al., 1981). The antioxidant activity of aqueous extracts of flours, concentrates, or isolates of soybean may not be a reliable indicator of their antioxidative potential when used as extenders or as functional ingredients (Pratt, 1980; Ziprin et al., 1981). Water as an extraction medium was not very effective; when using methanol in place of water, the antioxidant activity of the extractant increased (Rhee et al., 1981). Some major phenolic compounds in soy flour, such as caffeic acid, rutin, and chlorogenic acid, are water-soluble (Pratt, 1980; Shewfelt and Hultin, 1983). Presumably, they could be extracted and concentrated to provide an antioxidant fraction. Plant flavonols (cinnamic acid derivatives), and flavonol glycosides (genistein and diadzein) are among the most effective phenolic antioxidants (Pratt, 1980).

Our objectives were to measure and identify volatiles in a beef tissue model system containing a soy protein isolate antioxidant (SPIA) fraction stored at 4°C; to study the effects of SPIA fractions on peroxidation of membrane fatty acids; and to evaluate relationships among 2-thiobarbituric acid (TBA) value, sensory analysis, and flavor volatiles.

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MATERIALS & METHODS

Ground beef—SPIA model system

About 3 kg of beef chuck (Select grade beef, 10 days postmortem) was coarse-ground through a 0.95-cm plate; fat content was standardized to 8±1% (AOAC, 1980). Beef was reground through a 0.64-cm plate. Three 1-kg batches were prepared for three replications of all analyses. A 3-g sample of ground beef was used for each TBA test, sensory evaluation, and volatile analysis.

One gram of soy protein isolate (PP 610, Protein Technologies International, St. Louis, MO) (SPI) was extracted with 50 mL distilled

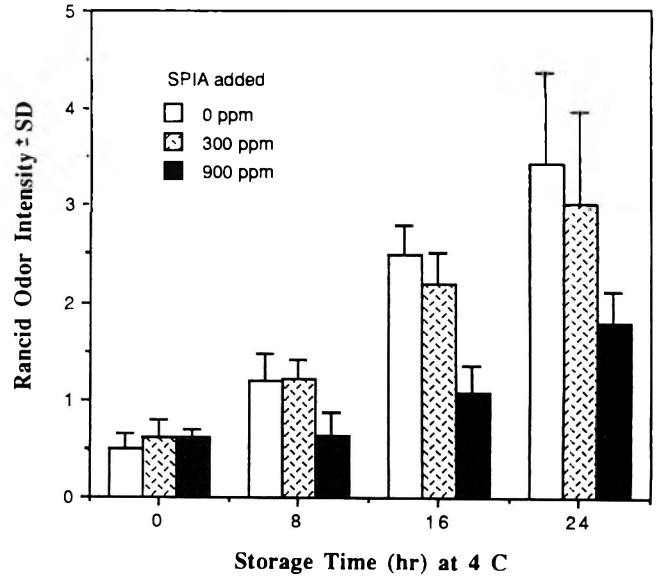

Fig. 1—Sensory Rancid odor scores of oxidizing ground beef as related to storage: 0 = none, 6 = intense.

water; pH was adjusted to 8 with 1N NaOH; extractant was centrifuged (490 × *g*, 10 min) and filtered (Whatman #1) to provide a clear filtrate. 1N HCl was added until a white precipitate began to form; then the sample was centrifuged (490 × *g*, 4 min). The pellet was collected and redissolved in 5 mL water. The preparation was then fractionated with a gel filtration column (20 mm × 200 mm, Sephadex G15, Fisher Scientific, Chicago, IL). Fractions were assessed for antioxidant activity as described by Wu and Brewer (1993). Aliquots (1 mL) of 10 pooled fractions were purified on a TLC plate (Uniplate, silica gel G, Analtech, Inc., Newark, DE) using butanol:acetic acid:water (10:3:7, v/v/v) as mobile phase solvent. The developed band with antioxidative activity (a fluorescent spot under long UV light) was scraped from the TLC plate and tested for antioxidant activity. Multiple plates were run until sufficient SPIA was collected for addition to the model system. SPIA, in water solution, was concentrated to 0.4% solids (w/v) by air-drying at room temperature (≈23°C).

Ground beef (3g) was placed in a 20-mL glass serum vial and SPIA solution was added to a final concentration of 300 ppm or 900 ppm (SPIA solid wt/total wt) for SPIA-treated samples; deionized water was added to controls. Oxidation was initiated by adding 0.1 mL of Fe^{2+}/Fe^{3+} (0.5 mM/0.5 mM) in 3 mL water. Vials were sealed with Teflon-lined caps and stored at 4°C.

Analyses

Vials were opened at 8-hr intervals and tested by the TBA test (Witte et al., 1970). Samples for GC analyses were prepared as described for TBA analysis. Vials were sealed and stored at 4°C; 3 mL of headspace gas was analyzed at 8-hr intervals. The vials were heated at 80°C for 3 min immediately prior to headspace sampling. Headspace gas was withdrawn from each vial only once. Volatiles were separated with a gas chromatograph (Hewlett Packard Model 5840, Hewlett Packard Co., Avondale, PA) equipped with a flame ionization detector (FID) using a Durabond (DB-5, 60m × 0.32 mm (i.d.) × 1 μm film) fused silica column (Supelco, Inc., Bellefonte, PA) and He as carrier gas. Operating conditions during analyses were: injector temperature, 200°C; splitless injection of 3 mL headspace gas; column pressure, 1.26 kg/cm²; temperature program starting at −20°C (4 min), increasing 5°C/min for 30 min, increasing 8°C/min to a final temperature of 280°C. Detector temperature was 300°C. Flow rates of FID gases were: air, 240 mL/min; hydrogen, 30 mL/min.

GC conditions for GC/MS were identical to those previously described except the column head pressure was 700 g/cm² He. Electron impact ionization was employed with the ion source set at 70 eV and source temperature 200°C. Scanning range was 20–200 M/Z. Identification of unknowns was by comparison of spectra with published data (NBS and Wiley Mass Spectral Libraries, Hewlett Packard Co., Avondale, PA) and by matching peak spectra of authentic compounds (Sigma Chemical Co., St. Louis, MO; Aldrich Chemical Co., Milwaukee, WI) based on retention times.

Microsomal model system

Basic assay system (BAS) containing 0.1 mg/mL beef microsomal protein was prepared as described by Slabyj and Hultin (1983) to contain 0.1 mM ADP, 0.1 mM NADH, and 0.025 mM Fe^{3+}. To 5 mL BAS, 0.02% linoleic acid in distilled water and 900 ppm (w/v) SPIA were added in vials; control samples contained distilled water in place of SPIA. Solutions were held at room temperature (≈23°C) for 30 min before sealing the vials. They were then stored at −20°C for 3 days prior to headspace gas analysis. Headspace gas (3 mL) was removed from the vial immediately after warming at 80°C for 3 min [to volatilize lipid peroxidation (LPO) products]. Headspace gas was analyzed using GC/MS.

Sensory analysis

Sensory analysis of volatiles was carried out by a six-member trained panel. Members were trained during three 1-hr periods using meat samples oxidized with iron for rancid meat-type odors and meat samples with water-diluted standards (hexanal, 200 ppb; *t*-2-octenal, 300 ppb; pentanal, 50 ppb; Aldrich Chemical Co., Milwaukee, WI) known to cause characteristic odors as outlined by Dupuy et al. (1976) and Jellinek (1985). A standard containing fresh ground beef with added hexanal (55 ppm), *t*-2-octenal (55 ppm), and pentanal (55 ppm) was dispensed (3g) into serum vials (20 mL), which were capped and stored at 4°C for 12 hr. A new standard was prepared for each evaluation session. Such standards were designated a rancid odor score of 6.

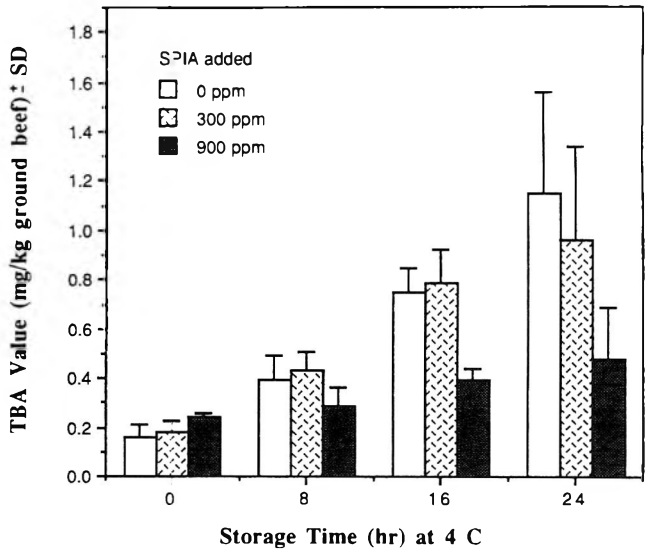

Fig. 2—TBA values of oxidizing ground beef as related to storage.

Table 1—Summary of probabilities of F for selected lipid peroxidation indicators in a ground beef model system

	Time effect[a] p-value	Treatment effect[b] p-value	Time × treat interaction[c] p-value
Undecane	0.001	0.021	0.078
t-2-Hexenal	0.042	0.008	0.140
Hexanal	0.001	0.001	0.009
t,t-2,4-Decadienal	0.001	0.231	0.677
Total volatiles[d]	0.001	0.001	0.001
TBA number	0.001	0.030	0.227
Sensory analyses	0.001	0.005	0.011

[a] Storage time = 0, 8, 16, and 24 hr.
[b] Treatment = 0, 300, or 900 ppm soy protein isolate antioxidant (SPIA).
[c] Time × treat = (0, 8, 16, and 24 hr) × (0, 300, and 900 SPIA).
[d] Total volatiles is the sum of the area count of all peaks.

Samples for sensory analyses were prepared as described for TBA analysis. Samples were coded with three-digit numbers and presented to evaluators in random order under incandescent light in sensory booths. Sensory samples were evaluated at 22°C, 55% relative humidity with positive air pressure. Panelists removed caps from vials, smelled samples, and scored each using a 15-cm line scale end-anchored with 0 = "none" and 6 = "intense" score for intensity of rancid odor at room temperature. Each sample was evaluated in duplicate by each panel member on a total of six separate occasions.

Data were subjected to two-way analysis of variance for main effects (SPIA treatment and time) and interactions. Means for significant ($p<0.05$) effects were separated using the Least Significant Difference (LSD) multiple range test (Steele and Torrie, 1980). Correlation coefficients were calculated for all pair-wise combinations of dependent variables using statistics software (StatView 512+ version 1.2, BrainPower, Inc., Calabasas, CA).

RESULTS & DISCUSSION

INTENSITY OF RANCID ODOR OF GROUND BEEF increased during 24-hr storage in which peroxidation was catalyzed by Fe^{2+}/Fe^{3+} ions (Fig. 1). After 16 hr incubation, rancid odor was more intense ($p<0.05$) in the control and 300 ppm samples than at 0 hr; rancid odor did not change during 24-hr incubation in the 900 ppm SPIA samples. Suppression of LPO probably was related to ratios among SPIA, Fe^{2+}/Fe^{3+} ions and ground beef lipids. SPIA at 300 ppm did not inhibit LPO while 900 ppm did, suggesting that inhibition was altered when the SPIA:substrate or SPIA:oxidation initiator ratio changed.

Table 2—Correlation coefficient matrix for selected indicators of lipid peroxidation in a ground beef model system

	TBA	Sensory	Total v.[a]	Hexanal	*t*-2-Hex	Decadienal	Undecane	Nonane
TBA	1.00							
Sensory	0.93*	1.00						
Total v.[a]	0.83	0.89	1.00					
Hexanal	0.88	0.89	0.92	1.00				
t-2-Hexenal	0.70	0.69	0.61	0.74	1.00			
Decadienal	0.86	0.81	0.76	0.81	0.68	1.00		
Undecane	0.79	0.84	0.73	0.74	0.80	0.68	1.00	
Nonane	0.85	0.81	0.71	0.77	0.64	0.87	0.68	1.00

* r values significant at $p<0.05$.
[a] Total v. = total volatiles.

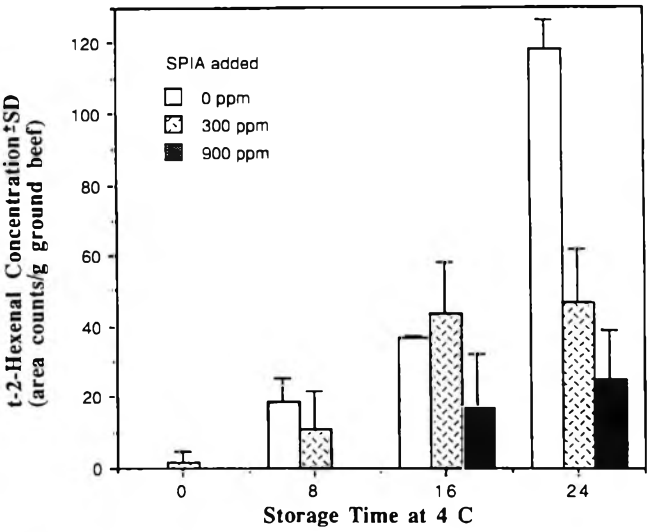

Fig. 3—*t*-2-Hexenal in oxidizing ground beef as related to storage.

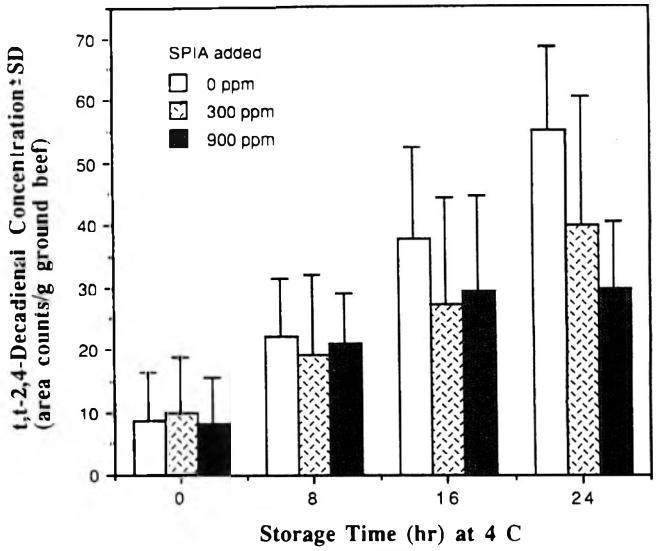

Fig. 4—*t,t*-2,4-Decadienal in oxidizing ground beef as related to storage.

Table 3—Hexanal concentration (area counts/g ground beef) from oxidized ground beef containing soy protein isolate antioxidant (SPIA)[a]

	Storage time (hr) at 4°C			
SPIA added	0	8	16	24
Control (0 ppm)	$78.0^{c} \pm 29.9$	$114.2^{c} \pm 41.7$	$219.6^{b} \pm 43.1$	$377.8^{d} \pm 44.6$
300 ppm	$33.8^{c} \pm 13.3$	$118.5^{c} \pm 37.1$	$198.7^{b} \pm 48.2$	$328.1^{d} \pm 33.2$
900 ppm	$19.2^{c} \pm 5.1$	$41.0^{c} \pm 9.7$	$54.0^{c} \pm 10.4$	$76.3^{c} \pm 20.1$

[a] Means are calculated based on three replications ± standard error.
[b,c,d] Means with different superscript letters are different ($p<0.05$).

Similar effects were observed for production of TBA-reactive substances (Fig. 2). The TBA values were higher in controls and samples treated with 300 ppm SPIA than in those containing 900 ppm SPIA after 16 and 24 hr. The difference in TBA values at zero time was smaller than differences in sensory scores. This may have been because the TBA-reactive substances are secondary lipid peroxidation products while some of the primary oxidation products were directly perceptible as "rancid odor."

By GC/MS, several compounds were identified in headspace gases of the oxidizing ground beef model system. These included pentane, hexane, heptane, octane, undecane, 2-pentanone, 3-pentanone, 2-pentylfuran, pentanal, hexanal, *t*-2-pentenal, *t*-2-hexenal, *t*-2-octenal, *t,t*-2,4-decadienal, propanol, 1-pentanol, hexanol, heptanol, and 1-octen-3-ol (data not shown). These volatiles have been consistently reported in oxidized beef (Grosch et al., 1992). Five compounds found consistently and associated with the treatment were *t*-2-hexenal, hexanal, undecane, *t,t*-2,4-decadienal, and nonane (Table 1). Storage time was related to ($p<0.05$) all characteristics except *t*-2-hexenal; SPIA treatment significantly ($p<0.05$) affected all characteristics except *t,t*-2,4-decadienal. Area counts of these compounds were closely related to SPIA level and to storage time (Tables 2 and 3; Fig. 1 through 6). As SPIA increased, area counts decreased and the longer the storage time, the greater their area counts. Among these volatiles, *t*-2-hexenal, hexanal, and *t,t*-2,4-decadienal have been commonly generated in cooked foods with MFD (St. Angelo et al., 1987; Drumm and Spanier, 1991; Lee et al., 1991), oxidized animal lipids (Brewer et al., 1992), and oxidized fatty acid model systems (Frankel et al., 1981). Their presence can be explained by the ubiquitous distribution of the precursor fatty acids (linoleic and linolenic acid) in tissues and their characteristic decomposition reactions. The double bonds in an unsaturated fatty acid are locked into position when oxygen reacts with the methylene group adjacent to the double bonds. Hydroperoxides form, then decompose to specific end products (Frankel, 1985).

Lipid peroxidation measured by TBA, sensory analysis, and headspace volatiles, changed ($p<0.05$) as storage time increased (Table 1). The SPIA treatment effect was most apparent with hexanal or total volatiles as the indicator, followed by sensory evaluation, *t*-2-hexenal, undecane, and TBA value. Although *t,t*-2,4-decadienal is a common lipid peroxidation product, it was not affected ($p>0.05$) by SPIA while hexanal and total volatiles were (Table 1).

Hexanal and total volatiles correlated highly with sensory evaluations of rancid odor and TBA value (Table 2), confirming findings of Dupuy et al. (1976), St. Angelo et al. (1987, 1990), and Spanier et al. (1992). Hexanal increased over storage for control and 300 ppm SPIA-treated beef but did not change (from 0 hr to 24 hr) for 900 ppm SPIA-treated sample (Table 3). Hexanal is produced from the breakdown of 13-hydroperoxide fatty acids, including linoleic, linolenic, and arachidonic acids (Moriarity, 1987; Simic and Taylor, 1987). *t*-2-Hexenal was essentially absent at 0 hr storage in all samples, but appeared after 8 hr in the control in 300 ppm SPIA

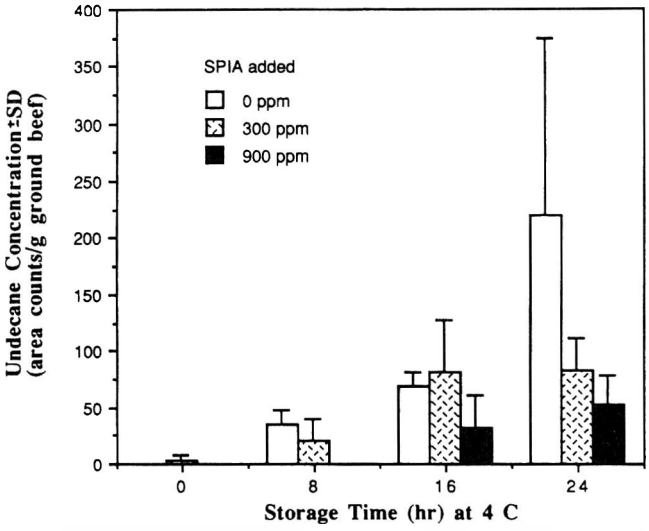

Fig. 5—Undecane in oxidizing ground beef as related to storage.

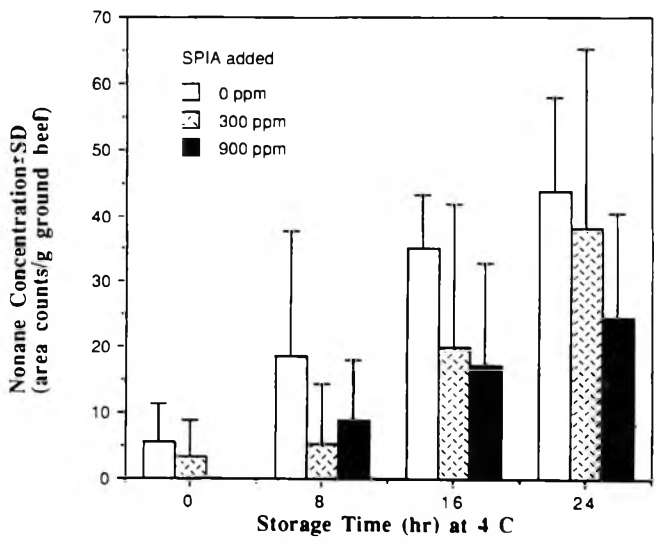

Fig. 6—Nonane in oxidizing ground beef as related to storage.

Table 4—Effect of soy protein isolate antioxidant (SPIA) on total volatile area counts of ground beef[a]

SPIA added	Storage time (hr) at 4°C			
	0	8	16	24
Control (0 ppm)	811.2[d] ± 126.8	1215.9[c] ± 208.1	2808.3[b] ± 278.5	5416.8[e] ± 235.0
300 ppm	786.2[d] ± 17.2	1018.9[c,d] ± 186.2	2502.4[b] ± 428.5	4886.4[e] ± 576.4
900 ppm	728.1[d] ± 107.7	882.0[c,d] ± 264.8	1206.2[c] ± 160.2	1496.6[c] ± 163.8

[a] Means of total area counts are calculated based on three replications ± standard error.
[b-e] Means with different superscript letters are different ($p<0.05$).

Table 5—Effect of soy protein isolate antioxidant (SPIA) on microsomal enzymatic oxidation of beef lipids[a]

	Control		SPIA (900 ppm)[b]	
Total volatiles[c]	20148.0 ± 238.7		5932.0 ± 117.0	
Hexanal	3864.0 ± 108	(19.18)[d]	124.0 ± 33.5	(20.90)
Decadienal[b]	38.0 ± 1.4	(0.19)	31.0 ± 3.1	(0.52)
Nonane[b]	120.0 ± 7.1	(5.96)	314.0 ± 12.7	(5.29)
Undecane[b]	46.0 ± 3.6	(0.27)	85.0 ± 3.2	(1.43)
t-2-Hexenal[b]	47.0 ± 4.0	(0.23)	36.0 ± 2.9	(0.61)
Number of volatiles	53.0 ± 1.7		38.0 ± 2.9	

[a] Data reported were for samples incubated 30 min at 22°C.
[b] All means in this column are significantly different ($p<0.05$) from those in the control column.
[c] Area counts based on three replications ± standard error.
[d] Percent of total volatile area counts.

samples, and after 16 hr in the 900 ppm SPIA samples (Fig. 3). *t*-2-Hexenal content of control samples increased rapidly between 16 and 24 hr while remaining constant in 300 and 900 ppm SPIA samples. *t,t*-2,4-Decadienal was in all samples at 0 hr (Fig. 4). Concentration increases of decadienal occurred over time with significant variability among samples as evidenced by high standard deviations. Significant differences due to time × treatment effects on decadienal concentration did not occur until samples had been held for 24 hr. Undecane concentration was essentially 0 until 8 hr in control and 300 ppm SPIA samples, and until 16 hr in 900 ppm SPIA samples (Fig. 5). Nonane concentrations tended to increase with storage time, but standard deviations within treatment groups were high (Fig. 6). Concentration of total volatiles was the same for all treatments at the beginning of storage (0 hr). After 16 hr, control and 300 ppm SPIA had higher ($p<0.05$) total volatiles than did 900 ppm SPIA samples (Table 4). After 24 hr, control and 300 ppm SPIA samples contained three times the total volatile concentrations of 900 ppm SPIA samples. Total volatiles followed a pattern similar to that for hexanal. The high reactivity of aldehydes, ketones, and alcohols could change the profiles of volatiles due to fragmentation, condensation, and esterification. The highest correlations with lipid oxidation occurred between hexanal and total volatiles, between sensory scores and hexanal or total volatiles, between TBA and hexanal, and between TBA value and sensory scores (Table 2).

The response of the beef microsomal LPO system to SPIA (Table 5) resulted in more than a three-fold reduction in total volatiles. The NADH-dependent enzymatic LPO system in the sarcoplasmic reticulum can catalyze LPO in the membrane lipids as well as the surrounding lipid compartment in the presence of NADH and iron (Rhee, 1988). The effect of SPIA on microsomal LPO may be different from that on ground beef LPO because more polyunsaturated fatty acids are in the membrane phospholipids than in the cytosol neutral lipids. Also, probably some localization occurred of activated oxygen species and lipid radicals generated in the membrane's lipophilic zone that would reduce effectiveness of SPIA. The oxidation by hydroxyl radical (HO•) is site-specific; the radical will attack at the site of its formation (Kanner, 1992). Site-specific reactions are not effectively inhibited by HO• scavengers, superoxide dismutase, or catalase (Kanner, 1992).

The SPIA treatment effect was significant ($p<0.05$) on total volatile area counts and numbers of volatiles generated. Control samples contained a greater number of different volatiles than did samples with SPIA. More lipids would be in the enriched membrane fraction than when diluted with whole cell constituents. A greater number of different volatiles were detected in the microsomal LPO system than in the ground beef system, possibly due to enhanced exposure of membranes to the oxidizing environment without the buffering effect of cellular protein. Although numbers and patterns of volatiles were different in the microsomal system from the ground beef lipid system, common LPO indicators such as hexanal, *t,t*-2,4-decadienal, and *t*-2-hexenal were present in both systems.

Volatiles generated during LPO can be influenced by several factors. Maruri and Larick (1992) reported that high-molecular-weight compounds such as heptane, undecane, dodecane, tridecane, tetradecane, pentadecane, hexadecane, and octadecane correlated well with beef flavor. Generated volatiles are related to the way LPO is induced. In the presence of metals, homolytic cleavage of the oxygen-oxygen bond of peroxy radicals results in formation of alkoxy and hydroxy radicals. Carbon-carbon cleavage of alkoxy radicals produces short-chain aldehydes, ketones, alcohols, hydrocarbons, esters, furans, and lactones (Frankel, 1984, 1985; Moriarity, 1987). The presence

of aldehydes has been attributed to thermal decomposition of monohydroperoxides hypothesized to be initial products of thermally oxidized fats. In addition, amounts of volatiles isolated and identified are related to vapor pressure affinity for the stationary phase (or traps), and analysis method (static headspace, sparging or purge-and-trap) (Vercellotti et al., 1992). We used Fe^{2+}/Fe^{3+}-induced LPO and headspace sampling. Results could be different with other techniques (Spanier et al., 1992; Vercellotti et al., 1992). Low-molecular-weight iron complexed with organic phosphates, inorganic phosphates, amino acids, or carboxylic acids can activate oxygen initiating lipid peroxidation through the hydroxyl radical. The rate of iron-catalyzed lipid oxidation in muscle foods is related to both the concentration and activity of iron and reducing systems which can promote formation of ferrous ions (Decker and Hultin, 1992; Kanner, 1992). The distribution of iron (heme or low-molecular-weight) is affected by storage and processing conditions (Kanner, 1992).

The volatile profiles, as well as total volatiles are expected to change during LPO with addition of an antioxidant, such as SPIA fractions. If the ratio of volatiles (e.g., hexanal:methyl 5-oxopentanoate:2,4-decadienal) formed without antioxidant were the same as with antioxidant, then the antioxidant did not notably alter the pathway(s) leading to formation of the major decomposition products. At 0 hr storage, hexanal amounted to 9.6%, 4.4%, and 2.5% of total volatiles in control, 300 ppm- and 900 ppm-containing samples, respectively. After 24 hr, hexanal was 6.8%, 6.8%, and 5.2% of total volatiles in respective samples. In the microsomal system, individual volatiles as percentage of total volatiles in headspace were relatively constant (Table 5).

The mechanism of action of SPIA prevention of beef lipid LPO is unclear. However, the major phenolic compounds identified in soy flour, including quercetin, caffeic acid, rutin, and chlorogenic acid (IV), may act as free radical scavengers by reacting with unpaired electrons from lipid molecules or oxygen species, or as chelators by complexing with ions such as Fe^{2+} and Fe^{3+} (Klausner et al., 1979; Shewfelt and Hultin, 1983). If free radical scavenging is the mode of action of SPIA they would probably be less effective in suppressing microsomal lipid oxidation than LPO in beef due to site specificity (Kanner, 1992). The water extract of soy protein isolate can be concentrated by chromatographic methods based on molecular weight and polarity. This concentrated fraction was an effective antioxidant under limited storage times.

CONCLUSIONS

GEL FILTRATION-SEPARATED WATER EXTRACTS of soybean protein isolate were antioxidative in linoleic acid emulsions, microsomal lipids, and ground beef lipids subjected to iron-induced oxidation as measured by sensory methods, TBA, GC/MS-detected volatiles, and total volatiles profile. Oxidation of meat fat correlated with sensory rancid odor, TBA, and GC/MS-detected volatiles such as hexanal, *t*-2-hexenal, and *t,t*-2,4-decadienal. High correlations occurred between LPO indicators and TBA.

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—Continued on page 738

Hot Processed Raw Materials and Fat Level Affect Physical and Sensory Characteristics of Ground Beef

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ABSTRACT

Sixteen treatment combinations of ground beef were evaluated (two lean types, four fat types, and two fat levels) to determine the characteristics of ground beef produced from hot fat and prerigor lean (HL). Half of each batch was immediately made into patties and the remaining chub pack stored (2°C). Fat type had no ($P>0.05$) effect on appearance or sensory characteristics of patties; however, all prerigor fat treatments and HL reduced ($P<0.05$) cooking loss. Fat smearing was greater ($P<0.05$) in HL patties, but no ($P>0.05$) difference was detected after chub pack storing (2°C). The HL improved ($P<0.05$) tenderness in stored ground beef.

Key Words: beef, fat content, color, appearance, flavor

INTRODUCTION

U.S. RETAILERS have reduced fat trim levels on marketed beef cuts to <6.4 mm (Savell et al., 1991). This has increased the demand by meat packers for leaner subprimals. An efficient method for producing closer trimmed subprimals is to remove excess fat on the slaughter floor (commonly referred to as hot fat trimming). Since USDA yield and quality grades are uncoupled (USDA, 1989), packers can now implement hot fat trimming and still quality grade carcasses. Hot fat trimming, however, results in an abundance of hot fat (prerigor fat). A typical yield grade 3 steer carcass would yield ≈12% of its hot carcass weight as hot fat (Savell et al., 1989; Ahmed et al., 1992). That would be 41 kg of hot fat for a 341 kg typical yield grade 3 carcass. For a processor slaughtering 5000 hd/d, 205,000 kg of hot fat would be produced/day introducing a fat-disposal problem.

An alternative to rendering prerigor fat (hot fat) that would add value to the raw material, would be to include it in a ground meat system. Prerigor lean raw materials have long been shown to improve tenderness, juiciness, cooking losses, and increase pH in ground beef (Cross et al., 1979; Jacobs and Sebranek, 1980; Cross and Tennet, 1981; Berry and Leddy, 1988). However, little information is available on the addition of prerigor fat or "hot fat" alone to ground beef. The use of prerigor pork fat has been investigated in further processed meat products. Bentley et al. (1988) reported luncheon loaves produced with hot boned pork fat had higher cook yields and lower moisture and fat loss than those with cold boned pork fat. If adding prerigor beef fat to ground beef would provide similar improvements in physical and sensory properties, then implementing hot fat trimming systems would be more feasible. Our objective was to evaluate the acceptability of ground beef produced from prerigor fat and lean raw materials at two different fat levels.

MATERIALS & METHODS

Design

Sixteen 4.5 kg batches of ground beef were produced in a 2 (lean type) × 4 (fat type) × 2 (fat level) factorial arrangement with two replicates using the following treatments: Prerigor (hot) lean (HL); Postrigor (control) lean (CL); Prerigor (hot) fat (HF); Postrigor (control) fat (CF); Chilled prerigor fat (ChF); Equal mixture of HF and CF (Mix); 10% fat level; and 20% fat level. The study was duplicated only once due to limited availability of live fed cattle for hot lean and fat source.

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Ground beef production

Lean and fat for CL and CF treatments were obtained from 4 USDA Choice IMPS 126A, 2-Piece Chucks, Boneless (NAMP, 1988). The HL and HF were obtained from the chuck region and subcutaneous depot, respectively, of crossbred steers immediately after slaughter (within 30 min of exsanguination, no electrical stimulation). The HL and HF were kept in insulated coolers to maintain temperatures until further processing (within 1 hr after removal). All lean and fat portions were separately ground (1.3 cm plate) and the lean tissue sampled for lipid determination using modified Babcock procedures (sulfuric acid and centrifugation) (Kelly et al., 1954). A sample of HF was ground (1.3 cm plate) and stored at 2°C for 24 hr for use as chilled prerigor fat. Appropriate amounts of each lean and fat type were determined for formulating each batch to the targeted fat percentages of 10 and 20% (Pearson and Tauber, 1984). Each 4.5 kg batch was hand mixed for 1 min, ground through a 0.6 cm plate, and temperature of the ground product was measured immediately after grinding with a hand held thermistor digital thermometer (Model TM99A-H, Hantover, Kansas City, MO). The 0.6 cm plate was selected over a smaller plate to help reduce fat smearing. After grinding, half of each batch was made into 113.6g patties using a Hollymatic Patty Machine (Hollymatic Corporation, Park Forest, IL), visually scored, blast frozen (−30°C) for 30 min, vacuum packaged in Cryovac® B620 barrier bags (Cryovac Division of W.R. Grace & Co, Duncan, SC) and frozen (−20°C) for later analysis. The remaining half was vacuum stuffed (Vemag Robot 500 type 128, Robert Reiser Co, Canton, MA) into E-Z-PAK® polyethylene meat bags (2 mil opaque white, Koch, Kansas City, MO), ≈2.2 kg, boxed and stored at 2°C for 7 days. After storage, chubs for each batch were made into patties and handled as described for fresh patties. Weights on all chub pack ground beef were collected prior to and after storage for purge determination. Purge was determined as the difference between weight of fresh chubs, minus bag weight, and stored ground beef (purge removed) weight, minus bag weight, divided by the original fresh chub weight expressed as a percentage.

Physical and proximate analysis

Prior to freezing, all patties were visually scored for fat smearing and color by trained personnel using a 4 point scale (fat smearing: 1=extreme, 2=moderate, 3=slight, 4=none; and color: 1=extremely light red, 2=slightly light red, 3=slightly red, 4=extremely red). Personnel were trained during a preliminary run of the experiment, which produced patties varying widely in fat smearing and color. After the trial run, personnel were comfortable with the scales and were within ±1 of raw mean scores.

Three raw patties from each treatment combination/storage time were thawed at 2°C overnight and homogenized in a Hamilton Beach Model 702W food processor (Hamilton Beach, Washington, NC). Moisture (oven drying method) and protein (Kjeldahl) were determined using AOAC (1990) procedures. Total lipid was determined by a modified method of Folch et al. (1957). The procedure was modified to accommodate lipid determination of fresh (wet) tissue instead of dried tissue. For each sample, 2.5g were homogenized in 15 mL of chloroform:methanol (1:2) and allowed to stand for 1 hr. Five mL each of chloroform and 1 M KCL were added, sample was then vortexed, incubated in an ice bath for 5 min, and centrifuged for 10 min at 2000 rpm. The final 1:1 chloroform to methanol ratio provided a more distinct biphasic layer for more accurate separation. The aqueous phase was aspirated off, the lipid phase transferred to aluminum pans, and the pellet discarded. Samples were evaporated to dryness at room temperature (≈23°C) overnight and then oven dried at 90°C for 15 min.

Table 1—Effects of fat and lean type on initial ground beef temperature

Effect[a]	Temperature, °C
Lean type	
HL	27.2[b]
CL	11.4[c]
SEM[e]	0.3
Fat type	
HF	19.9[b]
Mix	19.7[b]
CF	17.7[c]
ChF	19.8[b]
SEM[e]	0.4
Fat by lean type	
HF, HL	27.5[b]
HF, CL	12.4[c]
Mix, HL	26.8[b]
Mix, CL	12.6[c]
CF, HL	26.3[b]
CF, CL	9.2[d]
ChF, HL	28.2[b]
ChF, CL	11.4[c]
SEM[e]	0.6

[a] See text for description of variables.
[b,c,d] Means in the same column within an effect differ (P<0.05).
[e] Standard error of the mean.

Table 2—Proximate analysis of ground beef with different fat levels

	Fat level		
Item	10, %	20, %	SEM[a]
Fat	11.5[b]	18.8[c]	0.38
Moisture	67.9[b]	61.8[c]	0.35
Protein	19.6[b]	17.9[c]	0.18

[a] Standard error of the mean.
[b,c] Means in the same row differ (P<0.05).

Finally, samples were cooled at room temperature in a desiccator for 10 min and reweighed for lipid determination. All moisture determinations were performed in triplicate and fat and protein determinations in duplicate.

Sensory analysis

Patties from each batch and storage time were thawed overnight at 2°C and cooked on Farberware® open-hearth broilers (Farberware Company, Bronx, NY). Patties were cooked to 50°C on one side then turned and cooked to a final temperature of 80°C. That temperature was selected because it approximated the temperature at which patties were "well-done" displaying no pink coloration. The point at which no internal pink color was detected was chosen as the best method to determine a constant end point degree of doneness. Temperature was monitored with a hand held thermistor digital thermometer (Model TM99A-H, Hantover, Kansas City, MO). After cooking, patties were cut into 1.27 cm^2 samples and served warm to a six member trained descriptive textural attribute panel. Panelists were selected according to the methods of Cross et al. (1978). Training consisted of 5 sessions where panelists were served 6 samples from a wide array of treatments exposing them to a wide range of attribute scores (Civille and Szczesniak, 1973; Lyon et al., 1980). Training ended when panelists were comfortable with the scoring and each panelist was within ±1 score of the raw mean score. Panelists sat in isolated booths with green fluorescent lighting free from distractions. Members were served 8 samples/session, had one session/day and were instructed to cleanse their palate between samples with unsalted crackers, apple juice and room temperature water. Patties were scored for the attributes of springiness, cohesiveness, texture, and beef flavor, using an 8 point scale with; 8 = extremely springy, cohesive, coarse texture and flavorful, 1 = extremely nonspringy, noncohesive, fine texture, and unflavorful. Panelists also noted defects for patties being too tough, mushy, fine or having off-flavors. Panel members did not train for specific off-flavors, but were instructed to indicate if off-flavor was detected. Patties were weighed prior to and after cooking for calculation of cooking loss.

Data analysis

Data were analyzed by least squares fixed model procedures (SAS Institute, Inc., 1988). Main effects of lean type, fat type, fat level and replication and their interactions were included in the statistical model. No interactions higher than 2nd order were significant. Thus, only least squares means from significant two-way interactions and main effects are reported. When F-tests for fat type were significant, least squares means were separated using the least significant difference procedure in SAS.

Table 3—Effects of lean type, fat type and fat level on visual appearance and cooking loss of ground beef patties

Effect[a]	Color[b]	Fat smear[b]	Cook loss, %
Lean type			
HL	2.88[c]	2.00[c]	30.81[d]
CL	3.38[d]	3.63[d]	34.54[c]
SEM[e]	0.13	0.12	0.70
Fat type			
HF	3.13	2.75	30.39[d]
Mix	3.25	2.88	31.79[d]
CF	3.13	3.00	35.81[c]
ChF	3.00	2.63	32.72[d]
SEM[e]	0.18	0.17	0.99
Fat level			
10, %	3.69[c]	3.19[c]	32.27
20, %	2.56[d]	2.44[d]	33.08
SEM[e]	0.13	0.12	0.70

[a] See text for description of variables.
[b] Color: 1 = extremely light red, 4 = dark red; fat smearing: 1 = extreme, 4 = none.
[c,d] Means in the same column within an effect differ (P<0.05).
[e] Standard error of the mean.

RESULTS & DISCUSSION

TEMPERATURES OF THE DIFFERENT TYPES of ground beef initially after grinding varied (Table 1). Fat by lean type affected (P<0.06) the initial temperature of the ground beef. As anticipated, ground beef from HL raw material with any combination of fat type had higher (P<0.05) temperatures immediately after grinding than ground beef from CL. Fat type had little impact on temperature except for CF which had a lower (P<0.05) temperature than the other types, especially in combination with CL. The minimal effect of HF, Mix and ChF fat on temperature was most likely due to the relatively small proportion required in each batch to reach targeted levels. Lean and fat type interacted with replication for ground beef temperature. For both treatments temperatures were greater (P<0.05) for the second replicate (data not shown). Fat level had no (P>0.05) effect on temperature (data not shown). Initial temperatures were HL ≈30°C and HF ≈24°C.

Ground beef patties were within 1.5% of targeted fat levels of 10 and 20% fat (Table 2). As expected the proportion of protein and moisture in the patties decreased (P<0.05) as fat content increased (Cross et al., 1980; Ono et al., 1985; Hoelscher et al., 1987).

Physical traits

Lean and fat type, and fat level influenced the physical characteristics of ground beef patties (Table 3). Fat type had no (P>0.05) effect on visual red color; however, patties from CL were redder (P<0.05) than those from HL. Several factors (pH, cytochrome activity) could be responsible for the color variation in HL and CL patties. Visual evaluators noted the HL patties were scored lower on the color scale because they appeared more purple than red. Although pH was not measured, HL patties would be expected to have higher pH resulting in a looser myofibril structure, and more protein repulsion, thus altering visual properties (Lawrie, 1966). A significant interaction between fat and lean type for patty color revealed a greater (P<0.05) difference in red color for ChF in CL than HL compared to other fat/lean type treatments (data not shown).

Fat smearing was affected by lean type and fat level (Table 3). The HL patties had more (P<0.05) fat smearing than CL patties, likely due to the elevated temperature of the HL treat-

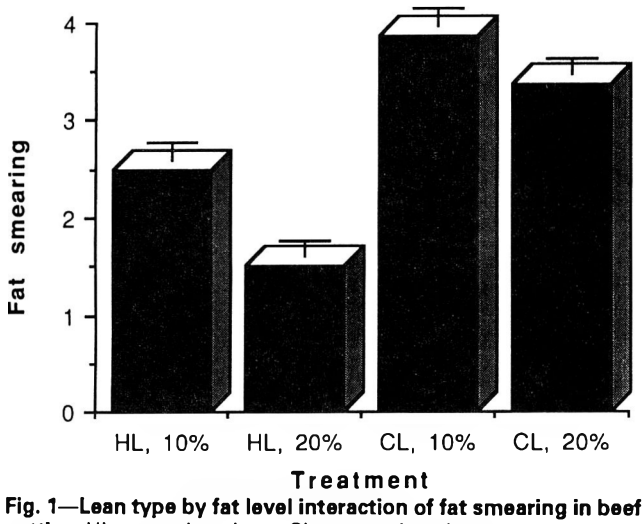

Fig. 1—Lean type by fat level interaction of fat smearing in beef patties. HL = prerigor lean, CL = postrigor lean.

ment causing the fat to be more liquid. The 20% fat patties had greater ($P<0.05$) fat smearing than 10% patties. This was primarily a function of greater fat content in the 20% patties. Noteworthy is the trend ($P<0.15$) for a lean type by fat level interaction for patty smearing. A greater degree of fat smearing occurred between fat levels for HL than CL patties (Fig. 1). The HL-20% patties had the highest degree of fat smearing. Van Laack and Smulders (1990) also reported a higher degree of fat smearing in beef patties produced from hot processed than conventional processed beef.

Patties of HL, HF, Mix, and ChF had lower ($P<0.05$) cooking losses than their CL and CF counterparts. Similar results were reported by Cross et al (1979), Jacobs and Sebranek (1980), Cross and Tennet (1981), Berry and Leddy (1988), and Van Laack and Smulders (1990). They reported beef patties from hot boned carcasses had reduced cooking losses and shrank less than those prepared from beef boned at 24 h postmortem. Furthermore, Bentley et al. (1988) reported addition of hot boned pork fat reduced moisture loss and increased cooking yields compared to cold boned fat in luncheon loaves. Results from our study indicate hot boned fat or lean may improve juiciness of beef patties based on reduced cook loss. Cooking loss was greatest ($P<0.05$) for CL patties in the second replicate compared with all other lean type treatment/replicate combinations (data not shown). Random variation in cooking may partially account for this, since no causes or effects were obvious.

Fat or lean type had no ($P>0.05$) affect on red color, fat smearing or purge for patties produced from ground beef stored for 7 days (Table 4). This indicated storage may help reduce color and smearing differences by allowing factors such as pH and temperature to equilibrate. A lean type by replication interaction existed for color in the chub stored ground beef. The HL chub stored patties in the first replication were redder ($P<0.05$) than those in the second replication; however, no ($P>0.05$) difference in color occurred for the CL treatment between replicates (data not shown). The 20% chub stored patties were less ($P<0.05$) red and had greater ($P<0.05$) fat smearing than 10% patties.

Sensory traits

Sensory characteristics for beef patties (Table 5) showed the only trait affected by lean or fat type was texture. Patties from HL had coarser ($P<0.05$) texture than CL patties. Parameters of overall tenderness in beef patties, cohesiveness and springiness, were not different ($P>0.05$) indicating similar tenderness across treatments. Contreras et al. (1981) also reported no

Table 4—Effects of lean type, fat type and fat level on visual appearance and purge of chub packed stored ground beef

Effect[a]	Purge, %	Color[b]	Fat smear[b]
Lean type			
HL	0.45	2.31	2.13
CL	0.36	2.31	2.31
SEM[e]	0.05	0.17	0.15
Fat type			
HF	0.41	2.38	2.25
Mix	0.34	2.25	2.12
CF	0.52	2.25	2.25
ChF	0.36	2.38	2.25
SEM[e]	0.07	0.24	0.21
Fat level			
10, %	0.46	2.69[c]	2.56[c]
20, %	0.36	1.94[d]	1.88[d]
SEM[e]	0.05	0.17	0.15

[a] See text for description of variables.
[b] Color: 1 = extremely light red, 4 = dark red; fat smearing: 1 = extreme, 4 = none.
[c,d] Means in the same column within an effect differ ($P<0.05$).
[e] Standard error of the mean.

Table 5—Effects of lean type, fat type and fat level on sensory traits

Effect[a]	Springiness[b]	Texture[b]	Cohesiveness[b]	Flavor[b]
Lean type				
HL	5.74	5.31[c]	5.07	5.74
CL	5.76	4.87[d]	5.31	5.50
SEM[e]	0.13	0.08	0.18	0.12
Fat type				
HF	5.60	5.11	5.15	5.86
Mix	5.79	5.17	4.99	5.49
CF	5.82	5.10	5.25	5.54
ChF	5.80	4.99	5.38	5.60
SEM[e]	0.18	0.12	0.25	0.17
Fat level				
10, %	6.00[c]	5.06	5.53[c]	5.76
20, %	5.50[d]	5.12	4.85[d]	5.48
SEM[e]	0.13	0.08	0.18	0.12

[a] See text for description of variables.
[b] 1-8 point scale, where 1 = extremely nonspringy, fine texture, noncohesive, and unflavorful. 8 = extremely springy, coarse texture, cohesive, and flavorful.
[c,d] Means in the same column within an effect differ ($P<0.05$).
[e] Standard error of the mean.

differences in tenderness of ground beef prepared from hot boned and conventional chilled lean. However, their patties had been formed from previously frozen product. A significant lean type by replication interaction showed in one replication HL patties were less ($P<0.05$) cohesive and CL patties were finer ($P<0.05$) textured than the other lean type/replication treatments (data not shown). Lack of improvement in tenderness contrasted with earlier results (Cross et al., 1979; Jacobs and Sebranek, 1980; Cross and Tennet, 1981; Berry and Leddy, 1988; Van Laack and Smulders, 1990) which indicated patties made from cold boned carcasses were less tender than those from hot boned carcasses. A possible reason we found no differences in tenderness may be the variation in processing temperatures between replicates. The replicate with the highest temperature during processing (replicate 2) produced HL patties that were less cohesive than CL patties. Variation between replicates may have masked statistical differences between treatments, which tended toward improved tenderness (less cohesive) for HL patties (Table 5). No differences ($P>0.05$) in beef flavor or off-flavors were detected across treatments. These results are in agreement with Jacobs and Sebranek (1980) and Cross and Tennet (1981), who reported no differences in flavor intensity of beef patties produced from hot and cold boned beef.

The 10% patties were springier ($P<0.05$) and more ($P<0.05$) cohesive than 20% patties, indicating they were less tender overall. Berry and Leddy (1984) found beef patties with 14% fat were less tender (sensory analysis) than those with 24% fat. Similarly, Kregel et al. (1986) reported the same response for sensory tenderness between 9.5 and 21.1% fat beef

Table 6—Effects of lean type, fat type and fat level on sensory traits of chub packed stored ground beef

Effect[a]	Springiness[b]	Texture[b]	Cohesiveness[b]	Flavor[b]
Lean type				
HL	5.04[d]	5.00[c]	4.22[d]	5.33[c]
CL	5.77[c]	4.26[d]	5.66[c]	4.70[d]
SEM[e]	0.12	0.08	0.11	0.15
Fat type				
HF	5.10	4.63	4.74	4.78
Mix	5.45	4.41	5.16	4.98
CF	5.49	4.63	4.89	4.93
ChF	5.59	4.86	4.99	5.37
SEM[e]	0.17	0.12	0.16	0.22
Fat level				
10, %	5.48	4.53	5.06	4.98
20, %	5.34	4.73	4.83	5.05
SEM[e]	0.12	0.15	0.11	0.15

[a] See text for description of variables.
[b] 1-8 point scale, where 1 = extremely nonspringy, fine texture, noncohesive, and unflavorful. 8 = extremely springy, coarse texture, cohesive, and flavorful.
[c,d] Means in the same column within an effect differ (P<0.05).
[e] Standard error of the mean.

patties. Other reports (Berry, 1992; Troutt et al., 1992; Berry, 1993) also found ground beef tenderness improved with increasing fat content over a wide range of fat levels. The level of fat had no significant effect on beef flavor. Berry (1992, 1993) and Troutt et al. (1992) reported a general increase in beef flavor with increasing fat for ground beef patties over a much wider range of fat levels than we found. Troutt et al. (1992) reported no difference in beef flavor between patties with 10 and 20% fat.

Sensory characteristics for beef patties from ground beef stored in chub packs for 7 days (Table 6) showed fat type or fat level had no (P>0.05) effect. As with patties from non-stored ground beef, those from HL were coarser (P<0.05) in texture than CL patties. However, in contrast to those from non-stored ground beef, lean type had a significant effect on tenderness characteristics. Patties from CL were springier (P<0.05) and more cohesive (P<0.05) than HL patties, indicating they were less tender. Our results agreed with those of other researchers who reported decreased tenderness for beef patties produced from cold boned vs hot boned beef (Cross et al., 1979; Jacobs and Sebranek, 1980; Cross and Tennet, 1981; Berry and Leddy, 1988; Van Laack and Smulders, 1990). The differences in springiness and cohesiveness probably occurred due to their reduction in the HL patties (Tables 5, 6) and the CL patties remained almost unchanged. Possibly, elevated temperature and pH early in the chilling process of HL ground beef brought about greater myofibrillar degradation or alteration of some functional properties. The exact mechanism could not be determined from our data. The HL chub stored patties had higher (P<0.05) beef flavor scores than CL patties. This may have resulted from a slight increase (P<0.05) in the incidence in off-flavors in the CL chub stored patties (data not shown). Off-flavors were only significantly different for CL patties in one replicate. All other lean type/replication combinations showed no (P>0.05) differences in off-flavors. This could also have resulted from differential initial temperatures between replications. Similar findings were reported by Abu-Baker et al. (1982) for beef franks from prerigor beef. They reported, in general, flavor scores were more acceptable for beef franks from stored prerigor beef than from stored postrigor beef.

Ground beef could be prepared from hot fat raw materials with almost no reduction in quality. Prerigor and chilled prerigor fat improved cooking loss, potentially enhancing juiciness of products, without detrimental effects on appearance or sensory traits. Hot fat (prerigor) trim could feasibly be used by processors to produce quality ground beef, thus increasing value of the hot fat. Use of hot fat may enhance some palatability traits. Addition of prerigor lean raw materials decreased cooking loss and improved texture although the patties had more fat smearing. Processors could store hot lean (prerigor) ground beef prior to forming into patties to alleviate fat smearing. No interactions of fat level with other treatments were detected. Thus, patties could be produced from hot fat raw materials across a wide range of fat levels.

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Differential Scanning Calorimetry of Beef/Kappa-Carrageenan Mixtures

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ABSTRACT

The thermal properties of kappa-carrageenan (KC) and/or beef under various ionic conditions were evaluated using Differential Scanning Calorimetry (DSC). The single endotherm observed for 2% aqueous KC (Tmax at 53°C) shifted to 54–59°C with addition of 1–3% NaCl and 0.35% sodium tripolyphosphate. Three endotherms were observed for post-rigor bovine semimembranosus meat (Tmax at 57, 66 and 80°C). Addition of salt/phosphate to beef had greater effects on Tmax than did 2% KC. On rescanning following 24 hr refrigerated storage, beef samples showed no thermal response, while KC treatments and beef/KC mixtures showed single endotherms at 53–63 and 69–76°C, respectively, indicating a wide shift in melting temperature of KC both in the presence of meat and at higher ionic strength.

Key Words: beef, carrageenan, differential scanning calorimetry

INTRODUCTION

THE PROTEIN MATRIX FORMED during heating of restructured meat products is important to product functionality. Stabursvik and Martens (1980) reported that the most important heat induced process in a meat system is the thermal denaturation of muscle proteins since that initiates other molecular processes such as protein-protein aggregations. Differential scanning calorimetry (DSC) is useful for studying the thermal behavior of meat proteins and protein mixtures (Stabursvik and Martens, 1980; Findlay and Stanley, 1984). DSC can be applied to study thermal changes and denaturation of proteins in solution as well as in insoluble suspensions or pastes. Thus it permits examination of proteins in realistic environments such as complex and relatively solids-concentrated systems of meat mixtures (Quinn et al., 1980).

Addition of polysaccharide gums such as carrageenan to meat products is of interest to processors for production of lean, economical structured beef products. Carrageenans are sulfated linear polysaccharides extracted from red seaweed (Modliszewski, 1984). The kappa and iota fractions form thermoreversible gels. After heating to solubilize, they gel on cooling and remelt on reheating. The lambda fraction does not gel. In general, in aqueous solutions, the remelt temperature is ≈10–20°C greater than the setting temperature (Anonymous, 1988) but exact values depend on concentration of the colloid as well as quantity and types of metallic ions present (Morris et al., 1980). Holmes et al. (1986) indicated that melting characteristics of hydrocolloid gels are important for their effects on flavor release, perception of "juiciness" and mouth retention times but may be difficult to determine accurately under realistic conditions. Properties of carrageenan have been investigated in model aqueous systems but no information is available regarding thermal properties of carrageenan in meat systems.

Few studies have reported meat protein-carrageenan interactions. Howell and Lawrie (1984) observed that kappa-carrageenan decreased the gel strength of porcine blood plasma, while Wills et al. (1988) found that kappa-carrageenan induced denaturation of metmyoglobin. Bater et al. (1992) reported a synergistic increase in gel strength of simulated turkey breast juice with addition of 0.5% kappa-carrageenan. However, Bernal et al. (1987) found no apparent interaction between carrageenan and a crude myofibrillar protein fraction in dilute model systems.

Our objective was to investigate the physiochemical and thermal properties of beef/carrageenan mixtures under various ionic conditions through the use of DSC.

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MATERIALS & METHODS

Materials

For each of two replications, fresh (24 hr postmortem) beef semimembranosus muscle (top round) was obtained from a local beef packer (Monfort, Inc., Greeley, CO). The beef was trimmed of visible fat and connective tissue and cut in small slivers (2 × 20 mm). The beef had a proximate composition of 72.1 ± 0.4% moisture and 4.0 ± 0.4% fat (pentane extractable), as determined by standard procedures (AOAC, 1984). Nonmeat ingredients included distilled/deionized water, sodium chloride (reagent grade, Sigma Chemical Co., St. Louis, MO), sodium tripolyphosphate (STP; FMC Corp., Chemical Products Group, Philadelphia, PA) and kappa-carrageenan (KC; Gelcarin ME911, FMC Corp., Marine Colloids Division, Philadelphia, PA). KC had a moisture content of 3–4% (dried under vacuum at 100°C for 12 hr).

Preparation of samples

Thermal properties of 2% KC aqueous systems were assessed under three ionic strength conditions (2% KC + water [KC-W], KC + 1% NaCl/0.35% STP [KC-L] and KC + 3% NaCl/0.35% STP [KC-H]). The L (low) and H (high) levels were selected to represent two possible ionic strength conditions encountered in meat processing (Table 1). For each replicate, KC (0.30 ± 0.05 mg) was weighed directly into Dupont polymer coated aluminum pans (parts 900796.901 and 900790.901; E. I. Dupont, Wilmington, DE). Water or appropriate salt/phosphate solutions (14.70 ± 0.50 mg) were added with a pipette or syringe. Pans were immediately hermetically sealed and reweighed. Samples were stored overnight at 3°C to allow for equilibration (Oates et al., 1987).

Six meat formulations were prepared in batches of 4g each (Table 1). Dry ingredients were added directly to 50 mL polycarbonate centrifuge tubes, followed by the meat and water. The tubes were covered with parafilm (American National Can, Greenwich, CT) and placed in an ice water bath. Each sample was homogenized (Tekmar Tissumizer, Cincinnati, OH) using 3–4 pulses (90% full speed, 5 sec each). Samples (15.0 ± 1.0 mg) were sealed in Dupont aluminum pans and stored overnight (3°C) prior to DSC. The meat mixture remaining in the tube (about 3 g) was divided in half, placed in aluminum dishes and weighed for subsequent moisture analysis (12 hr in a 65°C vacuum oven).

pH

For pH determination duplicate 4-g batches of each KC, meat and meat/KC formulation were prepared. Eight additional mL of distilled/deionized water were added and then the samples were homogenized. The pH of the homogenate was determined with a Corning pH meter (model 125, Medfield, MA) using a Corning combination electrode.

Table 1—Formulations and pH of kappa-carrageenan (KC), meat and their mixtures

Formulation	Batch size	Ingredients (% of formulation) Beef[a]	Water	KC	NaCl	STP[b]	pH	IS[c]
KC Treatments								
KC-W[d]	15 mg/4 g	0	98.00	2	0	0	7.37	0
KC-L	15 mg/4 g	0	96.65	2	1	0.35	8.89	0.24
KC-H	15 mg/4 g	0	94.65	2	3	0.35	8.74	0.61
Meat and Meat/KC Treatments								
Meat-W	4 g	50	50.00	0	0	0	5.32	0.13
Meat-L	4 g	50	48.65	0	1	0.35	5.89	0.40
Meat-H	4 g	50	46.65	0	3	0.35	5.97	0.82
Meat/KC-W	4 g	50	48.00	2	0	0	5.58	0.13
Meat/KC-L	4 g	50	46.65	2	1	0.35	5.94	0.41
Meat/KC-H	4 g	50	44.65	2	3	0.35	5.98	0.84

[a] Beef semimembranosus.
[b] STP=sodium tripolyphosphate.
[c] Ionic strength. Estimated as described in the text.
[d] W, L, H represent three ionic conditions: W (water alone), L (low) and H (high)

Table 2—DSC of kappa-carrageenan treatments under various ionic conditions

	Scan	Differential scanning calorimetry values[a] Tmax, °C	Δ H,J/g
KC Treatments[b]			
KC-W	1	52.6±2.6	0.96±0.13
KC-L	1	53.9±1.9	0.69±0.13
KC-H	1	59.2±1.1	1.03±0.27
KC-W	2	53.4±2.6	0.55±0.07
KC-L	2	56.3±1.8	0.45±0.07
KC-H	2	62.8±0.7	0.47±0.10
		$LSD_{(0.05)}$ 1.9	0.43

[a] Values are the means ± standard deviations of eight determinations (four from each of two replications).
[b] Formulations are shown in Table 1.

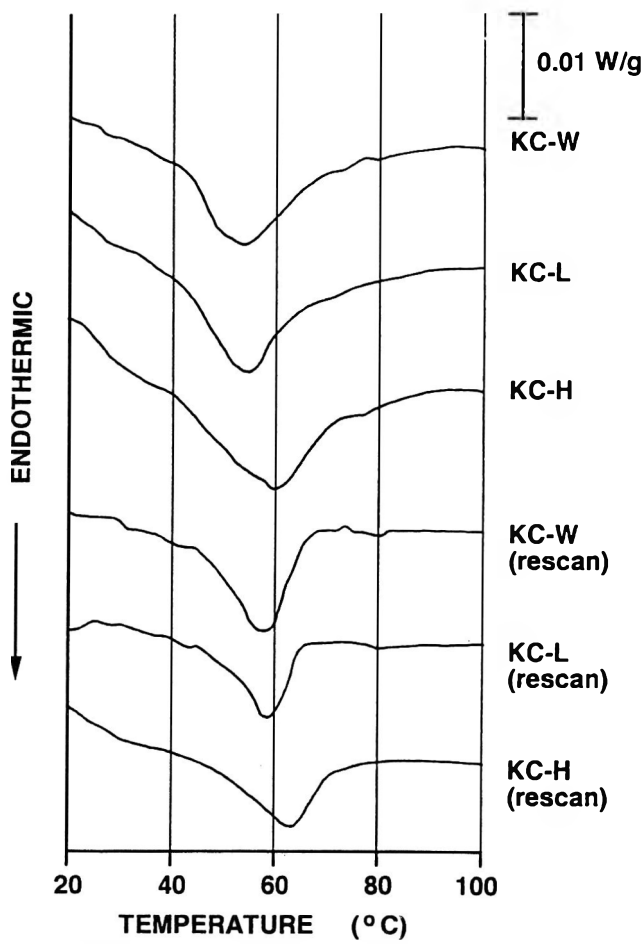

Fig. 1—Representative differential scanning calorimetry curves for kappa-carrageenan (KC) treatments under various ionic conditions. For formulations see Table 1.

Differential scanning calorimetry

A DuPont 910 Differential Scanning Calorimeter equipped with a DuPont 9900 Computer/Thermal Analyzer (E. I. DuPont, Wilmington, DE) was used. For each test, an equal weight of water was sealed in another pan and used as a reference. Before each test, the DSC cell was cooled using dry ice and allowed to warm at ambient temperature to 0–1°C. The sample and reference pans were then placed in the cell and the DSC test started when the cell temperature reached 3–4°C. These conditions allowed establishment of a stable baseline well in advance of any endothermic peaks. Thermal curves were developed over the range 10–110°C at a heating rate of 10°C/min with a nitrogen flush of 45 mL/min. After heating, the sample and reference pans were allowed to cool at room temperature (≈23°C) for 30–40 min, refrigerated overnight (3°C) and then rescanned the following day. Samples were reweighed to ensure no weight loss had occurred. Four samples/treatment were evaluated in each of two replications.

The instrument constant used to calculate enthalpies (ΔH) and calibration check of transition temperatures was obtained from thermal curves of indium (15–16 mg; enthalpy 28.45 J/g; melting point 156.6°C). The apparent transition heat, ΔH, was determined from the peak area using the internal curve integration program of the DSC instrument and expressed as J/g sample. For reproducibility, peak transition temperatures were recorded as Tmax (Hastings et al., 1985).

Statistical analysis

Randomized complete block designs were used to study effects of three ionic strength conditions and rescanning on a) KC gels and b) meat and meat/KC mixtures. Experiments were performed twice.

Analysis of variance (ANOVA) was run for the various factors under study using the General Linear Models procedure of SAS Institute, Inc. (1986). Main effects for the randomized complete block designs included: two replications (blocks), three or six treatments (for KC gels and meat treatments, respectively) and rescanning (KC gels only). For analysis of meat and meat/KC mixtures, some ANOVAS were run with treatment further classified as factorial combinations of the three ionic strength levels and two KC levels. When analysis of variance showed a significant ($P<0.05$) difference, the least significant difference (LSD) was computed to identify significant ($P<0.05$) differences among treatments.

RESULTS & DISCUSSION

KC treatments

The pH of 7.37 for the KC control (plus water) treatment (Table 1) was within the range (7–10) specified by the supplier. KC/STP treatments with 1 or 3% sodium chloride (KC-L and KC-H) had higher ($P<0.05$) pH values than the KC control treatment (KC-W) due to phosphate addition to the unbuffered samples. The estimated ionic strengths (IS) of the

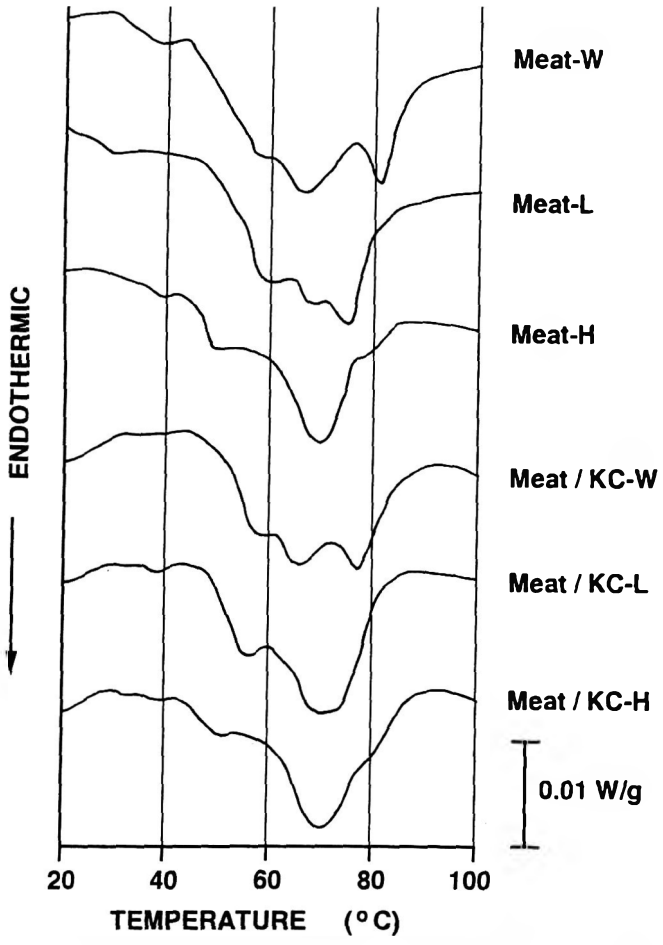

Fig. 2—Representative differential scanning calorimetry curves (scan 1) for beef and beef/kappa-carrageenan (KC) treatments under various ionic conditions. For formulations see Table 1.

various treatments were calculated based on molar concentrations of sodium chloride (aqueous phase) and the IS of STP (aqueous phase, according to Trout and Schmidt, 1986). The contribution of KC to IS was unknown.

DSC Tmax and enthalpy values for KC samples (Table 2) were compared with corresponding representative DSC thermal curves (Fig. 1) Representative curves are shown, based on two criteria: closeness of transition temperatures to the mean transition temperature for that treatment and general features representative of four or more of the thermal curves for that treatment. During the first DSC test, one endotherm was observed with Tmax at 52.6, 53.9 and 59.2°C for KC-W, KC-L and KC-H treatments, respectively. Commercial KC samples are dehydrated KC gels (Anonymous, 1989) and thus these transition temperatures likely represent the average melting temperature of KC gels. Above that temperature, enough thermal energy is in the system to destabilize the double-helix KC gel structure and to break hydrogen bonding to the water, resulting in complete solubilization of the KC (Morris et al., 1980; Anonymous, 1985).

Transition temperatures of KC treatments were increased ($P<0.001$) by inclusion of salt and STP. This confirmed observations by FMC (Anonymous, 1989) that increasing ionic concentration decreases carrageenan's cold water swelling property and increases the solvation temperature. Based on viscosity, they reported that the swelling maximum of KC in 2% NaCl solution occurred between 55–60°C with complete solubility at ≈65°C (compared to 50°C for KC in distilled water). Bater et al. (1992) found that increasing salt concentration increased both swelling temperature on heating and gelling temperature on cooling of kappa-carrageenan in simulated turkey breast juice (3% protein). Using model systems, Nishinari and Watase (1987) reported a shift of the gel-sol transition temperature of KC gels to higher temperatures upon addition of polyols.

On rescanning 24 hr later, thermal transition temperatures (Tmax) of KC gels increased by an average 2.3°C over that observed during the first DSC scan. However, the increases in Tmax were influenced by IS, with the KC-W treatment having the smallest increase (0.8°C) on rescanning while that with the highest IS (KC-H; KC/STP + 3% NaCl) had the greatest increase (3.6°C). During the first scan, the KC/STP treatment with 1% NaCl (KC-L) had significantly lower enthalpy values than the similar sample with 3% NaCl (KC-H). We could not readily explain this, as both pH and/or ionic effects may be involved. On rescanning, enthalpy values were similar among the three KC treatments and significantly lower than comparable values for the first scan. This suggested that the KC gel network formed after the first scan had fewer and/or weaker juncture zones which resulted in less energy needed to melt the gel network on rescanning. The small samples in the DSC pans possibly underwent rapid cooling which minimized gel network development. Further study of KC gels utilizing controlled cooling rates might help explain this.

Our results indicated that KC gels melt in characteristic manners which can be identified by DSC. Furthermore, inclusion of 3% NaCl and 0.35% STP shifted transition temperatures by 6–10°C higher than those observed for comparable 2% KC treatments in water alone, indicating a strong dependence of KC gel-sol transitions on IS.

Meat and meat/KC mixtures (scan 1)

The pH of the meat treatment (Meat-W) was 5.32 while addition of KC increased the pH by 0.26 pH units (Table 1). With added sodium chloride and STP, the pH of meat and meat/KC treatments was similar (pH 5.89 to 5.97). The moisture contents of the various treatments ranged from 81.3 to 86.4% and were similar to values predicted from the formulations (based on moisture of the meat and KC), indicating evaporative losses during portioning of the DSC samples were minimal. Estimated ionic strength (IS) values for the meat and meat/KC treatments (Table 1) were based on experimentally determined moisture and an IS contribution of the meat assumed to be 0.13 (50% of the IS of meat of 0.26 suggested by Dubuisson, 1950).

Thermal curves for samples of postrigor beef semimembranosus (Meat-W) indicated 3 apparent transition temperatures, a shoulder at 57.0°C (Tmax1) and peaks at 66.2°C (Tmax2) and 80.3°C (Tmax3) (Fig. 2 and Table 3). These transitions were similar to those previously reported (Martens and Vold, 1976; Stabursvik and Martens, 1980; Findlay and Stanley, 1984; Ensor et al., 1991). Martens and Vold (1976) stated that the three peaks derived from bovine sternomandibularis represented myosin (57°C), collagen (65°C) and actin (80°C). For beef at pH 5.4, Stabursvik and Martens (1980) assigned transitions at 58 and 65°C to myosin, 66°C to sarcoplasmic proteins, 67°C to collagen and 80°C to actin.

With addition of STP and 1% NaCl to the meat (Meat-L), the first transition was not affected by the changes in pH and IS (Table 3 and Fig. 2). However, the second transition became smaller (peak height) and shifted to a higher temperature (Tmax2 = 68.8°C); while the third peak shifted to a lower temperature (Tmax3 = 73.7°C). At the highest IS, the thermal curve for the Meat-H treatment showed two main responses—a shoulder at 49.7°C and a broad peak at 69.6°C. A very slight shoulder was noted at 79.2°C. Enthalpy values did not differ ($P > 0.05$) among the three meat treatments (Table 3).

In general, at the three ionic conditions, addition of KC had a very small negative (destabilizing) effect on transition temperatures of meat (Table 3 and Fig. 2). With addition of 2% KC, Tmax1 temperatures decreased by an average 0.9°C,

Table 3—DSC of meat and meat/kappa-carrageenan (KC) mixtures under various ionic conditions

		Differential Scanning Calorimetry Values[a]			
	Scan	Tmax 1,°C	Tmax 2,°C	Tmax 3,°C	Δ H,J/g
Meat and Meat/KC Treatments[b]					
Meat-W	1	57.0±0.3	66.2±0.9	80.3±0.3	1.73±0.44
Meat-L	1	57.8±1.3	68.8±0.3	73.7±1.1	1.80±0.39
Meat-H	1	49.7±0.8	69.6±0.7	79.2±2.4	1.74±0.34
Meat/KC-W	1	56.7±1.9	65.6±0.5	77.7±0.5	1.89±0.33
Meat/KC-L	1	56.1±1.5	68.5±0.3	77.1±1.3	1.99±0.39
Meat/KC-H	1	49.0±1.4	68.8±0.4	(78.6±1.9)[c]	1.87±0.51
	$LSD_{(0.05)}$	1.9	1.1	2.5	0.43

[a] Values are the means ± standard deviations of eight determinations (four from each of two replications).
[b] Formulations are shown in Table 1.
[c] Values from replication 1 only.

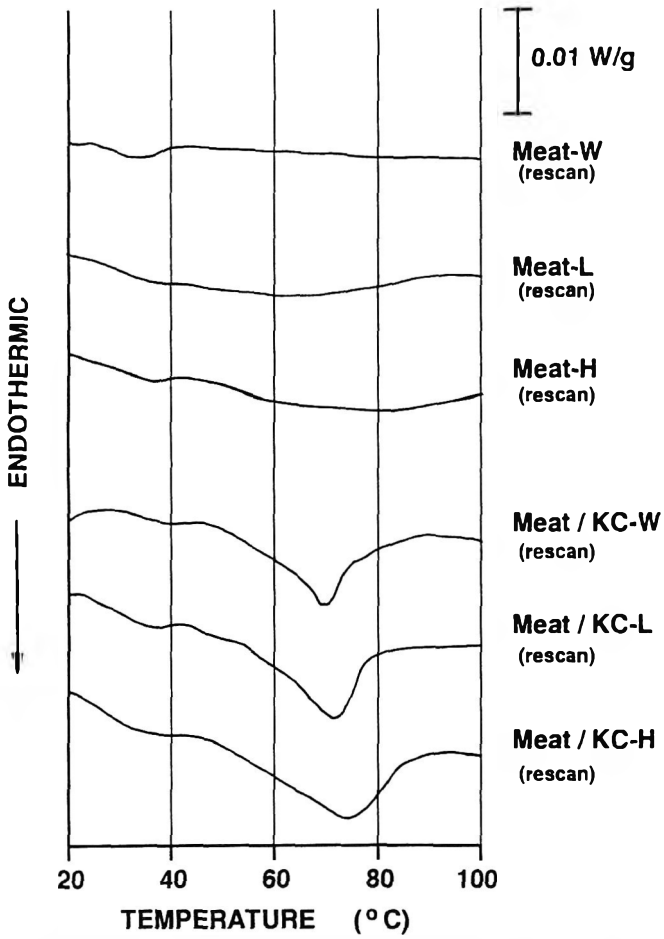

Fig. 3—Representative differential scanning calorimetry curves (scan 2) for beef and beef/kappa-carrageenan (KC) treatments under various ionic conditions. For formulations see Table 1.

Tmax2 by 0.5°C and Tmax3 by 2.6°C (Meat-W) to 0.6°C (Meat-L), respectively. The slight shoulder at Tmax3 for the Meat-H treatment was not comparable to the meat/KC-H treatment since this shoulder occurred in the first replication (mean of 78.6°C) but was not present in thermal curves of the second replication. Since a different sample of meat was used for each replication, small compositional differences in meat (e.g., collagen content) may have been the cause. The enthalpy of the meat transitions was slightly decreased due to KC addition (by 0.16 J/g sample).

Transitions attributable to melting of KC were not identified during the initial DSC scan of meat/KC mixtures (Fig. 2). Enthalpy (ΔH) values for meat/KC treatments tended to be higher than for comparable meat treatments, indicating a possible contribution of a KC gel-sol transition to observed enthalpies (Table 3). For meat/KC treatments, the corresponding KC thermal transition may have been obscured by meat transitions and thus not detectable on the DSC thermal curves.

No published reports were found on DSC of meat-carrageenan mixtures. Using model systems, Imeson et al. (1977) reported that the addition of specific anionic polysaccharides (alginate, pectate and carboxymethyl cellulose) decreased the thermal stability of myoglobin and bovine serum albumin and reduced the thermal transition temperatures by 2–5°C. Ensor et al. (1991) observed that the algin/calcium binder destabilized crude meat protein fractions, likely due to both polysaccharide effects and changes in ionic conditions. Bater et al. (1992), monitoring thermal scanning rigidity reported no influence of KC on gelling temperatures of simulated turkey breast juice (3% protein). Oates et al. (1987) reported that 2% high mannuronic acid alginate decreased transition temperatures of soy isolate by 2–3°C, while addition of other gums such as xanthan, guar, pectin and gum arabic had little or no effect. At moisture >60%, denaturation events (T onset, enthalpies) of soy or soy/alginate proceeded independently of moisture level. However, at lower moisture levels (<40%) the denaturation enthalpy of soy 7S globulin was decreased by alginate addition (Oates et al., 1987).

Meat and meat/KC mixtures (scan 2)

On rescanning 24 hr later, meat alone did not show transitions (Fig. 3), indicating a complete irreversible denaturation of meat proteins. Previous research has also shown that meat was permanently denatured under rapid heating to similar temperatures (Findlay and Stanley, 1984). However, rescanning of meat treatments with 2% added KC revealed a single endotherm at 69.2±0.5°C (meat/KC-W), 70.7±0.5°C (meat/KC-L) and 76.6±1.8°C (meat/KC-H), suggesting the possible remelting of a KC gel network in the meat samples with added KC. The enthalpy of these transitions (0.50–0.53 J/g) was similar to that for the 2% KC gels alone upon rescanning (Table 2).

The thermal transition temperatures of KC noted for the meat/KC mixtures on rescanning (69–77°C) were considerably higher than those for aqueous KC samples (53–63°C for scan 2; Table 2) They indicate that both IS and meat (or meat constituents) may strongly influence the gelling/melting properties of KC in mixed systems. These results may partially explain previous reports (Shand et al., 1994), which showed that the functionality of KC in restructured beef rolls was lowest at the highest salt concentration (3% NaCl) and lowest cooking temperature (63°C). If we assume that the melting and solubilization temperature of KC during initial heating of the meat/KC mixtures was a few degrees lower (perhaps 67 to 75°C) (based on results for pure KC gels [Table 2]), complete solubilization of the KC may not have occurred at the lowest temperature (63°C) described by Shand et al. (1994). In addition, at the highest salt concentration (3%), the increased ionic

strength may have delayed solubilization of KC in the meat system. Further investigations of meat protein/KC mixtures in model systems may help elucidate the influence of meat on KC melting temperatures. Bater et al. (1992) hypothesized that at high IS, KC dispersion in meat products may also be decreased because the meat proteins would begin denaturation before the carrageenan swelled and dissolved.

Ainsworth and Blanchard (1979) observed that carrageenan gel strength was related to both density of crosslinks and gel network chains. Morris and Belton (1982) stated that the gelation temperature of carrageenan gels could influence the kinetics of chain association and resulting gel strength. They hypothesized that a smaller difference between the maximum solution temperature and the gelation temperature (due to increases in gelation temperature with addition of specific cations) would allow less time for the system to reach "set" temperatures on cooling. This could result in less order in the gel, and consequently lower gel strength.

The specific mechanism of KC effects on meat product texture and interactions in a meat-carrageenan multicomponent gelling system is not understood. In addition to evaluations of melting temperatures, DSC thermal analysis during controlled cooling could provide information on set temperatures and effects of cooling rate on properties of meat systems with added polysaccharide gums. Further research with DSC may permit gum selection/modification to achieve maximum functionality for specific meat applications with fixed ionic strength and processing temperatures. In addition, melting temperatures of carrageenan in meat systems may also be important from a sensory perspective, since the temperature of the gel-sol transition may influence sensory perception of "juiciness", mouthfeel and texture.

CONCLUSIONS

DIFFERENTIAL SCANNING CALORIMETRY OF BEEF, kappa-carrageenan, and their mixtures indicated that kappa-carrageenan had a very small destabilizing effect on thermal transition temperatures of beef. However, addition of salt and phosphate strongly influenced the melting behavior of kappa-carrageenan. Under meat processing conditions where meat products are of high ionic strength (IS >0.60) and/or reach internal temperatures of <65–70°C, kappa-carrageenan may not be completely solubilized and may not achieve optimal gel network development on cooling.

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Restructured Reindeer Steak Quality as Affected by Antioxidants and Frozen Storage

R.B. SWANSON, M.P. PENFIELD, C.L. DORKO, and R.F. BARON

ABSTRACT

Restructured steaks were fabricated from forequarter muscles from field-slaughtered Alaskan reindeer. Steaks were formulated with an antioxidant (1:1 mixture of BHA and TBHQ with a propylene glycol carrier) at 0.00, 0.01 and 0.02% of the fat and 0.5% salt. Steaks were flash-frozen, vacuum-packaged and stored at −18°C. Instrumental and sensory testing was conducted after storage (2, 6, 10, 14, 18, 22 wk). Minimal effects occurred on moistness, greasiness, softness and chewiness. Gamy flavor intensity decreased and off-flavor intensity fluctuated with storage and antioxidant. Effects of antioxidants on color were not notable. Use of the antioxidant system is not justified by any oxidative or color effects. Frozen storage was not detrimental to restructured steak quality.

Key Words: reindeer, color, antioxidants, flavor, off-flavors

INTRODUCTION

RESTRUCTURING OF MUSCLES from the reindeer forequarter is feasible. Sensory panelists rated the resultant product acceptable on a 15-point scale (Penfield et al., 1992; Swanson and Penfield, 1992). Reindeer is harvested primarily during winter months (Swanson et al., 1990), and product supply is sporadic. However, restructured steaks are held frozen to maintain structural integrity and potentially could be available throughout the year.

Effects of frozen storage on sensory quality of restructured reindeer steaks are unknown. Oxidative rancidity of ground and restructured beef and pork products during frozen storage has been reported (Huffman et al., 1981; Mandigo and Booren, 1981). Salt, which was found to improve sensory properties of restructured reindeer (Penfield et al., 1992), has facilitated development of rancidity and caused deleterious color effects in beef and pork products (Huffman et al., 1981; Mandigo and Booren, 1981). Antioxidants have been used successfully to reduce the development of rancidity in restructured products (Wheeler et al., 1990). Some antioxidants including butylated hydroxyanisole (BHA) reportedly inhibit meat discoloration, whereas other such as tertiary butylated-hydroquinone (TBHQ) protect against flavor degradation. Combining the two may provide the best protection against oxidative changes (Chastain et al., 1982). Our objectives were: (1) to evaluate the effects of frozen storage on quality of restructured reindeer steaks; and (2) to investigate the effects of an antioxidant system (BHA:TBHQ) on development of off-flavor and changes in appearance.

METHODS

Fabrication

Ten frozen reindeer forequarters from steers at least 3 yr of age were shipped from Nome, AK to Knoxville, TN. After tempering at 2°C, external fat and meat were removed, cut into chunks, and flaked in a Urschel Comitrol (Model 2100, Valparaiso, IN) equipped with a 20-post cutting head with 4.06-cm openings. Following flaking, approximate fat content was determined using the modified Babcock method (Salwin et al., 1955). Antioxidant (1:1 mixture of BHA and TBHQ with a propylene glycol carrier) at 0.0%, 0.01% or 0.02% of the fat and 0.5% NaCl were added and mixed in a Leland Mixer (Model L-100DA, Detroit, MI) at 2°C for 10 min. Mixer speed was 26 rpm. Mixed muscle tissue was formed into 1.3-cm thick strip steaks with a Koppens food forming machine (Model VM 100, Bakel, Holland), frozen in a CO_2 freezer (10 min at −56°C), vacuum packaged, and stored in a freezer (−18°C).

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Table 1—Proximate composition of restructured reindeer steaks (%)•

Parameter	Uncooked	Cooked
Moisture	70.1±0.6	62.0±0.8
Fat	8.3±0.9	11.1±0.5
Protein	19.4±0.3	24.0±0.8
Ash	1.7±0.1	1.7±0.1

• n=9; mean ± standard deviations across steaks from three antioxidant levels; p>0.05 among samples differing in antioxidant level according to ANOVA.

Table 2—Restructured reindeer steak fatty acid profile

Fatty acid•	%
C14:0	1.87±0.10
C16:0	32.06±0.44
C16:1	1.01±0.25
C18:0	23.89±0.38
C18:1	36.36±0.33
C18:2	3.19±0.11
C18:3	0.22±0.01
C20:0	0.28±0.02
C20:1	0.34±0.02
C22:0	0.64±0.05

• n=9; means ± standard deviations across three antioxidant levels; p>0.05 among samples differing in antioxidant level according to ANOVA.

Cooking method

Steaks were thawed at 1–2°C for 16–19 hr in the packages. Thawed steaks were cooked at 177°C on a griddle (Hobart Electric Model HG2, Troy, OH) previously sprayed with vegetable oil cooking spray. Steaks were grilled for 4 min on one side, then turned and cooked to an endpoint temperature of 70-74°C in the geometric center. Sixteen steaks per antioxidant level were weighed prior to and after cooking for determination of cooking losses (Penfield and Campbell, 1990) at each storage interval.

Chemical analyses

Both raw and cooked composite samples from three steaks at each antioxidant level were analyzed in triplicate for moisture, protein, total fat and ash (AOAC, 1984) after 2 wk frozen storage. Fatty acid composition (Melton et al., 1979) and cholesterol were also determined (AOCS, 1983) on the uncooked composite samples in triplicate at each antioxidant level after 2 wks frozen storage. TBA analysis (Rhee, 1978) was conducted on samples from 8 steaks at each antioxidant level 2 wk after fabrication and every 4 wk thereafter for a total of 22 wk storage.

Instrumental analyses

Color was determined with a Hunter Color Difference Meter (Model D25, Hunter Associates Laboratory, Inc., Reston, VA.); a pink tile (C2-

Table 3—Effects of antioxidant level and storage time on Hunter color values of restructured reindeer steaks

Hunter value	Antioxidant level (% of fat)[a]			Storage (wk)[b]	
	0.00	0.01	0.02	10	64
L	26.1±1.5a	24.0±0.9b	24.7±1.4b	25.3±1.7a	24.6±1.4a
a	8.1±1.1a	8.2±0.9a	8.4±1.1a	7.6±0.6a	8.9±0.9b
b	3.1±0.4a	6.7±0.5b	7.3±0.4b	6.8±0.5a	7.2±0.4b

[a] n=8; means ± standards deviation across steaks from 2 storage periods. Means in a row within antioxidant levels followed by different letters are significantly different ($p<0.05$), according to Tukey's studentized range test.

[b] n=12; means ± standard deviation across antioxidant levels. Means in a row within storage followed by different letters are significantly different ($p<0.05$), according to Tukey's studentized range test.

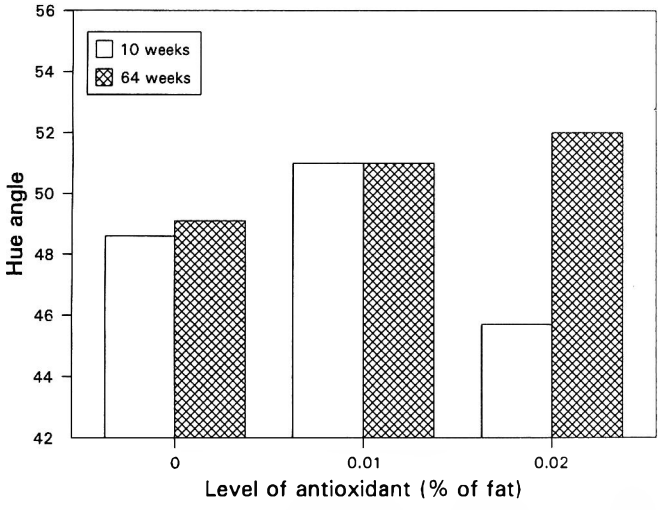

Fig. 1—Means of hue angle (n=8) on restructured reindeer steaks containing three levels of antioxidant with differing periods of frozen (−18°C) storage.

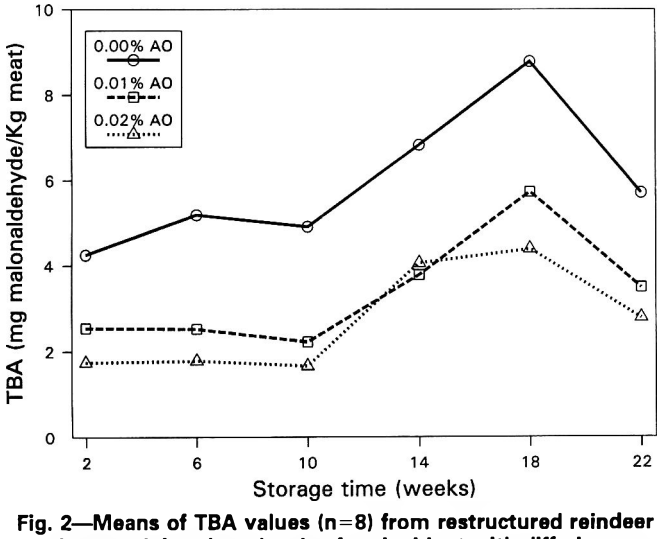

Fig. 2—Means of TBA values (n=8) from restructured reindeer steaks containing three levels of antioxidant with differing periods of frozen (−18°C) storage.

21126) was the reference. Steaks were allowed to thaw as described. Two measurements on 4 steaks per antioxidant level were taken immediately after removal from the package. Data were collected after 10 and 64 wk of frozen storage. pH was determined on 3 composite samples of flaked muscle prior to incorporation of the antioxidants. After 2 wk frozen storage and at the conclusion of the study, pH was determined on 2 steaks per antioxidant level. Tenderness of the cooked product was assessed with a Warner-Bratzler shear (GR Manufacturing Co., Manhattan, KS) equipped with a straight-edged blade. Three 1.3-cm thick strips cut-across-steaks were sheared from 16 steaks per antioxidant level at each storage interval. All samples were at room temperature (~23°C).

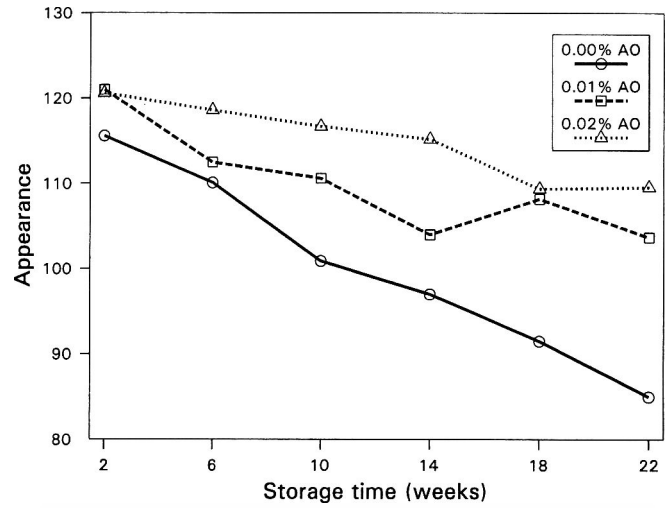

Fig. 3—Means of sensory scores assigned by 16 panelists across two replications for appearance of restructured reindeer steaks containing three levels of antioxidant (AO) and stored frozen (−18°C) for up to 22 wk prior to cooking. Steaks were evaluated on 150-mm line where 150=very well done and 0=rare.

Sensory analyses

Twenty-seven individuals who had previously consumed hunt-killed venison and were students, faculty and staff from the Dept. of Food Science & Technology at The Univ. of Tennessee, Knoxville served as sensory panelists. They scored the samples on a scorecard using procedures previously described (Penfield et al., 1992). Cooked samples were cut into 1.3 × 2.5-cm pieces and held in ceramic casseroles prior to presentation to panelists within 30 min of cooking. Panelists evaluated the room temperature samples in random order in individual booths equipped with white fluorescent lighting. Within each storage period, sensory evaluation was replicated two times. Data from the 16 panelists, who participated in the evaluation at least five of the six storage periods, were included in statistical analysis.

Statistical analyses

PROC GLM (SAS Institute, Inc., 1985) was used to determine the main effects of antioxidant level, storage time, replication within storage time and panelists and the two-way interaction between antioxidant level and storage. Tukey's studentized range test was used for mean separation when appropriate.

RESULTS & DISCUSSION

PROXIMATE COMPOSITION OF THE REINDEER STEAKS (Table 1) showed no differences among the steaks with differing levels of antioxidant ($p>0.05$). Composition was similar to that reported for uncooked composite forequarter samples from the Alaskan reindeer field-slaughtered in March (Swanson et al., 1990). Proximate composition of cooked steaks also did not differ with antioxidant level. The fatty acid composition (Table 2) revealed relatively low levels of polyunsaturated fatty acids, as reported (Swanson et al., 1990). Miller et al. (1986) suggested that the higher levels of polyunsaturated fatty acids

Table 4—Effects of antioxidant level and storage time on sensory evaluation of restructured reindeer steaks.

	Antioxidant level (% of fat)[a]			Storage time (wk)[b]					
Attribute[c]	0.00	0.01	0.02	2	6	10	14	18	22
Softness to tooth pressure	54	54	57	51	53	60	58	54	56
Moisture release	84	84	81	77	81	86	78	88	84
Chewiness	*80a*	*79a*	72b	*88a*	77b	71b	73b	76b	77b
Greasiness	61	65	60	63ab	66ab	67b	58ab	63ab	56a
Off-flavor	31a	26b	29ab	39a	29b	29b	22c	23c	30b
Overall acceptability	83	85	84	84	82	80	84	86	87

[a] Means of scores assigned by 16 panelists across steaks stored for six storage periods and two replications. Means in a row within antioxidant levels followed by different letters differ significantly ($p<0.05$), according to Tukey's studentized range test.

[b] Means of scores assigned by 16 panelists across steaks containing three antioxidant levels and two replications. Means within a row within storage time followed by different letters differ significantly ($p<0.05$), according to Tukey's studentized range test.

[c] Samples were evaluated on 150-mm line scales: Softness to tooth pressure, 150=very hard, 0=very soft; Moisture release, 150=great, 0=slight; Chewiness, 150=highly resistant, 0=yields readily; Greasiness, 150=very difficult to remove fatty film from palate, 0=not at all difficult; Off-flavor, 150=intense, 0=none; Overall acceptability, 150=extremely acceptable, 0=not acceptable.

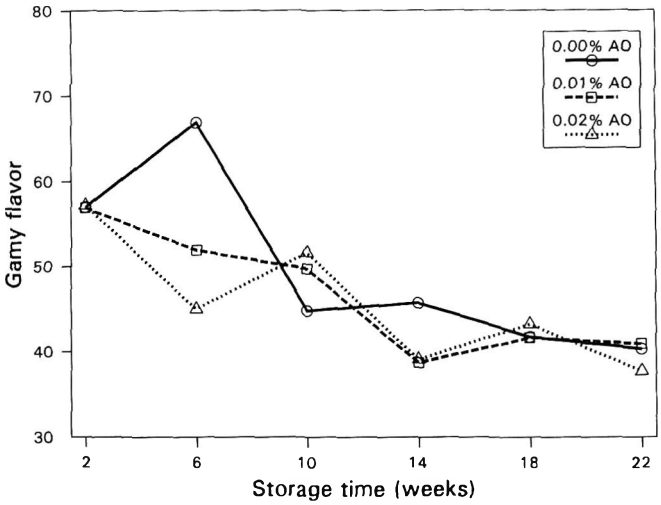

Fig. 4—Means of sensory scores assigned by 16 panelists across two replications for gamy flavor of restructured reindeer steaks containing three levels of antioxidant (AO) and stored frozen (−18°C) for up to 22 wk prior to cooking. Steaks were evaluated on 150-mm line where 150=intense and 0=none.

were responsible for the gamy flavor in less traditional meat species. These reindeer forequarter muscles were rich in stearic, palmitic and oleic acids. Cholesterol levels (77.5 ± 0.72mg/100g) approximated those in domestic red meats (USDA, 1983, 1986, 1989). *pH of the flaked muscle* was 5.6 ± 0.0; after fabrication pH was 6.2 ± 0.0 regardless of antioxidant level or storage time.

Cooking loss

Cooking losses, which ranged from 19.4% ± 0.48 to 22.4% ± 0.48, can be attributed primarily to loss of moisture (Table 1). A significant interaction was found between antioxidant level and storage time; however, no consistent trend occurred and the variation found was not notable. Wheeler et al. (1990) reported that the same antioxidant system at these levels did not affect cooking losses in beef restructured steaks stored at −20°C. Conversely, Chastain et al. (1982) indicated that cooking losses in a beef-pork restructured product increased after 20 wk frozen storage at −10°C.

Color

Both antioxidant level and storage time affected Hunter color values. Color intensity (Hunter L) values increased with incorporation of antioxidants at either 0.01 or 0.02% of the fat; however, no effect of storage was found (Table 3). No differences in redness due to antioxidant level (Hunter a) were detected. Longer storage time significantly increased redness and yellowness (Table 3). A significant interaction between storage time and antioxidant level occurred. Steaks with antioxidant at 0.02% of the fat had a significantly lower hue angle with extended storage (Fig. 1). Despite the effects found, the practical importance of these results is limited. The reindeer meat is very dark, suggesting that consumer education would be necessary if the product was marketed directly to consumers unfamiliar with reindeer meat.

Shear and TBA Values

No significant effects attributable to antioxidant level or storage time were found on Warner-Bratzler shear values (3.4 ± 1.1 kg). Little effect of antioxidant has been reported on textural properties of restructured products from domestic species (Wheeler et al., 1990; Chastain et al., 1982). Protection from rancidity by antioxidants was apparent from the TBA (Fig 2). At 18 wk storage, TBA of steaks with antioxidant approximated that of 2 wk stored steaks without antioxidant. Increasing the level of antioxidant apparently increased the protection. The data appeared to suggest that rancidity decreased with continued storage beyond 18 wk regardless of antioxidant, but this was likely an artifact of the determination of rancidity due to a secondary reaction (Tarladgis et al., 1960).

Sensory evaluation

Storage time and antioxidant level interacted to affect scores for appearance (Fig. 3). At 2 wk storage, scores were similar but, as storage progressed, cooked steaks appeared to be less well done. The decrease in apparent doneness was greater in steaks without antioxidant. Minimal effects of antioxidant level and storage time on textural characteristics were found (Table 4). Moisture release and softness to tooth pressure did not differ with antioxidant level. Chewiness varied with both antioxidant and storage (Table 4). Restructured reindeer steaks stored for two weeks were more chewy than those stored for longer. Those containing 0.02% antioxidant were less chewy than those containing 0.01% or no antioxidant. Only one difference in greasiness was found (Table 4); the 10-wk samples were perceived as more greasy than the 22-wk samples.

Gamy flavor intensity decreased with storage; the effect of storage was related to antioxidant level (Fig. 4). The sample without antioxidant that was stored for 6 wk was rated more gamy than samples with antioxidants. Data for off-flavor indicate an effect of antioxidant level and length of storage (Table 4). Off-flavor scores were highest at 2 wk storage and lowest at 14 and 18 wk storage. Intermediate values were found at 6, 10 and 22 wk storage. Steaks with 0.01% antioxidant had lower off-flavor scores than did steaks with no antioxidant. Off-flavor scores for steaks with 0.02% were intermediate and did not differ from other samples. In general,

scores were low for off-flavors and gamy flavor. Overall acceptability scores (Table 4) were above the mid-point. No differences in overall acceptability due to storage period or antioxidant were found. Inclusion of an antioxidant would not be of much potential benefit based on these results.

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THERMALLY PROCESSING SUB-FROZEN CANNED MEAT. . .From page 696

the model usually slightly overestimated lethalities possibly due to assumptions of perfect thermal contact at the can surface and zero come-up time.

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Heat-induced Gelation of Chicken Gizzard Myosin

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ABSTRACT

Chicken gizzard myosin solution formed a gel when heated above 40°C. The rigidity of the gel was constant above 65°C. Maximum pH for gel formation was 5.9 at 0.6M and 5.7 at 0.15M KCl. Higher rigidity of the myosin gel was observed at low ionic strength than at high ionic strength. Rigidities of myosin at 0.6M KCl increased by $(mg/mL)^{2.5}$ and at 0.15M $(mg/mL)^{1.4}$ myosin concentration. The strength of gizzard myosin gels was comparable to that of myosin gels from chicken breast muscle under similar conditions.

Key Words: chicken, gizzard, myosin, gelation

INTRODUCTION

THERMAL GELATION OF MYOFIBRILLAR PROTEINS from skeletal muscles is largely responsible for textural properties of processed meat products (Acton et al., 1983; Asghar et al., 1985). Myosin shows a greater gel-forming ability than most other myofibrillar proteins, and actin affects myosin gelation (Siegel and Schmidt, 1979; Asghar et al., 1985). Myosins from physiologically different types of skeletal muscle and cardiac muscle have characteristic ATPase activities and different isotypes of subunits (Sarkar et al., 1971; Hoh et al., 1976; Termin and Pette, 1991). Poultry breast (white) muscle shows higher binding capacity than leg (red) muscle (Asghar et al., 1984; Dudziak et al., 1988; Xiong and Brekke, 1989). Porcine cardiac muscle myosin forms much stronger heat-induced gel than skeletal muscle myosin (Samejima et al., 1985). These facts imply that the myosin isoforms from different sources may show different gelation characteristics upon heating.

Smooth muscle cells are found in many tissues such as vascular and gastrointestinal tracts of vertebrate animals, yet little has been published on the gelation of smooth muscle proteins. Although physicochemical properties of smooth muscle myosin are similar to those of skeletal and cardiac muscle myosin, some distinctions occur in the amino acid sequences, subunit composition, regulation of actin dependent ATPase activity, and other features (Murphy and Megerman, 1977; Hartshorne, 1987). A large amount of smooth muscle is produced as by-products from meat industries. Some is used as human foods and some is used as feed for animals. The study of the heat-induced gelation of myosin from smooth muscle is important to further elucidate the reason for differences in gelation of myosin isoforms and for exploring effective uses of animal products. Our objective was to evaluate the heat-induced gelation of chicken gizzard myosin and properties of the gel as affected by physico-chemical factors.

MATERIALS & METHODS

Proteins

Myosin was prepared from frozen chicken gizzards by the method of Ikebe and Hartshorne (1985); rabbit skeletal actin was prepared as described by Spudich and Watt (1971). Myosin and actin concentrations were measured spectrophotometrically with extinction coefficients of 0.454 (Ikebe and Hartshorne, 1985) and 1.1 $mg^{-1}cm^{-1}$ (Houk and Ue, 1974) at 280 nm, respectively.

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Polyacrylamide gel electrophoresis and gel strength

Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out on 10–20% polyacrylamide gradient slab gels in the presence of 0.1% SDS using the discontinuous buffer system of Laemmli (1970). The rigidity of heat-induced gels of myosin was measured with a band-type viscometer by a modified method of Yasui et al. (1979). Protein solution containing KCl and buffer in a glass spectrophotometer cell (10 × 10 × 40 mm) was placed in a cell holder with temperature controlled by circulating water. A rectangular thin plate (7 × 20 mm) of stainless steel attached to a force-electric signal transformer of a rheometer was immersed about 5 mm under the surface of the solution in the center of the cell. The surface of protein solution was covered by a layer of silicon oil to help prevent against drying. Temperature of the protein solution was changed by circulating water. Come-up time was < 5 min at 70°C. The force required for 1 mm upward shift of the metal plate was measured. We confirmed that the rigidity did not change after 15 min heating until 60 min and that longer heating caused drying. Equations and other procedures for gel strength measurement were reported by Yasui et al. (1979) and experimental conditions are shown in the figure captions. Experiments were replicated with freshly prepared protein samples. The data presented in the figures are the mean values derived from five or six replicates along with standard deviations. No standard deviation bar is shown when standard deviation is smaller than symbol.

Scanning electron microscopy

Protein solutions containing KCl and 40 mM potassium phosphate buffer were heated at 65°C for 30 min. Gel sections of < 1 mm^3 (prepared with a razor blade) were immersed in 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.0) for 2 hr at 4°C. All fixed specimens were dipped in deionized water and dehydrated in graded ethanol of 30, 50, 70, 90, 95, and 100% (twice) for 15 min per alcohol solution. They were transferred into isoamyl acetate and dried by the carbon dioxide critical point method (Anderson, 1951). Dried specimens were mounted on brass studs, coated with a layer of gold in a vacuum evaporator, and observed with a JEOL TSM-T20 scanning electron microscope at an accelerating voltage of 19 kV.

RESULTS & DISCUSSION

Effects of temperature, ionic strength and pH

The effects of temperature on the gelation of gizzard myosin at pH 5.9 (Fig. 1) show rigidity increased with temperature above 50°C, reached maximum at 65°C and after that remained constant. The temperature dependence was similar to that of rabbit skeletal muscle myosin (Yasui et al., 1979). However, the rigidity in 0.15M KCl increased from 40°C reaching maximum at 55°C and then decreased to a constant above 65°C. Similar transition of gel strength was reported by Egelandsdal et al. (1986) and Xiong and Blanchard (1993) for skeletal myosin gels. The decrease in rigidity above 55°C probably resulted from a redistribution of intra- and intermolecular interactions within the gel network based on conformation changes of myosin, suggested by Xiong (1993) and Kitabatake et al. (1989). Gel formed at 55°C was more transparent than those formed at > 55°C which supports the premise. The apparent absence of maxima around 55°C at 0.6M KCl could be due to the difference in gelling activity between monomeric

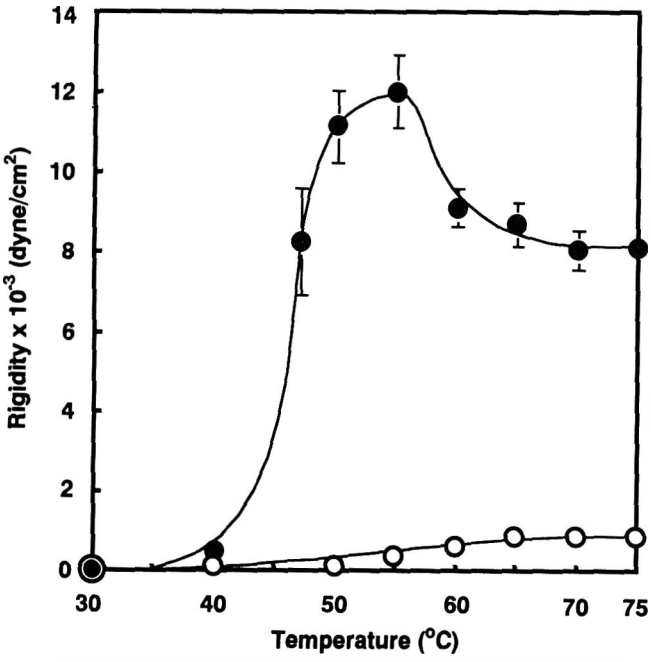

Fig. 1—Effect of temperature on rigidity of gizzard myosin gel. Myosin (4.0 mg/mL) dissolved in 0.6M KCl (○) or 0.15M KCl (●), and 40 mM potassium phosphate buffer (pH 5.9) was incubated for 25 min and rigidity of system measured.

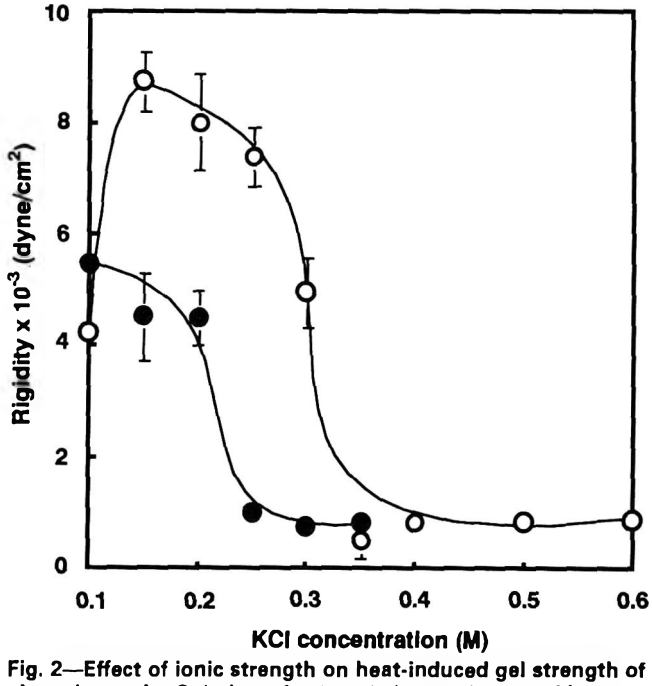

Fig. 2—Effect of ionic strength on heat-induced gel strength of gizzard myosin. Solution of 4.0 mg/mL myosin, 40 mM potassium phosphate buffer (pH 5.9), 0 mM (○) or 10 mM (●) ATP, and KCl was heated at 65°C for 25 min and rigidity measured at 65°C.

and polymeric myosins based on differences in rates of thermal denaturation, reported by Ishioroshi et al. (1983) and Egelandsdal et al. (1986).

Effect of ionic strength on rigidity of gizzard myosin gel (Fig. 2) showed rigidities at 0.15–0.25M KCl were much higher than those at 0.35–0.6M KCl. The rigidity at low ionic strength decreased by addition of 10 mM ATP just before heating. The rigidity of gels from filamentous skeletal muscle myosin at low ionic strength was higher than that of gel (monomer gel) formed at higher ionic strength (Ishioroshi et al., 1979, 1983). Rigidity of heat-induced gels of longer myosin filaments was higher than that of short ones (Hermansson et al., 1986; Yamamoto et al., 1988). Unphosphorylated gizzard myosin forms filamentous assemblies in the absence of ATP (Shoenberg, 1965; Kaminar, 1969), but the filaments change to folded monomer forms in the presence of ATP at low ionic strength (Suzuki et al., 1982; Trybus et al., 1982; Onishi and Wakabayashi, 1982). Therefore, the higher gel strength of gizzard myosin at low ionic strength was due to formation of filaments. The scattering of rigidity data at low ionic strength without ATP might be due to the various lengths of filaments formed under such conditions (Sobieszek, 1977). Decrease in rigidity by addition of ATP was probably caused by partial disassembly of myosin filaments reported by Trybus et al. (1982) and Craig et al. 1983).

The pH dependence of gelation (Fig. 3) indicates rigidity was maximum at pH 5.9 in 0.6M and 5.7 in 0.15M KCl. The rigidity markedly increased at pH < 5.5 in 0.6M KCl whereas no increase in rigidity occurred in 0.15M KCl. High rigidity of heat-induced gels of chicken skeletal muscle myosin at acid pH in 0.6M KCl was due to formation of filamentous aggregates before heating (Choe et al., 1989). The higher rigidity we observed at pH 5.5 in 0.6M KCl could be explained by similar reasoning. On the contrary, even in 0.15M KCl, the formation of high rigidity gel by heating was probably suppressed at pH around 5 because of coagulation of myosin assemblies induced by acidification (Sobieszek, 1977).

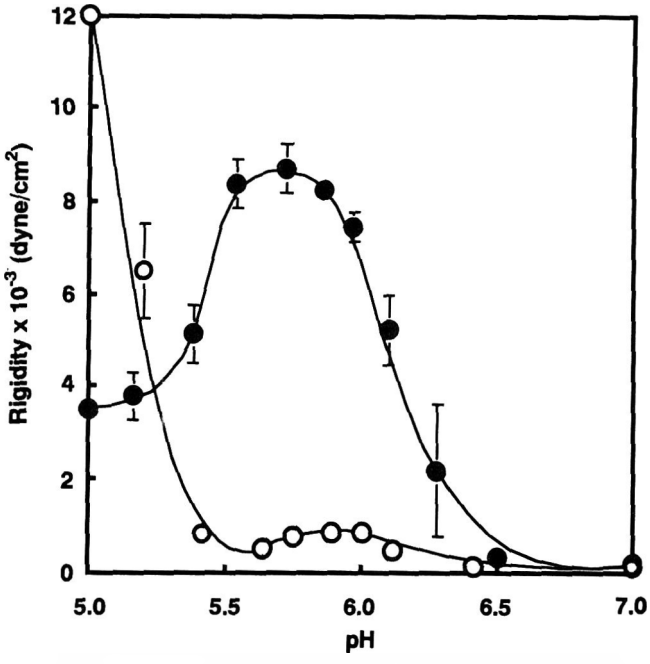

Fig. 3—Effect of pH on heat-induced gel strength of gizzard myosin. Solution of 4.0 mg/mL myosin, 0.6M KCl (○) or 0.15M KCl (●), and 40 mM potassium phosphate buffer (pH> 5.7) or acetate buffer heated at 65°C for 25 min and rigidity measured at 65°C.

Effect of protein concentration

According to the network theory of Flory (1953), Clark and Ross-Murphy (1987) showed that gel strengths of biopolymers increased exponentially by about the square of concentration. The effects of myosin concentrations from 1 to 10 mg/mL were examined by quantitative gelation of gizzard myosin (Fig. 4). The rigidity of gizzard myosin gel at pH 5.9 was proportional to $(mg/mL)^{2.5}$ and $(mg/mL)^{1.4}$ in 0.6M and 0.15M KCl, respectively. Rigidity of heat-induced gels of skeletal muscle myosin increased proportionally to $(mg/mL)^{1.8}$ at 0.6M KCl (pH 6.0) (Samejima et al., 1981). These results suggest that the mechanisms of cross-linking and network formation of gizzard myosin molecules on heating were different from

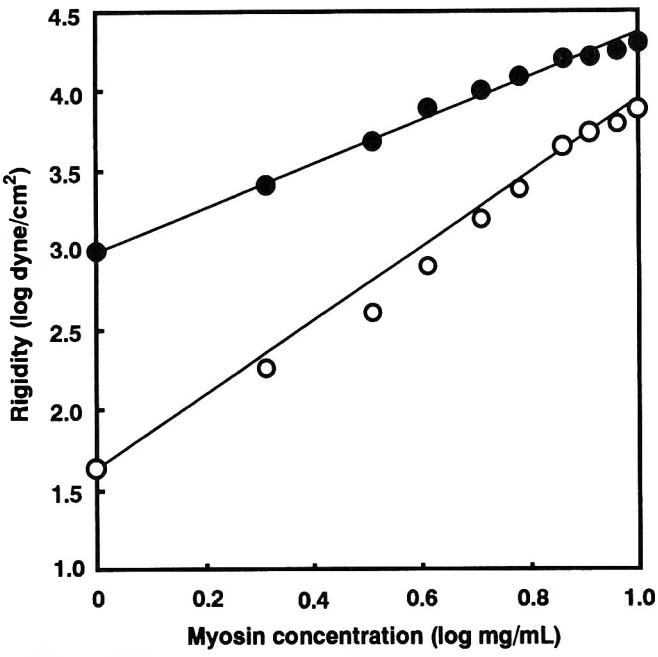

Fig. 4—Effect of myosin concentration on heat-induced gel strength of gizzard myosin. Solution of 0.6M KCl (○) or 0.15M KCl (●), 40 mM potassium phosphate buffer (pH 5.9), and myosin heated at 65°C for 25 min and rigidity measured at 65°C.

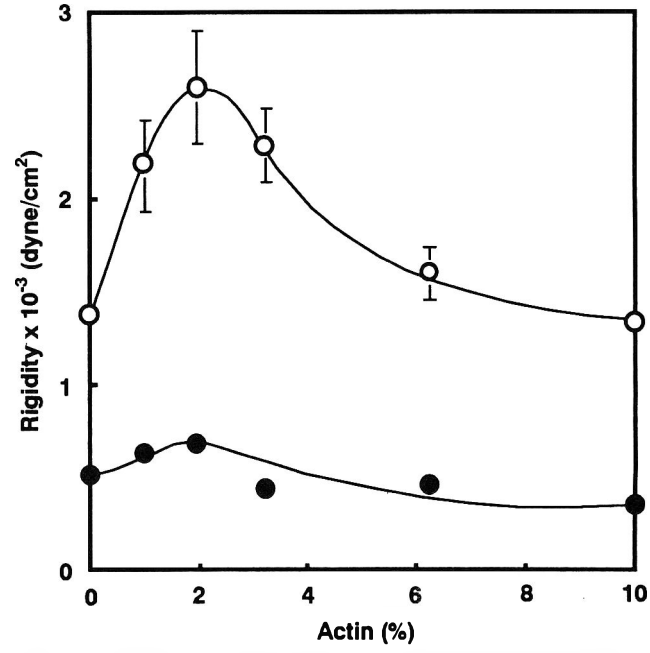

Fig. 5—Effect of F-actin on heat-induced gel strength of gizzard myosin. Solution of 8.0 mg/mL protein (myosin plus F-actin), 0.15M KCl, 40 mM potassium phosphate buffer, pH 6.5 (○) or pH 7.0 (●) was heated at 65°C for 25 min and rigidity measured at 65°C.

those of skeletal muscle myosin. An exponent >2.0 observed at 0.6M KCl in our study could be due to contributions from trapped entanglements of gel network, branching of network chains, or intermolecular associations between network chains of gizzard myosin (Clark and Ross-Murphy 1987). However, further investigations with more sensitive apparatus are necessary for explanation of the concentration dependence of myosin gelation.

Effect of F-actin

For skeletal muscle myosin, actin showed an enhancing effect on heat-induced gel strength of myosin (Siegel and Schmidt, 1979; Yasui et al., 1980, 1987). A two- to threefold increase in gel rigidity was observed when 6.3% (w/w) of F-actin was present in the myosin dissolved in 0.6M KCl and 20 mM phosphate buffer (pH 6.0) (Yasui et al., 1980, 1987). The effect was more apparent in red muscle myosin than white muscle myosin of chicken and was dependent on pH (Morita et al., 1987). F-actin was added to gizzard myosin solution at various myosin/actin weight ratios. No effects of F-actin were observed between pH 5.9 and 7.0 in 0.6M KCl or at pH 5.9 in 0.15 M KCl. However, increase in gel rigidity occurred at pH 6.5 and 7.0 in 0.15M KCl (Fig. 5). The optimum content of F-actin for the enhancement was about 2% (w/w). Based on those results, we re-examined the pH dependence of myosin rigidity with 2% F-actin in 0.15M KCl (Fig. 6). Gel strength was inhibited by F-actin at pH < 6.2 but enhanced at pH > 6.2. The decrease in gel strength by addition of F-actin at pH < 6.2 may be due to the increase in the dissociation constant of F-actin and gizzard myosin at lower pH as reported in skeletal actomyosin complex (Tonomura et al., 1962).

Scanning electron micrographs of heat-induced gel

Heat-induced gels of skeletal muscle myosin with higher rigidity had a finer and more regular network structure than those with lower rigidity (Yasui et al., 1979; Hermansson et al., 1986). Structures of heat-induced gels by scanning electron microscope (Fig. 7) showed gels in 0.6M KCl had a coarse and porous structure (Fig. 7a), whereas the network of gels in

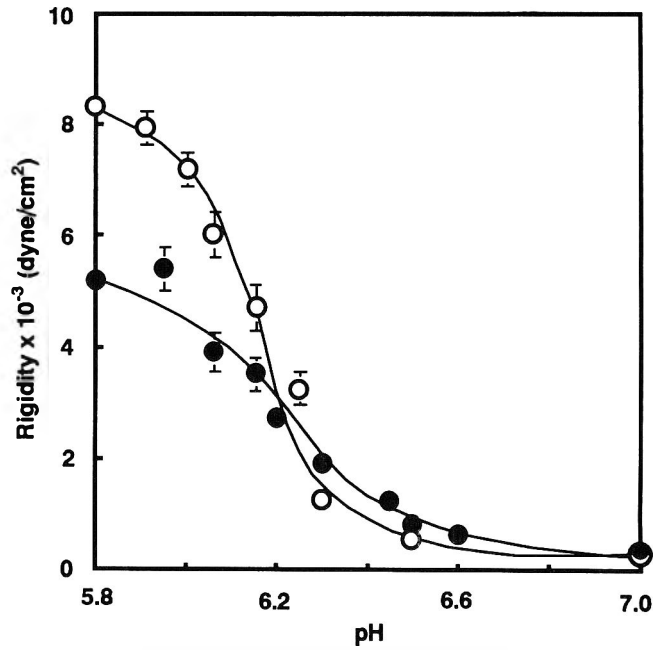

Fig. 6—Effect of pH on heat-induced gel strength of gizzard myosin and F-actin mixture. Solution containing 4.0 mg/mL myosin (○) or 3.92 mg/mL myosin plus 0.08 mg/mL F-actin (●), 0.15M KCl, 40 mM potassium phosphate buffer was heated at 65°C for 25 min and rigidity measured at 65°C.

0.15M KCl was finer, strands were thinner than gels at pH 5.9 (Fig. 7b). When gels were strengthened by addition of F-actin at 0.15M KCl and pH 6.5, the gel network became denser (Fig. 7c and 7d). Thus, gels of gizzard myosin with higher rigidity showed a finer and denser network structure than those with lower rigidity as reported for skeletal muscle myosin.

Effect of proteolysis on gel strength of myosin

Myosin from skeletal white muscle had far greater gel strength than that from red muscle under similar conditions

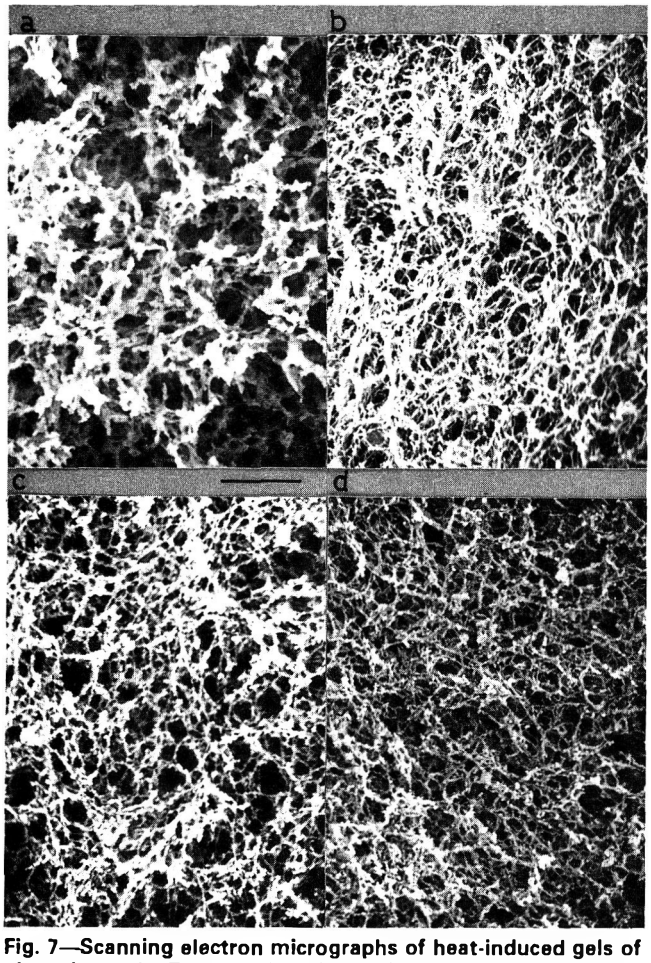

Fig. 7—Scanning electron micrographs of heat-induced gels of gizzard myosin. The samples of scanning electron micrographs containing 10 mg/mL myosin (a, b, and c) or 9.8 mg/mL myosin plus 0.2 mg/mL F-actin (d), 0.6M KCl (a) or 0.15M KCl (b, c, and d), 40 mM potassium phosphate buffer [pH 5.9 (a and b) or pH 6.5 (c and d)] were heated at 65°C for 30 min. Bar indicates 3 μm.

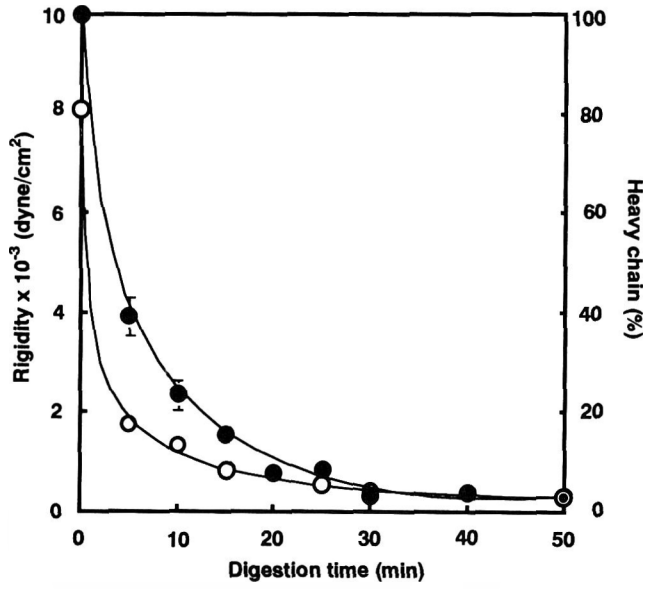

Fig. 8—Changes in strength of heat-induced gel and heavy chain content of gizzard myosin during papain digestion. Gizzard myosin (8 mg/mL) hydrolyzed at 25°C with activated papain (1/1,500 w/w) in 0.2M KCl, 30 mM Tris-HCl buffer (pH 7.5), 1 mM $MgCl_2$, 1 mM dithiothreitol, and 0.2 mM ethylene glycol bis(β-monoethyl ether)-N,-N,-N',-N'-tetraacetic acid (EGTA). Reaction stopped by addition of 5 mM iodoacetamide. Rigidity of digests (o) measured at 65°C after adjusting to 4 mg/mL protein, 0.15M KCl and 40 mM potassium phosphate buffer (pH 5.9) and gelation at 65°C for 25 min. Digests analyzed on SDS polyacrylamide gel electrophoresis to measure relative contents of myosin heavy chain (•).

(Asghar et al., 1984; Morita et al., 1987). However, the higher gel strength of white muscle myosin was seen only for intact myosin molecules but not for digested myosin. Choe et al. (1991) assumed that the difference in strength of heat-induced gels of these isoforms was caused by differences in filament-forming and in gel-forming activity of head and tail segments of myosins. Changes in the rigidity of heat-induced gel at 0.15M KCl and pH 5.9 of gizzard myosin during papain digestion (Fig. 8) showed rigidity was much reduced within 20 min of digestion. Correspondingly, polyacrylamide gel electrophoresis with SDS showed > 90% of the myosin heavy chains were degraded to 90-95 kDa and 130 kDa polypeptides. Further fragmentation of the polypeptides to smaller molecular weight units was not observed during our experiment since the weight ratio of papain to myosin was low (data not shown). These results for gizzard myosin were similar to those for chicken breast myosin (Choe et al., 1991). Since digestion with papain cleaves gizzard myosin molecules at subfragment 1-rod junction (Seidel, 1980; Ikebe and Hartshorne, 1984), these results suggested that head-head or head-tail interactions or cross-linking on heating are important for heat-induced gelation of gizzard myosin.

Samejima et al. (1981) reported that the rigidity of myosin rods from rabbit skeletal muscle was about 66% that of intact whole myosin under similar conditions of ionic strength, pH, or protein concentration. We prepared gizzard myosin rods by papain digestion and ethanol fractionation, but the gelation of the rod fragments was much less than that of intact myosin.

Gizzard myosin formed heat-induced gels basically similar to skeletal muscle myosin, i.e., strength of gels was almost the same as that of skeletal myosin gels under similar conditions (Yasui et al., 1979; Ishioroshi et al., 1979; Samejima et al, 1981). However, some properties of gels of gizzard myosin were different from skeletal muscle myosins. Enhancement of rigidity by F-actin was not found in 0.6M KCl, but only at 0.15M KCl and at pH > 6.2. Gelation of myosin rods was much less than that of skeletal muscle myosin rods. Smooth muscle cells contain much less myosin although they contain many other filamentous (intermediate filament) proteins besides actin (Murphy and Megerman, 1977). Effects of such proteins on heat-induced gelation of myosin may be important in evaluating functional properties of smooth muscles in meat processing.

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ULTRASONIC SPECTRAL ANALYSIS FOR BEEF SENSORY ATTRIBUTES. . .From page 701

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Mention of a company or trade name is for the purpose of description only.

Vegetable Oils Replace Pork Backfat for Low-Fat Frankfurters

E.D. PANERAS and J.G. BLOUKAS

ABSTRACT

Low-fat frankfurters (10% fat, 12.5% protein) with olive, corn, sunflower or soybean oils, compared to control (29.1% animal fat, 10.4% protein) had 67% lower total fat, 40-45% lower saturated fatty acids, 50-53% lower calories, reduced cholesterol and 20% higher meat protein. Although they had darker red color they were 6–7.2% lower in processing yield and had higher purge accumulation, were firmer and less juicy. The type oil had no effect ($P>0.05$) on these characteristics but affected fatty acid composition. Frankfurters with olive oil had 41.8% higher monounsaturated fatty acids and those with seed oils 5–7 times higher polyunsaturated fatty acids. Soybean oil increased linolenic acid content and negatively affected overall acceptability and shelf-life.

Key Words: Low-fat frankfurters, olive oil, corn oil, sunflower oil, soybean oil

INTRODUCTION

FRANKFURTER-TYPE SAUSAGES usually contain up to 30% fat. Fat-reduced and low-fat frankfurters are desirable from a diet/health standpoint as they provide reductions in calories, saturated fatty acids, and cholesterol content. Potential positive effects of low-fat frankfurters could be further increased by substituting the added animal fat with vegetable oils, which are free of cholesterol and have a higher ratio of unsaturated to saturated fatty acids. Because vegetable oils differ considerably in physical properties (color, flavor, free fatty acids, and fatty acid composition, Swern, 1964), they could differently affect the quality characteristics and nutritional value of meat products.

The direct incorporation of vegetable oils in meat products other than frankfurters has been reported. Riendeau (1990) incorporated canola oil into smoked sausages and reported that fat-reduced products (16% fat) were acceptable. Liu et al. (1991) evaluated the replacement of beef patty fat with partially hydrogenated oils from corn, cottonseed, palm, peanut and soybean. They found that only samples containing hydrogenated corn or palm oil were comparable to all-beef patties in cook loss and overall acceptability. A similar comparative study has not been done for low-fat frankfurter-type sausages, which have a different manufacturing technology.

Hammer (1992) incorporated olive oil and sunflower oil in frankfurter-type sausages containing 25% fat. At the high fat level no problems occurred in the processing of vegetable oils into frankfurter-type sausage even without use of blood plasma, emulsifiers, phosphate or salts of food acids. The products were lighter in overall color and the red shades in the cut surface were lighter. Marquez et al. (1989) produced beef frankfurters at 12, 20 and 29% fat levels substituting 60% beef fat with peanut oil. They reported that frankfurters with 12% fat content were less acceptable to a consumer panel than products with 20 or 29% fat, and the substitution of 60% of beef fat with peanut oil did not change overall acceptability. Park et al. (1989) incorporated high-oleic acid sunflower oil (HOSO) and fish oil in frankfurters. They found that frankfurters with 5% fish oil had very low sensory scores due to undesirable fish flavors. Sensory evaluation and texture profile analysis showed that reduction in total fat introduced texture problems. Park et al. (1990) reported that the simultaneous incorporation of maximum allowable levels of water and HOSO at a fat level of 14-16% in frankfurters resulted in products as acceptable as control frankfurters with 28% animal fat. Bloukas and Paneras (1993) incorporated olive oil in low-fat frankfurters (10% fat) formulated for 10%, 12% and 14% protein. Low-fat frankfurters with olive oil had similar flavor but lower processing yield and palatability, compared to the control (27.6% all animal fat, 10.9% protein); and among low-fat treatments, the 12% protein level had better quality characteristics.

For our study, low-fat frankfurters were prepared with ≈10% total fat and 12% meat protein by complete substitution of pork backfat with olive oil, corn oil, sunflower oil or soybean oil. Our objective was to compare processing parameters, sensory properties and composition of the products to a control (28% animal fat, 11% protein) and to evaluate composition in relation to dietary recommendations.

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Table 1—Formulations

	Control[a] (g)	Low-fat treatments[b] (g)
Beef lean (8% fat)	605	984
Pork lean (4.5% fat)	865	1398
Pork backfat	1535	—
Vegetable oil	—	290
Ice/water	2010	2342
Sodium chloride	95	87
Sodium nitrite	1.1	1.2
Sodium ascorbate	3	4
Sodium phosphate	12	12
Sodium caseinate	50	50
Potato starch	200	200
Seasoning	24	32

[a] Prepared with pork backfat formulated for 28% fat and 11% protein.
[b] Prepared with olive oil, corn oil, sunflower oil and soybean oil formulated for <10% fat and 12% protein.

MATERIALS & METHODS

Components and formulation

Frozen lean beef, lean fresh pork and fresh pork backfat were obtained from a local meat market. All meat materials were handled and analyzed as described previously (Bloukas and Paneras, 1993). Four commercial vegetable oils were used: olive oil (O), corn oil (C), sunflower oil (SF), and soybean oil (SB), all obtained from a local market. Representative samples of each were taken for measurement of color and free fatty acid content. Fatty acid compositions were provided by the oil manufacturing companies. All vegetable oils were pre-emulsified on the day of use. Eight parts hot water were mixed for 2 min with one part sodium caseinate. The mixture was emulsified with 10 parts oil for 3 min (Hoogenkamp, 1989a,b).

Five treatments were prepared. Batter formulations of the control and low-fat treatments are presented in Table 1. The control was produced using only pork backfat, formulated to 28% fat and 11% protein. These values approximately represent the mean fat and protein contents of commercial frankfurters in Greece (Bloukas and Paneras, 1986). Other treatments were produced only with vegetable oils, formulated to give final products with <10% fat and 12% protein. In low-fat treatments the added salt was reduced, while the amount of seasonings was increased as suggested by Wirth (1988, 1991) and Hoogenkamp (1989b). All treatments were replicated three times from separate meat, pork backfat and vegetable oil sources at three different time periods.

Table 2—Color and free fatty acid content of pork backfat and vegetable oils used

	Pork backfat	Oils			
		Olive	Caorn	Sunflower	Soybean
Hunter color					
Lightness: L	65.2[a]	37.8[b]	43.9[b]	45.9[b]	45.6[b]
Greenness: a (−)	5.1[a]	−7.2[b]	−8.8[b]	−5.3[b]	−8.2[b]
Yellowness: b (+)	10.2[a]	22.8[a]	3.8[b]	−2.5[b]	−0.9[b]
Hue angle: $\tan^{-1}$ b/a	63.3[a]	−72.7[c]	−20.9[b]	−2.3[b]	5.6[b]
Saturation index: $(a^2 + b^2)^{1/2}$	11.4[b]	23.9[a]	11.2[b]	6.8[b]	8.2[b]
Free fatty acids % (as oleic acid)	0.45[b]	0.92[a]	0.34[b]	0.35[b]	0.38[b]

[a-c] Means within same row with different superscript letters are different ($P<0.05$).

Table 3—Fatty acid composition of animal fats[a] and vegetable oils[b] used

		Fats		Oils			
Fatty acid %		Beef	Pork	Olive	Corn	Sunflower	Soybean
Palmitic	C 16:0	27.4	28.3	11.3	10.5	6.6	10.9
Stearic	C 18:0	21.1	11.9	2.4	1.8	4.4	3.7
Palmitoleic	C 16:1	2.0	2.7	0.7	0.2	0.1	0.2
Oleic	C 18:1	41.6	47.5	78.9	28.4	25.5	20.1
Linoleic	C 18:2	1.8	6.0	9.2	57.2	62.2	55.6
Linolenic	C 18:3	0.5	0.2	0.6	0.4	0.1	7.3
Total saturated		53.7	41.5	13.7	12.3	11.0	14.6
Total unsaturated		46.3	58.5	89.4	86.2	87.9	93.2
Total monounsaturated		43.6	50.2	79.6	28.6	25.6	20.3
M/S ratio[c]		0.8	1.2	5.8	2.3	2.3	1.3

[a] From Forrest et al., 1975.
[b] Provided by manufacturers.
[c] Total monounsaturated/total saturated ratio.

Frankfurter manufacture

Partially thawed lean meat was mixed with curing ingredients and dry chopped for 20–30 sec in a Laska 30L cutter at low speed. After dry-chopping about half the water was added in the form of ice and the chopping continued until a temperature of +3°C was reached. At that point the thawed pork backfat, pre-emulsified vegetable oil, seasoning and other ingredients, together with the remainder of the ice/water, were added and the batter was chopped at high speed until the temperature reached 12°C.

Immediately after chopping the batter of each treatment was vacuum-stuffed into 24-mm-diameter Novax cellulose casings. Each treatment was handlinked at 15-cm intervals and frankfurters were heat-processed and smoked in a smokehouse to an internal temperature of 72°C. The frankfurters were showered for 15 min and chilled at +2°C for 24 hr. After chilling the frankfurters were peeled, vacuum-packaged (650 mm Hg) in film pouches with reported oxygen permeability rate 116 cm^3/m^2/24 hr/1 atm (23°C, 0% RH) and stored in the dark in a cooler at +4°C until analysis.

Analytical methods

Moisture, fat, protein, ash, starch, sodium chloride and sodium nitrite tests, pH and color measurements, and processing yield, purge accumulation and rancidity determinations were conducted as described previously (Bloukas and Paneras, 1993). A five-member trained sensory panel evaluated the frankfurters on an 8-point scale the first week of storage for firmness (8=extremely firm, 1=extremely soft) and juiciness (8=extremely juicy, 1=extremely dry) and the first, third, and seventh week of storage for flavor intensity (8=extremely strong, 1=extremely weak to unpleasant). Each attribute was discussed and tests were initiated after panelists were familiarized with the rating scheme. Samples were prepared by steeping frankfurters in boiling water in individual pans for 2 min. Warm 2.5-cm-long pieces from each treatment were randomly distributed for evaluation. Tap water was provided between samples to cleanse the palate.

A 40-member untrained panel evaluated frankfurters the 1st week of storage for color and overall acceptability on a 6-point hedonic scale (6=like extremely, 1=dislike extremely). Panel members were selected from students, staff and faculty of the Dept. of Food Science & Technology. External color acceptability was evaluated during display of two links from each treatment. Samples for overall acceptability were prepared and provided to panelists as in the trained panel evaluation. Panelists were instructed to evaluate appearance, texture, flavor and juiciness of products and express overall acceptability.

Table 4—Proximate composition and calories of control and low-fat frankfurters made with vegetable oils

		Oils[b]			
	Control[a]	Olive	Corn	Sunflower	Soybean
Moisture (%)	53.6[c]	70.2[d]	71.1[d]	71.3[d]	71.1[d]
Protein (%)	10.4[c]	12.5[d]	12.7[d]	12.8[d]	12.6[d]
Fat (%)	29.1[c]	10.3[d]	9.6[d]	9.4[d]	9.3[d]
Ash (%)	2.6	2.8	2.8	2.5	2.8
Starch (%)	4.3	4.2	3.8	4.0	4.2
Sodium chloride (%)	1.8	1.8	1.9	1.9	2.0
Sodium nitrite (ppm)	148	162	171	159	163
Added water (%)[e]	12.0	20.2	20.3	20.1	20.7
Caloric content[f] (kcal/100g)	325	162	155	154	153
Caloric reduction (%)		50.1	52.3	52.6	52.9
Calories from fat (kcal/100g)	264	94	87	85	85
% calories from fat	81.4	57.8	56.3	55.5	55.3
Composition of fat (%):					
- Beef fat	3.8	18.3	18.3	18.3	18.3
- Pork fat	96.2	14.4	14.4	14.4	14.4
- Vegetable oil	—	67.3	67.3	67.3	67.3

[a] Prepared with pork backfat formulated for 28% fat and 11% protein.
[b] Low-fat treatments formulated for <10% fat and 12% protein.
[c-d] Means within same row with different superscripts are different ($P<0.05$).
[e] Added water (%) = % moisture − 4 × % protein (Claus et al., 1990).
[f] Calculations based on 9.1 kcal/g for fat and 4.1 kcal/g for protein and carbohydrates (Wirth, 1988).

Statistical analysis

Statistical analysis was performed using the MSTAT (1985) program. Data collected for product pH, purge accumulation, TBA and flavor intensity were analyzed by a split-plot design in a completely randomized system. Treatment was the whole plot and storage time and treatment by storage time was the sub-plot. All other parameters were analyzed by one-way analysis of variance. Means were compared by using the $LSD_{0.05}$ test.

RESULTS & DISCUSSION

PORK BACKFAT AND VEGETABLE OILS varied in color and free fatty acid content (Table 2). Pork backfat was ($P<0.05$) lighter, more red and had higher hue angle values than vegetable oils. Olive oil had higher ($P<0.05$) yellowness and saturation index values and lower ($P<0.05$) hue angle values than the other vegetable oils. Also, olive oil had more ($P<0.05$) free fatty acids than the other oils and the pork backfat. The lower free fatty acid content of C, SF, and SB was due to their refining processes. The pork backfat also had a low free fatty acid content because it was fresh.

Vegetable oils and beef and pork fats differed considerably in fatty acid composition (Table 3). Animal fats and especially beef fat had the highest total saturated fatty acids, while O had the most total monounsaturated fatty acid content. C, SF, and SB had the highest percentage of linoleic acid and SB had the most linolenic acid. O had the highest M/S ratio (5.8) while SB and the animal fats had the lowest. The fatty acid composition of the vegetable oils was similar to published values (Swern, 1964; Egan et al., 1981).

Control frankfurters had lower ($P<0.05$) percentage moisture and protein content and higher ($P<0.05$) fat than low-fat treatments (Table 4). Total fat was 67% lower and meat protein 20% higher in low-fat frankfurters compared to the control. No differences ($P>0.05$) were found in sodium chloride and sodium nitrite contents, although the added quantities in low-fat treatments were slightly different than in the control. Caloric content in the low-fat frankfurters was 50.1–52.9% lower than controls. Only 55.3–57.8% of total calories originated from fat in low-fat frankfurters compared to 81.4% in the control. In the control treatment all fat was of animal origin, while in the low-fat frankfurters 67.3% of the total fat was of vegetable origin. Control frankfurters had higher ($P<0.05$) batter pH than low-fat treatments (Table 5). This was due to the high pH of pork backfat which ranged from 6.5 to 6.7, while the pH of beef and pork meat ranged from 5.6 to 5.9. The pork backfat was added only to the control in quan-

Table 5—Effect of substitution of vegetable oils for pork backfat on batter physical factors during 7-wk vacuum storage at 4°C

	Batter pH	Processing yield (%)	Product pH	Purge accumulation %
Control[a]	6.58[d]	86.9[d]	6.49	1.3[c]
Oils				
Olive[b]	6.41[c]	80.9[c]	6.46	1.9[d]
Corn[b]	6.39[c]	80.5[c]	6.45	2.0[d]
Sunflower[b]	6.40[c]	80.6[c]	6.45	1.9[d]
Soybean[b]	6.38[c]	79.2[c]	6.30	2.1[d]
$LSD_{0.05}$	0.05	4.17	0.26	0.54
Storage time (wk)				
1			6.49[d]	1.5[d]
3			6.42[d]	1.8[d]
7			6.26[c]	2.3[c]
$LSD_{0.05}$			0.17	0.30

[a] Control treatment with 29.1% animal fat and 10.4% protein.
[b] Low-fat treatments with ~10% fat and 12.5% protein.
[c-d] Means within same column with superscripts are different (P<0.05).

Table 6—Visual and instrumental color evaluations of control and low-fat frankfurters (means)

	Visual evaluation[e]	Lightness (L)		Redness a(+)		Yellowness b(+)	
		Skin	Internal	Skin	Internal	Skin	Internal
Control[a]	1.7[c]	54.8[c]	55.4	15.4[c]	11.8	14.8	12.5
Oils							
Olive[b]	4.1[d]	44.3[d]	53.4	21.8[d]	13.6	15.4	12.3
Corn[b]	4.6[d]	44.1[d]	54.0	24.6[d]	13.9	15.3	12.4
Sunflower[b]	4.0[d]	44.5[d]	56.0	23.3[d]	13.2	15.5	12.4
Soybean[b]	4.5[d]	41.9[d]	54.4	24.2[d]	13.5	14.7	12.4
$LSD_{0.05}$	0.97	5.34	4.61	3.12	2.82	2.77	1.22

[a] Control treatment with 29.1% animal fat and 10.4% protein.
[b] Low-fat treatments with ~10% fat and 12.5% protein.
[c-d] Means within same column with different superscripts are different (P<0.05).
[e] External color evaluation scale : 6=like extremely, 1=dislike extremely.

Table 7—TBA and sensory quality characteristics and consumer acceptance of control and low-fat frankfurters

		Trained panel			Consumer panel
	TBA	Flavor intensity[e]	Juiciness[f]	Firmness[g]	overall acceptability[h]
Treatment[a]					
Oils					
Control[b]	0.96[cd]	5.2[c]	6.9[c]	3.0[d]	3.2[d]
Olive[b]	0.70[d]	5.4[c]	4.6[d]	5.0[c]	3.7[cd]
Corn[b]	0.76[d]	5.2[c]	5.2[d]	5.0[c]	4.1[c]
Sunflower[b]	0.85[cd]	5.3[c]	4.8[d]	5.4[c]	3.8[cd]
Soybean[b]	1.06[d]	4.3[d]	4.8[d]	5.6[c]	3.3[d]
$LSD_{0.05}$	0.28	0.6	0.7	0.7	0.7
Storage time (wk)					
1	0.78[d]	4.7[c]			
3	0.87[cd]	4.7[c]			
7	0.94[c]	3.5[d]			
$LSD_{0.05}$	0.13	0.5			

[a] Control treatment had 29.1% animal fat and 10.4% protein.
[b] Low-fat treatments with ~10% fat and 12.5% protein.
[cd] Means within same column with different superscripts are different (P<0.05).
[e] Evaluated 1st, 3rd and 7th week of storage with: 8=extremely strong, 1=extremely weak to unpleasant.
[f] Evaluated 1st week of storage with: 8=extremely juicy, 1=extremely dry.
[g] Evaluated 1st week of storage with: 8 =extremely firm, 1=extremely soft.
[h] Evaluated 1st week of storage with: 6=like extremely, 1=dislike extremely.

ties higher than beef and pork meat (Table 1). Thus, its pH value affected batter pH.

Processing yields for the control (about 87%) was 6–7.2% higher (P<0.05) than low-fat frankfurters with the 10% fat level, in confirmation of previous results (Bloukas and Paneras, 1993). Such processing yields for low-fat frankfurters with vegetable oils are considered low for the industry and need to be increased. Park et al. (1989) reported that control frankfurters, with nearly the same fat level, had 86.5% processing yield, while the yield of low-fat HOSO frankfurters, with 16.8–17.5% fat level, was 5–6% lower and ranged from 81.3–82.0%. The type of vegetable oil had no effect on processing yield in low-fat frankfurters. Processing yield was affected by fat level rather than by fat type. Reducing the fat content in frankfurters reduced processing yields (Sofos and Allen 1977; Wallingford and Labuza 1983; Marquez et al., 1989).

Low-fat frankfurters had more (P<0.05) purge accumulation than controls, while no differences in pH values occurred (Table 5). Claus et al. (1990) also found higher purge accumulation for low-fat, high-added water frankfurters which was attributed to the lower ionic strength of low-fat frankfurters compared to controls. As storage time (Table 5) increased pH decreased and purge accumulation increased (P<0.05). The correlation coefficient between pH and purge accumulation was $r = -0.58$ (P<0.05).

Control frankfurters had lower (P<0.05) scores for consumer panel (visual) color than all other treatments (Table 6). Significant differences in visual color scores among low-fat frankfurters were not found. Untrained panelists rated the external color of control frankfurters low because it was rather pale-red, while the external color of low-fat frankfurters was darker-red. The skin of control frankfurters had higher (P<0.05) L values and lower (P<0.05) a(+) values, indicating these products were light and less red than low-fat frankfurters, while lightness, redness and yellowness of internal colors were not different. Claus and Hunt (1991) found that bologna sausage with 30% fat had lower (P<0.05) visual color scores and a(+) values and higher (P<0.05) L values than the same products with 10% fat. Marquez et al. (1989) found that lightness of frankfurters was affected by fat content, while 60% substitution of beef fat with peanut oil had no effect (P>0.05). Differences in visual color scores and Hunter color values between controls and low-fat frankfurters could be attributed to the higher cured pigment concentration in the skin of low-fat frankfurters. The surface of frankfurters is exposed during heat processing to higher temperatures. This results in a more intensive coagulation of proteins in that area and the formation of a skin. In low-fat frankfurters, the protein matrix is more dense and the higher shrinkage increased the cured pigment content in the skin.

Differences occurred in TBA values between treatments (Table 7), although all were acceptable (<1.0) for rancidity (Ockerman, 1976) after 7 wks storage at 4°C. This was probably due to the presence of curing mixture ingredients, such as nitrite, phosphate and ascorbate, which act as antioxidants. Low-fat frankfurters with O had the lowest (P<0.05) TBA values, while those with SB had the highest. This is because SB had the highest concentration of polyunsaturated fatty acids. Olive oil, in addition to low linolenic acid (18:3) concentration, has tocopherols and phenolic substances with antioxidant activity. The storage time of frankfurters under vacuum at 4°C over 7 wks had a significant effect on TBA values but significant interactions occurred between treatment and storage time. These results were not in agreement with Marquez et al. (1989) and Park et al. (1989) who had not found significant increases in TBA values over 6 and 12 wks storage, respectively, in low-fat frankfurters produced with peanut oil or HOSO. This difference may be due to oxidation of SB which is less saturated than peanut or HOSO.

Low-fat frankfurters with SB had lower (P<0.05) cured flavor intensity than all other treatments (Table 7). Storage time affected flavor intensity but no significant interactions occurred between treatment and storage time. After the third week the flavor intensity declined and at the seventh week it was lower (P<0.05) than initially. The seventh week of storage the trained panelists characterized the flavor of low-fat frankfurters with SB as unpleasant, while all other treatments had very weak flavor. According to Egan et al. (1981) SB is prone to autoxidation and rancidity because of its high linolenic acid. The correlation coefficient between flavor intensity and TBA value was $r = -0.38$ (P<0.01). Thus the decline in flavor intensity can be partially attributed to oxidation of oils.

Control frankfurters had higher (P<0.05) juiciness and lower (P<0.05) firmness than low-fat frankfurters. Differences

Table 8—Calculated fatty acid composition of controls and low-fat frankfurters

	Fatty acids (%)						Total saturated	Total m/unsat.	Total polyunsat.	M/S ratio
	16:0	18:0	16:1	18:1	18:2	18:3				
Control[b]	28.2	12.2	2.6	46.2	5.8	0.2	40.4	48.8	6.0	1.20
Oils										
Olive[c]	16.7	7.2	1.2	67.6	7.4	0.5	23.9	68.8	7.9	2.87
Corn[c]	16.1	6.8	0.8	33.6	39.7	0.3	22.9	34.4	40.0	1.50
Sunflower[c]	13.5	8.5	0.8	31.6	43.1	0.2	22.2	32.4	43.3	1.47
Soybean[c]	16.4	8.0	0.8	28.0	38.7	5.0	24.4	28.8	43.7	1.18

[a] Calculations based on fatty acid composition of animal fats and vegetable oils (Table 3), and contribution of each type fat in total fat content of each treatment (Table 4).
[b] Control treatment with 29.1% animal fat and 10.4% protein.
[c] Low-fat treatments with ~10% fat and 12.5% protein.

were not found between low-fat treatments in these sensory characteristics. Control frankfurters were soft, while all low-fat frankfurters had a hard skin due to more shrinkage during heat processing. Overall acceptability, evaluated by consumer panel the 1st week of storage, was higher ($P<0.05$) for low-fat frankfurter with C than control. All other low-fat frankfurters had no different acceptability from the control. Overall acceptability scores for all treatments were low. This was attributed to the pale-red external color of controls and to the high firmness and low juiciness of the low-fat frankfurters. Calculated fatty acid compositions (Table 8) show substitution of vegetable oils reduced the percentage of saturated fatty acids from 40.4% in controls to 22.2–23.9% in low-fat frankfurters. Thus the total reduction in saturated fatty acids was 40–45%.

Incorporation of O into low-fat frankfurters increased the percentage of monounsaturated fatty acids to 68.8%, about 41.8% higher than controls. The O treatment had a higher monounsaturated/saturated ratio than all other treatments. Low-fat frankfurters produced with O, rich in oleic acid, are probably better quality from a nutritional point, since they have 2 × more total monounsaturated fatty acid than low-fat frankfurters produced with C, SF or SB. Monounsaturated fatty acids lower total plasma cholesterol and LDL-cholesterol levels when substituted for saturated fatty acids in the diet and may decrease coronary heart disease (NCEP, 1988). Considering also the stearic acid, which acts in the diet as a monounsaturated fatty acid (Bonanome and Grundy, 1988), the total beneficial fatty acids in olive oil frankfurters account for 76% of the total fatty acid content.

Low-fat frankfurters with C, SF, and SB had lower percentages of monounsaturated fatty acids than controls. The monounsaturated fatty acid content in the control was 48%, while in C, SF and SB frankfurters it ranged from 28.8 to 34.5%. However, the above treatments had a five to seven times higher content of linoleic acid (18:2) than controls and frankfurters containing O. Linoleic acid (18:2) has been implicated in blood platelet adhesiveness and thrombosis (Reiser and Shorland, 1990). Recommendations toward a high level of linoleic acid in the diet have been reduced to the same 10% level of calories as for saturated fatty acids.

The SB treatment had nearly the same M/S ratio as controls. Soybean oil, incorporated into low-fat frankfurters, increased more than 10-25 times the percentage in linolenic acid (18:3) compared to all other treatments. Linolenic acid, although nutritionally desirable, is polyunsaturated and is considered a cancer risk in high concentrations. It also leads to the oxidative loss of nutrients and the development of rancidity (Briggs and Schweigert, 1990).

CONCLUSIONS

COMMERCIALLY AVAILABLE VEGETABLE OILS, such as O, C, SF and SB were used for complete substitution of pork backfat in the production of low-fat (10% fat, 12.5% protein) frankfurters. Such products would be beneficial from a diet/health standpoint since they have lower total fat and higher protein, lower caloric value, reduced cholesterol and lower saturated fatty acids. The type of vegetable oil affected the fatty acid composition of frankfurters. Olive oil increased >40% the monounsaturated fatty acids, C, SF and SB increased six to seven times the linoleic acid (18:2) and SB more than 10 times the linolenic acid (18:3) of frankfurters. Low-fat frankfurters with vegetable oils had higher rated external and internal color than conventional frankfurters, but firmer consistency and lower juiciness. Frankfurters with O, C and SF had the same flavor intensity as controls and higher scores for overall acceptability. Frankfurters with SB had lowest scores for flavor and overall acceptability and the least storage stability.

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—Continued on page 733

Textural Properties of Chicken Frankfurters with Added Collagen Fibers

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ABSTRACT

Chicken frankfurters made with 0, 2, 4, 6, 8% added collagen fibers and 10, 15, 20, 25, 30% added water were evaluated for textural differences using a torsion test and sensory texture profile analysis. Frankfurters with high amounts of added water and low amounts of collagen fibers had lower shear stress values. Sensory analysis indicated that both collagen fibers and added water had significant influences ($P<0.05$) on hardness, springiness and juiciness. The addition of collagen fibers resulted in harder, springier, and less juicy frankfurters. Added water resulted in softer, less springy and juicier frankfurters. Response surface methodology demonstrated that for 2% added collagen, the optimum added water was ≈20%.

Key Words: poultry, collagen, chicken, sensory, frankfurters

INTRODUCTION

HISTORICALLY, RED MEAT FRANKFURTERS have dominated the sausage markets. Nutritional concerns about fat intake from red meat, combined with an abundance of comminuted poultry meat, and its moderate price, have resulted in an increasing market for poultry products. Texture is important for consumer acceptance of frankfurters, and some poultry frankfurters lack acceptable texture. Such poultry products are often evaluated as lacking firmness and bite. The protein gelation process determines, in part, the final texture of frankfurters and understanding the factors affecting gelation is fundamental to their formulation. Some factors influencing gelation are protein source, processing conditions and nonmeat ingredients (Amato et al., 1989; Montejano et al., 1984; Wu et al., 1985; Saliba et al., 1987).

Sensory evaluation has been the traditional method of determining acceptable texture, but may be costly and time consuming. Instrumental structural failure tests have been developed to lower the cost of textural evaluations (Diehl and Hamann, 1979). A torsion test that measures shear stress and strain at failure has been used to evaluate protein gels (Kim et al., 1986; Montejano et al., 1985). Shear stress at failure (the measure of gel strength) is sensitive to protein concentration, processing effects, and ingredients (Hamann, 1988b). Unlike shear stress, true shear strain at failure is a measure of a gel's ability to deform before rupture. It is not very sensitive to protein concentration nor to processing variables, but, is influenced mainly by protein quality and may be considered an evaluation of protein functionality (Hamann and Lanier, 1986). Both shear stress and strain at failure are good predictors of sensory properties such as springiness, hardness, and cohesiveness for meat gels (Daget and Collyer, 1984; Montejano et al., 1985; Lanier et al., 1985).

Our objectives were (1) to evaluate effects of collagen and added water on texture of frankfurters using sensory evaluation and torsion tests; (2) to evaluate optimum use of collagen in formulations of chicken frankfurters; (3) to determine correlations between sensory and torsion results and evaluate whether the torsion test could replace sensory evaluation in such textural studies.

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MATERIALS & METHODS

CHICKEN FRANKFURTERS were processed using two independent variables (collagen fibers and added water). A full 5×5 factorial design with orthogonally spaced treatments, using five levels of water (10, 15, 20, 25 and 30% of raw meat weight) and five levels of collagen fibers added (0, 2, 4, 6, 8% of raw meat weight) was used for 25 different treatments. The process was replicated three times. This was followed by instrumental and sensory evaluation for the 75 samples.

Processing

Mechanically deboned poultry meat (MDPM, 15–16% fat) (Proteins Foods Inc., Gainesville, GA) was used to manufacture frankfurters with a standard frankfurter manufacturing technology to include spices, additives and water. Collagen fibers (Stork Fibron B.V., Holland) were included at the beginning of the process. Twelve pounds of raw material were used for each batch. The meat was chopped and blended in a meat chopper (Kramer-Grebe, Germany) for 12–14 min to a final temperature of 5–6°C. Batters were then promptly vacuum stuffed (V-Mag Corp.) into clear fibrous casings (20 mm) (Teepak Co., Atlanta, GA). The sausages were cooked using a standard smokehouse schedule, then showered before being placed in a cooler (4°C). The sausages were kept at 4°C overnight, peeled, vacuum packaged in barrier bags (Cryovac Multivac Type AG500, Mash Nr 4106, Duncan, SC) and frozen until sensory evaluation and torsion testing.

Sensory evaluation

Panelists were recruited among university personnel. Selection of panelists was done using a demographic questionnaire, followed by ranking test for hardness of various food samples. People with allergies or dental devices were eliminated. The ability of panelists to determine textural properties was tested using a ranking test for sensory hardness (Meilgaard et al., 1987). Panelists were asked to rank four samples (yellow cheese, peanuts, olives, and hot dogs) from the less hard to harder.

Eleven experienced panelists, who had participated in other sensory panels, were selected and trained 10 hr for evaluation of frankfurters according to the Texture Profile Analysis training procedure of Civille and Szczesniak (1973). During training, judges were familiarized with different attributes used in the Texture Profile Analysis. The five characteristics used were hardness, cohesiveness, springiness, chewiness, and juiciness. These attributes were considered those most important to adequately describe frankfurter texture. Definitions for the attributes were as described by Civille and Szczesniak (1973) with the exception that juiciness was included. It was defined as the amount of liquid released from the sample when chewed. The 150 mm intensity scale was modified from Szczesniak (1963) by placing two laboratory manufactured frankfurter standards on the scale and removing Szczesniak's standards from the scale after the training period. These two standards, one beef and one poultry frankfurter, were expected to be quite different and to provide a wide range of texture. Scores attributed to the two standards were determined by panelists during the training period by comparing frankfurter standards with standards used by Szczesniak (1963) for each attribute. This modification was made to minimize the number of standards presented to judges during each session. The composition and description of the two standards manufactured in the laboratory were compared and their sensory scores were determined (Table 1). One batch of those standards was processed at the beginning of sensory evaluation and was used throughout the study.

Table 1—Composition and sensory scores[a] attributed to frankfurter standards

	Standard 1[b]	Standard 2[b]
Composition		
Raw material	beef 50/50	MDPM[c]
Water added	10%	10%
Scores		
Hardness (mm)	50	60
Cohesiveness (mm)	80	90
Springiness (mm)	90	105
Chewiness (chews)	18	18
Juiciness (mm)	30	55

[a] Sensory scores were determined by the panelists during the training period by comparing the frankfurter standards with the standards from Szczesniak (1963). The scores are expressed on a 150 mm scale.
[b] The standards were manufactured in the processing plant of the Univ. of Georgia at the beginning of the study. Those standards were used throughout the sensory analysis and the instrumental texture analysis.
[c] Mechanically deboned poultry meat.

Table 2—Means of shear stress values[a] for the different combinations collagen/water

Added water (%)	Added collagen fibers (%) 0	2	4	6	8	Means
10	26.3	33.8	50.3	68.8	70.1	49.8[f]
15	33.6	39.9	43.8	62.7	70.6	50.1[f]
20	25.8	29.9	39.3	49.1	73.0	43.4[b]
25	24.2	30.7	43.6	49.3	56.3	40.8[c]
30	23.9	23.3	39.2	47.6	57.1	38.2[d]
Means	26.8[a]	31.5[b]	43.2[c]	55.5[d]	65.4[e]	

[a] Shear stress values are expressed in KPa. ANOVA and protected LSD tests were performed to determine the effects of collagen and added water on true shear stress and strain at failure.
[b-f] Means in the same column or row with different superscripts differ significantly ($P<0.01$).

Torsion tests

Torsion tests were performed on the same sausage samples used for sensory evaluation. Frankfurters were cut into 28.7 mm long samples with a specially designed cutter (Univ. of Georgia). Plastic disks (Accu-Tool Corp. Cary, NC) designed to fit the torsion apparatus were glued onto the ends of the sample with cyanoacrylate glue (Richbond Super glue). The ends of each sample were dried with a napkin to allow the disk to stick properly to the fresh cut meat cylinder. Samples were machined (Accu-Tool Corp., Cary, NC) into dumbbell-shaped specimens with a final minimum diameter of 10.0 mm using the method described by Montejano et al. (1983). As reported by Diehl et al. (1979), the dimensions of the annular groove were critical to proper calculation of shear stress and strain at failure. Samples were placed in a modified Brookfield digital viscometer model DV-I (Torsion fixture attached to the viscometer) and twisted at 2.5 rpm until failure. Torque and time required to break the sample were recorded on a chart recorder (Sargent and Co., model SRG). Shear stress and strain at failure were calculated as described by Hamann (1983) using the following equations:

$$\text{Shear stress (Pa)} = 1580^{*}\text{torque in instrument units}$$

$$\text{Shear strain (dimensionless)} = \tau_{true} = \text{Ln}[1+\tau^2+\tau(1+\tau^2/4)^{1/4}]$$

where $\tau = 0.147^{*}$(chart travel/chart speed) $- 0.00848^{*}$instrument torque.

Statistical analysis

The experiment was conducted as a randomized block design (2-way ANOVA). The combinations of water/collagen were orthogonally spaced. The data were analyzed using SAS data analysis system. Protected Least Square Difference (LSD) tests were performed to test differences between treatments. A multiple regression model, including linear, quadratic and cross product effects was used to predict any effects of added collagen fibers and added water on frankfurter texture. Response surface methodology was used to determine optimum levels of collagen fibers and water. Correlation factors between torsion test results and sensory evaluation were calculated and compared.

Table 3—Means of true shear strain values[a] at failure for the different combinations collagen/water

Added water (%)	Added collagen fibers (%) 0	2	4	6	8	Means
10	1.46	1.37	1.44	1.57	1.49	1.47[b]
15	1.52	1.65	1.49	1.51	1.48	1.53[b]
20	1.49	1.42	1.48	1.45	1.49	1.47[b]
25	1.59	1.52	1.41	1.49	1.54	1.51[b]
30	1.46	1.41	1.54	1.56	1.44	1.48[b]
Means	1.51[b]	1.48[b]	1.47[b]	1.52[b]	1.48[b]	

[a] True shear strain values are dimensionless. ANOVA and protected LSD tests were performed to determine the effect of collagen fibers and water on true shear strain at failure.
[b] Means with different letters are significantly different ($P<0.05$) from each other.

RESULTS & DISCUSSION

Chemical analysis

Chemical analysis of raw material (MDPM) showed no differences ($P>0.05$) among treatments. This indicated uniformity of raw materials used to prepare the different treatments. Chemical analysis of the frankfurters showed some differences ($P<0.05$) among treatments. As the quantities of added collagen and water increased, fat content decreased and moisture and protein contents increased. This was a dilution effect, because the quantities of fat and water remained the same but their ratios were decreased because of added collagen.

Torsion

Means for shear stress at failure (strength) (Table 2) showed added collagen and added water had a highly significant effect ($P<0.01$) on true shear stress. As amount of added water increases, the shear stress decreased. These results confirmed that shear stress is influenced by protein concentration or ingredient variations (Kim et al., 1986; Montejano et al., 1984). Collagen had the opposite effect on true shear stress. Adding collagen fibers increased the concentration of total protein in the product and therefore increased the strength of gels. This confirmed results of Hamann (1988b) who reported that shear stress was primarily sensitive to protein concentration.

The means for true shear strain at failure (deformability) are presented in Table 3. As previously indicated, Hamann (1988b) found that shear strain was sensitive to protein functionality. Shear strain is influenced by types of proteins in the protein matrix of the meat batter. Because no significant differences ($P>0.05$) were found among treatments for shear strain (Table 3) we concluded that collagen fibers did not participate in formation of the gel matrix. We hypothesized that collagen fibers were denatured at 65°C, and acted as filler of the protein matrix. Therefore, the influence of collagen was only notable on product resistance but not on deformability. Lanier (1986) proposed that a plot of torsional rigidity (stress/strain) vs strain at failure would adequately describe textural properties of most protein gels and could also be used to show treatment effects on gel texture (Fig. 1). Each quadrant was associated with one characteristic that described texture of a gel.

The standard frankfurter recipe (010:0% of collagen added and 10% of water added) was used for reference to compare with other treatments. Note that all treatments with collagen added (even 2% added collagen) had changed textural properties. The treatments with 6 or 8% collagen added showed an undesirable toughness. Treatments with 2 or 4% collagen added showed increased torsional rigidity but in a more acceptable range than the 6% or 8% samples. The closest combinations to the reference were "220" and "225". The water holding capacity of collagen fibers allows water to be held in the product. Thus a 2% collagen combination allowed more water to be added to the product without a change of texture.

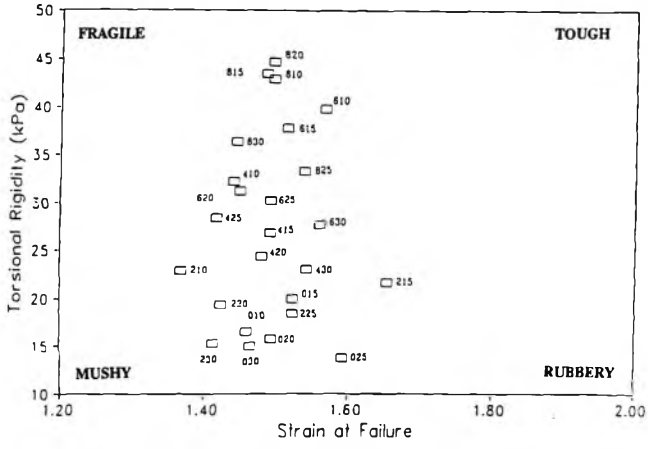

Fig. 1—Torsional rigidity vs strains at failure of frankfurters. "815" = 8% collagen fibers and 15% added water.

Sensory analysis

The effect of added water was most significant ($P<0.01$) on hardness and juiciness (Table 4). As water increased, hardness decreased and juiciness increased, consistent with results of Lanier et al. (1985). Added water had no influence on cohesiveness or chewiness. This confirmed conclusions of Lanier et al. (1985) who reported that water had little effect on cohesiveness because it did not influence the quality of proteins forming the protein matrix of the gel. Springiness was influenced ($P<0.05$) by the amount of water added. Treatments with 20% and more of added water were significantly less springy than those with 10 or 15% added water. Both hardness and juiciness were highly influenced ($P<0.01$) by the amount of collagen added to the meat batter (Table 5). An increase in collagen fibers increased toughness and decreased juiciness of the final product. This is important since hardness and juiciness are important textural attributes in determination of acceptable sausages. Added collagen had little influence on cohesiveness ($P>0.05$). A difference ($P<0.05$) was found only between treatments with no collagen fibers added compared to 8% added fibers. Collagen added in high proportion tended to decrease cohesiveness. Results by torsion test were verified by those of the sensory evaluation for this attribute. Collagen increased ($P<0.05$) chewiness of the final product. Chewiness was increased by almost one chew for those treatments with 8% added collagen fibers compared to treatments with 0% added collagen fibers.

Correlations between torsion and sensory analysis

Hamann and Lanier (1986) reported that shear stress most highly correlated with hardness (0.82) and shear strain most highly correlated with cohesiveness (0.84). Our results (Table 6) showed highest positive correlations between shear stress and hardness (0.76) and chewiness (0.57). A significant correlation between shear strain and cohesiveness was not found because different treatments were not significantly different for shear strain. Shear stress at failure most highly negatively correlated with juiciness (−0.89). The addition of collagen fibers decreased juiciness and increased shear stress. In contrast, the addition of water increased juiciness and decreased shear stress.

Response surface methodology

Multiple regression models were determined from sensory and torsion data to evaluate optima for hardness, cohesiveness, springiness, chewiness and juiciness from amounts of water and collagen added (Table 7). Because independent variables of the models were not all significant at the 10% level, the models were reduced using the stepwise procedure of SAS to retain the significant variables. Because of colinearity problems between independent variables, linear and quadratic effects were not kept in the same models. Quadratic effects often better described the data (higher R-squares) (Table 8). Good prediction models that explain most of the variation among treatments (high R-square) with reasonably good fits (no significant lack of fit) were found for hardness (0.82), chewiness (0.79) and juiciness (0.89). Models with relatively low R^2 values were found for cohesiveness (0.11) and springiness (0.58). Those models (Fig. 2 through 6) were predictors of the evolution of each characteristic when amounts of added water and

Table 4—Means of sensory scores[a] for different levels of water added

sensory attributes	Added water (%)				
	10	15	20	25	30
hardness	68.0[e]	66.6[e]	62.0[b]	58.9[c]	56.0[d]
cohesiveness	95.4[e]	94.7[be]	93.6[be]	92.6[b]	92.3[b]
springiness	106.5[e]	106.3[e]	103.2[b]	101.3[b]	100.5[b]
chewiness	18.5[e]	18.5[e]	18.3[e]	17.7[b]	17.5[b]
juiciness	43.6[d]	47.3[c]	49.4[bc]	50.7[b]	52.7[e]

[a] Sensory scores for hardness, cohesiveness, springiness and juiciness are expressed in millimeters (0 to 150 mm scale). Chewiness is expressed in number of chews. ANOVA and protected LSD tests were performed to determine the effect of added water on sensory scores.
[b-e] Means with different letters are significantly different ($P<0.05$) from each other.

Table 5—Means of sensory scores[a] for different levels of collagen fibers added

sensory attributes	added collagen fibers (%)				
	0	2	4	6	8
hardness	58.7[c]	59.5[c]	62.4[b]	65.3[f]	65.5[f]
cohesiveness	96.3[f]	94.4[bf]	93.4[b]	92.8[b]	91.8[b]
springiness	103.2[bf]	101.6[b]	103.6[bf]	105.4[f]	103.0[bf]
chewiness	17.7[c]	17.8[bc]	18.2[bf]	18.2[f]	18.4[f]
juiciness	66.9[f]	54.1[b]	46.5[c]	40.3[d]	36.7[e]

[a] Sensory scores for hardness, cohesiveness, springiness and juiciness are expressed in millimeters (0 to 150 mm scales). Chewiness is expressed in number of chews. ANOVA and protected LSD tests were performed to determine the effect of added collagen fibers on sensory scores.
[b-f] Means with different letters are significantly different ($P<0.05$) from each other.

Table 6—Correlation coefficients[a] between sensory analysis and torsion test results

	Torsional characteristics	
	shear stress	shear strain
Hardness	0.7614	0.4345
Cohesiveness	n.s.	n.s.
Springiness	0.4157	n.s.
Chewiness	0.5785	0.4028
Juiciness	−0.8986	n.s.

[a] Correlation coefficients reported in this table are Pearson product-moments, calculated using the means for each treatment (number of observations n=75).

Table 7—Full models[a] used to predict hardness, cohesiveness, springiness, chewiness and juiciness of chicken hot dogs using the independent variables collagen and water added

Regression equation:

$$Y = b_0 + b_1{*}c_2 + b_2{*}W + b_3CW + b_4{*}C^2 + b_5{*}W^2 + b_6{*}6\ C^2{*}W + b_7{*}C{*}W^2 + b_8{*}C^2{*}W^2$$

	Hardness	Cohesiveness	Springiness	Chewiness	Juiciness
R-sq	0.84	0.18	0.64	0.83	0.89
b_0	80.63	117.79	123.99	19.63	34.62
b_1	−2.09	−5.11	−3.42	−0.40	+0.12
b_2	−1.45	−1.94	−1.67	−0.12	+2.98
b_3	+0.13	+0.31	+0.25	+0.03	−0.78
b_4	+0.05	+0.07	+0.05	+0.01	+0.08
b_5	+0.02	+0.04	+0.03	+0.001	−0.06
b_6	+0.02	+0.01	+0.01	+0.001	+0.04
b_7	+0.001	−0.005	−0.003	−0.0002	+0.02
b_8	−0.001	−0.001	−0.01	−0.0001	−0.001

[a] All models were significant at 1% level. "C" stands for added collagen fibers (%) and "W" for added water (%). The regression models were determined using SAS (procedure RSreg).

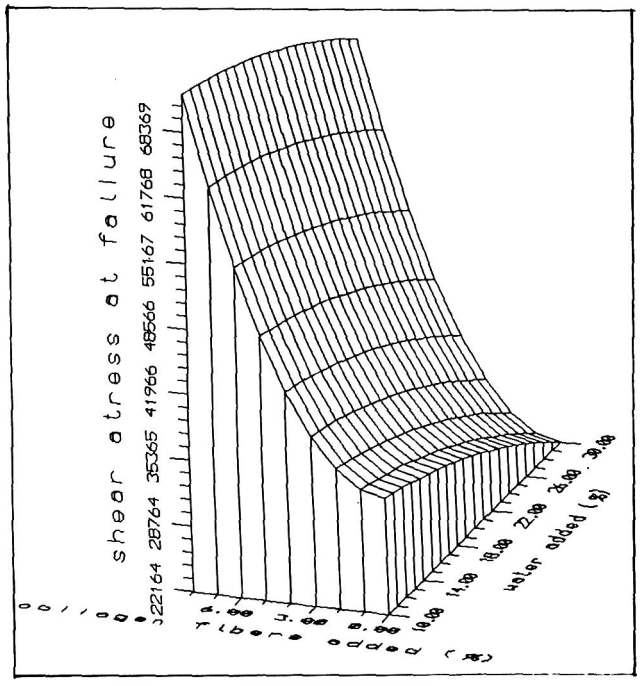

Fig. 2—Response surface for stress at failure.

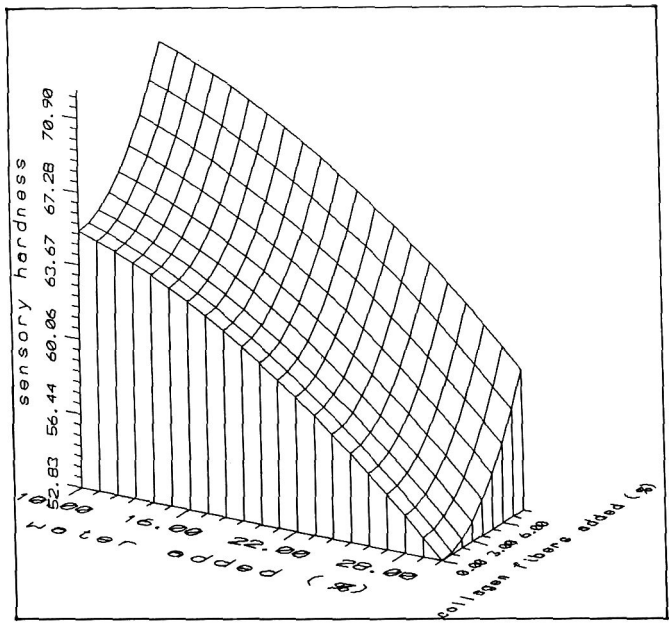

Fig. 3—Response surface for sensory hardness.

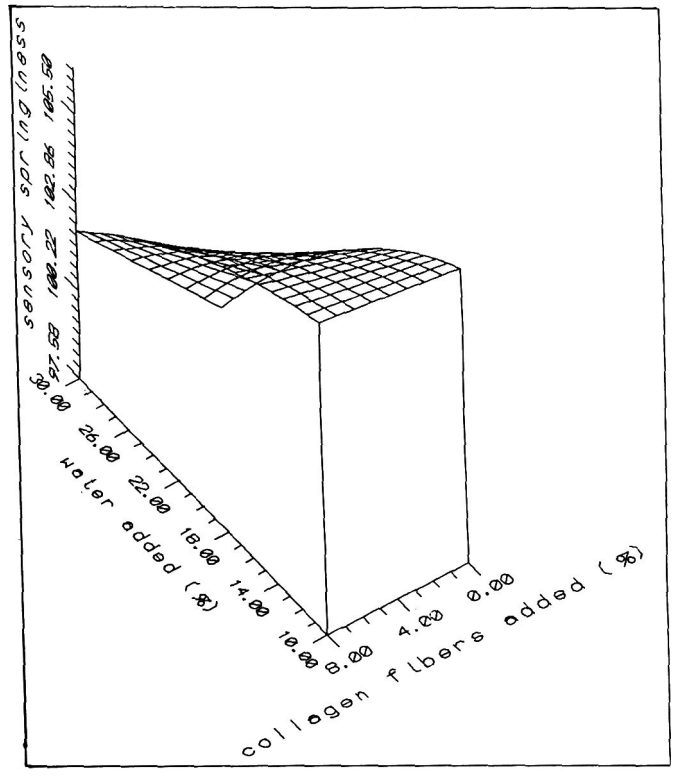

Fig. 4—Response surface for sensory springiness.

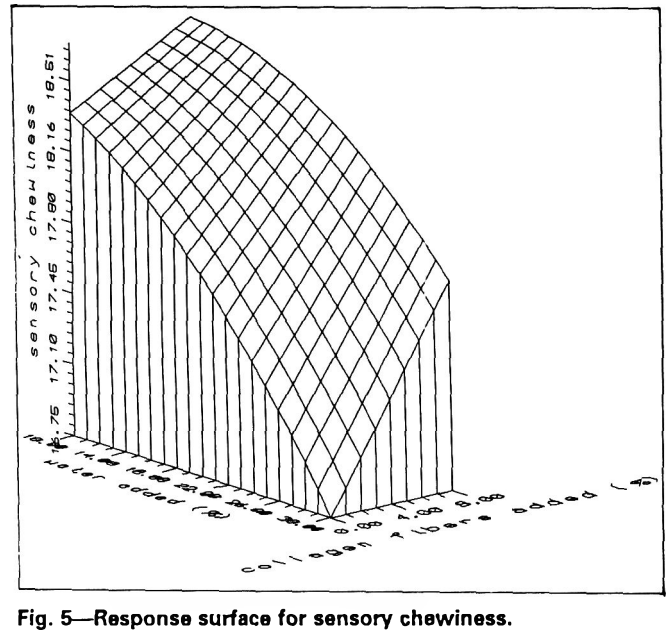

Fig. 5—Response surface for sensory chewiness.

Table 8—Reduced models[a] used to predict hardness, cohesiveness, springiness, chewiness, juiciness, shear stress and shear strain of chicken hot dogs using collagen and water added as independent variables

	R-squares
hardness = 66.96 + 0.107*C^2 −0.015*W^2	0.82
cohesiveness = 95.72 − 0.023*C*W	0.11
springiness = 107.53 − 0.011*W^2 + 0.0006*C*W	0.58
chewiness = 18.55 − 0.002*W^2	0.79
juiciness = 54.83 + 0.023*W^2 −0.288C*W	0.89
shear stress = 35229 + 599.85*C^2 − 14.51*W^2	0.85
shear strain : none of the variables were significant	

[a] All models were significant at 1% level. "C" stands for added collagen fibers (%) and "W" for added water (%). The models were reduced using the stepwise procedure for SAS. All the variables with a p-value < 0.1 were kept in the regression model.

collagen vary. All panelists noted that collagen fibers gave a strong objectionable taste to the final product when used at >2% level. Therefore, 2% appears a reasonable amount of collagen fibers to add. Based on 2% collagen addition, an optimum use of water was determined by using as reference the scores for the MDPM standard hot dog (no collagen fibers added and 10% added water). To reach a score of 60 mm for hardness, the amount of added water was determined to be 21.7%. To reach a score of 105 mm for springiness, the amount of added water should be 21.5%. To reach a score of 18 for chewiness, considering 2% addition of collagen, the added water should be 16.5%. Finally, to reach a score of 55 mm for juiciness, 28.6% of water should be added. Addition of 2% collagen fibers in the product would allow addition of

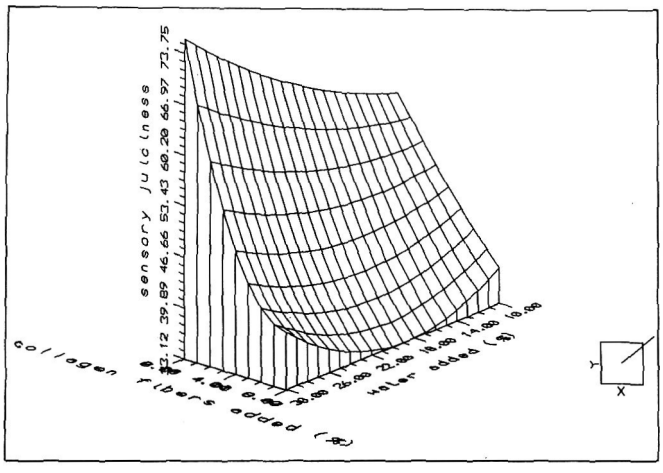

Fig. 6—Response surface for sensory juiciness.

≈20% water to make a final product comparable to the reference hot dogs.

CONCLUSIONS

COLLAGEN AND WATER in combination affected the texture of chicken franks. Adding collagen fibers increased the amounts of added water without changing notably the gel texture. Collagen gave a strong objectionable taste to the final product starting at 4% addition. Thus, the addition of collagen fibers should probably not exceed 2%. A plot of torsional rigidity vs strain at failure showed textural differences between the treatment samples. Water up to ≈20% could be added without diminishing textural acceptability of the standard hot dog. Similar results were verified using Response Surface Methodology.

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Myofibrillar Protein Gelation: Viscoelastic Changes Related to Heating Procedures

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ABSTRACT

Dynamic rheological properties were investigated during gelation of chicken myofibrillar protein as influenced by heating procedures, Thermal scan (1°C/min) of myofibril suspensions in 0.6*M* NaCl (*pH* 6.0) induced a major transition in storage modulus (G', peak 48°C), preceded by a transition in protein-protein aggregation (46°C) and accompanied by a marked reduction in actomyosin solubility. Preheating at 50°C diminished the transition and resulted in increased final G' value. Isothermal heating produced complex, temperature-dependent rheological changes (G' and phase angles), particularly within 43–58°C. The rheological transitions of myofibrillar protein were probably related to kinetic changes during formation of elastic gel networks. Such rheological data on gel formation can help predict and control muscle food responses to specific thermal processes.

Key Words: chicken muscle, myofibrillar protein, gelation, storage modulus, rheological transitions

INTRODUCTION

GELATION OF MUSCLE PROTEIN is a dynamic process which involves protein unfolding and aggregation prior to formation of three-dimensional network structures. During thermal gelation, myosin and salt-soluble myofibrillar protein (SSP) exhibit complex changes in rheological characteristics depending on specific temperatures and pH (Egelandsdal et al., 1986; Wang et al., 1990; Xiong, 1993; Xiong and Blanchard, 1993). When myofibrillar proteins are heated at a constant rate (e.g., 1°C/min), they usually show transitions in shear modulus development. Physical properties, such as rigidity and rupture strength, of resultant gels are influenced by heating rate and final gelling temperatures (Foegeding et al., 1986; Xiong and Brekke, 1991).

Storage modulus (G'), an elastic component of protein gels which can be measured with small-strain dynamic testing, is a useful rheological parameter for delineating gel network properties. The number of transitions and the transition temperatures for G' are influenced by pH, protein solubility and ionic conditions, and vary among proteins (e.g., myosin vs SSP) (Fretheim et al., 1986; Wang et al., 1990; Culioli et al., 1993; Xiong, 1993). Linear or gradient heating (thermal scanning) usually produces a single G' or shear modulus peak for myosin suspended in low-ionic-strength ($\mu < 0.3$) solutions (Egelandsdal et al., 1986; Fretheim et al., 1986). However, as ionic strength is increased to 0.5 or above, the peak becomes less pronounced (Egelandsdal et al., 1986; Fretheim et al., 1986, Wu et al., 1991; Culioli et al., 1993). Yet, at high ionic strengths ($\mu > 0.5$), myofibrils and SSP consistently show a prominent G' peak around 50-55°C and, additionally, a minor peak around 55-60°C (Wang et al., 1990; Xiong, 1993; Xiong and Blanchard, 1993). Such transitions in G' also appear to be related to the distribution of red and white fiber types (Fretheim et al., 1986; Culioli et al., 1993; Xiong and Blanchard, 1994).

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The complexity of the dynamic rheological curves (e.g., transitions in G') of the gelling myofibrillar proteins has not been fully explained. Egelandsdal et al. (1986) suggested that under conditions favoring myosin filament formation, G' transitions involved two types of reactions, i.e., reversible and irreversible associations of myosin filaments. Based on a kinetic study, Wu et al. (1991) concluded that the complex changes in shear modulus of myosin gels resulted from kinetic variations with different gelling temperatures (related to heating rate). For mixed myofibrillar proteins, such as the actomyosin complex and SSP, the multiple transitions in G' are less understood, and seem to involve more complicated inter- and intramolecular (filamental) reactions. Wang and Smith (1993) reported that the magnitude of G' transitions, as well as the shape of the G' curve for actomyosin, were closely related to the myosin to actin ratio.

Our objectives were to examine rheological changes in myofibril suspensions subjected to different heating procedures, and to explain differences in viscoelastic properties of gelling suspensions in relation to protein aggregation processes.

MATERIALS & METHODS

Preparation of myofibrils

Carcasses of commercial chicken broilers slaughtered at a local poultry processing plant were transported on ice to the University of Kentucky and used within 36–48 hr postmortem. Myofibrils were prepared from combined pectoralis muscles from two chickens as described (Xiong, 1993). Two replicated myofibril isolations, conducted at 2°C, were carried out on different days. Reported data are representative or means of the two replications. The isolation medium (pH 7.0) consisted of 0.1 M NaCl, 50 mM sodium phosphate (Na_2HPO_4/NaH_2PO_4) with 1 mM sodium azide as antimicrobial agent. Purified myofibril samples were suspended in 8 vol (w/v) 0.1M NaCl solution, adjusted to pH 6.0 with 0.1 N HCl, and then centrifuged at 2,000 X *g* for 15 min. Myofibril pellets were recovered, kept on ice, and used within 24 hr. Protein concentration was determined by the biuret method (Gornall et al., 1949) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard.

Protein solubility and gel electrophoresis

Myofibril suspensions (2 mg/mL protein in 0.6M NaCl, 50 mM sodium phosphate, pH 6.0) at 2°C were equilibrated to 20°C in a waterbath and heated at 1°C/min to final temperatures 30, 35, 40, 45, 50, 55, 60 or 65°C. As each final temperature was reached, duplicate samples were removed and chilled immediately in an ice slurry. Cooled samples were then centrifuged at 5,000 X *g* for 15 min to determine protein solubility, which was defined as the protein concentration in the supernatant divided by that in the original suspension then multiplying by 100.

Protein composition of the supernatants was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as outlined by Xiong (1993). The stacking gel contained 3% acrylamide and resolving gel 10% acrylamide. Protein bands were tentatively identified based on relative mobility in comparison with published data (Porzio and Pearson, 1977).

Thermal aggregation

Dilute myofibril suspensions (0.5 mg/mL protein in 0.6M NaCl, 50 mM sodium phosphate, pH 6.0) were heated: (1) from 25 to 75°C at

a 1.6°C/min temperature gradient, or (2) at constant temperatures of 40, 45, 50, 55, 60, 65 and 70°C. Heating was conducted with a temperature-controlled thermal unit mounted to a Model Response II UV/VIS spectrophotometer (Ciba Corning Diagnostics Corp., Oberlin, OH). Thermally induced protein aggregation was examined spectrophotometrically at 320 nm (Xiong, 1992).

Dynamic rheological testing

Myofibril suspensions (20 mg/mL protein in 0.6M NaCl, 50 mM sodium phosphate, pH 6.0) were subjected to thermal gelation with three heating regimens: (1) thermal scan with a linear heating rate (1°C/min) from 20 to 75°C; (2) isothermal heating at 40, 43, 45, 59, 53, 55, 58, 62, 65 or 70°C for up to 70 min; and (3) preincubating the protein suspensions at 20, 30, 35, 40, 45, or 50°C for 30 min, followed by linear heating (1°C/min) to the final temperature 75°C. Heating was conducted in a programmable thermal unit mounted to a Model VOR Bohlin rheometer (Bohlin Instruments, Inc., Cranbury, NJ). Sample temperature was verified using a Model PM20700 thermocouple (Barnstead/Thermolyne Corp., Dubuque, IA).

Dynamic rheological properties of protein suspensions were monitored simultaneously with heating by oscillatory measurements using a 30-mm-diameter parallel-plate geometry (Xiong, 1993). Samples were loaded in the gap (1 mm) between the two plates and the exposed edge was covered with a thin layer of silicone oil to prevent dehydration. Plates were preheated to specific heating temperatures before sample loading. The time elapsed between sample loading and beginning of rheological testing was about 50 sec. For heating regimen 1, where initial temperature of sample plates was low (20°C), initial exposure of sample to the plate temperature was not critical in regard to protein changes. Therefore, a general 5-min pre-measurement setting was allowed to ensure sample equilibration. However, the heating regimens 2 and 3, where initial temperatures of sample plates were high enough (≥ 35°C) to cause protein changes, rheological measurements were begun immediately following sample loading (within 50 sec).

Preliminary stress-strain tests for gels formed at 65°C indicated that a 0-0.02 strain range was well within the linear viscoelastic region. Therefore, shear was applied at a fixed frequency of 100 mHz with a maximum strain of 0.02 to ensure integrity of gel networks. Dynamic rheological parameters used to evaluate the gel networks were: 1) storage modulus, G', a parameter representing the elastic feature of the gel, and 2) phase angle, δ, which ranges from 0 (elastic response) to 90° (viscous response), and can be calculated from the relationship $\delta = \tan^{-1}(G''/G')$, where G" is loss modulus of the gel.

RESULTS & DISCUSSION

Thermal scan (linear heating)

Thermally induced changes in dilute (0.5 mg/mL) and concentrated (20 mg/mL) myofibrillar protein suspensions (Fig 1) indicated that in dilute solution, protein-protein interaction began at about 43°C which led to formation of aggregates that scattered light. In concentrated solutions, a gel formed upon heating, exhibiting complex changes in G' with temperature. The initial change in G' was detected at 41°C which was lower than the onset temperature for protein aggregation, probably due to the higher protein concentration and lower heating rate (1°C/min instead of 1.6°C/min) for the gelling suspension. Furthermore, the dissociations of several native myofibrillar complexes, e.g., F-actin helix into single chains, myosin (filaments) into light and heavy chains, and possibly, actomyosin into myosin and actin, are hypothesized to commence around 40°C (Ziegler and Acton, 1984). Such myofibril structural changes would probably result in an increase in fluid viscoelasticity without initiating crosslinks or aggregates of proteins. Both G" (a viscous attribute of the gel) and δ values began increasing at about 39°C (results not shown) where no major changes in protein conformation were expected, suggesting possible dissociation of some native myofibril structures.

The necessity of protein aggregation for formation of three-dimensional gel networks is well recognized (Hermansson, 1979; Foegeding et al., 1986; Xiong and Brekke, 1991) and our results confirmed this. Several "shoulder" peaks were produced on the turbidity (optical density) curves, closely corresponding to occurrences of those on the G' curve (Fig. 1).

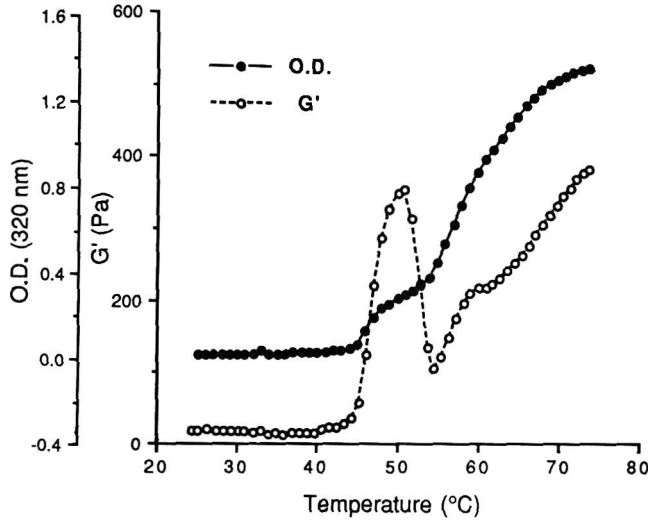

Fig. 1—Changes in turbidity and storage modulus (G') during linear heating of myofibrillar protein suspensions (in 0.6M NaCl, 50 mM sodium phosphate, pH 6.0). For turbidity measurement, samples (0.5 mg/mL protein) were heated at 1.6°C/min; for gelation (G'), samples (20 mg/mL) were heated at 1°C/min O.D. = optical density.

First-derivative plots showed three transitions in protein aggregation and two transitions in gelation, but temperature for the first transition was lower in aggregation (46°C) than in G' (48°C) (curves not shown). The observed multiple transitions may have been due to complexity of myofibril composition as well as myosin domains. As the protein suspension was heated, protein molecules continuously aggregated, resulting in a steady increase in turbidity. However, the magnitude of G', after reaching a maximum at 50°C, decreased sharply to a minimum at 55°C (Fig. 1). Thus, it appears that formation of an elastic gel matrix was influenced less by the degree of aggregation, than by the way protein molecules crosslinked.

Myofibril suspensions heated to specific temperatures were centrifuged to determine which constituents contributed to gel networks. Protein solubility was essentially unchanged in heating from 20 to 40°C. However, it markedly decreased from 73 to 24% as temperature increased from 40 to 45°C, and reached a minimum (11%) at 50°C (Table 1). Protein composition of the supernatant was unaffected by heating at < 45°C, but after heating to 50°C, myosin heavy chain and actin (possibly as the actomyosin complex) became almost totally insoluble and formed sediments (Fig. 2), while tropomyosin, troponin-T and -I, and some myosin light chains remained in the supernatant. Because some major structural changes in actomyosin occur in the 45-50°C range (Wright et al., 1977), partial denaturation and coagulation, resulting in insolubilization of the actomyosin complex, would be expected. This temperature range was also the region where protein-protein interactions (aggregation, gelation) were accelerated (Fig. 1). Hence, polymerization and aggregation of protein molecules or filaments probably occurred resulting in less protein solubility.

Complex rheological characteristics of myofibrillar proteins (similar to those in Fig. 1) have been observed on SSP (Wang et al., 1990; Xiong, 1993; Xiong and Blanchard, 1993) and myosin (Egelandsdal et al., 1986; Fretheim et al., 1986; Wu et al., 1991; Culioli et al., 1993) from avian and bovine skeletal muscles. The decline in G' around the 50-55°C region was of particular interest and has been investigated in myosin gelling systems. Egelandsdal et al. (1986) suggested that the initial G' increase at < 50°C involved crosslinking of myosin filaments due to denaturation of heavy meromyosin, and the G' decrease at > 50°C was attributed to denaturation of light meromyosin, leading to increased filamental "fluidity". The G' increase thereafter (> 60°C) probably resulted from formation of more

Table 1—Relationships between temperature, solubility and aggregation of myofibrillar protein

	Temperature (°C)									
	20	30	35	40	45	50	55	60	65	70
Solubility (%)	74.5	73.5	73.5	73.0	24.0	11.0	11.0	10.5	10.5	—
Turbidity[a]										
"0" min heating	—	—	—	0.48	0.52	0.81	0.90	1.20	1.74	1.79
5 min heating	—	—	—	0.49	0.87	1.01	1.61	1.93	2.05	1.92

[a] Turbidity = optical density at 320 nm.

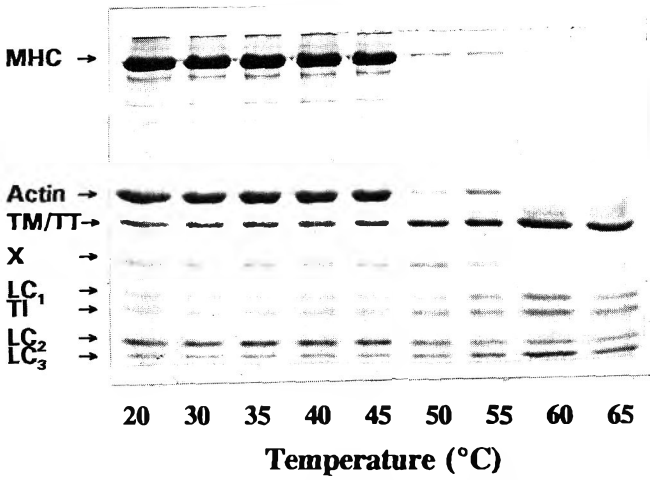

Fig. 2—SDS-polyacrylamide gel electrophoretic patterns of supernatant protein from temperature-solubility test. MHC = myosin heavy chain; TM/TT = tropomyosin/troponin-T; X = known protein; TI = troponin-I; and LC_1, LC_2 and LC_3 = myosin light chains 1, 2 and 3. All electrophoretic samples contained 10% β-mercaptoethanol, and 20 μg of protein was loaded in each lane.

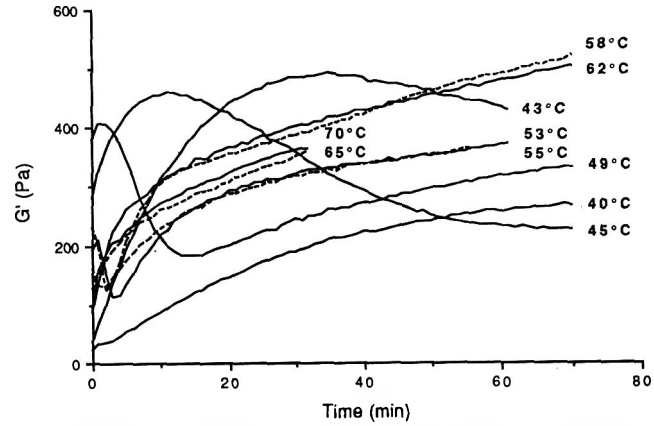

Fig. 3—Typical rheological curves illustrating complex changes in storage modulus (G') of myofibril suspensions (20 mg/mL protein in 0.6 M NaCl, 50 mM sodium phosphate, pH 6.0) during heating at constant (isothermal) temperatures.

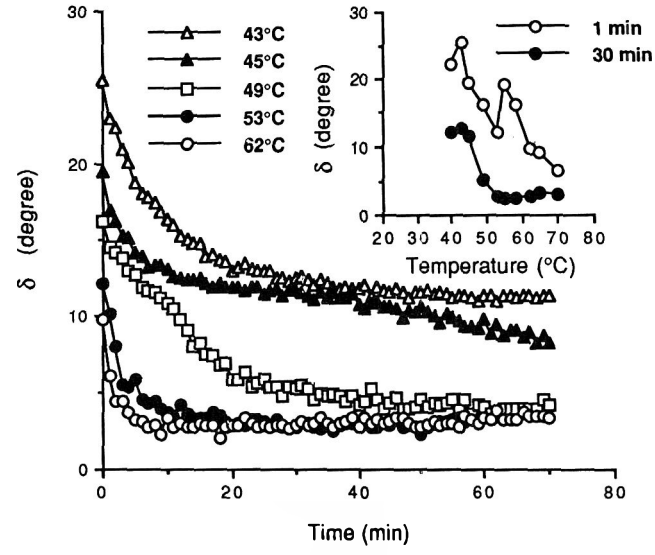

Fig. 4—Changes in stress-strain phase angle (δ) of gelling myofibril suspensions (20 mg/mL protein in 0.6 M NaCl, 50 mM sodium phosphate, pH 6.0) during heating at constant (isothermal) temperatures. Insert: δ-temperature plots for samples heated for 1 and 30 min.

permanent, irreversible myosin filaments or complexes. Wu et al. (1991) demonstrated that the decrease in G' (from 50 to 60°C) was due to kinetic limitations for gel network formation at increased rate constants. For actomyosin and SSP, the mechanism of viscoelastic changes appears more complicated. Wang and Smith (1993) showed a general decline in G' above 50°C for reconstituted actomyosin, but the extent of G' reduction was closely related to the myosin to actin ratio (w/w). That is, the "dip" became smaller as the ratio increased.

Isothermal heating

To determine whether kinetic variations were responsible also for multiple transitions in G' for myofibrils, suspensions were heated at constant temperatures corresponding to those where major viscoelastic changes had occurred (see Fig. 1). The induction of gel and development of gel elasticity were closely related to temperature and heating time (Fig. 3). The "zero time" was operationally impossible to achieve; about 50 sec were required for loading the sample and setting up the testing unit before dynamic viscoelastic measurement was begun. Thus, "zero min" (Fig. 3) actually represents 50 sec heating at the specific temperatures. Furthermore, the thermal "come-up time", i.e., time required for sample (0°C) to reach designated heating temperatures, was about 46 ± 12 sec from preliminary tests with a thermocouple.

At either low (40°C) or high (≥ 62°C) temperatures, G' increased steadily without reaching an equilibrium plateau in 70 min. The G' values at 65 and 70°C for > 30 min heating were variable (mostly ascending sharply at > 40 min, presumably due to sample dehydration), and, therefore, those data were not included in Fig. 3. Between 43 and 58°C, complex changes in G' were evident. In particular, at 43°C, G' increased within the first 30 min and then gradually decreased; the G' change at 45°C followed a similar trend with the reduction in G' occurring much earlier (about 10 min). As temperature increased to 49, 53, 55, and 58°C, the onset time for G' decrease was further lessened. At these discrete high temperatures, the G' decline was followed by a steady increase (Fig. 3), probably indicating formation of permanent elastic networks.

Gelation of muscle protein results from transformation of an amorphous viscous solution to a three-dimensional, relatively elastic network structure. Hence, dynamic gelation process could be monitored by measuring changes in stress-strain phase angle during oscillatory testing. While G' exhibited rather complex responses to temperature (Fig. 3), δ decreased steadily during isothermal heating, reaching a minimum after

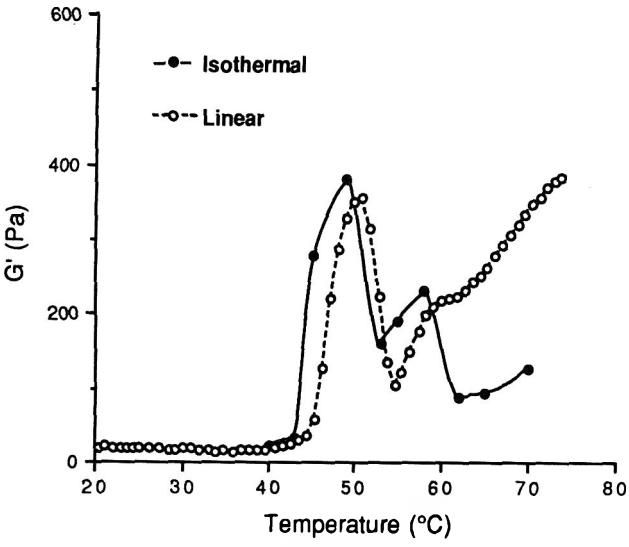

Fig. 5—Storage modulus (G') values generated during linear heating (at a 1°C/min gradient) and isothermal heating of the myofibril suspensions (20 mg/mL protein in 0.6M NaCl, 50 mM sodium phosphate, pH 6.0).

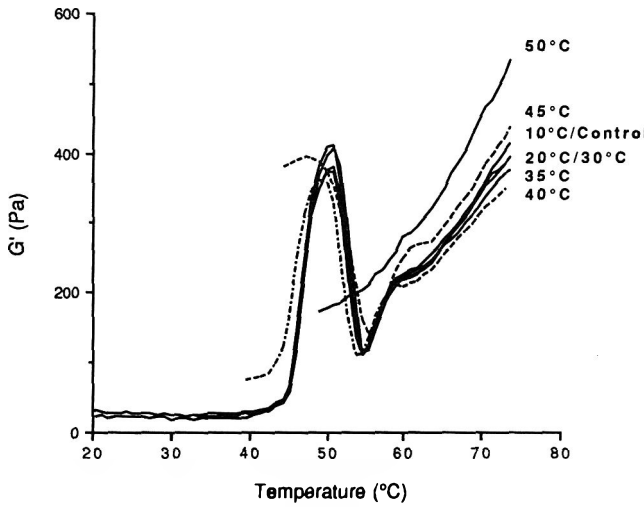

Fig. 6—Effect of preheating on storage modulus (G') of myofibril suspensions (20 mg/mL protein in 0.6 M NaCl, 50 mM sodium phosphate, pH 6.0) during thermal gelation. The myofibril suspensions were incubated 30 min at different temperatures (specified by curves) before further heating to 75°C with a temperature gradient of 1°C/min. Control = heating from 20 to 75°C at 1°C/min without preincubation.

30 min incubation at all temperatures (Fig. 4). Note that the magnitude of δ after 30 min heating was inversely related to temperature (Fig. 4, insert), indicating that gels induced at low temperatures were more "viscous" than gels formed at high temperatures. However, initially (1 min heating) two δ maxima (at 43 and 55°C, Fig. 4, insert) were observed which preceded the two G' peaks (50 and 59°C) during thermal scan (Fig. 1). Such data reflect that formation of a gel network is subject to kinetic limitations. The equilibrium elasticity modulus (E_e, similar to G') of thermotropic protein gels is determined by temperature and time of heating, which can affect kinetic gelling processes, i.e., protein denaturation, diffusion, and hydrolytic destruction (Grinberg et al. 1992).

Our results partially agreed with the report of Wu et al. (1991) for myosin in that shear modulus of gels was most sensitive to "intermediate" temperatures. They differed in that we did not observe an equilibrium after 25 min. The discrepancy was probably caused by the compositional difference of the gelling solutions (SSP vs myosin). Presumably, in the "intermediate" temperature zone (within 45–55°C), the myosin head portion (S-1 group) was initially denatured. This would result in temporary association of myosin filaments, thereby increasing the "gel" elastic response. As heating time was prolonged, G' decreased, probably caused by denaturation of the myosin tail portion (light meromyosin) which resulted in the redistribution of inter- and intramolecular forces. Such physicochemical changes could affect an increase in internal viscosity of myosin and therefore, fluidity of the bulk system, thereby weakening the elastic component of the gel. This process was presumably mediated by actin (Morita et al., 1987; Wang and Smith, 1993), and possibly other myofibrillar components.

To compare effects of different heating regimens, G' values from the first 50 sec ("0 min") at constant heating temperatures (Fig. 3) were superimposed over the G' curve from thermal scan (Fig. 1). The curves were extremely similar, especially at < 58°C (Fig. 5). As indicated previously, sample thermal "come-up time" was 46 ± 12 sec. Hence, within the initial 50 sec, a sample (at 0°C) heated at a high temperature was likely subject to a faster heating rate with greater heat input than a sample heated at a low temperature. Thus, we could assume that temperature-related kinetic variations (energy, reaction rate) were contributing factors for variations in G'. Temperatures around 50°C would probably thermodynamically favor the formation of elastic protein networks, compared with temperatures above or below 50°C. Possibly when the heating rate was too fast, many segments of denatured protein molecules did not form network structures, and therefore, the density of ordered gel crosslinks was relatively low. The results may account for the G' complexity in relation to continuous gradient heating.

Isothermal heating induced complex changes in viscoelasticity but protein aggregation (turbidity) increased steadily with heating temperature (curves not shown). The "zero time" was again difficult to attain; it varied but ranged from 65 to 75 sec. The optical density values at "zero time" and at the end of 5-min heating (beyond which no notable further optical density change was observed) were compared (Table 1). The initial heating rate, as well as heat input, were presumably greater at higher heating temperatures. Under such conditions, heating at high temperatures would probably cause more random coagulation and, therefore, more light scattering than would heating at low temperatures. Evidently, protein aggregation and gelation had different temperature relationships. For gelation, the heating process must be controlled so that it allows a high degree of coordination among protein functional groups for network formation. The manner by which protein molecules associate to form a network is more important than the degree of coagulation or aggregation.

Preincubation

The temperature sensitivity of G' was further illustrated from a pre-heating experiment in which myofibril suspensions were incubated at specific temperatures for 30 min before further heating to 75°C at 1°C/min (Fig. 6). Incubations at 10, 20, 30, and 35°C did not alter G' (compared with control). An incubation at 40°C increased the initial G' value but did not change the final G'. With a 45°C incubation, not only the initial but also the final G' values increased considerably, and after incubation at 50°C the transition peak was eliminated and the resultant gel had the highest elasticity. These results were confirmed by observations from both linear heating (Fig. 1) and isothermal heating (Fig. 3). Incubation of protein suspensions at high temperatures could cause structural modifications without extensively unfolding the protein (i.e., 45–50°C). This would probably increase coordination of protein-protein associations, thereby facilitating formation of ordered, elastic gel matrixes.

CONCLUSIONS

SLOW HEATING (1°C/min) and preincubation at ≥ 45°C of myofibril suspensions produced more elastic final gel networks than isothermal heating. Transitions in storage modulus during thermal gelation of myofibrillar proteins occurred due to alterations in heating rate and temperature. Aggregation of myofibrillar proteins was required for development of gel networks; however, aggregation did not fully account for the complexity of rheological changes. Viscoelastic functionalities of myofibrillar proteins in processed muscle foods are subject to kinetic variations with specific cooking procedures.

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Bacteriological Safety of Swine Carcasses Treated with Reconditioned Water

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ABSTRACT

Swine carcass microflora were evaluated for selected foodborne pathogens after exposure to reconditioned water during scalding, dehairing, and polishing operations. Reused water had been reconditioned and chlorinated. Rodac plates applied to hams were used to assess carcass microflora. Water samples were enumerated using membrane filtration or spiral plating. Sampling was at mid-week throughout the year. Total aerobic plate counts on hams were unaffected by treating with potable or reconditioned waters. No differences were observed for staphylococci, enterics, fecal streptococci, *Listeria monocytogenes*, coliforms, and *Aeromonas* levels. A preevisceration potable water carcass wash reduced the bacterial load, regardless of initial treatment. Bacterial counts on carcasses paralleled those in water. Reuse is an alternative to potable water for initial slaughter operations without diminishing bacteriologic safety.

Key Words: pork, reconditioned water, bacteriology, aerobic plate counts

INTRODUCTION

WATER CONSERVATION is an important issue in pork processing, where water is consumed in large volumes for unit operations. A need to reduce water consumption has resulted from wastewater discharge restrictions as well as from water shortages (Steffen, 1981). Inplant water conservation would be effective to reduce operating costs as well, since water reuse programs could potentially reduce total water consumption and effluent BOD. Such programs have been generally limited to poultry processing and inedible by-product operations. Research has indicated potential value for reconditioned/recycled water use on edible poultry products (Carawan et al., 1974; Lillard, 1978a, 1978b). For water reuse by the pork industry, its efficacy and safety must be demonstrated. Our objective was to determine if the use of reconditioned water would alter the microbiological flora of swine carcasses in a pork processing plant.

MATERIAL & METHODS

Swine slaughter

All swine microflora data were collected at Hatfield Quality Meats, Hatfield, PA. Pigs were of mixed breeds and weighed 109 kg (on average). A summary of the slaughter unit operations is shown in Fig. 1. Stunning was by electrocution, exsanguination was performed by severing the carotid arteries, and scalding was by immersion in 60°C potable or reuse water for 6 min. No additives were included in the scalding trough, which had an overflow rate of 22.7 L/min. Dehairing was done with 3 dehairing and 2 polishing machines (Nijhus, The Netherlands); potable or reuse water for these unit operations was applied by spraying. Carcass and water sampling was done on Wednesdays at about 10 a.m., 4 hr after slaughter commencement; data were obtained during all seasons between January, 1991 and May, 1992.

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Water reconditioning and reuse

Prior to treatment, the pH of the waste water was adjusted to 5.7–6.0. Reuse water was treated (Fig. 2) by flocculation, dissolved air flotation, anaerobic denitrification, aerobic nitrification (dissolved oxygen [DO] is 1–4 mg/L), clarification, sand filtration (following this step, the DO was 5+ mg/L), and chlorination (following chlorination, the oxidation-reduction potential was 600–800 mV). For all experiments, potable water was the control. Switchover between the two systems occurred ≥48 hr prior to sampling.

Bacteriological media

The following plating media were used: Tryptone glucose extract (TGE; Difco, Detroit, MI), Baird-Parker (BP; Difco), Hektoen Enteric (HEK; Difco), KF-Streptococcus (KF; Difco), MacConkey (MAC;

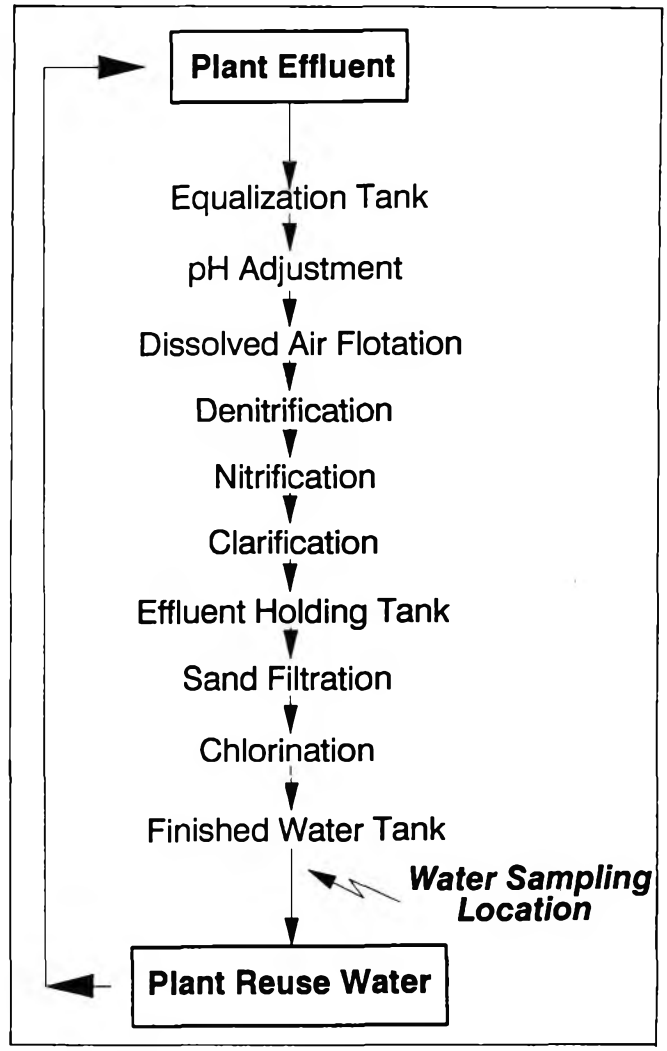

Fig. 1—Steps in swine slaughter operation with reuse water and carcass sampling locations. (1) and (2)—Carcass sampling locations.

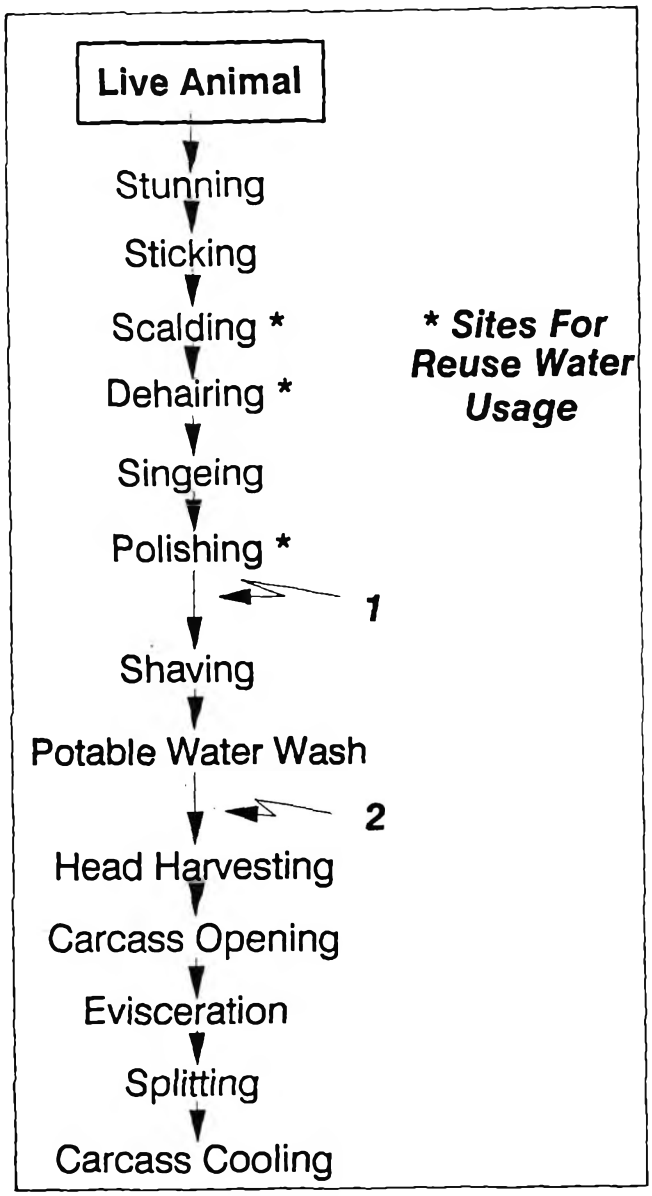

Fig. 2—Steps for reconditioning water for reuse (details in Materials & Methods).

Difco), Modified Vogel-Johnson (MMVJ; Smith and Buchanan, 1990) and Starch Ampicillin (SAA; Palumbo et al., 1985) agars. Media, presumptive organisms they detect, incubation conditions, and typical colony characteristics are described in Table 1.

Carcass sampling and bacteriological analysis

The first experimental series was performed to determine the most suitable sampling sites on carcasses. Samples were obtained after dehairing and polishing (Fig. 1, location 1) from the ham, belly, and shoulder portions of hanging carcasses to determine if anatomical location affected bacterial population density. Ten random carcasses were sampled, each at the 3 sites. For all studies involving carcass sampling, Rodac (Becton Dickinson, Lincoln Park, NJ) dishes (65 mm diameter) were used which contained various media. Rodac dishes were held in contact with carcasses for 2 sec and transported at ambient temperature to the laboratory for incubation (see Table 1) and subsequent enumeration. The ready incorporation into processing operations was another reason for using Rodac dishes instead of swabbing or excision methods which are more time consuming or would damage the carcass. Preliminary experiments indicated that 2 sec was the optimal contact period. For each trial 30 Rodac plates were used for each of seven media. Counting was manual.

The second experimental series compared the effect of using potable or reuse water during initial stages of slaughter on the carcass surface microflora. All sampling was after polishing, using either of the two hams as the sampling site (Fig. 2, location 1). Ten trials were performed consisting of five each, for potable and reuse water treatments. Thirty Rodac plates were employed for each of the seven media. Pigs from multiple lots (210 total) were used for each trial.

In a third experimental series the effect of the final potable wash on carcass bacterial microflora was assessed. Samples were obtained after dehairing/polishing (Fig. 2, location 1) with reuse or potable water, and after the final potable carcass wash (Fig. 2, location 2), prior to evisceration. Six trials were performed consisting of three each of potable and reuse prewash treatments. Fifteen Rodac plates were employed for the seven media. The 210 carcasses used for each treatment trial (105 for prewash sampling and 105 for postwash sampling) were randomly chosen.

Water sampling and bacteriological analysis

Quality of reconditioned water was evaluated immediately after treatment, prior to its return to the plant for reuse. Potable samples were obtained from water lines within the plant, immediately before use in slaughter operations. Lines were purged for at least 5 min prior to collection of samples into sterile 4L plastic containers. Water samples were obtained within 1 hr after carcass sampling. Samples were obtained generally in duplicate for the ten trials, consisting of five each for potable and reuse water. Each container was prepared with 0.1 mL of a 10% solution of sodium thiosulfate per 100 mL of water sample, to neutralize residual chlorine (Greenberg et al., 1981). Water samples were cooled immediately on ice, and transported to the laboratory for processing and analysis within 2 hr after collection. Samples were plated onto media (Table 1). The analyses were performed by either surface plating using a Spiral Plater (Model D, Spiral Systems, Cincinnati, OH) or by filtering 50 mL of water through a 0.45 μm nitrocellulose filter (#130-4045, Nalgene Co, Rochester, NY), then aseptically transferring the filter to the surface of standard Petri dishes (100 × 15 mm) which contained various media. Incubation conditions of media were identical to those used for carcass samples (Table 1). Enumeration was performed by either electronic counting using a Laser Colony Counting System Model 550A (Spiral Systems), or manual counting.

Statistical analysis

Viable cell counts from potable or reconditioned water samples and carcasses (Fig. 1, sampling site 1) were analyzed using a general linear models procedure (SAS™ Institute Inc, Cary, NC). Analyses comparing water sources were performed using nested models, with the sampling date nested within the water source as the error term for water source effects. Studies to evaluate the effects of a pre-evisceration potable carcass wash (Fig. 2, sampling site 1 vs site 2; Table 4) were analyzed using a randomized complete block design. Blocks in this case consisted of sampling date, thus date × location (i.e., block × treatment) was the error term.

RESULTS

No differences occurred ($P>0.05$) in bacterial flora on ham surfaces washed with the two water sources (Table 2). Mean population densities for total aerobic plate counts were <50 CFU/cm^2 for potable and reconditioned water and coliforms were <10. No differences occurred ($P>0.05$) in other measures of microflora quality or safety. Coagulase positive staphylococci on carcasses from potable and reconditioned treatments were very low as were total enteric bacterial loads and fecal streptococcal and *Aeromonas* levels. No *L. monocytogenes* were detected on any of the carcasses.

Similarly, no differences occurred ($P > 0.05$) between bacteriological quality of the potable and reconditioned waters (Table 3). Total aerobic plate counts were below 200 for potable water and for reconditioned water and total coliforms <15. Coagulase positive staphylococcal levels were <6, total enteric bacterial <2, fecal streptococci <0.1, and *Aeromonas* levels <0.2 in all cases from either water. No *L. monocytogenes* was observed in any water samples.

Improvements in the bacteriological quality of ham surfaces occurred as the result of a final potable water carcass wash

Table 1—Bacteriological media, incubation conditions, and colony identifications for water and carcass samples

Presumptive organism	Agar medium	Incubation conditions	Typical colonies[a]
Total Aerobic Plate Counts (TAPC)	Tryptone Glucose Extract (TGE)	24 hr, 28°C	Nonselective
Coag(+) staphylococci	Baird-Parker (BP)	48 hr, 37°C	Black, shiny, convex, clear zone
Total enterics	Hektoen Enteric (HEK)	24 hr, 37°C	*Shigella:* green, moist, raised *Salmonella:* blue green or black *Coliforms:* salmon pink-orange
Fecal streptococci	KF-Streptococcus (KF)	48 hr, 37°C	Red
Coliforms	MacConkey (MAC)	24 hr, 37°C	Pink, red, or colorless
Listeria monocytogenes	Modified Vogel-Johnson (MMVJ)[b]	48 hr, 37°C	Black[b]
Aeromonas	Starch Ampicillin (SAA)[c]	24 hr, 28°C	Honey-colored (3-5mm), amylase positive[c]

[a] Difco (1985), except where noted.
[b] Smith and Buchanan (1990).
[c] Palumbo et al., (1985).

Table 2—Bacterial counts of ham surfaces washed with potable vs. reconditioned water

	Potable			Reconditioned		
Presumptive organism	Mean[a]	SE[a]	n[b]	Mean[a]	SE[a]	n[b]
TAPC[c]	44.1	12.3	147	49.4	14.0	146
Coag (+) staphylococci	0.4	0.1	151	2.5	1.6	156
Total enterics	1.0	0.5	152	2.1	0.8	143
Fecal streptococci	<0.1	<0.1	151	0.5	0.4	154
Coliforms	3.3	1.1	151	8.7	5.1	150
Listeria monocytogenes	N.D.[d]	N.D.	149	N.D.	N.D.	157
Aeromonas	<0.1	<0.1	148	0.5	0.4	145

[a] CFU/cm^2.
[b] Total observations in five replicate experiments.
[c] TAPC = Total Aerobic Plate Count.
[d] N.D. = None Detected.

Table 3—Bacterial content of potable vs reconditioned water

	Potable			Reconditioned		
Presumptive Organism	Mean[a]	SE[a]	n[b]	Mean[a]	SE[a]	n[b]
TAPC[c]	187.1	68.5	8	100.3	31.5	10
Coag (+) staphylococci	<0.1	<0.1	8	6.0	1.9	10
Total enterics	1.7	0.9	8	<0.1	<0.1	10
Fecal streptococci	<0.1	<0.1	8	<0.1	<0.1	10
Coliforms	8.2	4.8	8	12.0	4.8	10
Listeria monocytogenes	N.D.[d]	N.D.	8	N.D.	N.D.	10
Aeromonas	<0.1	<0.1	8	0.2	0.1	10

[a] CFU/ml.
[b] Total observation in 5 replicate experiments.
[c] TAPC = Total Aerobic Plate Count.
[d] N.D. = None Detected.

Table 4—Bacterial counts after a final potable wash on ham surfaces initially processed with potable or reconditioned water

		Location					
		Prewash[1]			Postwash[2]		
Organism	Water source	Mean[b]	SE[b]		Mean[b]	SE[b]	F-Value[a]
TAPC[c]	Potable	75.9	27.5		24.8	11.9	
	Reuse	44.7	9.0		15.6	6.5	6.85*
	F-Value			1.58			
Coag(+)Staphylococci[d]	Potable	0.2	0.2		0.03	0.03	
	Reuse	0.9	0.5		0.2	0.1	1.79
	F-Value			1.24			
Enterics	Potable	3.5	2.1		0.3	0.1	
	Reuse	3.9	1.0		0.8	0.3	9.49*
	F-Value			0.34			
Coliforms	Potable	9.8	5.4		0.9	0.3	
	Reuse	18.3	6.7		3.1	1.5	7.79*
	F-Value			1.51			
Aeromonas	Potable	1.1	0.7		0.03	0.02	
	Reuse	1.0	0.6		0.3	0.2	3.12
	F-Value			0.0			

[a] Values followed by * are significantly different ($P < 0.05$).
[b] CFU/cm^2; SE = $s^2(p)/45 + s^2(d)/3$, where 45 = reps (15) × sampling days (3); $s^2(p)$ = within day variability; $s^2(d)$ = between day variability.
[c] Total Aerobic Plate Count.
[d] Coagulase positive staphylococci.
[1] 1/2 locations 1 and 2 in Fig. 1.

(Table 4). Reductions ($P<0.05$) occurred in total aerobic plate count (67%), total enteric bacteria (91%), and coliforms (91%) when the carcass was washed with potable water after scalding and dehairing. When reconditioned water was used for initial slaughter operations, the potable carcass wash reduced bacterial levels by 65%, for total aerobic plate count, 79% for total enteric bacteria, and by 83% for coliforms. No differences ($P>0.05$) occurred in microflora after carcasses were treated with a final potable wash.

The analysis of variance indicated a significant effect on total aerobic plate count ($P < 0.05$) due to sampling date. Major sources of variation appeared to be ambient temperature and operational changes in water reconditioning.

DISCUSSION

Considering maintaining carcass flow on the processing line, use of contact plates (Rodac dishes) on the ham surface would provide an adequate procedure to assess the influence of water source on microbiological quality of hog carcasses. To detect and quantify the different pathogens and indicator groups surveyed, various selective media were useful (Table 1). Pathogens as well as most other bacteria can be injured by various treatments such as heating (singeing and scalding), chlorine, etc. (Ray, 1989) and selective media typically do not provide quantitative recovery of injured bacteria. Most procedures we used would preclude detection of injured cells equally, regardless of treatment with reconditioned or potable water. Two of the selective media, (BP and MMVJ), were formulated to enhance recovery of injured cells of *Staphylococcus aureus* and *L. monocytogenes*, respectively (Baird-Parker, 1962; Smith and Buchanan, 1990).

Use of recycled water has been reported in the poultry industry for inedible products, and was shown experimentally to be efficacious and safe for certain edible products. For example, poultry chiller water was recycled after filtration though diatomaceous earth and chlorination (Lillard, 1978b). The safety and quality of the water and product were maintained when poultry necks were flumed with reconditioned water (Lillard, 1978a). Carawan et al. (1974) showed that flushing gizzards with water from the chiller and final washer had no detrimental effect on bacteriological quality of the gizzards.

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—Continued on page 746

Properties of Low-Fat, Nonbreaded Pork Nuggets with Added Gums and Modified Starches

B.W. BERRY

ABSTRACT

Low-fat (8%) pork nuggets were prepared with gums, modified food starches, and 90% pork. Sodium alginate with calcium-lactate or corn starch creme resulted in the greatest increases in cooking yield and tenderness. Convection oven cookery resulted in improvements in sensory and instrumental tenderness values compared with precooking and reheating. Nuggets with corn starch creme had notable distortions in shape after precooking and reheating. Gums and modified starches can improve tenderness in low-fat pork nuggets.

Key Words: pork nuggets, low-fat, gums, starch, sensory

INTRODUCTION

THE USDA HAS PROMOTED REDUCTION of fat from foods under the Special Nutrition Program (USDA, 1990; USDA and USDHHS, 1990). The program has included testing and selection of low-fat beef patties for National School Lunches (USDA, 1991). Products selected for that program had either iota-carrageenan or oat bran and oat fiber added. Other studies have shown improvements in sensory properties of cooked low-fat beef patties following use of dietary fiber, iota-carrageenan or modified food starches (Egbert et al., 1991; Brewer et al., 1992; Troutt et al., 1992; Berry and Wergin, 1993).

Reports have been published on fat reductions in pork products (Ahmed et al., 1990; Reitmeier and Prusa, 1990; Rhee et al., 1990; Huffman et al., 1992; Bradford et al., 1993). School foodservice systems have an interest in low-fat pork products of "finger-food" types. Foods such as "nuggets" have usually been processed to contain 20% or more fat, contain batter or breading, and are often cooked by deep fat frying. Our objective was to determine whether gums or modified starches could be effective fat replacers in nonbreaded pork nuggets cooked in oil-free systems.

MATERIALS & METHODS

Processing

Fresh, closely trimmed ham knuckles and trimmed jowls (from sow carcasses) were obtained from a local processor. Preliminary mixtures of the two materials were tested for fat content with an Anyl-Ray fat tester (The Kartridge Pak Co., Davenport, IA) to determine combinations providing 8% fat content. Six pork nugget treatments which included gums and modified food starches as fat replacers were processed. They were: all pork (AP), modified tapioca starch (MTS, Slenderlean™, National Starch and Chemical Co., Bridgewater NJ), modified potato starch (MPS, Pen Plus® FT 16, Penwest Foods Co., Englewood, CO), iota carrageenan (IC, Viscarin® ME 389, FMC Corp., Philadelphia, PA), sodium alginate and calcium lactate (AL, A7B609 sodium alginate, CaL-135E-7S, Kelco Div., Merck and Co., San Diego, CA), and corn starch creme with modified corn starch (CSC, Stellar™, STA-SLIM™ 171, A.E. Staley Mfg. Co., Decatur, IL) (Table 1). Pork comprised at least 90% of all formulations in order to be similar to requirements of the low-fat beef patty program for the School Lunch Program (USDA, 1991). Flaked salt (Premier Flake Salt, Cargill, Inc., Minneapolis, MN) and encapsulated salt (Cap-Shure® SC-165-85 FT, Balchem Corp., Slate Hill, NY) were also used.

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The pork materials, ground through a 1.25-cm plate and appropriate amounts of ground ham knuckles and jowls were mixed (Hobart Model 4346-SS Mixer Grinder, Hobart Corp., Troy, OH) for 30 sec. Batch weights of 27.2 kg were used including meat, water and dry ingredients. In the case of AP, MTS and MPS products, dry ingredients were added and mixed in the mixer-grinder for 60 sec. Water was added followed by another 60 sec mixing. For all formulations, after mixing was completed, half the mixture was ground through a 0.32 cm plate, and the remaining half was ground through a 0.47 cm plate. Previous results (Berry and Kotula, 1994) showed a school-age consumer panel preferred nuggets processed from a combination of 0.32 cm and 0.47 cm ground pork in equal amounts over other grind sizes. Thus, following final grinding, pork from the two plate sizes was mixed together for 45 sec.

Deviations in the procedure for the IC treatment consisted of first mixing carrageenan with the meat for 30 sec, followed by addition of water and another 45 sec mixing, then salt was added followed by a final 30 sec mixing. For the AL product, the salt was added first followed by 45 sec mixing, then water was added with another 45 sec mixing. Sodium alginate was added and the mixture was blended 90 sec before calcium lactate was added with a final 90 sec mixing.

For the CSC product, 20% of the meat block, 13% of the water, all of the corn starch creme and 33% of the salt were emulsified together (Stephan Cutter/Mixer Model 3188, Stephan Machinery Corp., Columbus, OH). The remainder of the meat, half the water, and all the salt were mixed for 60 sec. The emulsion, modified corn starch, and remainder of the water were added, followed by a final 60 sec mixing.

Mixtures were stuffed into 9.9 cm diameter, oxygen impermeable, polyethylene casings (Abar Plastics, Inc., Beltsville, MD) and placed in a −29°C freezer. Following 120 hr of frozen storage, the logs were peeled and cut with a band saw into 1.27 cm thick (34–38g) nuggets. They were vacuum-packaged in high oxygen barrier (30–40 cc O_2/m^2/24 hr) bags and returned to the −29°C freezer for 1 wk before evaluations.

Since our objective was to reduce fat in pork products for foodservice, we tried to obtain high-fat pork nuggets for comparison. Pork nuggets are seldom used in school foodservice. Thus, a precooked high fat (20%) breaded beef nugget (BB), used in State of Maryland school foodservice, was obtained and used for many comparisons.

Cooking procedures

Nuggets from all treatments were subjected to two cooking procedures (one-time cooking, precook-reheat). Nuggets cooked once from raw frozen to fully cooked state were cooked on trays (15/tray) to 71°C in a 177°C convection oven (Blodgett CTE-1, G.S. Blodgett Co., Burlington, VT). According to treatment, cooking times required to consistently produce a 71°C internal temperature were: AP = 7 min, 20 sec; MTS = 7 min, 25 sec; MPS = 7 min, 35 sec; IC = 7 min, 55 sec; AL = 7 min, 55 sec; and CSC = 7 min, 10 sec. After trays were removed from the oven, nuggets were turned and left on the tray 45 sec to brown the original top surface. At that time, internal temperatures were determined with iron constantan thermocouples, (Honeywell DPR 3000 recorder, Honeywell Inc., Ft. Washington, PA). Any nugget with < 71°C temperature was not further evaluated.

Precooking was in a Lincoln heat impinger oven (Model 1130, Lincoln Food Service Products, Inc., Ft. Wayne, IN). Nuggets were precooked on a belt moving through the oven set at 149°C. Precooking times to reach 61°C and maintain that temperature for 60 sec were: 9 min, 40 sec for IC and AL; 9 min, 50 sec for AP, and MPS and 10 min for MTS and CSC. Precooked nuggets were held at 25°C for 15 min, placed in high oxygen barrier bags (not under vacuum) and held at 25°C another 15 min before placing in a −29°C freezer. After 48 hr, frozen precooked nuggets were vacuum-packaged and stored at −29°C. Preliminary tests indicated placement of nuggets into the

Table 1—Pork nugget formulations (%)

	Primary functional ingredient					
	All pork (AP)	Modified tapioca starch (MTS)	Modified potato starch (MPS)	Iota carrageenan (IC)	Alginate and lactate (AL)	Corn starch creme (CSC)
Pork ham-knuckles	89.50	80.50	80.50	80.50	80.50	80.50
Pork jowls	10.25	9.50	9.50	9.50	9.50	9.50
Flaked salt	0.125	0.125	0.125	0.125	0.125	0.125
Encapsulated-salt	0.125	0.125	0.125	0.125	0.125	0.125
Water		8.75	8.75	9.25	8.95	3.00
Modified tapioca starch		1.00				
Modified potato starch			1.00			
Iota carrageenan				0.50		
Sodium alginate					0.40	
Calcium lactate					0.40	
Corn starch creme						6.00
Modified corn starch						0.75

Table 2—Cooking, compositional and juiciness properties of pork nugget treatments

Primary functional ingredient in formulation[a]	Cooking yield (%)	Raw nugget		Cooked nugget		Juiciness score[b]
		Moisture (%)	Fat (%)	Moisture (%)	Fat (%)	
All-pork (AP)	68.5^{de}	71.4^{f}	9.6^{c}	60.6^{e}	12.8^{c}	4.8^{d}
Modified tapioca starch (MTS)	67.5^{e}	73.3^{d}	8.8^{d}	63.6^{d}	11.4^{d}	5.4^{c}
Modified potato starch (MPS)	64.5^{f}	73.6^{d}	8.6^{de}	61.6^{e}	12.2^{c}	5.2^{cd}
Iota-carrageenan (IC)	68.3^{de}	74.3^{c}	8.0^{e}	64.3^{d}	11.2^{d}	5.4^{c}
Alginate and lactate (AL)	69.5^{cd}	74.3^{c}	8.2^{de}	66.0^{c}	10.2^{e}	5.2^{cd}
Corn starch creme (CSC)	70.5^{c}	72.4^{e}	8.5^{de}	63.0^{d}	11.4^{d}	5.2^{cd}
S.E.	0.50	0.15	0.16	0.14	0.12	0.12
Breaded high-fat beef (BB)	91.8			46.3	21.7	4.8^{d}

[a] Details regarding formulations are given in Table 1.

[b] Score based on 8-point system when 8 = extremely juicy and 1 = extremely dry.

[c-f] Means in the same column lacking a common superscript letter differ ($P < 0.05$). Breaded high-fat beef (BB) nuggets were not compared to other treatments for cooking yield, cooked moisture and fat content due to intended differences in composition. BB nuggets evaluated only in precooked-reheated form and thus, the 91.8 value is only reheating yield.

Table 3—Properties of pork nuggets cooked by different methods

Property	Cooking method		S.E.
	One-time cooking from raw frozen state to fully cooked state	Raw frozen to pre-cooked state, refrozen and reheated from frozen state	
Cooking yield (%)	74.2^{c}	68.1^{d}	0.43
Cooked nugget			
Moisture (%)	65.1^{c}	61.2^{d}	0.13
Fat (%)	11.1^{d}	12.0^{c}	0.10
Sensory panel			
Juiciness[a]	5.4^{c}	4.9^{d}	0.15
Force[a]	6.1^{c}	5.7^{d}	0.13
Instron shear force		5.3^{c}	0.17
Peak load (kg)	3.5^{d}		
Energy to peak (kg-mm)	19.6^{d}	30.3^{c}	0.93
Post-peak energy (kg-mm)	5.3^{d}	7.3^{c}	0.41
Instron compression			
Springiness (cm)	0.56^{d}	0.62^{c}	0.11
Cohesiveness[b]	0.38	0.39	0.01

[a] Scores based on 8-point system where 8 = extremely juicy and 1 = extremely dry and 8 = very minimal force required and 1 = extreme force required in 2-chew molar contact.

[b] Cohesiveness = ratio of second compression total energy to first compression total energy.

[cd] Means on the same line with different superscripts are different ($P < 0.05$) in analysis of variance.

freezer earlier than 30 min post-cooking occasionally resulted in toughening and moisture condensation in bags during freezing.

Frozen precooked nuggets were reheated in the 177°C convection oven to 71°C internal temperature. Reheating times were: AP = 7 min, 35 sec; MTS = 7 min, 25 sec; MPS = 7 min, 15 sec; IC = 7 min, 15 sec; AL = 7 min, 15 sec; CSC = 7 min, 25 sec; and BB = 6 min. Nuggets were again turned on the platters just after removal from the oven and maintained in that position for 45 sec before determining internal temperatures.

Cooking measurements

Total cooking yield was determined for one-time cooked and for precooked-reheated nuggets. For each treatment, four trays per cooking method, each with 15 nuggets, were monitored for weight loss. During the precooking phase, individual weights were recorded, while group weights were measured once nuggets were cooked or reheated on trays. Fifteen nuggets/treatment/cooking method were individually measured for thickness in the raw, frozen state and in the cooked or reheated state to determine any reduction in nugget thickness. In cooked and reheated states, nugget thickness was determined at the thinnest location.

Visual appraisal for the degree of distortion in nuggets resulting from one-time cooking and precooking-reheating was scored by a three-member trained panel. Panelists had 6 hr training for scoring nugget distortion prior to this study. The panel used photographic standards of nugget distortion which were developed for this study. Standards were developed where 8 = no distortion and 1 = extreme distortion. In scoring for distortion, panelists selected the photographic standard that most closely resembled nuggets being evaluated.

Compositional properties

Fat and moisture determinations were made on four separate blendings of four nuggets/blending for each treatment in the raw, one-time cooked and precooked-reheated forms. Blendings (20 sec) were made in a Waring commercial Blendor at low speed. Moisture was determined after 12 hr drying at 102°C in a vacuum (50.8 cm Hg) oven (Precision Scientific Model 524, Precision Scientific Co., Chicago, IL). Fat was determined by weight loss after 16 hr extraction in a Soxhlet apparatus with petroleum ether (AOAC, 1980).

Sensory evaluation

A ten-member trained sensory panel evaluated one-time cooked and precooked-reheated nuggets. During a 3-month training period, the panel leader and panelists identified sensory properties important in low-fat pork nuggets and developed protocols for measuring such properties. The panel had evaluated pork nuggets in 2 previous studies.

Panelists received samples within 150 sec of removal from the oven. Panelists received half-nugget pieces for evaluation. Force was evaluated by placing the sample on the molars and applying two chews. The size of pieces was evaluated after five and after ten chews. During the first five chews, panelists were instructed to keep the sample as much as possible on the molars. Size of chewed pieces was classified as small, medium or large. Panelists were provided visual pictures of

Table 4—Interaction of treatment and cooking method on dimensional changes of pork nuggets during cooking

Primary functional ingredient in formulation[a]	Reduction in nugget thickness at thinnest location (%)		Distortion score[b]	
	One-time cooking[c]	Precook-reheat[c]	One-time cooking[c]	Precook-reheat
All-pork (AP)	27.0^{gh}	37.0^{de}	4.6^{d}	4.8^{d}
Modified tapioca starch (MTS)	27.1^{gh}	29.5^{fgh}	5.5^{d}	4.8^{d}
Modified potato starch (MPS)	28.3^{fgh}	33.3^{ef}	5.0^{d}	4.7^{d}
Iota carrageenan (IC)	26.1^{h}	35.4^{de}	5.0^{d}	4.0^{d}
Alginate and lactate (AL)	28.5^{fgh}	32.4^{efg}	4.8^{d}	5.0^{d}
Corn starch creme (CSC)	27.2^{gh}	40.5^{d}	4.8^{d}	2.9^{e}
S.E.	1.9		0.37	
Breaded high-fat beef (BB)				7.1

[a] Details regarding formulations are given in Table 1.

[b] Scores based on 8-point visual evaluation where 8 = no distortion and 1 = extreme distortion.

[c] One-time cooking = nuggets cooked once from the raw frozen state to fully cooked state; Precook-reheat = nuggets cooked from raw frozen state to precooked state, refrozen and reheated from frozen state. Since breaded high-fat beef nuggets were not obtained in raw-frozen form, thickness reductions were not obtained.

[d-h] Means within a dimensional change lacking a common superscript letter differ ($P < 0.05$).

chewed nugget pieces to assist in size evaluation. In these pictures, small = 16 mm^2, medium = 36 mm^2, and large = 64 mm^2. Panelists were not allowed to remove chewed pieces from their mouths for examination. Panelists were permitted to record any and all of the three sizes if present.

Juiciness was evaluated after five chews. Panelists were instructed to keep samples on molars of one side of the mouth as much as possible. All three evaluations occurred on three separate half-nugget pieces. Force and juiciness were rated on 8-point structured scales where 8 = very minimal force and extremely juicy and 1 = extreme force and extremely dry. Four samples representing different formulation-cooking method combinations were served in a session. Each formulation-cooking method combination was evaluated in 6 different sessions.

Instron shear measurements

Thirty nuggets/treatment/cooking method were held at 25°C for 30 min following one-time cooking or reheating. Each nugget was cut with a knife into a 2.54 cm wide section and sheared in two places with a rounded straight edge blade attached to an Instron Universal Testing Machine (Model 1122, Instron Corp., Canton, MA). Both crosshead and chart speeds were set at 250 mm/min. Instrumental values from the shear force test included peak load, peak energy (total work expended in shearing until peak load was reached) and post-peak energy (total work from peak load to the end of shearing).

Instron compression measurements

Instron compression measurements were made on 15 nuggets/formulation/cooking method. Nuggets were held at 25°C for 30 min after cooking or reheating prior to sampling. A single 2.54 cm diameter core was removed from the center of each nugget. Each core was compressed twice to 75% of its original height using a 7.5 cm diameter, circular, flat-surface disk and an Instron Universal Testing Machine. A full scale load of 100 kg with a crosshead speed of 100 mm/min and a chart speed of 200 mm/min were used. Measurements included: hardness = peak force (kg) of first compression, springiness = distance in cm sample recovered after first compression; cohesiveness = total energy (area under the curve) of second compression ÷ total energy of first compression; gumminess = hardness × cohesiveness; and chewiness = hardness × cohesiveness × springiness.

Statistical analyses

The study was replicated twice. Data were analyzed using analysis of variance through the General Linear Model Program (SAS, 1988). Data were treated as a split-plot with treatment as the main plot and cooking system as the split-plot. Differences between mean values were tested with the Tukey's Studentized Range (HSD) Test.

RESULTS & DISCUSSION

PORK NUGGETS MANUFACTURED WITH CSC had higher cooking yields than AP, MTS, MPS and IC nuggets (Table 2). The slightly shorter cooking time for CSC nuggets to reach 71°C probably influenced the improved cooking yields. Inclusion of 20% of the meat in an emulsified form for CSC nuggets may have also affected cooking yields. Use of MPS reduced cooking yields over other treatments. With low-fat beef patties, Berry and Wergin (1993) reported the addition of 3.0% MPS and 5.0% water improved cooking yields to that observed for all-beef, low-fat beef patties. Values for BB nuggets were strictly for precooking-reheating and thus, they were not included in statistical analyses.

AP nuggets had slightly lower raw moisture and higher raw fat than those formulations with added ingredients. AL nuggets retained more moisture during cooking and thus, had the highest cooked moisture and lowest cooked fat values. With pork sausage patties formulated to 8% fat, Huffman et al. (1992) found the substitution of pork with 0.35% carrageenan and 20% added water decreased cooking yield, but, still provided more moisture in cooked patties compared to all-pork controls. Inclusion of 0.5% modified food starch, 0.5% carrageenan and 6% water in an 8% fat beef patty formulation produced higher cooking yields in contrast to an 8% all-beef patty (Brewer et al., 1992). The use of MPS did not improve cooking yields in our current study. Berry and Wergin (1993) reported MPS generated higher cooking yields while reducing fat in cooked beef patties. They attributed the results to heat stability of the MPS gel and its ability to hold and conduct heat during cooking.

Nugget formulations with MTS and IC were rated higher ($P < 0.05$) in juiciness than AP and BB products. IC was pre-

Table 5—Sensory panel force scores and Instron shear force and compression values of pork nugget treatments

Primary functional ingredient in formulation[a]	Sensory force score	Shear force			Compression	
		Peak load (kg)	Energy to peak (kg-mm)	Post peak energy (kg-mm)	Springiness (cm)	Cohesiveness
All pork (AP)	4.6^{g}	5.4^{d}	31.8^{d}	8.4^{d}	0.67^{d}	0.40^{e}
Modified tapioca starch (MTS)	6.0^{de}	4.1^{ef}	24.3^{f}	5.8^{ef}	0.58^{e}	0.37^{ef}
Modified potato starch (MPS)	5.5^{f}	5.2^{d}	26.6^{ef}	7.1^{de}	0.61^{e}	0.44^{d}
Iota-carrageenan (IC)	5.6^{ef}	4.5^{e}	27.2^{e}	6.0^{ef}	0.68^{d}	0.40^{e}
Alginate and lactate (AL)	6.2^{d}	3.6^{f}	20.2^{g}	5.0^{f}	0.49^{f}	0.34^{fg}
Corn starch creme (CSC)	6.4^{d}	3.6^{f}	19.7^{g}	5.4^{f}	0.48^{f}	0.32^{g}
S.E.	0.10	0.15	0.81	0.43	0.08	0.01
Breaded high-fat beef (BB)	5.9^{def}	2.3	13.3	5.2	0.53	0.28

[a] Details regarding formulations are given in Table 1.

[b] Score based on 8-point system when 8 = very minimal force required and 1 = extreme force required in 2-chew molar contact.

[c] Cohesiveness = ratio of second compression total energy to first compression total energy.

[d-g] Means in the same column lacking a common superscript letter differ ($P < 0.05$). Breaded high-fat beef nuggets were not compared to other treatments with the exception of sensory force scores since they were evaluated only in the precooked-reheated form.

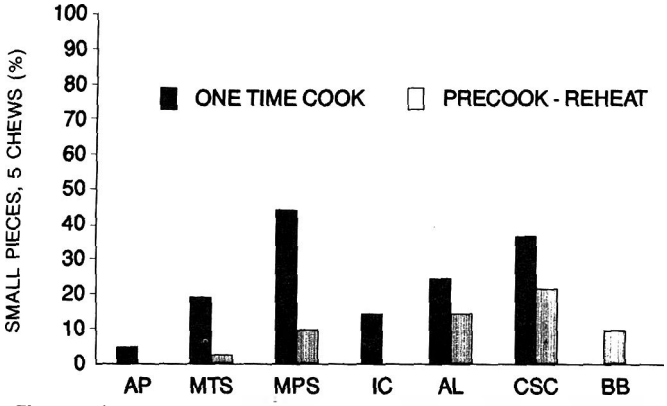

Fig. 1—Frequency distribution of small size pieces after five chews according to formulation and cooking method. Chi sq = $P < 0.0001$ for frequency of all sizes of chewed pieces. Acronyms are identified in Table 1.

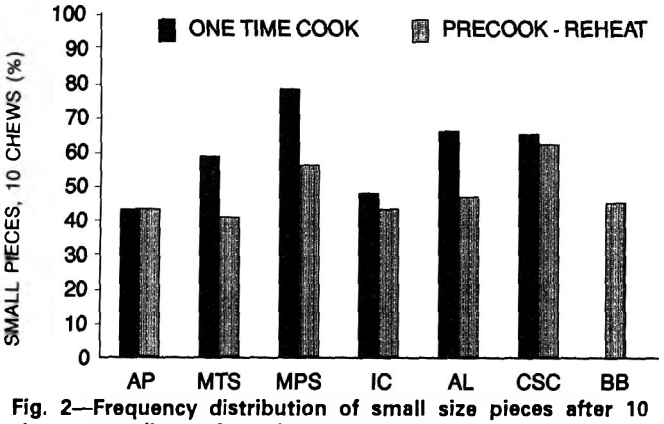

Fig. 2—Frequency distribution of small size pieces after 10 chews according to formulation and cooking method. Chi sq = $P < 0.0001$ for frequency of all sizes of chewed pieces. Acronyms are identified in Table 1.

viously reported to improve juiciness in low-fat beef patties (Egbert et al., 1991; Brewer et al., 1992), but, to have no influence on juiciness of low-fat pork patties (Huffman et al., 1992). Berry and Wergin (1993) reported reductions in juiciness when MPS was added to low-fat beef patty formulations. They attributed the lower juiciness to high water binding properties of MPS, making moisture unavailable for early juice release during mastication.

One-time cooking increased ($P < 0.05$) cooking yields, cooked nugget moisture and juiciness scores, while reducing cooked nugget fat in comparisons with precooking and reheating (Table 3). Extensive studies comparing one-time cooking with precooking-reheating for thin, small products like nuggets have not been reported. However, Rhee et al. (1988) reported 6 to 7% reductions in cooking yield during reheating in a prefrying-reheating approach for pork nuggets. The reheating phase increased fat values by 3%, while reducing moisture by 5.5%.

Various tenderness properties, determined by sensory panel, shear-force and compression, were reduced as a result of precooking-reheating compared to one-time cooking (Table 3). For maintaining and enhancing sensory and tenderness properties of low-fat nonbreaded pork nuggets, precooking-reheating is not recommended. This approach was included based on consultations with school foodservice officials. They indicated that the same cooking procedures and final internal temperatures may be frequently used for both raw and precooked items to minimize the number of cooking methods needed in the school-kitchen environment. However, with thin, low-fat, nonbreaded nuggets, reheating times from frozen state to 71°C appear to be similar to those required for single cooking from the raw-frozen state to 71°C. Obviously, this would contribute to reductions in sensory and instrumental tenderness determinations.

Precooking and reheating produced greater reductions in nugget thickness than one-time cooking for AP, IC and especially CSC products (Table 4). This interaction ($P < 0.05$) appeared to be due mainly to differences in the two cooking procedures for CSC nuggets. While CSC resulted in similar nugget reduction values to other formulations during one-time cooking, it produced the greatest reductions in nugget thickness during precooking-reheating ($P < 0.05$) over all other products, (except AP and IC). Likewise, for visual distortion, the significant ($P < 0.05$) interaction was due to different results produced by the two cooking methods for CSC nuggets. Substantial distortion was observed for the CSC treatment when precooking-reheating was employed, while no differences in distortion were noted among treatments when one-time cooking was used. No differences in distortion were detected between cooking methods for any other treatments. Additional research effort is necessary to ascertain factors responsible for distortion when modified corn starch and CSC were used together (CSC treatment) with precooking-reheating.

Formulation affected various tenderness measurements (Table 5). All primary functional ingredients improved tenderness of nuggets as measured by sensory force, peak load, post-peak energy and springiness compared to AP nuggets. The AP product did not differ from MPS nuggets in peak load and post-peak energy, or from IC nuggets in compression measurements of springiness and cohesiveness. Berry and Wergin (1993) reported the addition of MPS greatly improved tenderness of low-fat beef patties, while Huffman et al. (1992) and Bradford et al. (1993) reported higher tenderness scores when IC was incorporated into low-fat (8%) pork sausage patties. Bradford et al. (1993) further noted reductions in sensory-determined cohesiveness attributable to addition of IC.

Generally, the use of CSC and AL produced improvements in tenderness compared to other added ingredients. Similar sensory force scores were found for AL, CSC and BB nuggets. BB nuggets, although only available precooked were, nevertheless, compared to other nugget products in all sensory sessions. Patterns of differences between treatments for tenderness measurements, were similar regardless of measurement.

Although not rated as one of the highest in tenderness properties, one-time cooked MPS nuggets had a high frequency of small pieces following both 5 and 10 chews (Fig. 1, 2). CSC nuggets also produced a high incidence of small chewed pieces. Use of 20% emulsified meat in the CSC treatment may have contributed to the number of small size chewed pieces. The most prevalent of size categories was the small size, which also displayed the most differences between treatments. Precooking followed by reheating produced fewer small size pieces compared to one-time cooking especially for MPS nuggets.

Compression hardness values between the cooking methods were not different ($P > 0.05$) for AP and CSC products (Table 6). Precooking and reheating increased hardness over one-time cooking for all other nugget formulations. MTS, AL and CSC products had lowest hardness values with one-time cooking, while CSC had the lowest values with precooking-reheating. For gumminess and chewiness, only MTS and MPS had higher values as a result of precooking-reheating. MPS exhibited similar gumminess values to AP and IC with one-time cooking, but had higher gumminess with precooking followed by reheating. MTS was lower in chewiness than AP, MPS, and IC nuggets but was similar to AL and CSC with one-time cooking. With precocking-reheating, MTS was similar to AP and IC nuggets in chewiness, but higher than AL and CSC products. The notable difference in chewiness of MTS nuggets be-

Table 6—Interaction of treatment and cooking method on Instron compression values of pork nuggets

Primary functional ingredient in formulation[a]	Hardness (kg)		Gumminess[b]		Chewiness[b]	
	One-time cooking[c]	Precook reheat[c]	One-time cooking[c]	Precook reheat[c]	One-time cooking[c]	Precook reheat[c]
All-pork (AP)	30.8[e]	31.9[e]	12.9[e]	12.4[e]	8.7[de]	8.3[e]
Modified tapioca starch (MTS)	18.6[g]	32.0[e]	6.3[fg]	12.8[e]	3.2[fg]	8.4[e]
Modified potato starch (MPS)	29.3[ef]	37.4[d]	12.9[e]	16.8[d]	7.5[e]	10.8[d]
Iota carrageenan (IC)	26.8[f]	31.6[e]	11.0[ef]	12.6[e]	7.1[e]	9.0[de]
Alginate and lactate (AL)	18.8[g]	25.8[f]	6.2[fg]	9.0[f]	2.9[fg]	4.6[f]
Corn starch creme (CSC)	16.9[g]	21.1[g]	5.2[g]	7.2[fg]	2.4[g]	3.7[fg]
S.E.	0.83		0.49		0.38	
Breaded high-fat beef (BB)		12.7		3.6		1.9

[a] Details regarding formulations are given in Table 1.

[b] Gumminess = product of hardness × cohesiveness; chewiness = product of hardness × cohesiveness × springiness.

[c] One-time cooking = nuggets cooked once from the raw frozen state to fully cooked state; precook-reheat = nuggets cooked from raw frozen state to precooked state refrozen and reheated from frozen state.

[d-g] Means within a compression value lacking a common superscript letter differ ($P < 0.05$). Since breaded high-fat beef nuggets were not obtained in raw-frozen form, they were not part of the treatment-cooking method interaction.

tween the two cooking procedures was largely responsible for this significant ($P < 0.05$) interaction.

CONCLUSIONS

REPLACING 10% OF PORK in low-fat nonbreaded pork nugget formulations with gums and gels (especially CSC and AL) and added water provided a "softening" effect on tenderness, while maintaining normal cooking yields. Precooking-reheating procedures like those studied are not recommended for this type product due to reductions in total cooking yield and tenderness, while sometimes creating considerable appearance distortion (in CSC nuggets).

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Dietary Iron in Swine Rations Affects Nonheme Iron and TBARS in Pork Skeletal Muscles

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ABSTRACT

Fifteen crossbred pigs (mean wt = 25 kg) were allocated to three groups and fed to market weight (mean wt = 103 kg) on corn-soy based diets containing either 62, 131, or 209 ppm iron. After slaughter, the longissimus dorsi (LD) and rectus femoris (RF) muscles were dissected, cooked, and stored in oxygen-permeable bags for 12 days at 4°C. Cooking increased thiobarbituric acid reactive substances (TBARS) but did not affect nonheme iron (NHI) or α-tocopherol. NHI and TBARS increased continuously during storage while α-tocopherol decreased. NHI and TBARS were higher in cooked pork from pigs fed high-iron diets. Liver iron correlated with muscle iron ($p<0.05$).

Key Words: pork, nonheme iron, TBARS, muscle

INTRODUCTION

DEVELOPMENT OF WARMED-OVER FLAVOR (WOF), a specific defect that occurs in cooked, reheated meat products following short-term refrigerated storage, has been directly linked to autoxidation of highly unsaturated, membrane-bound phospholipids (Younathan and Watts, 1960; Igene and Pearson, 1979) and to the catalytic properties of nonheme iron (NHI) (Sato and Hegarty, 1971; Love and Pearson, 1974; Igene et al., 1979). NHI in muscle foods includes storage iron (ferritin and hemosiderin) and a low molecular weight iron (LMW) fraction. However, it is unclear which form of NHI is most responsible for catalysis of lipid peroxidation in meat systems. Kanner et al. (1991a, b) suggested that LMW may be an important initiator in lipid peroxidation. Catalysis by ferritin has also been demonstrated but only when reducing agents were present (Decker and Welch, 1990; Kanner and Doll, 1991; Seman et al., 1991).

Theoretically, dietary iron intake should influence muscle iron stores (Ullrey et al., 1960). Hence, dietary iron may affect muscle iron, which in turn may catalyze lipid oxidation in muscle foods. Kanner et al. (1990) demonstrated that removal of the iron supplement from turkey rations 3–7 wk prior to slaughter reduced lipid oxidation in dark turkey muscle by 50% but had less effect on white turkey muscle. Iron supplementation is a common livestock feeding practice in the U.S. Swine rations are typically fortified with iron beyond the National Research Council (NRC) requirement level (Miller et al., 1994). Ewan (1985, 1986) analyzed 89 diets formulated by swine producers and reported that they exceeded the NRC (1988) recommendation for iron by 6.8 times.

Our objective was to test the hypothesis that reducing iron in swine diets decreases NHI concentration in muscle tissue and minimizes lipid oxidation in cooked pork.

MATERIALS & METHODS

Feeding trial

Details of dietary treatments, housing, feeding, growth, and blood status of the pigs were previously reported (Miller et al., 1994). Briefly, 15 barrows were divided into three groups. The groups were randomly allocated to one of three treatment diets (62, 131, and 209 ppm iron). Each group of five pigs was fed one of the three diets for about 13 wk. The protocol was approved by the Institutional Animal Care and Use Committee at Cornell University. Pigs were slaughtered according to standard commercial practices.

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Samples

Longissimus dorsi (LD) (from right carcass side) and rectus femoris (RF) muscles (from right and left carcass sides) were excised from each pig 24 hr postmortem (4°C), trimmed, and cut into about 1.3 cm cubes. Cubes were mixed by hand, vacuum-packaged in lots of 25–30g (samples for lipid analysis) or 50–75g (remaining samples) and frozen at −70°C (pork for lipid assays) or −40°C (remaining pork). Vacuum packaging and cooking procedures followed those of Miller et al. (1994).

Storage

Bags (Ziploc® pleated sandwich with zipper, Dow Brands, Indianapolis, IN) of LD and RF were stored on trays in single layers at 4°C for 0–12 days (cooked) and 0–16 days (raw). One bag of LD and one bag of RF from each pig were removed on each of five storage days (day 0, 2, 4, 8, 12) for NHI and thiobarbituric acid reactive substances (TBARS) analyses. Total iron (TI) was determined on day 0 using freshly cooked LD and RF and was assumed constant throughout storage. Tocopherol content was measured on days 0 and 12 in cooked RF and on days 0 and 16 in raw RF. Prior to analysis, all LD and RF muscles were pre-blended (15 sec for cooked; 20–30 sec for raw) in a 50 mL stainless steel cup blender (Waring, New Hartford, CT) to form a homogeneous paste.

Iron and TBARS analysis

NHI was determined according to the method of Schricker et al. (1982) as modified by Rhee and Ziprin (1987) as described previously (Miller et al., 1994). Total iron (TI) was measured using the combined wet/dry ashing procedure detailed in Miller et al. (1994).

Modifications of the TBARS extraction method (Witte et al., 1970), denoted by Willemot et al. (1985) and Miller et al. (1994), were used. One mL butylated hydroxytoluene (BHT) solution (0.4 mg/mL in ethanol) was added (0.1 mg BHT/100 mg fat, 4% fat level assumed) to reduce oxidation during blending (Pikul et al., 1989).

Lipids

Raw LD and RF were thawed at 4°C and analyzed. The extraction method of Hara and Radin (1978) as modified by Willemot et al. (1985) was used for total lipids. For total phospholipids, aliquots (25 μL) of dried lipid extracts, dissolved in hexane (5 mL hexane/100 mg lipid), were pipetted into clean, screw-topped test tubes (15 mL), dried under nitrogen, and quantified spectrophotometrically by the method of Stewart (1980). Fatty acid methyl esters (FAMEs) from total lipid and phospholipid fractions were analyzed using pork from two randomly selected pigs in each treatment. The extraction method of Bligh and Dyer (1959) was used. Samples were saponified and methylated according to the procedure of Metcalfe et al. (1966) using pentadecanoic acid (Fisher) as internal standard. Phospholipid fractions were isolated on TLC plates. GC conditions were as detailed in Miller et al. (1994).

Table 1—NHI and TBARS in raw and freshly cooked (day 0) LD and RF muscles (±SE)[a]

Muscle	Treatment	NHI (μg/g) Raw	NHI (μg/g) Cooked	TBARS (μg/g) Raw	TBARS (μg/g) Cooked
LD	Low Fe	1.81±0.18	2.08±0.19[de]	0.04±0.02	0.05±0.01[e]
	Med Fe	2.02±0.17	2.14±0.09[d]	0.04±0.01	0.10±0.02[f]
	High Fe	2.50±0.33	2.78±0.21[e]	0.03±0.01	0.10±0.02[ef]
	Pooled[b]	2.11±0.15	2.39±0.14	0.04±0.01	0.09±0.01[h]
	p[c]	0.05	0.02	N.S.	N.S.
RF	Low Fe	3.53±0.44	3.64±0.32[fg]	0.04±0.02	0.11±0.02
	Med Fe	3.92±0.58	3.63±0.18[f]	0.04±0.01	0.15±0.01
	High Fe	4.40±0.25	4.95±0.48[g]	0.03±0.01	0.11±0.02
	Pooled[b]	3.95±0.26[i]	4.07±0.25[i]	0.04±0.01	0.12±0.01[hi]
	p[c]	N.S.	0.02	N.S.	N.S.

[a] n=15 pigs for each muscle analysis except n=12 for Cooked LD (NHI and TBARS) and n=14 for Cooked RF (TBARS). Muscles analyzed in triplicate for each pig (μg/g wet wt).
[b] Pooled means for all three treatments using each pig IGM.
[c] Significant linear trend with increasing dietary Fe level ($p<0.05$). N.S.=trend not significant.
[d-g] Treatment means in the same column for a given muscle marked with different superscripts differ ($p<0.05$).
[h] Significantly different from raw pooled mean (NHI or TBARS) in same row ($p<0.05$).
[i] Significantly different from LD pooled mean in same column ($p<0.05$).

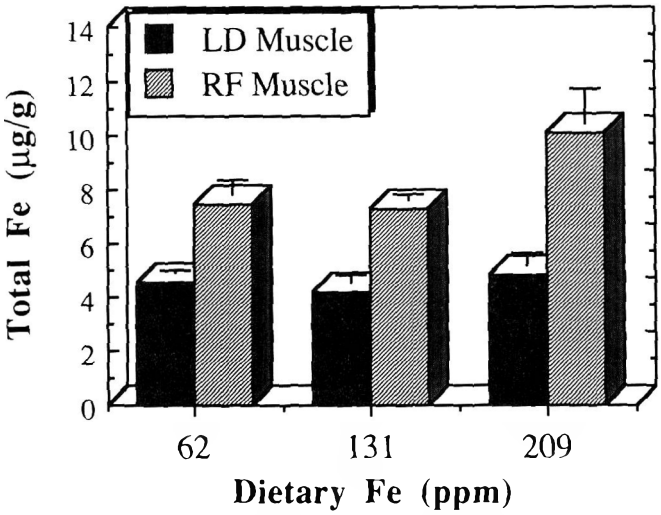

Fig. 1—Effect of dietary iron on mean TI (±SE) in freshly cooked LD and RF (day 0). RF TI regressed against dietary Fe was linearly significant ($p<0.05$). Treatment means (n=5) within a muscle type were not different ($p>0.05$).

α-Tocopherol content

Under dim lighting, 2.5g of pre-blended RF was homogenized in 7.5g buffer (0.005M Tris/maleate, 0.001M EDTA, 0.15M KCl, pH 7.4, stored at 4°C) and 0.1 mL ethanolic BHT (0.25% w/v) in a 56.75g plastic cylindrical vial (Capital Vial, Inc., Fultonville, NY) on setting 4 for 10–15 sec (Kinematica®, Model PT 10/35, Brinkmann Instruments Co., Westbury, NY). From each sample, duplicate homogenates (about 1.2 mL) were transferred to two plastic vials with rubber seal caps, flushed with nitrogen, capped, wrapped in aluminum foil, and stored at −70°C until further extraction. From each duplicate vial, 1 mL homogenate was pipetted into a disposable glass screw-topped tube, and 1 mL sodium dodecyl sulfate (SDS) (400 mM) was added. After mixing on a Vortex, 2 mL ethanolic BHT (0.1% w/v) and 2 mL BHT (0.025% w/v) in heptane were added. Tubes were capped, mixed on a Vortex for 2 min, and centrifuged (IEC Model HNS2, International Equipment Co., Needham Heights, MA) for 5 min at full speed. About 1.6 mL of the upper (heptane) phase was recovered, placed in a glass conical centrifuge tube, and dried under nitrogen in a 30–40°C water bath. Ethanol (200 μL) was added to each tube. Tubes were capped and vortexed for 10 sec. Samples were filtered into vials with Waters Intelligence Sample Processor (WISP) automatic sampler. Analyses were performed using high-performance liquid-liquid partition chromatography (HPLC) with a reverse-phase column described by McShane et al. (1991).

Statistics

A split-plot design with each pig as experimental unit was used (Miller et al., 1994). Outliers were excluded based on residual plots. Individual grand means (IGMs) for each pig (average of days 0, 2, 4, 8, and 12) were grouped according to treatment, and differences between treatments were determined by one-way analysis of variance (ANOVA) and two-sample independent t-tests with $\alpha=0.05$. Statistical analyses were conducted using MINITAB Statistical Software (State College, PA) as outlined in Miller et al. (1994).

RESULTS & DISCUSSION

NHI and TI in muscle

Increasing dietary iron in swine rations during the last 13 wk prior to slaughter did not affect hematological iron status indicators and only slightly improved growth (Miller et al., 1994). Nonetheless, NHI concentrations (μg/g wet wt) in LD and RF increased with dietary iron level, except in raw RF (Table 1). LD and RF were studied because they are classified as white (<30% red fibers) and red (>40% red fibers) porcine muscles, respectively (Beecher et al., 1965). RF had significantly more TI (Fig. 1) and NHI (Table 1) than did LD.

We have reported that cooking increased NHI in meats and hypothesized that the increase was due to oxidative cleavage of the porphyrin ring, allowing release of heme iron (Schricker and Miller, 1983). In this study, however, NHI did not significantly differ between raw and freshly cooked pork muscles (Table 1). Since pork was cooked in vacuum-sealed oxygen-impermeable bags, oxidative cleavage of the porphyrin ring may have been limited by low oxygen concentration. The short cooking time (about 15 min) may also have been a factor.

NHI increased gradually in cooked LD and RF during storage at 4°C (Table 2). Since cooked pork was stored in oxygen-permeable bags, oxidative cleavage of the porphyrin ring may have occurred. NHI release in meats appears to be related to time, temperature, and oxygen during cooking (Buchowski et al., 1988; Han et al., 1993; Schricker and Miller, 1983).

Low- and medium-iron fed pigs had similar muscle TI levels. The same pattern was observed in liver TI and NHI as well as in cooked ground pork NHI (Miller et al., 1994). NHI in liver has long been used as an indicator of iron status. As previously reported (Miller et al., 1994), we found that muscle NHI and TI were significantly associated with liver NHI and TI, respectively (Table 3).

Lipid oxidation in cooked pork

Dietary iron did not affect TBARS in raw or freshly cooked (day 0) RF or raw LD and only slightly influenced TBARS in cooked LD (Table 1). Yet cooking increased TBARS in both muscles. Based on slopes (SLPs) and treatment grand means (TGMs), cooked RF stored at 4°C for 12 days oxidized faster than cooked LD (Table 4). Since both TI and NHI were higher in RF, muscle iron concentration may have been responsible for the more rapid oxidation in the RF. Higher total phospho-

Table 2—Relationship of storage and dietary iron treatment to NHI (±SE) in cooked LD and RF

		NHI[a]				
		Days storage				
Treatment	NHI TGM[b]	0	2	4	8	12
LD[c]						
Low Fe	2.79±0.12[de]	2.08±0.19*	2.45±0.20	2.67±0.18	3.29±0.28*	3.27±0.20
Med Fe	2.52±0.14[d]	2.14±0.09	2.12±0.12	2.14±0.16	2.63±0.11	3.58±0.48
High Fe	3.12±0.15[e]	2.78±0.21	2.73±0.20	2.69±0.16	3.98±0.13	3.39±0.29
RF						
Low Fe	5.09±0.25[fg]	3.64±0.32*	4.75±0.43*	5.22±0.31	6.03±0.41*	5.81±0.29
Med Fe	4.70±0.38[f]	3.63±0.19	4.09±0.32	4.16±0.57	5.18±0.47	6.45±1.09
High Fe	6.21±0.49[g]	4.95±0.48	6.02±0.67	5.40±0.48	7.50±0.77	6.66±0.22

[a] μg Fe/g wet weight.
[b] Treatment grand mean (TGM) - an IGM for each pig was calculated by averaging NHI (μg/g) from five storage days. TGM is the mean of all pigs in the same treatment. TGMs in the same column within a muscle type with different superscripts differ ($p<0.05$).
[c] LD SLPs and IGMs regressed against dietary iron and dietary iron squared showed significant ($p<0.05$) linear and quadratic trends.
[d-g] Treatment means in the same column for a given muscle marked with different superscripts differ ($p<0.05$).
* Treatment means from a given storage day and muscle type were significantly different ($p<0.05$).

Table 3—Correlation coefficients (r)[a]

Relationship tested	r
Liver TI vs. LD TI[b]	0.539
Liver TI vs. RF TI[b]	0.681
Liver NHI vs. LD NHI[c]	0.633
Liver NHI vs. RF NHI[c]	0.634
LD TBARS vs. LD NHI[d]	0.624
RF TBARS vs. RF NHI[d]	0.728

[a] n=14 pigs for correlations except n=13 pigs for LD TBARS vs LD NHI and n=15 pigs for liver NHI vs RF NHI. All correlations were independent of time ($p<0.05$).
[b] TI (LD and RF) measured on day 0.
[c] NHI (LD and RF) taken from IGM of 5 storage days for each pig.
[d] TBARS (LD and RF) and NHI (LD and RF) taken from IGM of 5 storage days for each pig.

lipids in the RF also may have increased TBARS (Table 5). Since red muscle is composed of more fibers per unit area, it contains more cell membranes rich in phospholipids. RF contained less total lipid than LD. Although red muscle usually is higher in fat than white muscle, Beecher et al. (1965) reported that RF was lower in lipid than other porcine red muscles. In general, our values for phospholipids and total lipids agreed with published data (Morrison and Campbell, 1971; Wilson et al., 1976).

NHI and TBARS correlated when the time factor was disregarded (Table 3). These data agreed with our previous findings in cooked ground pork (Miller et al., 1994). Although TBARS were higher in LD and RF from the high-iron group, the effect was not as great as reported for cooked ground pork from these same pigs (Miller et al., 1994) nor as Kanner et al. (1990) reported for turkey.

α-Tocopherol in RF

RF was analyzed for α-tocopherol (Vitamin E) because higher concentrations were shown in dark pork muscle than in white muscle (Yamauchi et al., 1980). Although α-tocopherol in RF did not change immediately after cooking, significant degradation occurred in both raw and cooked RF stored at 4°C (Table 6). A fat-soluble vitamin present in cell membranes, vitamin E is unstable to heat, light, and oxygen. Because pork was stored in oxygen-permeable packages (polyvinyl chloride for raw pork; polyethylene for cooked pork), tocopherol content was expected to decrease. Cooking predisposed pork to faster α-tocopherol losses in a shorter storage period.

No difference in plasma or RF α-tocopherol was detected between treatments perhaps because dietary tocopherol was similar in all three treatment diets (Miller et al., 1994). Cooked RF on day 12 revealed a faster rate of tocopherol degradation in RF from the high-iron group, suggesting the need for more α-tocopherol to quench free radicals in lipid membranes from the high-iron treatment. Vitamin E is destroyed *in vivo* when excess iron is consumed (Lannek et al., 1962).

CONCLUSIONS

THE EXTENT OF LIPID OXIDATION in cooked pork appeared to differ with different muscle cuts. Dark porcine RF muscle oxidized at a faster rate than light porcine LD muscle. There appeared to be a threshold for dietary iron level (between 131-209 ppm dietary iron) above which muscle and liver NHI and TI and muscle TBARS began to increase with increasing dietary iron. Hence, dietary iron may influence NHI and oxidative stability in cooked meats. However, the effects were slight and may be below consumer perception limits.

NOMENCLATURE

BHT	butylated hydroxytoluene
FAMEs	fatty acid methyl esters
IGMs	individual grand means
LD	longissimus dorsi
LMW	low molecular weight iron
MDA	malondialdehyde
NHI	nonheme iron
NRC	National Research Council
RF	rectus femoris
SE	standard error
SLPs	slopes
TBARS	thiobarbituric acid reactive substances
TGMs	treatment grand means
TI	total iron
WOF	warmed-over flavor

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Table 4—Relationship of storage and dietary iron treatment to TBARS (±SE) in cooked LD and RF

		TBARS[a]				
		Days storage				
Treatment	TBARS TGM[b]	0	2	4	8	12
LD[c]						
Low Fe	1.76±0.08[d]	0.05±0.01	1.30±0.11*	2.02±0.17*	2.64±0.15	2.74±0.15*
Med Fe	1.80±0.05[d]	0.10±0.02	1.35±0.01	1.92±0.09	2.62±0.11	2.99±0.07
High Fe	2.04±0.06[e]	0.10±0.02	1.57±0.03	2.34±0.03	2.89±0.15	3.33±0.11
RF[x]						
Low Fe	2.89±0.08[f]	0.11±0.02	2.31±0.13	3.39±0.11	4.03±0.12*	4.62±0.11
Med Fe	3.00±0.04[g]	0.15±0.01	2.37±0.12	3.57±0.14	4.09±0.09	4.84±0.16
High Fe	3.17±0.06[g]	0.11±0.02	2.59±0.04	3.48±0.09	4.54±0.16	5.11±0.13

[a] μg MDA/g wet weight.

[b] Treatment grand mean (TGM) - an IGM for each pig was calculated by averaging TBARS (μg/g) from five storage days. TGM is the mean of all pigs in the same treatment. TGMs in the same column with different superscripts differ ($p<0.05$).

[c] LD and RF SLPs and IGMs each regressed against dietary Fe showed significant linear trends ($p<0.05$)

[d-g] Treatment means in the same column for a given muscle marked with different superscripts differ ($p<0.05$).

* Treatment means from a given storage day were significantly different ($p<0.05$).

Table 5—Total lipid and phospholipid content of raw LD and RF muscles (±SE)

Muscle	Treatment	Total lipid (%)	Phospholipid (% lipid)[a]	Phospholipid (% wet tissue)[b]
LD[c]	Low Fe	3.11±0.45	11.28±1.66	0.32±0.02
	Med Fe	2.47±0.29	14.49±2.69	0.33±0.02
	High Fe	2.35±0.24	14.58±1.66	0.33±0.01
	Pooled[d]	2.64±0.20	13.45±1.17	0.32±0.01
RF[c]	Low Fe	1.71±0.26	25.92±2.87	0.41±0.02
	Med Fe	1.57±0.16	29.18±3.07	0.44±0.02
	High Fe	1.92±0.14	26.65±3.63	0.49±0.04
	Pooled[d]	1.73±0.11[e]	27.25±1.75[e]	0.45±0.02[e]

[a] mg phospholipid/100 mg lipid.

[b] mg phospholipid/100 mg wet tissue.

[c] n=15 pigs for each muscle (in duplicate). Columns within each muscle were not different ($p>0.05$).

[d] Pooled means for all three treatments using each pig IGM.

[e] Significantly different from LD pooled mean in same column ($p<0.05$).

Table 6—α-Tocopherol (ng/g) in raw and cooked RF muscles stored at 4°C (±SE)[a]

	Treatment	Day 0	Day 16
Raw	Low Fe	438.7±21.6	384.2±21.7
	Med Fe	379.6±63.2	260.8±47.5
	High Fe	434.4±27.0	326.0±25.7
	Pooled[b]	417.6±23.4	323.7±22.4[e]
	p[c]	N.S.	0.04
	Treatment	Day 0	Day 12
Cooked	Low Fe	459.2±34.5	33.1±20.5
	Med Fe	370.7±29.7	4.6±4.6
	High Fe	453.1±32.9	0.0±0.0
	Pooled[b]	427.6±20.4	12.6±7.6[de]
	p[c]	0.05	N.S.

[a] n=15 pigs for each muscle and storage period. n=5 pigs per treatment.

[b] Pooled treatment mean using each pig IGM.

[c] Significant quadratic trend with increasing dietary Fe level squared. N.S.=trend not significant. Treatment means within each muscle for the same storage period were not different ($p>0.05$).

[d] Significantly different from raw pooled mean in same column ($p<0.05$).

[e] Significantly different from day 0 pooled mean in same row ($p<0.05$).

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Lipid Oxidation and Warmed-Over Aroma in Cooked Ground Pork from Swine Fed Increasing Levels of Iron

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ABSTRACT

Fifteen crossbred feeder pigs were fed to market weight on corn-soy rations containing either 62, 131, or 209 ppm iron. After slaughter, pork was ground, cooked, and stored at 4°C for 12 days. Heavily fortifying swine rations with iron (≥200 ppm) increased nonheme iron (NHI) and thiobarbituric acid reactive substances (TBARS) in cooked, stored ground pork (GP) but did not increase warmed-over aroma (WOA) ($p>0.05$). NHI, TBARS, and WOA increased during storage. TBARS strongly correlated with WOA during storage ($r=0.903$) and with NHI ($r=0.901$).

Key Words: pork, iron, lipid oxidation, warmed-over flavor

INTRODUCTION

WARMED-OVER FLAVOR (WOF) IN MEATS is of concern to food processors because of the demand for convenient, precooked meat entrees for home and food service markets. WOF, defined by Tims and Watts (1958), occurs when cooked meat products are stored at refrigeration temperature. The off-flavor develops after refrigerated cooked meat has been stored for ≥ 48 hr (Pearson and Gray, 1983). WOF has been hypothesized to be caused by the degradation of aroma compounds and peroxidation of muscle lipids (Vercellotti et al., 1989). Membrane-bound phospholipids high in polyunsaturated fatty acids are susceptible to autoxidation and contribute to WOF (Pearson and Gray, 1983). Therefore, any process that disrupts muscle membranes, such as cooking, chopping, or restructuring would enhance WOF (Gray and Pearson, 1987).

Both heme and NHI may be important to WOF due to their catalytic effects (Love, 1983; Rhee et al., 1987). However, specific mechanisms are not fully understood. Forms of NHI such as ferritin, lactoferrin, cytosolic iron-dependent enzymes, and low molecular weight (LMW) chelatable iron ions enhance lipid peroxidation in meat (Decker and Hultin, 1990; Graf and Panter, 1991; Kanner et al., 1991) and *in vivo* systems (Gutteridge et al., 1979; Halliwell and Gutteridge, 1986). Human and animal studies have linked high iron stores with *in vivo* lipid peroxidation (Dougherty et al., 1981; Lee et al., 1981), atherogenesis (Murray et al., 1991; Salonen et al., 1992), and coronary heart disease (Salonen et al., 1992). Oxidative modification of low density lipoprotein (LDL) may be catalyzed by metal ions such as iron (Steinberg et al., 1989).

U.S. swine producers usually feed iron-supplemented rations. The National Research Council (NRC, 1988) recommends 40–80 mg iron/kg feed for maintaining adequate growth in 10–110 kg swine. Removal of the iron supplement from turkey rations reduced lipid oxidation by 50% in dark turkey muscle stored at 4°C for 7–14 days post-slaughter (Kanner et al., 1990). If lower levels of iron in swine rations would yield similar reductions in lipid oxidation in pork, then it may be practical for swine producers to alter supplementation practices.

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Table 1—Composition of treatment diets

	Low Fe		Med Fe		High Fe	
	Protein level					
Ingredient (%)[a]	16%	14%	16%	14%	16%	14%
Ground corn	76.39	82.12	76.04	81.92	76.04	81.92
Soybean meal	21.25	15.88	21.25	15.88	21.25	15.88
Ca phosphate	0.00	0.00	0.96	0.54	0.96	0.54
RG $CaHPO_4$	0.89	0.48	0.00	0.00	0.00	0.00
Ca carbonate	0.87	0.92	1.15	1.06	1.15	1.06
Salt	0.35	0.35	0.35	0.35	0.35	0.35
+Fe vit/min mix[b]	0.00	0.00	0.00	0.00	0.25	0.25
−Fe vit/min mix[c]	0.25	0.25	0.25	0.25	0.00	0.00
Nutrient[d]						
Iron (ppm)	68.9	55.7	148.8	113.0	226.6	191.1
Mean iron (ppm)	62.3		130.9		208.8	
α-toco (IU/kg)	24.5	25.1	22.9	24.1	19.5	20.4
Mean α-toco (IU/kg)	24.8		23.5		20.0	

[a] By formulation.
[b] Swine Premix A.
[c] Swine Premix B.
[d] By analysis. Mean iron: n=8, SE≤5.7%; Mean α-toco: n=2, SE≤2.6%.

Our objective was to test the hypothesis that iron supplementation of swine rations increases lipid oxidation in cooked ground pork (GP), thereby exacerbating the problem of WOF.

MATERIALS & METHODS

Animals and diets

The experimental protocol was approved by the Institutional Animal Care and Use Committee at Cornell University. Fifteen crossbred barrows were selected on a similar weight basis (24.7 ± 1.3 kg) and allocated to one of three groups so that each group had similar mean body weights. Groups were randomly assigned to the three treatment diets (Table 1). Prior to the study, swine were injected with 100 mg iron dextran at 3 days of age, weaned at 21 days of age, and fed commercial creep pellets (Vigortone® VigorStart NT Pig Starter Medicated, Vigortone® Ag Products, Inc., Cedar Rapids, IA) containing 580 ppm iron (by analysis) up to 25 kg mean body weight.

Corn-soy rations were formulated to contain 16% protein for the first 6 wk (59 kg mean body weight), and 14% protein for the remaining 7 wk, thus meeting NRC (1988) requirements (Table 1). Mean dietary iron level for each treatment diet was calculated based on the average iron content in the 16% and 14% protein rations. These values were used in statistical analyses. The high-iron diet was formulated using ingredients and levels commonly fed by U.S. swine producers. The medium iron diet was identical to the high-iron diet except that an iron-free vitamin/mineral mix was used. The low-iron diet was provided by using iron-free vitamin/mineral mix as well as reagent grade calcium phosphate in place of feed grade calcium phosphate which includes appreciable quantities of iron. By formulation, all diets had a Ca:P ratio of 0.55:0.44 and contained 30 IU vitamin E/kg. Due to time constraints, diets were analyzed for vitamin E several months after the feeding study. Therefore, analytical α-tocopherol values (Table 1) may have been lower than the α-tocopherol content fed.

Ingredients in the diet formula were: shelled yellow corn = 8.2% protein, 0.03% Ca, 0.28% P, 0.003% Fe, 7.9 mg/kg α-tocopherol; dehulled soy meal = 48% protein, 0.26% Ca, 0.64% P, 0.016% Fe, 2.03 mg/kg α-tocopherol; Nutrena® dicalcium/monocalcium phosphate = ≥15% Ca, ≥20.5% P, 1.29% Fe (Cargill, Minneapolis, MN); reagent grade dibasic calcium phosphate = 29.46% Ca, 22.77% P, 0.0135% Fe (Sigma, St. Louis, MO); granular $CaCO_3$ = ≥38% Ca, 0.0144% Fe (Limestone Products Corp., Sparta, NJ); iodized salt (Agway, Ithaca,

NY); swine premix (A) = >3.2% Fe (as ferrous sulfate), >0.4% Cu, >0.012% Se, 8430 IU/kg α-tocopherol, and swine premix (B) = 0.179% Fe (as ferrous sulfate), 0.4% Cu, 0.012% Se, 7900 IU/kg α-tocopherol (Carl S. Akey, Inc., Lewisburg, OH).

Housing and feeding

Animals were individually housed at ≥21.1°C in a temperature-regulated building on unbedded concrete floors in 2.44 × 0.91m epoxy-coated (IronClad®, Benjamin Moore & Co., Montvale, NJ) galvanized metal pens equipped with automatic stainless steel nipple water units and 30 kg capacity wooden feeders. A 5-micron water filter (W.W. Grainger, Inc., Lincolnshire, IL, Cartridge #110, 24.77 × 6.99 cm diam cartridge for 37.15 cm pump filter) kept iron contamination in the water < 2 ppm by analysis. Feed and water were provided *ad libitum*, and pens were rinsed daily. Weekly feed intakes, rates of gain, and feed efficiencies were calculated throughout the 13-wk study.

Hematology

Blood was drawn biweekly from the anterior vena cava using both 3 mL heparinized and 7 mL nonheparinized tubes (Vacutainer™, Becton Dickinson Co., Rutherford, NY). Swine were partially restrained by a snout snare during collection. Serum was separated by 15 min centrifugation at 850-1000 RCF (2400 rpm) (CRU-5000 International Equipment Co., Needham Hts., MA). Samples were refrigerated and analyzed in duplicate within 24 hr for serum iron (SI) and total iron-binding capacity (TIBC) (Sigma Chemical Co., St. Louis, MO). Hemoglobin (Hb) (Sigma) and packed cell volume (PCV) determinations were made in triplicate using heparinized blood. Plasma tocopherol (PT) was determined at slaughter (McShane et al., 1991).

Slaughter and ground pork

Slaughtering took place on three separate days within the 13th week. On a given slaughter day, five pigs were fasted overnight and killed by humane electrical stunning and exsanguination. Slaughter order was based on body weight and treatment, starting with the heaviest animal within each treatment. Venous blood was collected at slaughter for SI, TIBC, Hb, PCV, and PT determinations. Carcasses were eviscerated, split, and chilled at 4°C overnight. Unilateral cryptorchid was detected at time of slaughter in two pigs from the high-iron group. Although this was a concern, these carcasses were retained in the experiment.

Tenth rib backfat and loin eye were measured in each carcass half. Approximately 6.81 kg ground pork (GP) was prepared from deboned, 0.32 cm fat-trimmed Boston Butt and Picnic cuts from the right carcass half. Pork from each animal was ground in two batches in a stainless steel bowl chopper for 60 sec. Both batches of chopped pork were then blended in a paddle mixer for 60 sec. Poorly ground pieces high in connective tissue were discarded.

Packaging

Samples were vacuum-packaged (Multi-Vac vacuum package machine, type AG5, Sepp Haggenmuller KG, W. Germany) in pouches (FreshPak 500™, Koch Supplies, Inc., Kansas City, MO) in portions of 25–30g (lipid determinations), 50–75g (all other chemical asays) or 100 g (sensory panel), and stored at either −70°C (for lipid analysis) or −40°C. The pouches were made from laminated nylon/polyethylene with the following specifications (by manufacturer): 15.24 × 21.59 cm o.d., 3 mils, O_2 transmission rate=3.5 cc/645.16 cm²/24 hr at 21.1°C, water vapor transmission rate=0.6 g/645.16 cm²/24 hr at 37.8°C.

Cooking procedure

One day prior to cocking, pork was thawed overnight at 4°C, and pouches were examined for seal and vacuum imperfections. Poorly sealed or poorly evacuated bags were repackaged before cooking. Pouches were flattened to about 1.9 cm thicknesses, slit about 1.3 cm above the vacuum seal (without breaking seal), slid onto 0.3 cm rods in a handmade cooking rack apparatus, and submerged in water held at 80°C in a steam-jacketed, 189.4 L stainless steel rectangular cheese vat. The vacuum seal on one extra bag of pork was punctured, and internal temperature was monitored using a digital probe thermometer (Omega Engineering, Inc., Stamford, CT). Samples were removed from the bath 10 min after reaching internal temperature 76°C. (Final meat temperature at time of removal was ~80°C). Cooking time averaged 15 min/batch.

Storage study

After cooking, each bag was opened and its contents sealed in a prelabeled pleated sandwich bag with zipper (Ziploc®, Dow Brands, Indianapolis, IN, 16.51 × 14.92 cm, polyethylene, 1.15 mil, O_2 transmission rate=424 cc/645.16 cm²/24 hr at 22.8°C, water vapor transmission rate=1.0-1.5 g/645.16 cm²/24 hr at 37.8°C and 90% relative humidity). Bags of cooked pork were stored in non-overlapping layers on trays at 4°C for 0-12 days. One bag from each pig was removed on each of 5 storage days (day 0, 2, 4, 8, 12) for chemical analyses and 3 bags for the same 5 days were removed for sensory evaluation.

TBARS test

The method of Witte et al. (1970), as modified by Willemot et al. (1985), was used to measure lipid oxidation in triplicate samples from each pig. The procedure was further modified by adding butylated hydroxytoluene (BHT) (0.1 mg/100 mg fat) prior to blending to minimize sample oxidation during assay (Pikul, 1989). A 20% fat level was assumed when calculating BHT concentration. Five grams crumbled, cooked GP was weighed into a 250 mL glass beaker and the following reagents were added: 1 mL BHT solution (1 mg/mL in ethanol) and 44 mL extracting solution (10% TCA in 0.1M H_3PO_4). Each sample was blended directly in the beaker (30 sec, high speed) using an upright hand blender (Rival Ultra Blend™, Kansas City, MO). Recovery was measured in three augmented pork samples containing 12 mL 1,1,3,3-tetraethoxypropane (TEP) standard (10^{-5} M), 1 mL BHT, and 32 mL extracting solution. Sample blanks were analyzed for each sample. A standard curve was prepared using 0–5 mL TEP solution (10^{-5} M). Test tubes were incubated in the dark for 15-17 hr at room temperature (≈23°C). TBARS were expressed as μg malondialdehyde (MDA)/g sample (wet weight) with total volume adjusted according to sample moisture: total volume = 45 mL + mL water in 5g cooked pork.

Iron analysis

All glassware was acid-washed in 1 N HCl and rinsed with distilled water prior to use. NHI concentration was measured by modifications of methods of Schricker et al. (1982) and Rhee and Ziprin (1987). Two hundred μL of 0.39% $NaNO_2$ was added prior to incubation to stabilize the heme complex and thereby minimize release of heme iron during 20 hr incubation at 65°C. After heating, each sample was assayed as follows: 1 mL extract was combined with 1 mL hydroxylamine hydrochloride (3% w/v in distilled water, Sigma), vortexed, and held 10 min. Bathophenanthroline disulfonic acid (1.5 mL of 0.3 mg/mL in 3M sodium acetate, Sigma) was added as color reagent, and, after 15 min, absorbance was recorded at 533 nm. Sample blanks (3M sodium acetate replaced bathophenanthroline) were analyzed to correct for dark-colored extracts. A standard curve was prepared using an iron atomic absorption standard (Sigma).

Total iron (TI) was determined colorimetrically following ashing. Prior to ashing, 2g cooked pork was weighed into tared Pyrex® ignition tubes in triplicate, and moisture was determined after 24 hr at 95°C. Sample tubes were transferred to a heating block and dry-ashed in a muffle furnace for 2 hr at 250°C, followed by 20 hr at 450°C. Concentrated HNO_3 (4 mL) was added, and tubes were heated to 90–95°C in an aluminum foil-wrapped heating block on a temperature-controlled hot plate until dry. Wet ashing with HNO_3 was repeated. The dry/wet ashing procedure was repeated, where necessary, until a gray-white residue remained. Concentrated HCl (0.5 mL) was added to each tube. Tubes were vortexed, covered, and held ≥ 2 hr. The volume was brought to 10 mL with distilled water. Sample aliquots (2 mL each) were taken, and iron was measured colorimetrically as described for NHI. National Bureau of Standards bovine liver yielded 95% recovery.

Lipid anlayses

Lipid measurements were made in duplicate using raw GP from each of 15 pigs, unless otherwise stated. Lipids were extracted from the GP samples by the method of Hara and Radin (1978) as modified by Willemot et al. (1985). The extracting solution (hexane:isopropanol, 3:2, v/v) contained BHT (0.01% w/v) to prevent sample autoxidation.

Total lipids were determined gravimetrically. Total phospholipids were quantified using dried lipid extracts dissolved in hexane (3 mL

Table 2—Relationship of storage and dietary iron to TBARS (±SE) in cooked GP

		TBARS[a]				
		Days storage[b]				
Treatment	TBARS TGM[c]	0	2	4	8	12
Low Fe	3.76±0.05[d]	0.10±0.00	3.66±0.21*	4.76±0.12	5.15±0.08*	5.13±0.06
Med Fe	3.79±0.05[d]	0.09±0.01	3.86±0.05	4.78±0.10	5.14±0.10	5.09±0.06
High Fe	4.21±0.07[e]	0.11±0.02	5.57±0.04	5.07±0.08	5.74±0.09	5.73±0.11

[a] μg MDA/g wet weight.
[b] Regression analysis of transformed SLPs showed higher (p=0.006) rates of TBARS increase over time as function of increasing dietary iron levels.
[c] Treatment grand mean (TGM) - an IGM for each pig was calculated by averaging TBARS (μg/g) from 5 storage days. TGM is the mean of all pigs in the same treatment.
[d] TGMs with different superscripts differ (p<0.05).
* Treatment means from a given storage day were significantly different (p<0.05).

Table 3—Relationship of storage and dietary iron to NHI (±SE) in cooked GP

		NHI[a]				
		Days storage[b]				
Treatment	NHI TGM[c]	0	2	4	8	12
Low Fe	4.98±0.20[d]	3.34±0.29*	4.96±0.33*	5.11±0.17*	5.86±0.17*	5.64±0.18*
Med Fe	4.84±0.06[d]	3.04±0.09	4.62±0.18	5.29±0.25	5.74±0.11	5.56±0.07
High Fe	6.30±0.21[e]	4.12±0.34	6.32±0.07	6.29±0.18	7.40±0.11	7.36±0.46

[a] μg Fe/g wet weight.
[b] Regression analysis of transformed SLPs showed higher (p=0.014) rates of NHI increase over time as function of increasing dietary iron levels.
[c] Treatment grand mean (TGM) - an IGM for each pig was calculated by averaging NHI (μg/g) from 5 storage days. TGM is the mean of all pigs in the same treatment.
[d-e] TGMs with different superscripts differ (p<0.05).
* Treatment means from a given storage day were significantly different (p<0.05).

hexane/100 mg lipid). Aliquots of 15 μL (0.5 mg fat) were transferred to the bottom of a screw-topped test tube (Pyrex, 15 mL) and dried under nitrogen. Phospholipids (mg) were quantitated by the ferrothiocyanate method (Stewart, 1980) using a standard curve prepared with dipalmitoyl DL-α-phosphatidyl choline (Sigma). Phospholipid content was expressed as % total lipid as well as % wet tissue weight.

Fatty acid compositions of total lipid and phospholipid fractions were analyzed from two randomly selected pigs/treatment. Raw GP (2.5 g) was homogenized in 10 mL saline (0.9% NaCl in deionized water) and 0.1 mL ethanolic BHT (0.25% w/v) for 10–15 sec on setting 4 (Kinematica®, Model PT 10/35, Brinkmann Instruments Co., Westbury, NY). Aliquots of homogenate (0.8 mL) were transferred to each of four screw-topped test tubes, and lipids were extracted using the Bligh and Dyer (1959) method. Phospholipids from two tubes were isolated on TLC plates (Krackeler Scientific, INC., Albany, NY) developed in a chloroform:methanol (8:1, v/v) solvent system. Phospholipid scrapings and dried total lipid (in remaining two tubes) were saponified/methylated by a modification of Metcalfe et al. (1966) with pentadecanoic acid (Fisher, EK-102–4876) as internal standard. A megabore fused silica column (J & W Scientific, DB-23 series, 125-2332, 30 m, 0.75 mm ID, 0.20 μm df) and 5710A Hewlett Packard gas chromatography (GC) apparatus equipped with a 3390A Hewlett Packard integrator were used for detection of fatty acid methyl esters (FAMEs). GC conditions were: injection temperature=140°C; final oven temperature=240°C; program rate=8°C/min; carrier gas=nitrogen; nitrogen flow rate=30 mL/min. FAME standards (NuChek Prep, Elysian, MN, GLC-68B and GLC-79) were used to determine retention times. Fatty acids were calculated on a mole % basis.

Sensory evaluation

Ten panelists (plus one alternate) were selected and trained after achieving ≥ 75% accuracy rate in four consecutive-day triangle tests using samples of freshly cooked and reheated 14 day or 16 day stored, cooked GP. Training was conducted in ten 30 min sessions over a 2 wk period using a group discussion format. WOA was described using known quality descriptors summarized by Melton et al. (1987) and additional terms generated by the panel. Trainees rated WOA intensity in cooked GP stored for 2, 4, 8, and 12 days against a freshly cooked reference (labeled "R") using a 9-point scale (0=weak; 8=strong). Panelists were instructed to assign "0" or "1" to the reference as a calibration from day to day.

The sensory experiment was replicated on three alternate days. A randomized block design of 10 panelists and 15 pigs was used. Each of two panelists was paired with three pigs (one from each of the three treatment diets) and asked to evaluate three sets of samples on each day of testing. Each set contained five samples from the same pig stored as follows: reference (freshly cooked), 2, 4, 8, and 12 days. On each test morning, cooked, stored GP (~30 g) was spooned from each sandwich bag to two glass jars coded with three-digit random numbers, topped with pre-cut aluminum foil liners, and screw-capped. A headspace of about 5 cm was in each jar. Jars were refrigerated at 4°C and warmed in a 70°C water bath 30 min prior to panelists' arrival. Format and ballot were the same as used during training. WOA scores were averaged from the two panelists' triplicate replication (n=6) so that each pig had five scores which corresponded to days 0, 2, 4, 8, and 12.

Statistical analysis

The experimen: followed a split-plot design with each pig representing one experimental unit, dietary iron as the whole plot factor, and storage time and bags of pork as subplot factors. MINITAB Statistical Software (State College, PA) was used for all analyses.

Single-day data

Means and standard error of the mean (SE) were calculated for each pig, and outliers were excluded on the basis of residual plots. Data were separated according to the three iron treatments (n=5 values/treatment unless stated otherwise) and analyzed by analysis of variance (ANOVA) and independent two-sample t-tests when ANOVA was significant ($p<0.05$). Regression analysis of chemical and/or sensory data was used to detec: linear trends or quadratic fits as a function of iron level or iron level squared, respectively ($p<0.05$).

Over-time data

Treatment data from the 12-day storage study was analyzed for time effects and absolute differences. Slopes (SLPs) of NHI, TBARS, and WOA plotted vs storage days were calculated using the best linear transformation of the x-axis [$\log_{10}$ (days + 1), $\sqrt{}$(days), or (days + 1)$^{-1}$]. Absolute differences between pigs were corrected for time by averaging the five chemical or sensory values for days 0, 2, 4, 8, and 12 for each pig [individual grand mean (IGM)]. Treatment grand means (TGMs) were also computed. SLPs and IGMs were sorted by dietary iron treatment level and analyzed by ANOVA, t-tests, and regressions (as described under single-day data).

Correlations

Three separate methods were used for each of the following correlations: TBARS vs WOA, TBARS vs NHI, and NHI vs WOA. First, the factor of time was removed by regressing IGMs to show between-animal relationships. Secondly, within-animal deviations (IGM minus single-day response) were correlated, revealing relationships over time while removing animal-to-animal variations. Thirdly, correlations through time were made by using the five single-day data points for each animal, leaving animal-to-animal variation uncorrected.

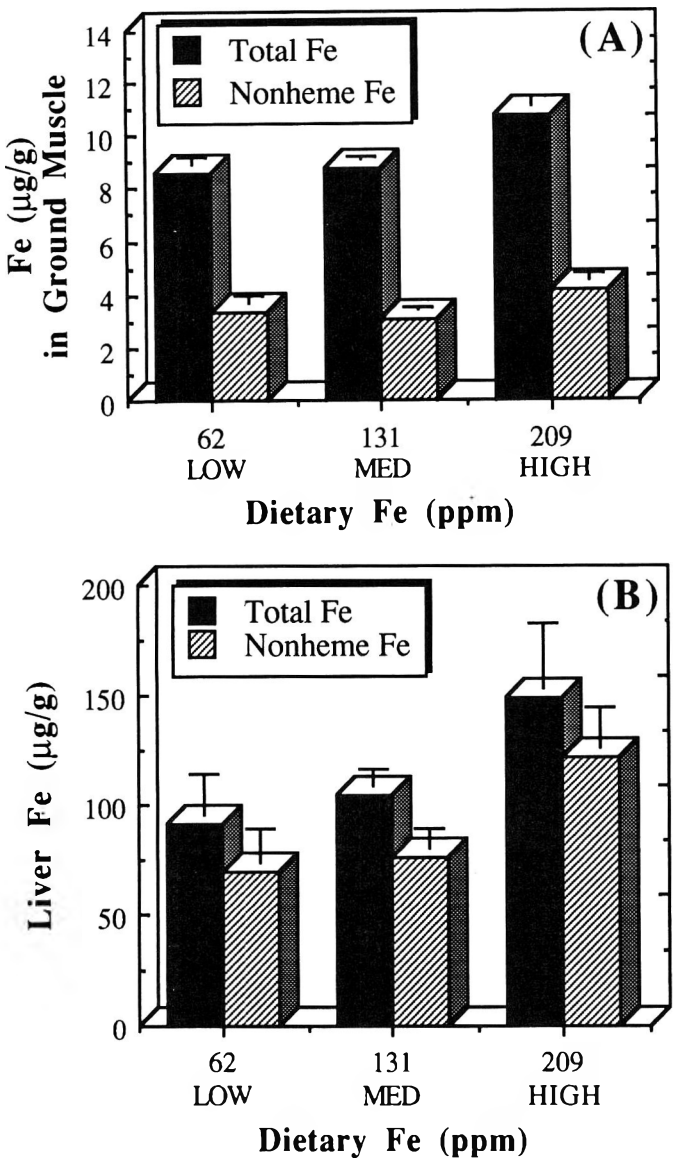

Fig. 1—Effect of dietary iron on iron in: (A) freshly cooked (day 0) GP and (B) raw liver. Values are means ± SE. Treatment bars for GP TI (n=4) and GP NHI (n=4) with different superscripts differ ($p<0.05$). Liver NHI (n=15, $p=0.024$) increased with respect to dietary iron level according to linear regression analysis.

Table 4—Correlation coefficients (r)

Relationship	r[a]
Liver TI vs muscle TI[b]	0.816
Liver NHI vs muscle NH[c]	0.643
TBARS vs NHI[d]	0.901
TBARS vs WOA[e]	0.903
NHI vs WOA[f]	0.754

[a] n=12 pigs for all correlations except n=10 pigs for TBARS vs NHI ($p<0.05$).
[b] Muscle TI from day 0 and liver TI at time of slaughter (independent of time correlation).
[c] Muscle NHI from GM of five storage days for each pig (independent of time correlation).
[d] GMs of five storage days for each pig (independent of time correlation).
[e] Five values for each pig (over-time correlation).
[f] Five values for each pig (over-time correlation).

RESULTS & DISCUSSION

Growth and hematology

Swine fed high-iron diets had slightly higher mean final weights (106.1 ± 1.0 kg) than pigs from the medium-iron group (100.3 ± 1.3 kg) but not the low-iron group (98.5 ± 2.6 kg). Weight increased with increasing dietary iron, but probably the large SE associated with the low-iron group masked any statistical weight differences between the low-iron group and the other two groups. When final weights were regressed against dietary iron level, the slope was significant ($p<0.05$). SI, %TS, Hb, %PCV, and PT were not affected by dietary iron treatment (data not shown), and no signs of iron-deficiency were evident.

Chemical analyses

Both TBARS and NHI increased significantly during the 12-day storage period (Table 2 and 3). These data suggest that some disruption of the porphyrin ring occurred during storage, especially in the early stages. Iron released from the heme complex as a result increased NHI concentration and this may have increased TBARS formation. However, TBARS in freshly cooked GP (day 0) and pork stored 4 and 12 days did not differ between treatments, while NHI treatment differences were significant on all five storage days. Increases in TBARS and NHI were more pronounced in pork from the high-iron treatment group (Table 2 and 3).

TI in freshly cooked GP was also higher in the high-iron group compared to the other two (Fig. 1). A significant quadratic trend in muscle TI ($p=0.029$) as a function of dietary iron level squared was observed, suggesting a threshold for the relationship between muscle iron concentration and dietary iron. Above this threshold of 131–209 ppm dietary iron, muscle TBARS, NHI, and TI increased, but below the threshold dietary iron concentration did not affect tissue iron or TBARS.

Homeostatic regulation of total body iron operates at the site of absorption: iron-deficient animals absorb a higher percentage of dietary iron that iron-adequate animals (Bothwell et al., 1979). Apparently this regulation was partially overridden in the high-iron group since tissue iron concentrations were greater in that group. A threshold was also evident for liver TI and NHI (Fig. 1). Both muscle and liver from the low- and medium-iron fed swine did not differ in TI and NHI concentration. Hence, the low- and medium-iron treatment diets appeared to be in the range where homeostasis was effective. Liver TI and NHI correlated with the respective TI (day 0) and NHI (IGMs) concentrations in ground muscle (Table 4). This was direct evidence that changes in iron status were reflected in muscle iron concentrations.

Swine rations contain a wide range of iron levels. However, few analyzed values have been reported. Analyses of rations from one producer and from the Cornell Swine Farm revealed that total dietary iron varied between 302-737 ppm (Miller, 1992). This confirmed published analyses of 89 swine diet samples formulated by swine producers (Ewan, 1985, 1986) and of a basal diet fed by Pond et al. (1989). We approximated our high-iron treatment diet to contain an iron level representative of that used by commercial producers. However it probably was formulated at a lower iron level because of special precautions with respect to cleanliness when mixing diets and precision when weighing micronutrients.

In our study, differences in pork lipid peroxidation due to dietary iron treatment were not as apparent as Kanner et al. (1990) reported for turkey. This may be partially due to the precautions mentioned. Turkey is also higher in polyunsaturated fatty acids, such as arachidonic acid, and total phospholipids when compared to pork (Ang, 1988; Wilson et al., 1976). Thus, WOF and lipid oxidation in general have been more of a problem in poultry than in red meat (Wilson et al., 1976).

Dietary iron treatment did not affect FAMEs of total lipid or phospholipid fractions of raw GP (data not shown). Raw pork lipid content varied from 8-20% between pigs. Treatment means were: low-iron=13.53 ± 2.09%; medium-iron=13.21 ± 1.64%; high-iron=12.84 ± 2.04% lipid. No differences in total lipid content were observed due to dietary iron treatment. This wide

Table 5—Relationship of storage and dietary iron to WOA sensory scores (±SE) for cooked GP

		WOA score				
		Days storage				
Treatment	WOA TGM[a]	0	2	4	8	12
Low Fe	3.34±0.13	0.13±0.06	2.77±0.43	4.53±0.38	4.67±0.41	4.60±0.49
Med Fe	3.49±0.19	0.27±0.04	2.83±0.36	4.07±0.23	4.93±0.32	5.37±0.39
High Fe	3.63±0.13	0.27±0.10	2.77±0.13	4.93±0.44	4.97±0.21	5.20±0.49

[a] Treatment grand mean (TGM) - an IGM for each pig was calculated by averaging WOA from five storage days. TGM is the mean of all pigs in the same treatment. No differences were found between TGMs, treatment means on any given storage day, rates of WOA increase (SLPs), or IGMs corrected for time ($p>0.05$).

range of total fat was expected due to similar variability in tenth rib backfat between animals.

Phospholipids in raw GP ranged from 2.92–3.48% of total lipid or 0.36–0.42% based on pork wet weight. Phospholipid content was not significantly different in pork from the three treatments. Since GP was prepared with both intramuscular fat and adipose tissue, phospholipid content was lower than we found in lean muscle.

Warmed-over aroma

WOA was rated instead of WOF for the general safety of the panel and for sensitivity reasons. Cooked meats held at refrigeration temperature become rancid after 8 days (Anonymous, 1984) and may be unpalatable. The human nose is sensitive to oxidation by-products commonly found in cooked, refrigerated meats (Dupuy et al., 1988; Frazzalari, 1978). Other researchers have reported meat aroma responses instead of flavor scores (Poste et al., 1986; Tellefson et al., 1982).

Trained panelists detected WOA in cooked GP throughout the storage study (Table 5). On storage day 4, sensory scores reached a plateau which corresponded with the plateau of MDA production (Table 2). Unlike TBARS, however, the panel found no differences in WOA intensity among the three treatments. Our results suggested that manipulating dietary iron levels in swine at or below 209 ppm may have reduced lipid oxidation but not at consumer-perceivable levels.

Inability to detect differences may be explained by the manner in which panelists were trained. The panel generally utilized only 3/4 of the WOA intensity scale on the sensory ballot. During training and testing, the most intense WOA samples were stored for 12 days. However, panelists were pre-selected based on triangle tests where freshly cooked and 14- or 16-day stored pork was used. Thus, panelists may have been influenced by experiences in pre-screening. Unjudged stimuli may affect judgment scales by anchoring either end (Sarris and Parducci, 1978).

Another possible explanation for the inability to distinguish differences in WOA between treatments may relate to Weber's Law. As stimuli intensity increases, a larger incremental change in concentration is required to detect barely noticeable differences (Stevens, 1971). Poste et al. (1986) reported inabilities to rate WOA in cooked pork after 8 days storage because of rancidity odor overlap. Our panel may have confused WOA with rancidity, thus rating WOA the same in cooked pork stored 4 or more days.

Tarladgis et al. (1960) reported a TBARS threshold of 0.5 to 1.0 μg MDA/g for detectable WOA in pork by trained panelists. However, TBARS values vary by method despite high correlations. For example, the distillation method (Tarladgis et al., 1960) produced higher TBARS than the extraction technique (Melton, 1983). Since we used a modification of the White et al. (1970) extraction, our TBARS threshold may have been slightly lower than Tarladgis' range. Our TBARS values for cooked GP were higher than 1 μg MDA/g on day 2 and after. Thus, based on Tarladgis' TBARS threshold range, our panelists should have detected WOA in all cooked GP treatments at day 2 and beyond. Since trained panelists did not distinguish WOA differences according to dietary iron treatment, withdrawing iron supplements from swine rations to protect against lipid oxidation and WOA may be ineffective.

Correlations

Several significant correlations were found (Table 4). Muscle NHI correlated significantly with TBARS and, to a lesser extent, with WOA. Since our trained panelists did not detect differences in WOA among the three treatment groups, WOA did not significantly correlate with TBARS or NHI when the factor of time was disregarded. However, strong through-time correlations occurred between all three variables. Our study confirmed that chemical tests, such as TBARS and NHI, were valuable indicators of WOA over time. Such chemical measurements were more applicable for large numbers of stored samples, and probably are more sensitive, consistent, reliable, and economically feasible than trained consumer panels.

NOMENCLATURE

BHT	butylated hydroxytoluene
FAMEs	fatty acid methyl esters
GP	ground pork
Hb	hemoglobin
IGMs	individual grand means
LMW	low molecular weight iron
MDA	malondialdehyde
NHI	nonheme iron
NRC	National Research Council
PCV	packed cell volume
PT	plasma tocopherol
SE	standard error
SI	serum iron
SLP	slope
TBARS	thiobarbituric acid reactive substances
TGMs	treatment grand means
TI	total iron
TIBC	total iron binding capacity
WOA	warmed over aroma
WOF	warmed-over flavor

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Stability of Membrane-Sterilized Bovine Immunoglobulins Aseptically Added to UHT Milk

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ABSTRACT

Lyophilized immunoglobulin (IgG) of 56% purity isolated from Cheddar cheese whey was reconstituted with distilled water to a protein content of 60–70 mg/mL. The solution was membrane-sterilized (0.2 μm) and aseptically injected into cartons of UHT milk. This method could be used for fortification of UHT products without denaturing IgG. Concentration of IgG remained constant in milk stored at 4, 25 and 35°C over 5 mo. However, when stored at 25 and 35°C, the milk thinned probably due to residual enzymes in the IgG solution. D values measured between 62 and 80°C for thermal destruction of IgG in UHT milk appeared to confirm shelf life results.

Key Words: immunoglobulin, milk, cheddar cheese, ultra high temperature

INTRODUCTION

SEVERAL STUDIES HAVE REPORTED that the ingestion of immunoglobulins (Ig) from the colostrum of immunized cows can prevent rotavirus and enterotoxigenic *Escherichia coli* infection in infants and adults (Hilpert et al., 1977, 1987; Ebina et al., 1985; Brüssow et al., 1987; Tacket et al., 1988). Yolken et al. (1985) found Ig in raw and pasteurized milk from non-immunized cows also had anti-rotavirus activity. Since infant formulas are deficient in immunologic activity, researchers have suggested fortifying infant formulas with bovine Ig (Goldman, 1989; Facon et al., 1993).

A method is needed to fortify and package Ig in such products. Conventional heat sterilization cannot be used since Ig are heat-denatured at 70°C (Glover, 1985). Commercial systems can aseptically inject membrane-sterilized solutions like flavoring compounds and lactase solutions into ultra-high temperature (UHT) products (Johnson, 1987). Such a system may be applicable for fortifying products with Ig, since membrane sterilization, being relatively gentle, would probably not denature Ig.

The stability of membrane-sterilized bovine Ig aseptically added to UHT products needs to be determined. The Ig must be active to provide protection. Although studies on thermal destruction of human milk Ig have been reported (Morgan et al., 1986), little information is available for bovine milk IgG. Our objectives were to determine the feasibility of membrane-sterilizing IgG and aseptically adding it to UHT products, as well as to determine the stability of IgG in such a product.

MATERIALS & METHODS

Materials

Immunoglobulins were isolated from Cheddar cheese whey using ultrafiltration and immobilized metal affinity chromatography (IMAC) using the method of Fukumoto et al. (1994). The IgG was lyophilized and reconstituted with distilled water to a protein concentration of 60–70 mg/mL, of which 56% was IgG.

Membrane sterilization and aseptic injection

The IgG solution was sterilized by prefiltering through 1.6-μm GF/A filter paper (Whatman Ltd., England) and then filtering through a presterilized 37-mm syringe filter, Acrodisc 37 GF (Gelman Scientific, Ann Arbor, MI). The filter had a borosilicate glass fiber prefilter and a 0.2-μm Supor membrane. The filter-sterilized IgG solution was collected in sterile 25-mL microflex flasks with silicone septums (Kontes Scientific Glassware/Instruments, Vineland, NJ) that had been swabbed with 70% ethanol prior to injection of the IgG solution.

UHT cartons (250 mL) containing 2% fat milk were obtained from Dairyland Foods (Burnaby, B.C.). Carton tops were swabbed with 70% ethanol and then a piece of 19-mm-wide Scotch Brand Magic Tape (3M Canada Inc., London, ON) was applied. The tape was swabbed with 70% ethanol, and a door and window silicone caulking plug (General Electric, Mississauga, ON) was applied. The silicone was allowed to cure for at least 24 hr. Using a sterile syringe and a 3.8 cm 20G needle (Becton Dickinson & Co., Rutherford, NJ), sterilized IgG solution was added to each of 12 cartons through the silicone plug to provide a final milk IgG concentration of 1.2 mg/mL. All operations were carried out in a horizontal laminar flow hood Model EV424 (Envirco, Albuquerque, NM). Four injected cartons and four cartons not injected were incubated at 4, 25, and 35°C each.

Radial immunodiffusion for IgG concentration

Radial immunodiffusion (RID) plates were prepared according to Ingild (1983) with modifications. Each RID plate was prepared by using 0.06–0.18 mL of anti-bovine IgG (whole molecule) developed in rabbit (Sigma Chemical Co., St. Louis, MO, or ICN ImmunoBiologicals, Lisle, IL) with phosphate buffer-saline (PBS: 0.15M NaCl in 10 mM sodium phosphate buffer at pH 7.0) to make a total volume of 2.0 mL. Fifty mg of agarose Type IV (Sigma Chemical Co., St. Louis, MO) was mixed with 4.6 mL of PBS and 0.4 mL of 0.35% NaN_3, and boiled to dissolve the agarose. Both solutions were equilibrated in a 55°C waterbath and then mixed together before pouring into an RID plate (ICN ImmunoBiologicals, Lisle, IL). Plates were allowed to solidify at room temperature for 15 min and were stored in a moist chamber at 4°C. Plates were prepared by cutting 3.0-mm-diameter holes and 3.0 μL of sample was added to each well. Bovine IgG from serum (Sigma Chemical Co., St. Louis, MO) was used for standard solutions in concentrations from 0.1–1.5 mg/mL prepared using an extinction coefficient of 13.7 for a 1% solution in a 1-cm cell at 280 nm (Fasman, 1976). RID plates were incubated at room temperature (≈23°C) in a moist chamber until there was no further increase in precipitin ring diameter, (about 48 hr). A standard curve was plotted with the square of the diameter of precipitin ring as a function of IgG concentration.

Protein determination

Protein content was measured by the biuret method using Sigma Diagnostics Total Protein Reagent (Sigma Chemical Co., St. Louis, MO). The recommended protocol was modified, so readings could be made with an ELISA reader at 550 nm. To each well in an Immulon 2 flat-bottomed, polystyrene microtiter plate (Dynatech Laboratories

Table 1—Changes in microbial counts and IgG concentrations during membrane sterilization steps

Step in membrane sterilization	Microbial count (CFU/mL)[a]	IgG conc (mg/mL)[b]
Trial 1 IgG solution		
Initial	2.70×10^5	23
After prefiltration through GF/A	2.6×10^4	24
After filtration through Acrodisc 37 GF	0	26
Trial 2 IgG solution		
Initial	5.09×10^5	39
After prefiltration through GF/A	7.1×10^4	41
After filtration through Acrodisc 37 GF	0	40

[a] Bacterial counts measured by spread-plating 0.1 mL of sample on standard plate count agar. Plates were incubated at 25°C for 48 hr. Averages of duplicates. Differences between duplicates were <10%.

[b] IgG concentration measured using radial immunodiffusion; averages of duplicates. Differences between duplicates <10%.

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Table 2—Changes in IgG concentration in white 2% UHT milk injected with membrane-sterilized IgG solution and stored 5 mo

Temp.		IgG concentration (mg/mL)[b]					
(°C)	Package[a]	0 time	1 mo	2 mo	3 mo	4 mo	5 mo
4	1 IgG	1.22	1.29	1.25	1.19	1.23	1.19
	2 IgG	1.23	1.28	1.25	1.21	1.28	1.22
	1 no IgG	ND[c]	ND	ND	ND	ND	ND
	2 no IgG	ND	ND	ND	ND	ND	ND
25	1 IgG	1.25	1.34	1.39	1.26	1.32	1.42
	2 IgG	1.22	1.26	1.16	1.15	1.19	1.17
	1 no IgG	ND	ND	ND	ND	ND	ND
	2 no IgG	ND	ND	ND	ND	ND	ND
35	1 IgG	1.27	1.36	1.25	1.28	1.30	1.26
	2 IgG	1.23	1.34	1.30	1.25	1.24	1.36
	1 no IgG	ND	ND	ND	ND	ND	ND
	2 no IgG	ND	ND	ND	ND	ND	ND

[a] Packages consisted of two injected with IgG solution and two without IgG (controls).
[b] IgG concentration measured using radial immunodiffusion; averages of duplicates. Differences between duplicates <10%.
[c] Not detectable.

Inc., Chantilly, VA), 50 μL of sample and 200 μL of Total Protein Reagent were added in triplicate. The plate was incubated at room temperature (≈23°C) for 10 min before reading. Protein standard solution containing bovine serum albumin (Sigma Chemical Co., St. Louis, MO) was used as a standard.

Microbiological assay

Microbial counts were measured using pour plates and spread plates with standard plate count agar (Difco, Detroit, MI). For pour plates, 1.0 mL of sample was used; and for spread plates, 0.1 mL of sample was used. Plates were incubated at 25°C for 48 hr. Samples with high counts were serially diluted in sterile 0.1% peptone water (Difco, Detroit, MI)(1.0 mL sample + 9.0 mL 0.1% peptone water). Yeasts and molds were determined using pour plates with potato dextrose agar (Difco, Detroit, MI) acidified to pH 3.5 with 10% tartaric acid. Plates were incubated at 25°C for 48 hr.

Thermal destruction curves

Membrane-sterilized IgG in UHT milk at 1.2 mg IgG/mL was placed in 100 μL glass micropipettes (VWR Scientific Inc., London, ON) which were then heat-sealed. A constant-temperature waterbath Model MB-1120A-1 (Blue M Electric Co., Blue Island, IL) was used to heat the micropipettes at 62, 66, 70, 74, 78, and 80°C (±0.3°C). A come-up time of 4 sec was determined using a thermocouple. Micropipettes were inserted into the waterbath and withdrawn after a determined period and immediately immersed in ice water. For each temperature, at least 8 micropipettes were used.

Assuming first-order destruction of IgG, the D (decimal reduction time) values at each temperature were calculated from the slope (−k/2.303) of the regression equation:

$$\log c = \log c_1 - kt/2.303,$$

where c = initial concentration, c_1 = concentration after time (t), and t = time.

D values were extrapolated by using the thermal death time model. The thermal death time curve used was:

$$\log D = \log D_o + (T_o - T)/z \text{ (Stumbo, 1973)},$$

where D and D_o are D values at temperatures T and T_o, and z is the temperature change needed to change D by a factor of 10.

RESULTS & DISCUSSION

THE IgG SOLUTION was pH 6.89 and was dark green-brown. The color was probably due to lactoperoxidase and was readily masked when added to milk. The solution also had a protein concentration of 62.0±2.0 mg/mL (mean ± SD, n=3) of which 56% was IgG. At that concentration, the solution was filtered efficiently. Hilpert (1984) membrane-sterilized a milk immunoglobulin concentrate (MIC) from bovine milk through a 0.45-μm membrane filter at a similar concentration. That MIC solution contained 7–8% total protein (70–80 mg/mL) and 2–3% Ig.

Microbial counts and IgG concentrations after prefiltration and final filtration in two trials were compared (Table 1). Membrane sterilization reduced counts to zero. The IgG concentration was not affected by membrane sterilization. About 9.0 mL of membrane-sterilized IgG solution had been added to cartons containing 250 mL UHT milk (final assayed IgG 1.2 mg/mL). RID of unfortified UHT milk indicated negligible IgG. Kummer et al. (1992) reported 0.003 mg/mL IgG in UHT milk by enzyme-linked immunosorbent assay (ELISA).

IgG concentration of the UHT cartons over 5 mo storage at 4, 25, and 35°C were compared (Table 2). No change was detected in IgG concentration. Fluctuations in values were because of fluctuation in the RID assay. Milford-Ward (1981) stated the coefficient of variation of RID was about ±5–8% for an experienced technician. Also, no microbial growth was noted. The expected shelf life of UHT milk is about 4 mo at room temperature (Amantea, 1983) thus IgG added by this process should be stable as long as the product.

The activity of the IgG was not determined. The IgG solution was obtained from whey of milk from cows not immunized against a specific antigen. Thus the IgG solution would likely have activity against a wide range of antigens without high activity to any specific antigen, which complicates measuring of activity. RID is specific for detecting the IgG, since anti-bovine IgG antibodies bind only to bovine IgG. If binding sites for anti-bovine IgG antibodies were deactivated, such as when IgG is denatured, RID would indicate a loss of IgG. Such loss of binding sites on IgG would indicate changes in structure which could affect the ability of the IgG to bind to its specific antigen.

In control cartons stored at 25 and 35°C, slight thickening of the milk was observed after 4 mo storage. This thickening could be associated with gelation that was reported in UHT-processed milk (Mehta, 1980). In IgG-injected cartons stored at 25 and 35°C, physical changes occurred in the milk after 3 mo storage. The milk became watery and had a cheesy odor. Yeast and mold counts, and anaerobic incubation of pour plates were done to determine if the changes were due to microbial contamination but results were negative. The changes were probably due to residual enzymes in the IgG solution. Since the IgG was only 56% pure, small amounts of indiginous or microbial enzymes from the whey may have been present.

Storage at 4°C may have reduced the activity of such enzymes in IgG-injected cartons, which could explain why no changes were observed in those samples. Control cartons stored at 4°C also showed no changes. Residual enzymes in the IgG solution might be inactivated at low temperatures that would not inactivate IgG. Refining the IgG isolation method to provide highly pure IgG could reduce the presence of residual enzymes also.

The thermal stability of IgG was determined using the thermal death time method. Thermal destruction of IgG was assumed to be first-order. Destruction of IgG at 62, 66, 70, 74, 78, and 80°C was a first order reaction (Fig. 1). Destruction over one log cycle was not completed at 62 and 66°C due to thickening and browning of samples. D values obtained for IgG in UHT milk were 6.6 min at 80°C, 12.9 min at 78°C, 81.6 in at 74°C, 702 min at 70°C, 8543 min at 66°C and 76 418 min at 62°C.

From Fig. 1, at 70, 74, 78, and 80°C, the first few points indicate the slope should be steeper, whereas the latter points indicate it should be flatter. This discrepancy probably reflects limitations in the sensitivity of RID at lower concentrations. Assuming the initial points were more accurate, the D values should be lower. Using the first six points D values would be 4.3 min at 80°C, 7.9 min at 78°C, 38.8 min at 74°C, 329 min at 70°C, 5714 min at 66°C and 58 992 min at 62°C. Further research on thermal destruction of IgG using a more sensitive detection method like ELISA may resolve this discrepancy.

The D values we calculated were greater than those reported for IgA in human milk by Morgan et al. (1986). They found

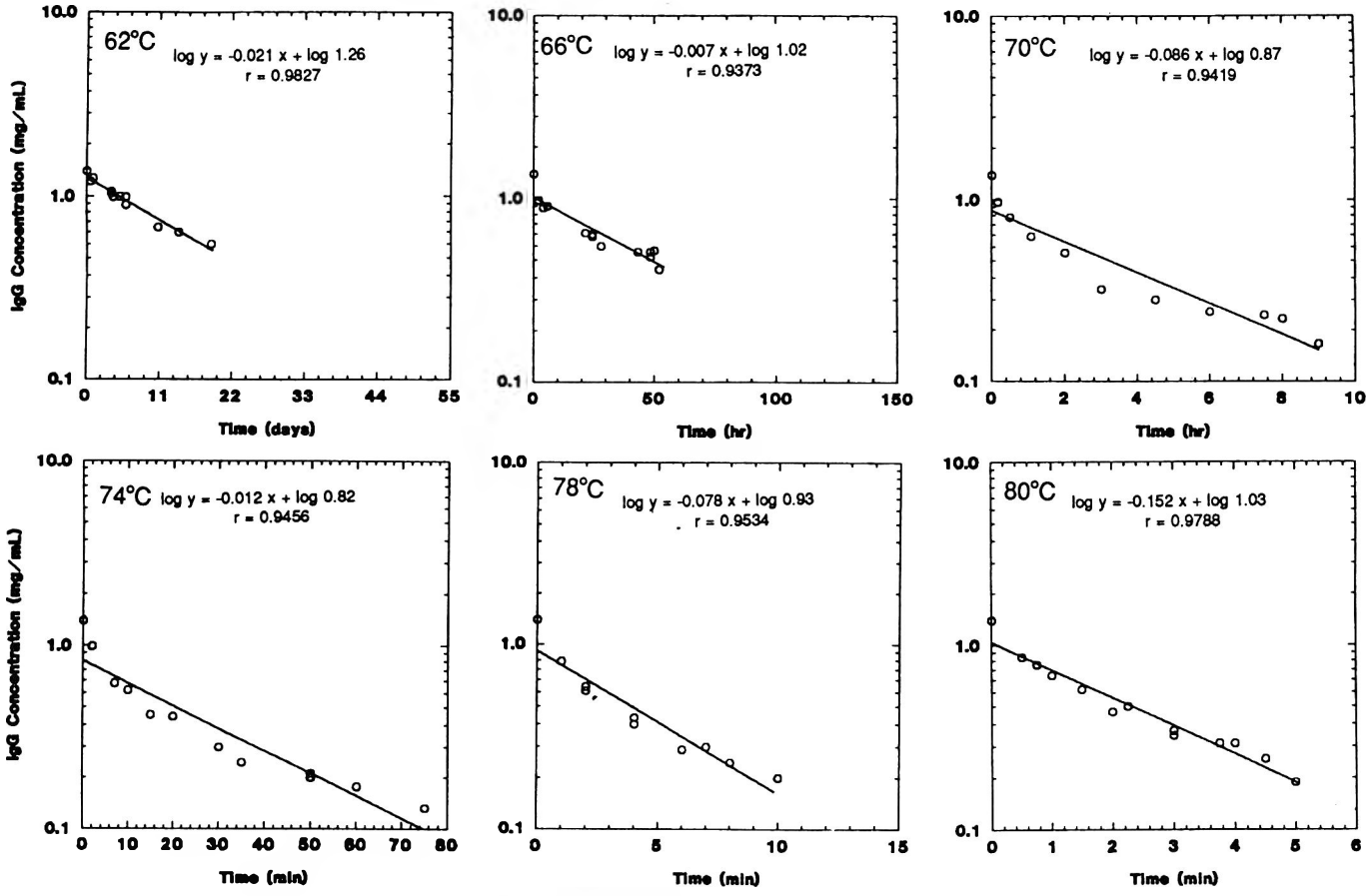

Fig. 1—First-order destruction of IgG in UHT milk fortified with IgG.

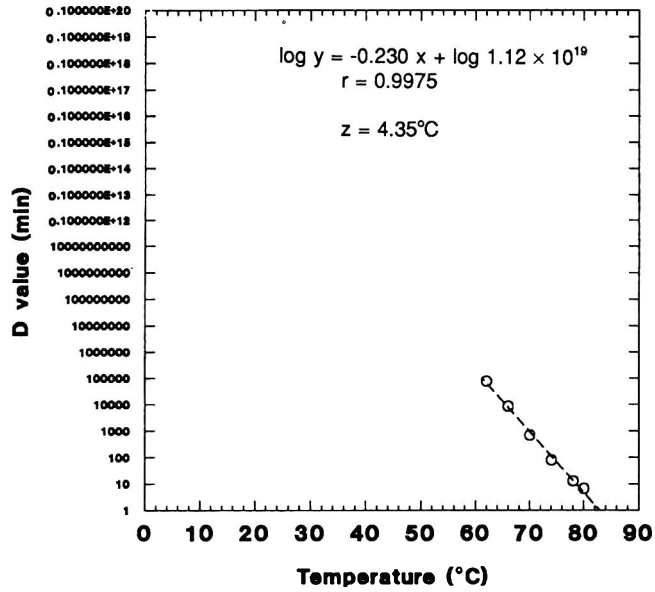

Fig. 2—Thermal death time curve for IgG in UHT milk fortified with IgG.

D values of 25.7 sec at 78°C, 59.6 sec at 76°C, 319 sec at 72°C and 1707 sec at 68°C. The composition of bovine milk, or the structure of IgG, may make it more stable than IgA.

The thermal death time curve for IgG in UHT milk was compared using D values obtained using all points from first-order destruction plots (Fig. 2). Extrapolated D values were 2.57×10^{12} yr at 4°C, 3.85×10^{7} yr at 25°C, and 1.94×10^{5} yr at 35°C. The value of z was 4.35C°. Such extrapolated D values are not accurate since they are for temperatures far beyond the range tested (Ramaswamy et al., 1989). Nonetheless, they imply that IgG would be stable at 4, 25, and 30°C.

CONCLUSIONS

AN IGG SOLUTION containing 60–70 mg/mL protein was membrane-sterilized and aseptically added to UHT products. The method did not affect IgG concentration, but removed microorganisms. The membrane-sterilized IgG should be stable in such a product at 4, 25, and 35°C for 5 mo or longer.

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—Continued on page 762

Listeria monocytogenes Survival Model Validated in Simulated Uncooked-Fermented Meat Products for Effects of Nitrite and pH

R. C. WHITING and M. O. MASANA

ABSTRACT

Previous modeling studies in broth cultures demonstrated that acidity and nitrite increased the inactivation rate of *Listeria monocytogenes*. To validate this effect during storage of simulated uncooked-fermented meat products, lean beef was ground with salt, adjusted to pH 4.0–5.1, and treated with nitrite at 0–300 μg/mL. Samples were immediately inoculated with *L. monocytogenes* (10^7 CFU/g) and survivors were enumerated over 21 days storage at 37°C. The time to achieve a four log decline as greatly affected by pH, ranging from 21 days at pH 5.0 to < 1.0 day at pH 4.0. Growth occurred at pH 5.1 after a long lag period. Nitrite additions did not affect survival, suggesting that the effective concentration was the rapidly decreasing residual nitrite level.

Key Words: listeria, meat, fermentation, nitrite, acidity

INTRODUCTION

THERE IS GREAT PUBLIC HEALTH CONCERN about *L. monocytogenes* because it can grow at refrigeration temperatures and low numbers can be infectious to susceptible people. Many traditional and ethnic ready-to-eat meat products are not cooked: these products include nonfermented (Proscuitti, Westphalian hams) and fermented and dried sausages (pepperoni, some salami). *L. monocytogenes* is a bacterial hazard in such products and is frequently present in the environment.

Processors add nitrite to such products for flavor, color fixation, antioxidant effect, and inhibition of *Clostridium botulinum*. Nitrite may also hasten destruction of other bacteria; the experimental data used to develop the ARS predictive models indicate that nitrite decreases survival times of *L. monocytogenes* (Buchanan et al., 1994) as well as slows growth (Buchanan and Phillips, 1990). The ARS model predicts that in a system at pH 4.7, 3.0% NaCl and 37°C the time for *L. monocytogenes* populations to decrease 4 log cycles (T_{4D}) would be 185 hr with no nitrite and 23 hr with 150 ppm nitrite. Note: For the Pathogen Modeling Program, contact Dr. R.L. Buchanan, Microbial Food Safety Research Unit, USDA, ARS, ERRC, 600 E. Mermaid La., Philadelphia, PA 19118, USA. However, because the nitrite level rapidly declines after contacting meat (Nordin, 1969; Dethmers et al., 1975; Tompkin, 1983), it is necessary to determine what nitrite level(s) (initial vs residual) are representative for the model.

Inoculated pack studies usually found reduced numbers of *L. monocytogenes* after fermentation and drying, however, survivors were also detected (Berry et al., 1990; Farber et al. 1993; Johnson et al., 1988; Glass and Doyle, 1989; Karches and Teufel, 1988; Junttila et al., 1989; Sabel et al., 1991). Surveys of fermented meat products confirmed the presence of *L. monocytogenes* in finished products (Bunčić, 1991; Comi et al., 1992; Farber et al., 1988; Trüssel, 1989; Johnson et al., 1990). Currently, comparisons between predictions by models based on broth systems and results of inoculation studies may be questionable because of the dynamic nature of the pH, nitrite levels and water activity during fermentation and drying.

The fermentation process should either kill bacteria or damage them so that any survivors would be destroyed during drying and storage. We determined *L. monocytogenes* survival in meat batters where the pH was controlled and the microorganisms were added immediately after the nitrite, simulating contamination of the product from the plant environment. Our objective was to help establish the validity of the broth model and quantitatively evaluate effects of pH and nitrite under circumstances closer to those of an actual uncooked, fermented meat product.

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MATERIALS & METHODS

Microorganisms

Stock cultures of *L. monocytogenes* strains (Scott A, V7 and HO-VJ) were from the culture collection at the USDA (Philadelphia, PA) and were the same cultures used in developing the broth models (Buchanan and Phillips, 1990; Buchanan et al., 1993, 1994). They were maintained at 4°C on Brain Heart Infusion (BHI) agar (Difco, Detroit, MI). Cultures were grown individually overnight in 25 mL of BHI broth on a rotatory shaker at 37°C. A mixture of equal volumes of the 3 strains was diluted in sterile 0.1% peptone water for inoculation.

Meat samples

Beef round meat, purchased in a local retail store, was chopped in a food processor (Cuisinart, CFP-9). Sodium chloride was added to give a final concentration of 3.0% (w/w) (3.2% brine). The pH was adjusted during chopping by adding a 50% (v/v) lactic acid solution. The relationship between added lactic acid and pH had been previously determined by intermittently adding acid to samples of meat as they were chopped in the food processor. pH was measured by a combination electrode with direct readings. The acidified meat samples (50 or 100g) were placed in Stomacher bags (Seward Medical UAC, London), frozen and irradiated with 3 kG from a ^{137}Cs source. Samples were kept frozen for ≥ 24 h before use. On the day of inoculation, samples were thawed and pH determined. Filter sterilized sodium nitrite was added to give initial concentrations of 0, 150 or 300 ppm. Sterile water was added where necessary to compensate for lactic acid and nitrite additions. Samples were immediately blended in a Stomacher for 3 min, sufficient inoculum was added to provide ≈10^7 CFU/g *L. monocytogenes* and the samples remixed for another 2 min. The contents of the Stomacher bags were then aseptically transferred to sterile 250 mL polycarbonate bottles (Nalgene).

Incubation and sampling

Inoculated meat samples were stored at 37°C. Periodically 2-4 g samples were aseptically transferred to Stomacher bags. Samples were diluted with 0.1% peptone water (1:10 w/v), mixed and plated on tryptose soy agar (Difco) with a Spiral Plater (Model D, Spiral Systems Instruments, Cincinnati, OH). After incubation for 24–48 hr at 37°C, colonies were counted with either a Bacteria Colony Counter (Model 500A, Spiral Systems Instruments) or manually. Nitrite analyses were conducted on an identically processed but uninoculated set of samples by the AOAC (1990) procedure of section 973.31. Six samples were testing having pH 4.0 or 4.7 with 0, 150 or 300 μg/g nitrite.

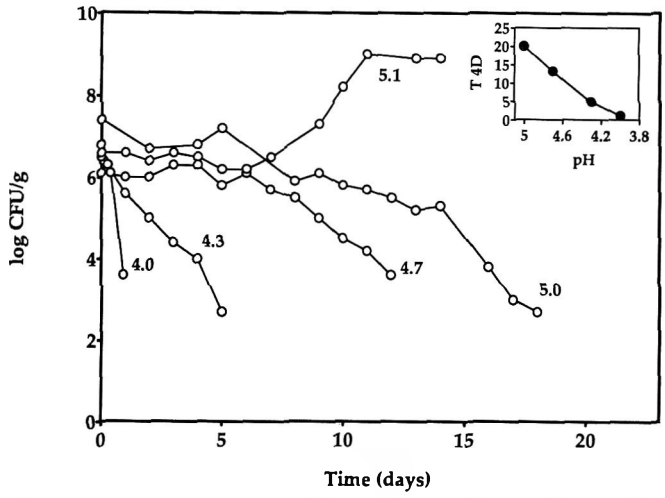

Fig. 1—Survival of *L. monocytogenes* at 37°C in meats (3% NaCl) acidified to various pH values and with 150 ppm added nitrite. Inset, effect of pH on T_{4D}.

Modeling

The complete design of 5 pH levels by three nitrite levels was replicated twice. Bacterial counts from samples with declining populations (non-growth) were fitted to a nonlinear inactivation model (Buchanan et al., 1994; Whiting, 1993) using the curve fitting program of Sigma Plot version 4.0 (Jandel Scientific, Corte Madera, CA). Values of lag time, initial decimal reduction time (D value), and time for four logs decline (T_{4D}) were determined (Whiting, 1993). Analyses of variance and polynomial regressions were performed with the RS/1 statistical program (BBN Software Products Corp., Cambridge, MA). Samples where growth occurred were modeled with the Gompertz equation (Buchanan and Phillips, 1990).

RESULTS & DISCUSSION

AT PH 5.1 AN EXTENDED PERIOD with a slow decline of 1 log or less in *L. monocytogenes* numbers was followed by growth. The inactivation model was fitted to the declining period and the Gompertz equation to the growth period. At that pH, the lag time was much longer and the growth rate slower than predicted by the growth model. Concurrently, the observed T_{4D} times during the initial period were much longer than those predicted by the inactivation model. At pH 5.0 and 4.7 the increases in numbers predicted by the growth model were not observed in the batters. The observed T_{4D} times were longer than expected although within the confidence range of the model. This may also reflect a slight amount of growth in an environment favoring overall decline. At pH ≤ 5.0, inactivation was observed with no growth. The survival data with 150 ppm nitrite showed that as pH decreased, inactivation rates increased (Fig. 1, Table 1). Lag, D and T_{4D} values were shorter at each lower pH ($p \leq 0.01$). T_{4D} values decreased from 22 to 11 days within the usual pH range of fermented meat products (pH 5.0 to 4.7). The regression equation to estimate the T_{4D} for this meat system, with the highest F and R^2 values was

$$T_{4D\ 37°C} = 68.99 - 46.81pH + 7.42pH^2 + 0.000012[NO_2]^2$$

where time is in days and nitrite in ppm ($R^2 = 0.95$ and $F = 120.1$). Evidently, from the equation, the nitrite levels had limited effects on estimated inactivation times. The amount of nitrite added to the batter had no significant effect upon the parameters by analysis of variance ($p > 0.05$).

The predicted T_{4D} values were calculated from the nonlinear-pH quadratic model of Buchanan et al. (1994). T_{4D} values from the model based upon lactic acid concentration instead of pH values were also compared (not shown) but agreement was not as good, probably because any added amount of acid would lower pH much further in the broth than in the meat. The model did not include 300 ppm nitrite within its range, therefore, these values were not calculated. Predictions of the inactivation model for 0 ppm nitrite batters were within the precision expected of a model which encompasses D values from a few hours to months. Lowering the pH below 5.0 greatly hastened the decline (as well as slowing growth) indicating that effective fermentation is important in ensuring control of *L. monocytogenes* in such uncooked products.

The approximately tenfold decrease in survival times predicted by the model with addition of 150 ppm nitrite was not observed. The nitrite analyses showed 56 ppm residual nitrite after 1 day in pH 4.0 and 4.7 samples and 28 and 84 ppm after 2 days, respectively. This generally agreed with the model for declining nitrite by Nordin (1969) and the measurements in thuringer sausage by Dethmers et al. (1975) which indicated the residual nitrite in this product would be expected to be < 20 ppm after 1 to 2 days. Junttila et al. (1989) concluded that nitrite and nitrate additions to a meat product at officially approved levels did not cause elimination of *L. monocytogenes*. The observed T_{4D} were closer to calculated T_{4D} with residual nitrite at pH 4.0. They were closer to no residual nitrite at pH 4.7 than to added nitrite.

Table 1—Predicted and observed survival of *Listeria monocytogenes* in an acidified meat batter with nitrite at 37°C and 3.2% brine

		Observed			Predicted T_{4D}[a]		Observed growth Gompertz equation		Predicted growth	
pH	Added $NaNO_2$ (ppm)	Lag (days)	D value (days)	T4D (days)	Value (days)	Lower, upper 95% CL (days)	Lag period (days)	Growth rate (log(CFU/mL)/day)	Lag period (days)	Growth rate (log(CFU/mL)/day)
5.1	0	—[b]	12.4	53.2	12.5	3.9, 39.8	9.2	0.30	1.2	5.5
	150	—[b]	10.8	46.6	1.3	0.3, 5.7	8.4	0.24	4.1	5.2
	300	—[b]	8.6	37.2	[c]		15.6	0.73		
5.0	0	9.7	3.0	21.6	11.9	4.0, 35.1			1.6	4.5
	150	7.6	3.1	20.0	1.3	0.3, 4.9			5.9	4.5
	300	7.2	3.5	21.0						
4.7	0	6.1	1.3	11.4	9.6	4.0, 22.9			4.4	2.4
	150	5.8	1.8	13.2	1.0	0.3, 3.2			20.0	2.6
	300	6.6	2.1	15.0						
4.3	0	0.00	1.2	5.3	5.9	3.0, 11.3				
	150	0.10	1.1	4.9	0.7	0.3, 1.7				
	300	0.19	1.4	5.9						
4.0	0	0.01	0.10	0.5	3.3	1.9, 5.8				
	150	0.05	0.25	1.1	0.5	0.2, 1.0				
	300	0.02	0.19	0.8						

[a] T_{4D} time for 4 logs decline. Values calculated from the nonlinear-pH model for *Listeria* survival given in Buchanan et al. (1994).
[b] Decline before growth. Lag period not distinguishable from decline (D).
[c] Blank values in prediction columns represent conditions outside the ranges of the models.

The studies of *L. monocytogenes* survival during fermentation showed several logs decline after the fermentation period of 1 to 2 days, particularly when starter cultures were used (Glass and Doyle, 1989; Sabel et al., 1991, Farber et al., 1993). The modeling studies in broth (Buchanan et al., 1993, 1994) and meat (this study) showed longer survival times than reported in fermented products. In particular, a lag period before the decline began was observed in both types of model studies. There are several possible explanations for the apparent absence of *L. monocytogenes* after fermentation in meat products. Accurate quantitative detection of reduced populations ($<10^2$ CFU/g) from inoculations in raw product of 10^4 CFU/g is difficult. Acid injured cells are unable to grow on selective media used in product studies (Siragusa and Dickson, 1992). There is potential for production of bacteriocins by the lactic acid bacteria of the starter cultures (Berry et al., 1990; Nettles and Barefoot, 1993). Additional studies with these types of products are needed before the models can be relied upon for accurate predictions and their use should be limited to acquiring first estimates of the likely behavior of the pathogen.

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Egg Yolk Antibody (IgY) Stability in Aqueous Solution with High Sugar Concentrations

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ABSTRACT

Effect of sugars on the stabilization of hen egg yolk immunoglobulin (IgY) under various processing conditions was investigated. By adding 30–50% (w/v) sucrose or invert sugar to an IgY solution heat denaturation of the IgY antibody at 75–80°C was markedly suppressed. A high concentration of sugar was also effective to retain the IgY activity under acidic conditions of pH 3 or high pressure of 5,000 kg/cm² at 60°C. Addition of high concentrations of sucrose may be a simple means to stabilize IgY for processing and preservation.

Key Words: chicken, egg yolk, immunoglobulin, antibodies

INTRODUCTION

HEN EGG YOLK provides a good source of antibodies, probably because one egg contains 100–200 mg of an IgG-type immunoglobulin termed IgY (Leslie and Clem, 1969). Large amounts of antibacterial or antiviral antibodies can be daily obtained from eggs by immunizing hens with bacterial or viral antigens. Bleeding the animals is not necessary and the antibodies can be isolated from egg yolk by simple precipitation methods (Polson and von Wechimar, 1980; Hatta et al., 1990; Akita and Nakai, 1993).

New types of food with protective activity against gastrointestinal infection, diarrhea and other diseases using such antibodies are being developed. Remarkable preventive effects of IgY antibodies against gastrointestinal infection by rotavirus (Yolken et al., 1988; Ebina et al., 1990) and dental caries formation by *Streptococcus mutans* (Otake et al., 1991; Hamada et al., 1991) have been reported using mice and rats as experimental animals. Prevention of diarrhea in piglets by oral uptake of anti-*E.coli* antibody IgY was also reported (Yokoyama et al., 1992).

For practical use of IgY antibodies as food supplements, however, stability of IgY under food processing and preservation conditions must be determined. We previously reported on the molecular stability of IgY antibodies in comparison with that of mammalian IgG antibodies (Shimizu et al., 1988, 1992, 1993), and found that heat (>75°C) or acid (<pH 3.0) treatment reduced the antibody activity of IgY. Developing a simple method for improving stability of IgY under processing and preservation conditions could help enhance the use of IgY in various food industries. Our objective was to study the effects of sugar addition on the stability of IgY subjected to three processing conditions, heat, acid and high-pressure treatment. No information is available regarding the stability of immunoglobulins under high pressure.

MATERIALS & METHODS

Materials

Anti-bovine serum albumin antibodies were prepared by immunizing hens with bovine serum albumin as described previously (Shimizu et al., 1992), and were purified by the procedure described by Hatta et al. (1990). The concentration of purified IgY was determined by measuring absorbance at 280 nm of an appropriately diluted IgY solution based on 13.5 as extinction coefficient at 280 nm of 1% solution of IgY (Tenenhouse and Deutsch, 1966). Affinity-purified rabbit anti-chicken IgG antibody conjugated with alkaline phosphatase was purchased from Cappel (West Chester, PA). Sucrose, glucose, fructose and other chemicals were of reagent grade. Invert sugar was prepared by mixing equal amounts of glucose and fructose.

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Antibody activity

Antibody activity (antigen-binding effectiveness) was measured by indirect enzyme-linked immunosorbent assay (ELISA). The indirect ELISA was carried out as described previously (Shimizu et al., 1988), except that a polystyrene microtiter plate was coated by bovine serum albumin (Sigma Chemical Co., St. Louis, MO).

High-pressure treatment

An antibody solution (5 mg protein/mL phosphate buffered saline (PBS), pH 7.2; 1L buffer contains 11.7g Na_2HPO_4, 5.0g KH_2PO_4, and 2.34g NaCl)) was diluted with 4 volumes of sugar solution to give 0.1% final protein and 0–50% w/v sugar concentrations. The mixture was compressed to 7,000 kg/cm² by using a pressure generation system (Model ITP-70, Ishikawajima-Harima, Tokyo, Japan) for 30 min. After treatment, the antibody solution was diluted with PBS containing 0.05% Tween 20 (PBS-Tw) and antibody activity was measured by ELISA.

Heat treatment

An antibody solution (5 mg protein/mL of 50 mM phosphate buffer, pH 7.0) was diluted with 4 volumes of sugar solution to give final protein 0.1% and sugar concentrations 0–50% (w/v). The mixture was incubated at different temperatures for 15 min and the antibody activity was measured by ELISA after appropriately diluting with PBS-Tw.

Acid treatment

An antibody solution (5 mg protein/mL of 50 mM phosphate buffer, pH adjusted to 7.0, 3.5 or 3.0 with HCl) was diluted with 4 volumes of sugar solution, pH adjusted to give final protein of 0.1% and sugar concentrations 0–50% (w/v). The mixture was incubated at 37°C overnight and then the antibody activity was measured by ELISA after appropriately diluting with PBS-Tw.

Storage of IgY solution

An 0.1% IgY solution in the presence or absence of sugar was placed in a test tube, sealed and incubated in a water bath at 50°C. An aliquot of solution was removed periodically and antibody activity measured by ELISA.

Fluorescence measurement

IgY (final concentration 0.05%) was dissolved in 50 mM phosphate buffer containing 0–50% sucrose, (the pH adjusted to pH 7.0 or 3.0 with HCl). The solution was excited at 296 nm and the wavelength at which the emission fluorescence was maximal was measured by a Jasco FP777 fluorescence spectrophotometer (Tokyo, Japan).

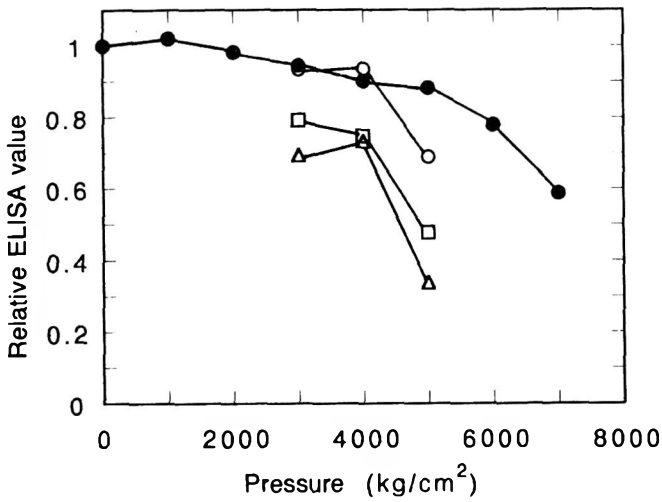

Fig. 1—Effect of high pressure on antibody activity of IgY. An IgY solution was pressurized at 15°C (●), 40°C (○), 50°C (□) or 60°C (△) for 30 min. Antibody activity is expressed as a relative ELISA value. The ELISA value for the IgY solution incubated at 15°C without pressure was used as control. Each value is the average of three determinations.

RESULTS & DISCUSSION

High-pressure effect on IgY activity

High-pressure treatment has become a useful procedure in food processing. Coagulation or gelation of protein foods by high pressure provides new types of processed foods with novel and useful properties (Hayashi, 1992). High pressure also induces inactivation of unfavorable enzymes and kills bacteria, suggesting that high-pressure treatment may help prevent deterioration of processed foods. However, the effect of high pressure on antibodies has not been reported.

High pressure caused a reduction of 10–20% of the antibody activity of IgY at various temperatures (Fig. 1). When an IgY solution was pressurized at 15°C for 30 min, the treatment up to 4,000 kg/cm² did not affect the antibody activity. When the treatment was performed at higher temperatures, however, reduction of antibody activity was accelerated particularly with pressures higher than 5,000 kg/cm². Although the stability against high pressure varies among proteins, many enzymes have been reported to lose enzymatic activity after being pressurized at 2,000 kg/cm² or below (Jaenicke, 1981). No detectable inactivation of IgY by pressure up to 4,000 kg/cm² indicated that the IgY antibody had fairly good stability against pressure.

Stabilizing effect of sugar on IgY

High concentrations of sugar are known to stabilize protein molecules in aqueous solutions. Sugars and such compounds as glycerol are often used as stabilizers for proteins during heating and freezing processes. The possible stabilizing effect of sugar on IgY denaturation by heat, acid or high pressure was thus included in this study.

As reported previously (Shimizu et al., 1988, 1992, 1993; Otani et al., 1991; Hatta et al., 1993), IgY has lost its activity by heating at 75°C or above. Different concentrations of invert sugar or sucrose were added to an IgY solution to determine whether heat inactivation of IgY could be suppressed by sugar addition. Invert sugar and sucrose both suppressed inactivation of the IgY antibodies (Fig. 2) in a concentration-dependent manner. In the presence of 50% sucrose, for example, the IgY activity was almost 100% even after heating at 80°C. The activity of IgY heated at 60–65°C in the presence of 30–50% sucrose showed higher ELISA values than that of nonheated

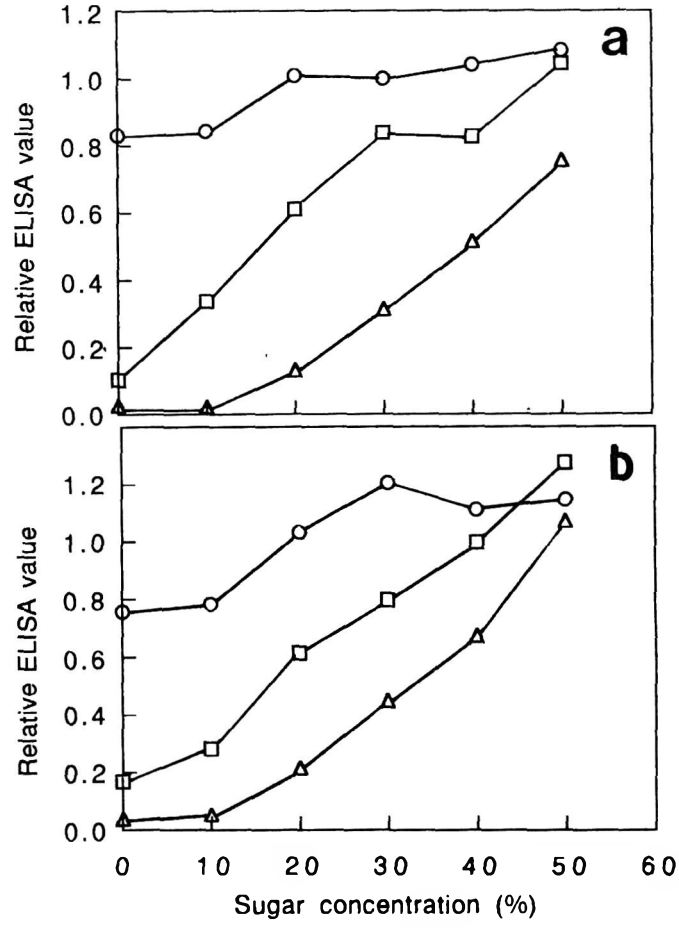

Fig. 2—Effect of invert sugar (a) and sucrose (b) on antibody activity of IgY heated at 70°C (○), 75°C (□) or 80°C (△) for 15 min. Antibody activity is expressed as a relative ELISA value. The ELISA value for the IgY solution incubated at room temperature without sugars was used as control. Each value is the average of three determinations.

IgY without sugar (Fig. 2b). Although the cause of such protection is unknown, possibly heating with high concentrations of sucrose induced slight conformational changes of IgY, resulting in increased reactivity of IgY with the secondary antibody (anti-IgY antibody).

Acid-induced inactivation of IgY (Shimizu et al., 1988, 1992, 1993; Otani et al., 1991; Hatta et al., 1993) was also suppressed in high sugar concentration. Invert sugar (>30%), sucrose (>30%) or such polysaccharides as dextran (>20%) almost completely stabilized IgY at pH 3.0 (data not shown).

The effect of sugar addition on stability of IgY under high pressure conditions was investigated at 60°C. In the absence of sugar, activity of IgY was greatly diminished under a pressure of 5,000 kg/cm² or higher. Addition of sucrose, however, suppressed the inactivation of IgY at this high pressure (Fig. 3).

Effect of sugar addition on stability of IgY during storage was investigated at 50°C for 3 wk at pH 7.0 or 3.0. At pH 7.0, IgY was fairly stable irrespective of sugar concentration, the antibody activity being only slightly decreased after 3 wk at 50°C (Fig. 4a). When IgY was stored at pH 3.0, however, inactivation of IgY occurred rapidly because of its acid labile properties (Shimizu et al., 1988, 1992, 1993; Ohtani et al., 1991; Hatta et al., 1993). Addition of sucrose retarded this inactivation in a concentration-dependent relationship (Fig. 4b). Addition of invert sugar also slightly retarded inactivation of IgY stored at pH 3.0 (data not shown). However, invert sugar enhanced inactivation of IgY when stored at pH 7.0 for longer periods of time. This was probably due to the amino-

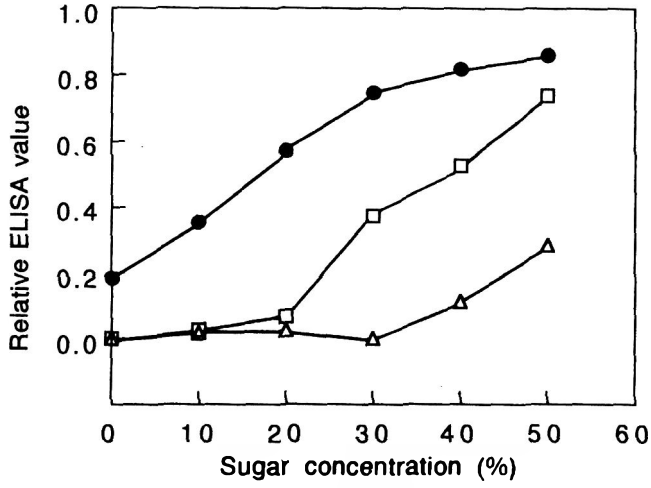

Fig. 3—Effect of sucrose on antibody activity of IgY pressurized at 5,000 (●), 6,000 (□) or 7,000(△)kg/cm² and at 60°C for 30 min. Antibody activity is expressed as a relative ELISA value. The ELISA value for the IgY solution incubated at room temperature without pressure was used as control. Each value is the average of three determinations.

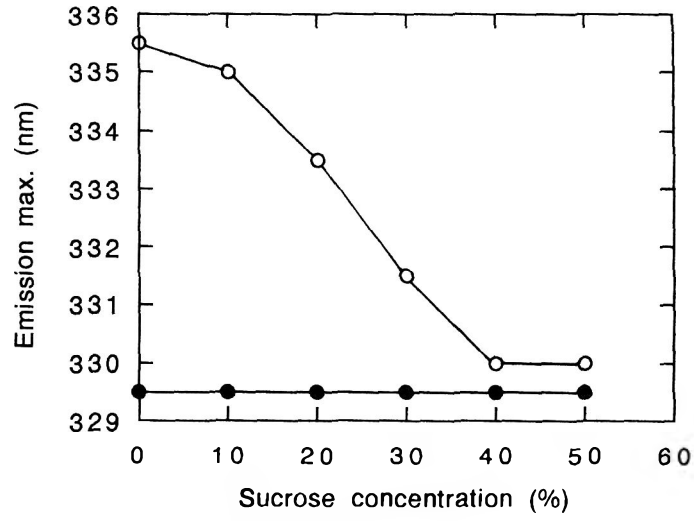

Fig. 5—Effect of sucrose on the fluorescence of tryptophan residues in the IgY molecule. The fluorescence spectrum was taken after Incubating IgY solutions at pH 7.0 (●) or at pH 3.0 (○) for 30 min, and the wavelength for the emission maximum was plotted.

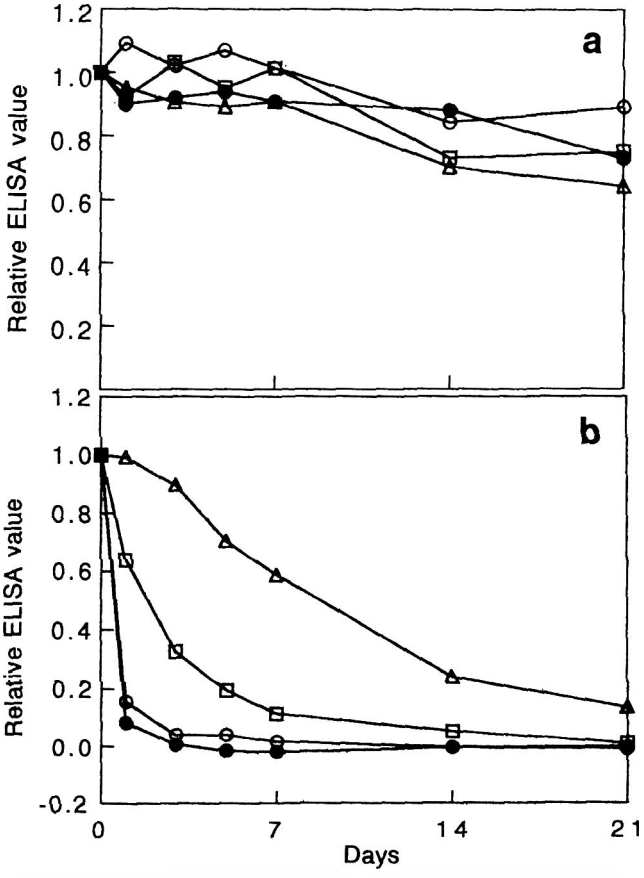

Fig. 4—Changes in the antibody activity of IgY during incubation at 50°C for 3 wk at pH 7.0 (a) or at pH 3.0 (b). An IgY solution was incubated without sugar (●) or in the presence of 10% (○), 30% (□) or 50% (△) sucrose. Antibody activity is expressed as a relative ELISA value. The ELISA value for the IgY solution at pH 7.0 without storage was used as control. Each value is the average of three determinations.

carbonyl reaction between IgY and invert sugar that had occurred during storage at 50°C, since the solution became progressively brownish (data not shown).

These results demonstrated the remarkable stabilizing effect of sugars, especially a nonreducing sugar such as sucrose, on IgY molecules under various physical and chemical conditions. Increased hydrophobic interactions in the protein molecules (Back et al., 1979) and changes in preferential solvation of protein molecules (Ismond et al., 1988; Timasheff, 1993) in sugar solutions may relate to the stabilization effect. We previously reported that IgY had fairly flexible conformation compared with mammalian IgG (Shimizu et al., 1992, 1993). Incubation under acidic conditions was observed to eliminate the activity of IgY, which was accompanied by conformational changes of the molecule. Acid-induced conformational changes in the presence or absence of sucrose were therefore evaluated by measuring fluorescence changes of internal tryptophan residues (Fig. 5). The wavelength of the emission maximum for IgY showed a marked blue shift with increases in sucrose. This suggested that the tryptophan residues slightly exposed to the hydrophilic environment in the absence of sugar were more structurally blocked when placed in sugar solutions. Reduction of the exposed hydrophobic moiety of IgY in sugar solutions could occur because of increased interactions between hydrophobic groups inside the protein molecule, as described by Back et al. (1979).

Addition of high concentrations of sugar also reduces the water activity. A sucrose solution of 50% (w/w), for example, lowers the water activity to 0.91. Under such conditions, growth of such bacteria as *E. coli, Pseudomonas, Bacillus, Salmonella* and *Vibrio* is inhibited (Beuchat et al., 1981). Thus, addition of high concentrations of sugar, especially the nonreducing sugar sucrose, to an IgY solution probably not only stabilized the IgY molecule under various conditions, but also kept the IgY solution well preserved from bacterial action. A sucrose syrup containing a high concentration of IgY may be useful for preservation of IgY when refrigeration facilities are not practical.

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—Continued on page 772

Cholesterol and Other Lipid Extraction from Egg Yolk Using Organic Solvents: Effects on Functional Properties of Yolk

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ABSTRACT

Cholesterol extraction from dehydrated egg yolk, using petroleum ether or petroleum ether-ethanol (35:65) resulted in decreased cholesterol content with petroleum ether and almost complete removal with petroleum ether-ethanol. Yolk extracted with petroleum ether gave emulsions of similar rates of coalescence to those prepared with dried yolk and mayonnaise-like emulsions of higher rheological properties. Yolk extracted with petroleum ether-ethanol gave emulsions of lower stability and did not lead to preparation of mayonnaise-like emulsions. Both yolk protein concentrates showed better foaming activity and foam stabilizing ability than dried yolk.

Key Words: egg yolk, cholesterol, extraction, organic solvents, emulsion stability

INTRODUCTION

EGGS CONTAIN PROTEINS of high biological value and other nutrients such as vitamins, minerals and phospholipids and other lipids. Egg constituents have important functional (emulsifying, foaming, textural, etc.) properties which make eggs indispensable in many foods, such as mayonnaise, salad dressings, meringue and bakery products (Shepherd and Yoell, 1976; Kiosseoglou and Sherman, 1983a; Baldwin, 1986). However, egg consumption has greatly declined because of their cholesterol content. Food cholesterol and its oxidation products, which may appear during dehydrated egg storage (Missler et al., 1985) are generally believed to be involved in cardiovascular diseases.

Egg cholesterol is found in the yolk. Removal of cholesterol from the yolk is feasible only if the functional properties, and especially unique emulsifying activities in products such as mayonnaise and salad dressings, are mostly unaltered. The extracted lipid material could possibly find use as a fat in human milk substitutes (Tokarska and Clandinin, 1985), or as an additive in cosmetics (Blackwelder and Pike, 1990).

Larsen and Froning (1981) using solvent extraction fractionated yolk into oil, protein and aqueous components. None of the fractions from hexane extraction, alone or in combination, gave emulsions as stable as those prepared with native egg yolk. Chung and Ferrier (1991) studied the emulsifying properties of egg yolk partially extracted with several organic solvents. They reported that protein solubility and emulsifying activity of extracted yolk decreased significantly following extraction.

Our objective was to study the influence of petroleum ether extraction (alone or in mixtures with ethanol) on the stability of egg yolks against oil droplet coalescence of o/w (oil-in-water) emulsions. We also investigated the rheological properties of mayonnaise-like emulsions from the extracted yolk powder. Additionally, the influence of extraction on such properties, as foaming and foam stability was also investigated. These functional properties could be of importance in the preparation of bakery products (Shepherd and Yoell, 1976).

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MATERIALS & METHODS

Materials

Liquid yolk was obtained by separating it from the albumen and rolling the yolk on absorbent tissue to remove traces of albumen. The vitelline membrane was then punctured and the liquid yolk collected. The yolk was freeze-dried and the dehydrated powder used in extraction experiments. The oil used for preparation of emulsions was locally purchased corn oil. Petroleum ether and absolute ethanol were of analytical grade (Riedel-de-Haen, Seelze, Germany). Cholesterol and 5α-cholestane standards were obtained from Sigma Chemical Co. (St. Louis, MO).

Extraction of egg yolk

Dried egg yolk (50g) was extracted with 500 mL petroleum ether or mixtures of petroleum ether-ethanol under continuous stirring for 2.5 hr using a propeller-type mechanical stirrer model RW14H, IKA Instruments, Staufen im Breisgau, Germany) operated at 1200 rpm. The yolk-solvent mixture was then filtered under vacuum using a Duran Scott sintered glass funnel (No. 3). The yolk was washed with 200 mL solvent and the resulting egg yolk powder was dried for 6 hr at 55°C in a vacuum oven to obtain egg yolk protein concentrate.

Dry matter, lipids, cholesterol, and lipid phosphorus

Total solids content was determined by drying samples in an oven at 98-100 °C (AOAC, 1975). Total lipid content was determined by extracting with chloroform-methanol (AOAC, 1975). Cholesterol of yolk samples was determined by gas-liquid chromatography (GLC) using the method of Beyer et al. (1989) on the total lipid extract obtained by chloroform-methanol extraction. A Hewlett-Packard model 7620A gas chromatograph equipped with a flame ionization detecter was used. The chromatograph was fitted with a 2-m glass column coated with OV-1 on WAW, 60-80 mesh. The column was operated at 260 °C. Lipid phosphorus was quantified using the method of IUPAC (1987) on the total lipid of samples extracted with chloroform-methanol.

Preparation of emulsions

Emulsions (o/w) were prepared by adding, at 20 mL/min, 30 mL corn oil into 70 mL of a dispersion containing 1% egg powder solids with a pH 3.8 (0.02N di-sodiumhydrogen citrate, 0.2M NaCl), while mixing continuously for 3 min using a propeller-type mechanical stirrer model RW14H, IKA Instruments) at about 1600 rpm.

The crude emulsion was then homogenized using an Ultra-Turrax T25 homogenizer (IKA Instruments) equipped with a S25KG-25F dispersing tool at 13,500 rpm for 1 min. A small amount of penicillin (0.001 g/100g) was added as preservative and the emulsions were stored at 5 °C to study stability.

Mayonnaise-like emulsions were prepared by adding, at 25 mL/min, 75 mL corn oil into 25 mL of a dispersion of pH 3.8 containing 10% egg powder solids, mixing for 3 min with the RW14H mechanical stirrer at 1600 rpm. The resulting emulsion was then homogenized using the Ultra-Turrax T25 homogenizer operated at 13,500 rpm for 1 min.

Stability of o/w emulsions

The stability against coalescence of oil droplets of the o/w emulsions was studied by following the change with time of oil droplet size distribution patterns, determined using the microscope method (Mita et al., 1974). The sizes of more than 1000 droplets were determined

Table 1—Effect of solvent extraction on some lipid components of egg yolk

	Dry matter (%)	Lipids[a] (%)	Cholesterol[a] (mg/g)	Lipid phosphorus[a] (mg/g)
Liquid egg yolk	48.5 ± 0.31[b]			
Dried egg yolk	98.8 ± 0.40[b]	65.1 ± 0.21[b]	18.7 ± 0.31[b]	5.4 ± 0.25[b]
Extracted with petroleum ether	96.2 ± 0.15[c]	32.7 ± 0.16[c]	5.7 ± 0.01[c]	6.4 ± 0.14[c]
Extracted with petroleum ether-ethanol (35:65)	97.7 ± 0.28[c]	3.8 ± 0.50[c]	<1.0	1.2 ± 0.20[c]

[a] Based on dry matter.
[b] Mean values ± standard deviation of three determinations.
[c] Mean values ± standard deviation of three extracted samples.

per sample and the mean volume diameter (D_v) of the droplets was derived using the equation

$$D_v = \sqrt[3]{\frac{\Sigma n_i D_i^3}{\Sigma n_i}} \quad (1)$$

where n_i is the number of the droplets with diameter D_i.

The rate of droplet coalescence (K) was calculated from the equation (Sherman, 1968)

$$N_t = N_o \exp(-Kt) \quad (2)$$

where N_t and N_o are the numbers of droplets per mL of emulsion at times t and t=0, respectively, and

$$N_t = 6 \frac{\emptyset}{\pi D_v} 10^{12} \quad (3)$$

where Ø, is the oil phase volume (0.3).

Rheological properties of mayonnaise-like emulsions

Shearing stress rate of shear diagrams were obtained with a Brookfield DV-II LV viscometer using the small sample adapter (SC4-25/13R) at 25 °C.

Foaming studies

Egg yolk powder sample dispersions (100 mL) of pH 7 (Na_2HPO_4, KH_2PO_4 buffer) were whipped in a kitchen-type Sunbeam Mixmaster mixer (Sunbeam Electric Co., Glasgow, UK) at 1000 rpm, and resulting foams were then carefully transferred to a 1-L glass measuring cylinder. Foam expansion (FE) and foam liquid stability (FLS) were determined as follows (Poole et al., 1984)

$$\% \text{ FE} = \frac{\text{Foam volume (mL)}}{\text{Initial liquid volume}} \times 100 \quad (4)$$

$$\%\text{FLS} = \frac{\text{Vol. of liquid (mL) retained in foam after 30 min}}{\text{vol. of liquid prior to whipping}} \times 100$$

RESULTS & DISCUSSION

COMPOSITION OF THE DRIED EGG YOLK, yolk extracted with petroleum ether and yolk extracted with a mixture of petroleum ether-ethanol (35:65) was compared (Table 1). Extraction with petroleum ether for 2.5 hr resulted in a significant reduction of initial yolk lipid. Preliminary experiments showed that extraction for longer time periods did not affect the lipid extraction. Petroleum ether extraction of yolk greatly decreased its cholesterol content, while organic phosphorus was retained to a large extent. This suggested that the lipid fraction of the resulting yolk protein concentrate consisted mainly of phospholipids. Extraction of dried yolk with a mixture of petroleum ether-ethanol resulted in greater reduction in the lipid content of yolk than did petroleum ether extraction alone. Only a trace of cholesterol in the resulting concentrate was observed while the amount of phospholipids was markedly reduced.

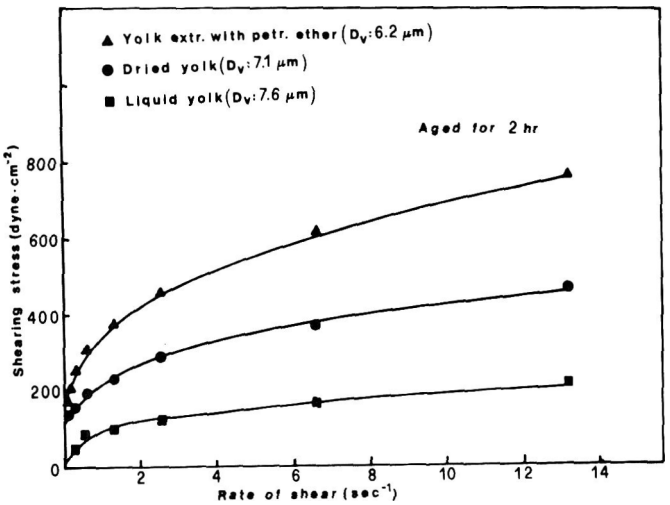
With storage time the mean volume diameter (Dv) increased in emulsions prepared with dried egg yolk and with yolk protein concentrates obtained by extraction with petroleum ether or a mixture of petroleum ether-ethanol (35:65) (Table 2).

Table 2—Effect of solvent extraction on stability against droplet coalescence of o/w emulsions

Aging time (hr)	Emulsion 1[a]	Emulsion 2 Dv (μm)	Emulsion 3
1	17.8[b]	18.7	16.9
24	18.3	20.0	17.6
144	19.0	21.6	21.3
240	19.6	22.1	22.1
330	19.8	22.7	25.6
510	20.3	23.1	28.9
$K \times 10^7$ (sec^{-1})	2.2	2.3	8.8

[a] Emulsion 1: Prepared with dried yolk. Emulsion 2: Prepared with yolk extracted with petroleum ether. Emulsion 3: Prepared with yolk extracted with a mixture of petroleum ether-ethanol (35:65).
[b] Mean values of two emulsions. Experimental error 0.12 μm.

Rates of drop coalescence for the same emulsions were also calculated from the slope of the straight line of plots of ln N vs time. From results the mean volume diameter of the emulsion prepared with petroleum ether-ethanol extracted yolk obviously increased faster than the diameter of the other two emulsions following aging for 510 hr. This was more apparent when comparing drop coalescence rates of emulsions. The rate for the emulsion with yolk extracted with the mixture of petroleum ether-ethanol was about 4 times greater than the rates of the others. Extraction with only petroleum ether did not seem to influence the stabilizing activity of yolk. This was probably because the main stabilizer of yolk, i.e., the protein-phospholipid complex, was not greatly affected by extraction and could adsorb at the o/w interface. There, following intermolecular association, it formed a cohesive and mechanically strong film which protected the oil drops from coalescing (Kiosseoglou and Sherman, 1983b, c). Extraction with a mixture of petroleum ether-ethanol would likely result in removal of a great amount of phospholipids. The protein-phospholipid balance would be upset and the emulsions prepared would be less stable. The influence of yolk extraction with petroleum ether was obtained on the shearing stress-rate of shear behavior of mayonnaise-like emulsions after 2 hr and 24 hr storage (Fig 1). The rheological behavior of mayonnaise emulsions prepared with liquid yolk were also compared. It was not possible to prepare mayonnaise-like emulsions with yolk extracted with a mixture of petroleum ether-ethanol because the crude emulsion tended to invert during homogenization. This was probably due to the low content in phospholipids which, being of low molecular weight, could adsorb faster than the protein molecules thus conferring stability to newly formed emulsion droplets. In absence of phospholipids the protein molecules cannot function effectively to cover all freshly formed interfaces, leading to coalescence and emulsion breakdown. The emulsions prepared with yolk extracted with petroleum ether were more viscous than the other two (Fig. 1). According to Kiosseoglou (1982), the rheological properties of emulsions stabilized by egg yolk depend on interdroplet interactions where protein segments of molecules adsorbed on different droplets are involved. The higher rheological values of the emulsion prepared with petroleum ether-extracted yolk could have been due to its greater protein content which resulted in more segments involved in interdroplet interactions. However,



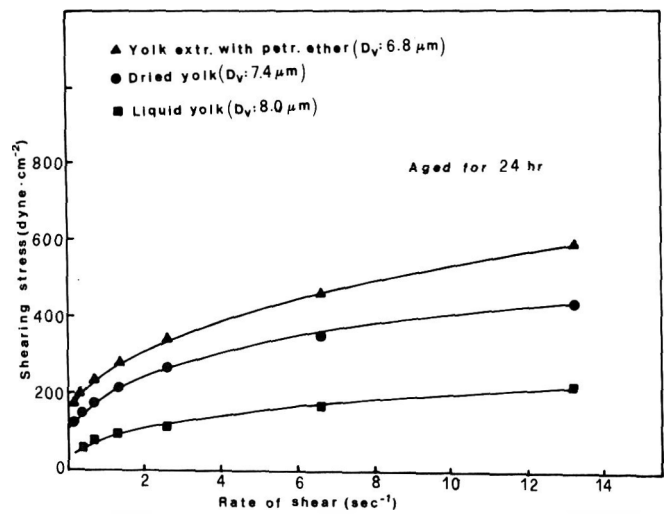


Fig. 1—Relation of shearing stress to rate of shear for mayonnaise-like emulsions prepared with egg yolk and solvent-extracted yolk and stability after 24 hr. Points represent means of three emulsions.

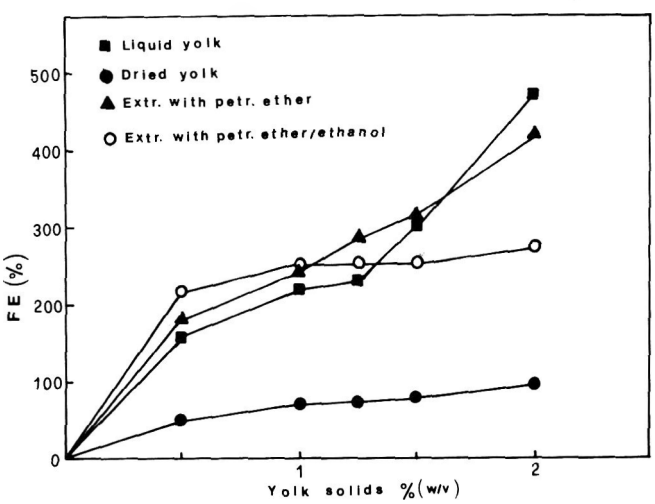


Fig. 2—Influence of solvent-extraction of egg yolk on foam expansion (FE %). Points represent means of three foam systems.

the smaller droplet size could have also been responsible, to some extent (Sherman, 1968).

Finally, the influence of solvent extraction of yolk was observed on foam expansion (Fig. 2) and foam liquid stability

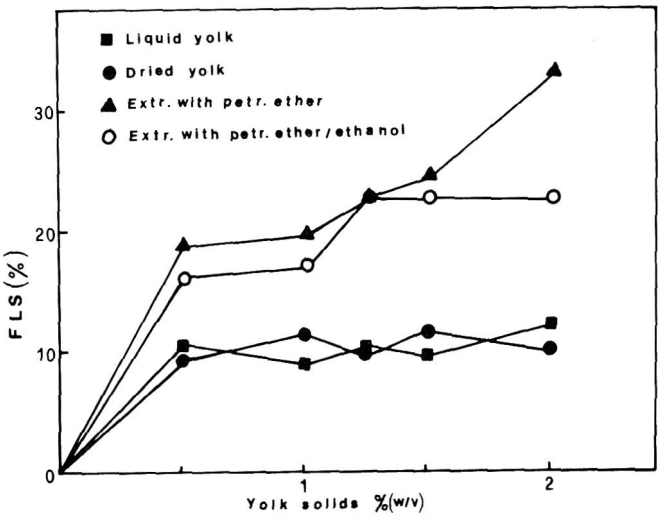


Fig. 3—Influence of solvent-extraction of egg yolk on foam liquid stability. (FLS %). Points represent means of three foam systems.

(Fig. 3). Yolk extracted with petroleum ether or petroleum ether-ethanol was a more effective foaming agent compared to dehydrated yolk. Furthermore, the stability of foams prepared with extracted yolk was higher. The higher foaming activity of extracted yolk could be explained by the fact that a large amount of lipids, known to depress foaming ability of proteins, were removed following extraction (Poole et al., 1986).

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Simple and Rapid Method for Measuring Turbidity in Gels and Sols from Milk Whey Protein

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ABSTRACT

Protein isolates prepared from acid whey concentrate and cheese whey concentrate, and β-lactoglobulin were studied. Turbidity of samples was measured using a 96-well microplate and a microreader. This method allowed many (>100) small samples (< 250 μL) to be treated at the same time. At low ionic strength and at neutral to alkaline pH, samples were transparent after heating. Transparent gels could also be prepared in this region with a small amount of NaCl.

Key Words: milk, whey protein, gels, turbidity, transparency

INTRODUCTION

MILK WHEY PROTEIN is an important source of food protein mainly produced as a by-product of casein and cheese manufacture. Large amounts of whey protein concentrate (WPC) are produced and commercially available. Products vary both in functionality and protein content (Zall, 1992; Pearce, 1992).

Whey protein is a major source of functional proteins with various uses in foods. Heat-induced gelation and increase in viscosity are two useful functionalities. The properties of heat-induced gels and sols are sensitively affected by various factors of the medium, including pH, ionic strength and protein concentration (Kinsella, 1984; Mangino et al., 1987; Xiong, 1992; Lupano et al., 1992). Most commercially available whey proteins give turbid products on heating and, thus, may not always be suitable for food materials. Therefore, there is need to understand why they give turbid products on heating and how this can be controlled.

The formation of a heat-induced gel and/or viscous sol from a globular protein results from the denaturation and aggregation of protein molecules (Hatta et al., 1986; Kitabatake et al., 1987). When the interaction between denatured protein molecules is restricted and the coagulation of protein molecules is inhibited, a linear aggregate of denatured protein molecules is formed (Kitabatake and Doi, 1993). Hydrophobic interaction seems to be important in the linking of molecules to form the aggregate. The appearance and mechanical properties of such heated products depend mostly on the size and form of the aggregate. Turbidity and transparency of gels are closely related to their mechanical properties (Kitabatake et al., 1989).

Our objective was to determine the effects of medium conditions, i.e., pH and ionic strength, on gel properties of whey protein using a new simple and rapid method. The gel properties of heated whey protein were reported to be influenced by heating conditions (Kinsella, 1984). Schmidt (1981) reported on the effects of pH and ionic strength, as well as ionic species, on turbidity and strength of whey protein gels. Dunkerley and Hayes (1980) described effects of pH on turbidity of whey protein gels. They measured turbidity by reflectance. Barbut and Foegeding (1993) described the effects of pH, NaCl and $CaCl_2$ on appearance and rheological properties of whey protein gels.

We introduced a simple and rapid method for measuring turbidity. This method uses a 96-well microplate and a microreader system which makes it possible to analyze a large number of samples in a short time.

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MATERIALS & METHODS

Materials

Acid whey concentrate (Lacprodan™, Danmark Protein A. S., Videbaek, Denmark) containing 22.0% protein, as measured by the Kjeldahl method, 0.3% ash by the gravimetric method (550°C), 1.0% lactose by HPLC, 1.4% crude fat by the extraction method, and 74.0% moisture by the gravimetric method (105°C), was used. Cheese whey concentrate (Lacprodan 80™) containing 24.2% protein, as measured by the Kjeldahl method, 0.8% ash by the gravimetric method (550°C), 1.3% lactose by HPLC, 3.1% crude fat by the gravimetric method (105°C), and 71.6% moisture, was also obtained from Danmark Protein A. S. β-Lactoglobulin from bovine milk (3× crystallized and lyophilized) was purchased from Sigma Chemical Co., (St. Louis, MO).

Preparation of protein samples

Whey protein isolates were prepared from acid whey and cheese whey concentrate as follows. Whey concentrates were diluted with an equal volume of distilled water. The pH was adjusted to 4.5 by addition of 1N HCl. The sample was centrifuged at 10,700 × *g* (8,000 rpm, Hitachi RPR 9-2-981 rotor) for 30 min at 4°C. The supernatant was decanted. Care was taken to avoid contamination by the floating fat layer. The supernatant was filtered (No. 2 filter paper, Toyo, Tokyo) and ammonium sulfate was added to 75% saturation. After stirring for 30 min at 4°C, the sample was centrifuged at 10,700 × *g* (8,000 rpm) to collect the precipitate. The precipitate was dissolved and exhaustively dialyzed against distilled water at 4°C. The dialysate was centrifuged at 10,700 × *g* (8,000 rpm) for 30 min at 4°C. The clear supernatant was adjusted to a given pH value and NaCl concentration by addition of 2N NaOH and 5M NaCl. Sodium azide (0.02%) was added to the supernatant as a preservative. The final protein concentration was adjusted to 70 mg/mL. When necessary to increase protein concentration, the clear supernatant was concentrated by centrifugation with Centri Cell-60 (MW 30,000, Polysciences, Inc., Warrington, UK) at 1,600 × *g* for 5 hr at 4°C, and then the pH and protein concentration were adjusted. The β-lactoglobulin powder was dissolved in distilled water and pH, and NaCl and protein concentrations were adjusted as for the whey protein isolates.

Protein concentration

Protein concentrations of whey protein isolates, dissolved in 20 mM sodium phosphate buffer, pH 7.5, were measured from absorbance at 280 nm based on $E^{1\%}_{1\,cm} = 11.7$, calculated from the composition of β-lactoglobulin (65.0%, $E^{1\%}_{1\,cm} = 9.6$), α-lactalbumin (15.2%, $E^{1\%}_{1\,cm} = 20.1$), and bovine serum albumin (10%, $E^{1\%}_{1\,cm} = 6.7$) (Townend et al., 1960; Leonard et al., 1963; Kronman and Andreotti, 1964).

Turbidity measurements

Test samples (250 μL) were placed in the wells of a 96-well microplate (Nunc, Inc., Roskilde, Denmark) or a microwell 1 × 8 module (Nunc-Immune Module MaxiSorp F8, Nunc, Inc.). Turbidity was expressed by absorbance at 590 nm. The absorbance of each sample was measured with a microwell reader (Model VH 450, UBE Handy Reader, UBE Industries, Ltd., Tokyo) using a 590-nm filter. For heating the sample, the microwell module, which was held by a frame (Nunc, Inc.), was covered with a seal (Linbro/Titertek, Flow Laboratories, Inc., McLean VA) and placed in an air-tight box containing some water. This closed box was placed in an oven at 80°C for 1 hr.

Samples were then cooled at room temperature (≈23°C) for 2 hr. The absorbance of each well was measured as described. Each experiment was done with five samples, and the mean was calculated. Contour graphs were prepared by a computer (NEC 98VM, NEC, Tokyo) equipped with a printer (EPSON HG-4000, EPSON, Tokyo) using software (DDIP-C and INTPOL, Success Plan Ltd., Tokyo) designed for contour lines.

RESULTS

PROTEIN ISOLATE SOLUTIONS from acid whey concentrate adjusted to pH 2 to 11 and NaCl concentrations from 0 to 200 mM were heated in the wells of a 96-well microplate. Protein isolate samples were clear, except those at pH 3.5 and 150 mM NaCl, pH 3.5 and 175 mM NaCl, and at pH 3.5 and 200 mM NaCl. After heating, samples at pHs 4.0 to 6.0 were turbid at all of NaCl concentrations (from 0 to 175 mM) (Fig. 1A). The absorbance at 590 nm, was recorded as related to pH and NaCl concentration by contour curves (Fig. 1B) and three-dimensional curves (Fig. 1C). With pH >6.0 or <4.0, the sample remained transparent when the concentration of NaCl was ≤ 100 mM. Transparent samples were obtained (Fig. 1) when the pH was away from the isoelectric point (pI) of a protein; i.e., pI of β-lactoglobulin, 5.18 (Cannan et al., 1942), and at lower NaCl concentrations. Samples became turbid, and absorbance sharply increased at the critical zone. When the NaCl concentration was low, the sample was transparent even at pH 6.5. However, turbidity increased with an increase in NaCl concentration and/or lowering of the pH. A slight change in the acidic pH region directly influenced the turbidity of the sample: i.e., from pH 4 to pH 3, turbidity increased sharply with slight changes in pH. A similar effect was observed in the neutral and slightly alkaline pH region: i.e., an increase in NaCl concentration and/or lowering of pH induced turbidity. However, a change in pH under that conditions had less effect than that in the acidic pH region.

The microplate method is a useful and convenient means of measuring many samples at the same time. The microplate was heat-stable and did not influence absorbance after heating. Each well held 250 μL of sample. However, a smaller volume could be used. The samples at pH 7 and 50 mM NaCl, and at pH 7 and 100 mM NaCl, were transparent before heating. After heating, the sample at 100 mM NaCl was turbid (Fig. 1). The turbidities of these samples was also related to the volume (Fig. 2). With the 100 mM NaCl sample, turbidity increased linearly with increase in volume and absorbance was > 2.0 above 100 μL. This indicated that 250 μL was sufficient volume for measuring turbidity of the sample. The volume could be reduced to 50 μL or less, indicating that 3.5 mg or less of protein (70 μg/μL × 50 μL) was adequate to measure turbidity.

Cheese whey (nonacid or sweet whey), a by-product of hard cheese manufacture, is another source of whey protein. Results were similar to those with acid whey protein isolate. Before heating, cheese whey protein isolate resulted in transparent solutions in most cases. The range of turbidity after heating was broader than that with acid whey protein isolate. Cheese whey protein may contain some hydrolyzed protein, whereas acid whey protein, as prepared, was not hydrolyzed or denatured.

β-Lactoglobulin is a major component in the protein fraction of whey. To examine the importance of β-lactoglobulin in turbidity and gel formation with heating, a similar experiment was carried out using the same protein concentration of β-lactoglobulin as with acid and cheese whey proteins. Before heating, most samples at pH 4.0 and 5.0 were turbid (Fig. 3A). After heating, samples at lower NaCl concentrations and at pHs further from the pI of β-lactoglobulin became transparent, as with the whey protein fraction. However, the range of turbidity was wider than that for either acid whey or cheese whey protein isolate. This difference was apparently due to contimaniation by other protein components in the whey protein,

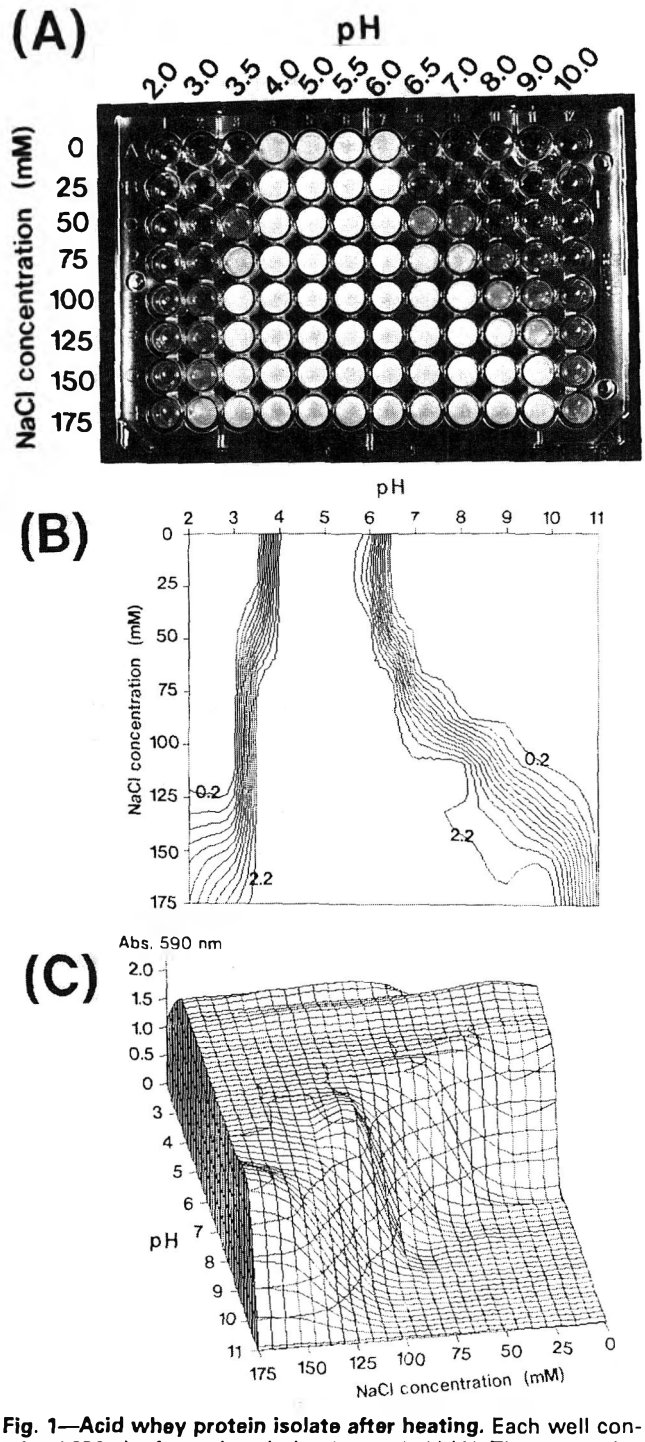

Fig. 1—Acid whey protein isolate after heating. Each well contained 250 μL of protein solution (70 mg/mL) (A). These samples were heated at 80°C for 1 hr. Absorbance at 590 nm indicated turbidity and is shown by a contour graph (B) and a three-dimensional graph (C). Acid whey prote n isolate was dialyzed against distilled water, and then pH, NaCl concentration and protein concentration adjusted.

such as α-lactalbumin, serum album, etc., which are much less sensitive to aggregation than β-lactoglobulin (data not shown).

Each lane of the 96-well microplate (vertical lane) could be detached from the frame. The lanes for pH 2, 5, 7, and 10 were detached before and after heating and slanted to show the appearance and gelling of samples. Before heating (Fig. 4A), both transparent and turbid samples were liquid, while after heating (Fig. 4B) the samples at pH 2, 7, and 10 gave transparent liquids, transparent gels, and turbid gels. At pH 5, all samples were turbid gels.

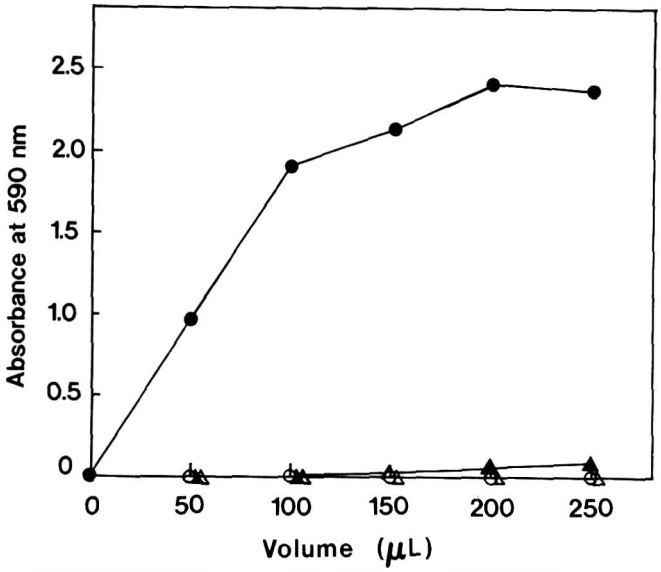

Fig. 2—Volume as related to acid whey protein isolate turbidity. Protein solutions (pH 7.0, 70 mg/mL) before heating with 50 mM NaCl (△) and 100 mM NaCl (○), and after heating (80°C, 1 hr) with 50 mM NaCl (▲) and 100 mM (●) are shown. Acid whey protein isolate was dialyzed against distilled water, and pH, NaCl and protein concentrations were adjusted.

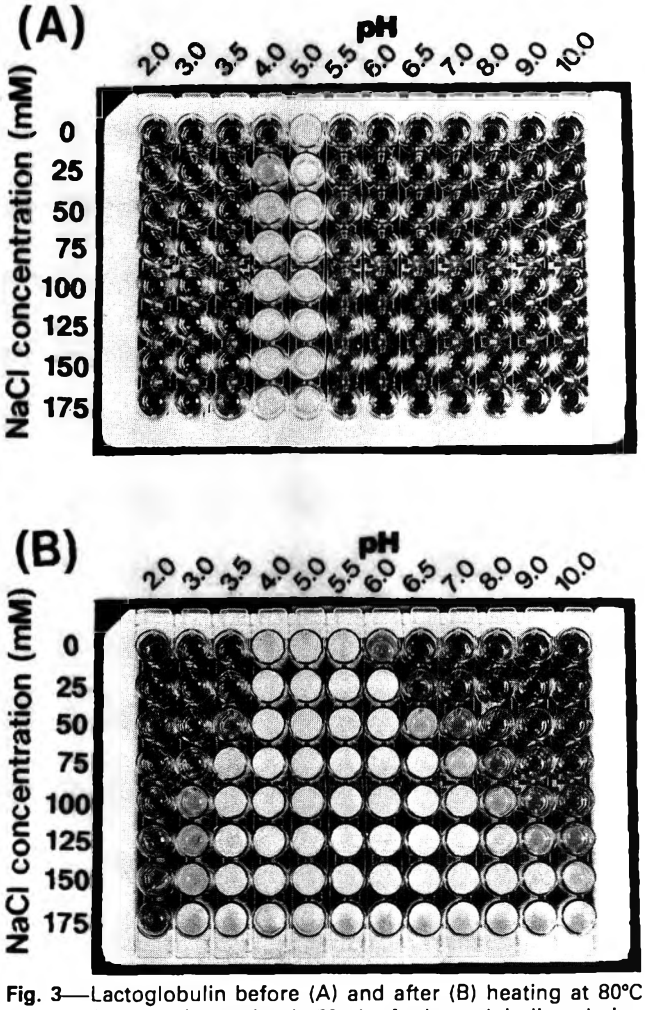

Fig. 3—Lactoglobulin before (A) and after (B) heating at 80°C for 1 hr. Each well contained 250 μL of β-lactoglobulin solution (70 mg/mL).

DISCUSSION

FROM OUR RESULTS THE PROPERTIES of a heated protein were influenced by heating conditions, pH and ionic strength. The

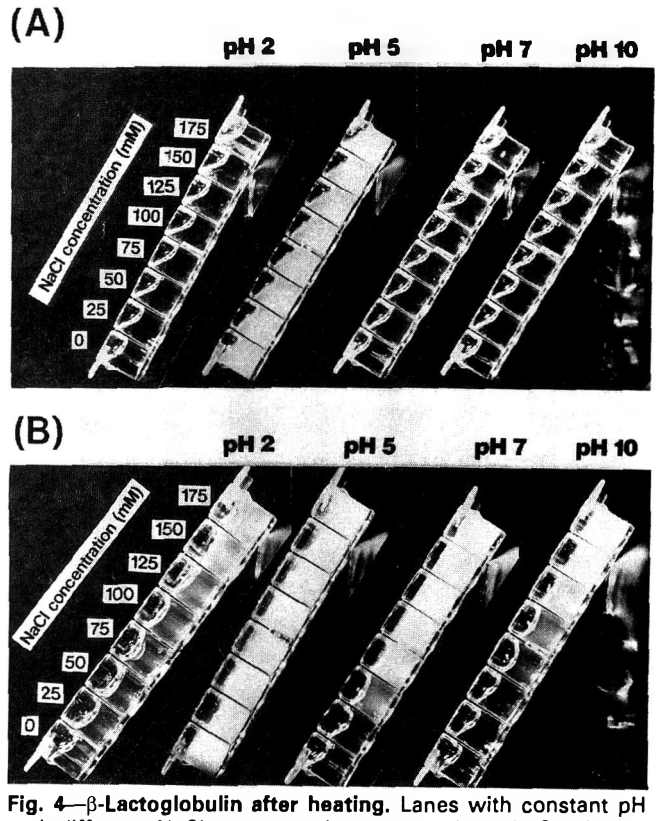

Fig. 4—β-Lactoglobulin after heating. Lanes with constant pH and different NaCl concentrations were slanted. Conditions same as Fig. 3.

major protein components in the protein fraction of whey, i.e., β-lactoglobulin, α-lactalbumin, and serum albumin, are globular proteins, and each has its own conformation. When heated, the protein conformations change, but do not collapse. Hydrophobic areas that were originally enclosed inside the protein molecule appeared to be partially exposed by heating. These denatured protein molecules then associated with each other by noncovalent hydrophobic interaction. Generally, protein molecules aggregate randomly to form a large coagulum which scatters light and causes turbidity. Molecular interactions between denatured molecules are influenced not only by hydrophobic attraction, but also by electrostatic repulsion, which is affected by pH and salt concentration. When electrostatic repulsion is relatively strong, molecular interaction is restricted and the number of contact areas on the denatured molecules may be reduced. When there are effectively two contact areas per molecule, such molecules link together and form a soluble linear aggregate. These aggregates have been observed with various proteins by transmission electron microscopy (Clark et al., 1981; Koseki et al., 1989; Kitabatake and Doi, 1993). When the pH is not near the isoelectric point of the protein or when the salt concentration is low, electrostatic repulsion is relatively increased and transparent gels or sols are formed. Protein molecules are charged under such conditions, as was clearly demonstrated by our results. The soluble linear aggregates are cross-linked and form a network to produce a viscous liquid or gel structure. Since there is no large aggregate, i.e., coagulum, the gel is transparent. When the protein concentration is low, such soluble linear aggregates do not form a gel network, but form a highly viscous transparent liquid. Whey protein gave transparent gels and liquids after heating over a wide range of pHs and NaCl concentrations. Whey protein gave transparent samples, especially at neutral and weak acid pH, which is the region often used in food processing and cooking.

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From page 765

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Rheological Behavior and Potential Cross-Linking of Pacific Whiting (*Merluccius productus*) Surimi Gel

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ABSTRACT

Gelation behavior and potential cross-linking of Pacific whiting (*Merluccius productus*) surimi were affected by setting temperatures and an enzyme inhibitor. Gels of Pacific whiting surimi with salt and beef plasma protein were compared with those containing guanidine hydrochloride, sodium dodecyl sulfate, and β-mercaptoethanol. The strongest gels were formed at 25°C setting followed by 90°C heating. Hydrogen and hydrophobic bonds appeared to strongly influence gel formation, while the influence of disulfide bonds was moderate. Viscosity scanning during setting at different temperatures was also useful to estimate effects of enzymes and inhibitors.

Key Words: surimi, rheology, setting, cross-linking, whiting

INTRODUCTION

WHEN SALTED SURIMI PASTE from Alaska pollock is kept at a constant low temperature below 40°C for 30 min to 20 hr prior to heating to 90°C, greater elasticity and higher water-holding capacity of surimi gels are produced (Kimura et al., 1991). This is called "setting." Surimi-based products which need a molding process or fiber development are greatly influenced by setting. The control of setting is very important for quality control. Kawashima et al. (1970) and Nishioka et al. (1983) reported the elasticity of surimi-based gel was influenced by both the quality and quantity of salt soluble proteins. Kim (1987) showed species differences between Alaska pollock and Atlantic croaker during low temperature setting (0 and 4°C).

Research has been conducted on Pacific whiting earlier, but commercial surimi manufacture with it began in 1992. Pacific whiting represents a major resource for the U.S. with a potential near-future annual harvest to reach 250,000 metric tons (OCZMA, 1994). But whiting's soft texture and inherent heat-activated protease have generally limited it to lower-valued markets. Research has demonstrated the value of enzyme inhibitors (Morrissey et al., 1993) and the characteristics of the enzyme (An et al., 1994). A notable development was an application of beef plasma protein to inhibit enzymes which are very active at 50–60°C. Morrissey et al. (1993) reported such activity was inhibited by about 90% with 1% BPP when Pacific whiting surimi was used as a substrate, followed by 60% inhibition with 1% egg white, and 50% with 1% potato extract. Due to the nature of the enzymes and limited information on rheological characteristics, Pacific whiting surimi has not been preferred commercially (Lester, 1993). Hydrogen and other polar bonds in fish gels have been reported to destabilize at high temperatures but stabilize at low temperatures, in contrast to hydrophobic bonds (Nemethy, 1968; Niwa et al., 1982a). Hydrogen bonds and hydrophobic bonds in the gelation of actomyosin or myosin have been studied extensively (Hamada, 1992; Niwa, 1975; Niwa et al., 1982a, 1986a; Wicker et al., 1989). Niwa (1975) and Niwa et al. (1983, 1986a) have emphasized the importance of the hydrophobic bond in setting and gelation of fish actomyosin. Hydrophic interaction occurs when nonpolar molecules are introduced into the polar environment of water (Busk, 1984). During gelation, disruption of the protein conformational structure occurs enabling exposure of the uncovered hydrophobic regions to the solvent (Lanier et al., 1982). This increase in surface exposure of hydrophobic regions is likely involved in intramolecular interactions of gel formation. Studies of covalent bonding in heat-induced protein gels have focused on disulfide (-SS-) linkages. Heat treatment can result in cleavage of existing -SS- bond structures or exposure of covered sulfhydryl (-SH) groups through unfolding of the protein. Such newly formed or exposed SH groups can form new intermolecular bonds, and affect final gel characteristics (Kim, 1987). The gelation mechanisms of Pacific whiting are not well understood. Study of potential cross-linking of gels is very important to understand the nature of gelation. Investigation of the rheological behavior and potential cross-linking of Pacific whiting surimi gels can lead to opimizing the functional characteristics of this new surimi. Our objective was to examine fracture shear stress and shear strain as affected by setting temperatures and added compounds, to elucidate the types of cross-linking in Pacific whiting surimi gels. The viscosity of surimi paste was also investigated as affected by setting temperatures and an enzyme inhibitor.

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MATERIALS & METHODS

PACIFIC WHITING (*Merluccius productus*) surimi (10 kg/frozen block), without enzyme inhibitor, was obtained from American Seafood Company (Seattle, WA). This surimi contained usual cryoprotectants (4% sugar, 5% sorbitol, 0.3% sodium tripolyphosphate). Randomly selected surimi blocks (10 kg) were cut into about 900g portions. These were packaged in food grade plastic bags, vacuum-packed, and kept at −18°C. The moisture content of the surimi was 76 ± 0.5%. For gel preparation, paste was prepared with 79.5% surimi, 2% salt, and 1.2% beef plasma protein (BPP; AMPC, Inc., Ames, IA) for treatments with enzyme inhibitor. A control group contained the same ingredients as the treated group but no BPP. Each treatment was mixed with additional compounds as described below. All treatments were made to 100% with ice water to bring final moisture to 78%. For viscosity measurement, two batches of surimi paste were prepared: one with 30% surimi, 2% salt, and 1.2% beef plasma protein (BPP) as enzyme inhibitor; the other with 30% surimi and 2% salt without BPP. The experiment was repeated twice.

Gel preparation

Frozen surimi, thawed at room temperature for 2 hr, was cut into small pieces (about 2.5 cm cubes). Surimi cubes were placed in a Stephan vertical vacuum cutter (model UM 5 Universal; Stephan Machinery Corp., Columbus, OH) connected with a NesLab constant temperature circulating chiller (model RTE-100LP; NesLab Instruments, Inc., Portsmouth, NH). Surimi was chopped for 1.5 min to a very small particle size (≈2 mm cubes). Salt was sprinkled in and the surimi paste was chopped for 1 min. Ice/water, BPP, and compounds were added and chopping was continued for 30 sec. The paste was chopped under vacuum at 0.5 bar for 3 min to remove air pockets from the surimi paste. Temperature was maintained below 8°C. A sausage stuffer (The Sausage Maker, Buffalo, NY) was used to stuff raw paste into stainless steel tubes (inner diameter = 1.86 cm, length = 17.5 cm) with stainless steel screwable caps. The interior wall of the tubes was coated with a film of PAM cooking spray (Boyle-Midway, Inc., New York, NY).

Treatments with and without BPP were divided into four different setting and heating categories: (1) 5°C setting for 24 hr followed by heating at 90°C for 15 min; (2) 25°C setting for 3 hr followed by

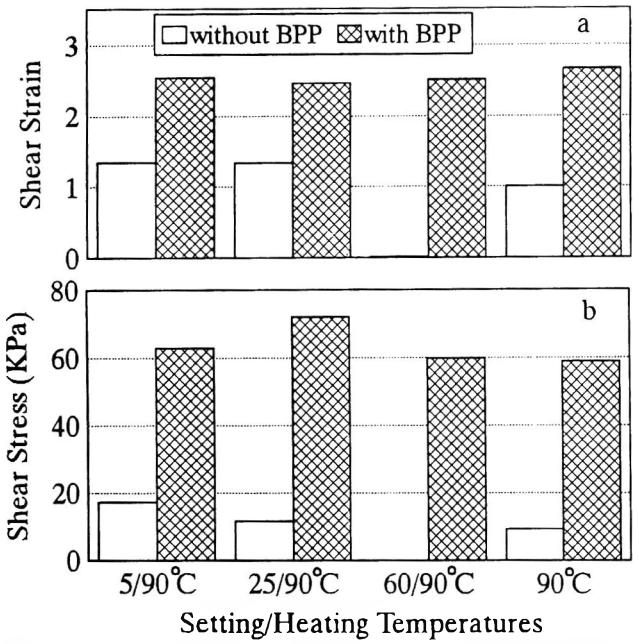

Fig. 1—Effects of beef plasma protein (BPP) and setting/heating temperatures on gel deformability (a) and hardness (b) of Pacific whiting surimi.

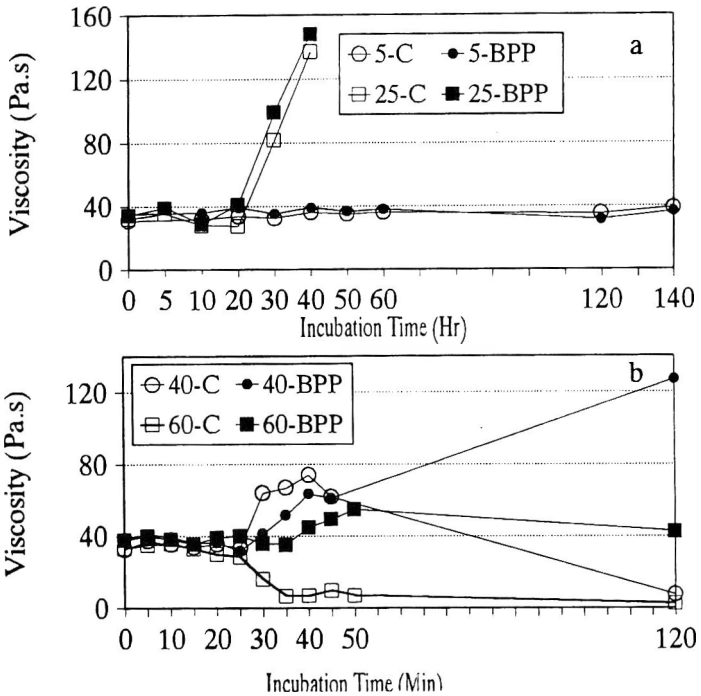

Fig. 2—Viscosity of Pacific whiting surimi paste during incubation. 5, 25, 40, and 60 denote incubation (setting) temperature (°C); C = control; BPP = treated with beef plasma protein.

heating at 90°C for 15 min; (3) 60°C setting for 30 min followed by heating at 90°C for 15 min; (4) 90°C heating for 15 min without setting. For the 90°C treatments, the tubes were submerged in a 95°C water bath, and immediately the water temperature was adjusted to 90°C using ice. Heated gels were immediately chilled to <5°C in ice water 1 hr before storing at 5°C overnight.

Fracture stress and fracture strain by torsion method

The cooled gels (5°C) were kept at room temperature (22–25°C) for 2 hr to reach higher stress and strain levels (Howe et al., 1994). Ten gels were milled into a dumbbell shape (length = 2.87 cm, end diameter = 1.86 cm and minimum diameter = 1 cm) and then subjected to torsional shear on a device adapted from a Brookfield viscometer set at 2.5 rpm (NFI, 1991). Shear stress and shear strain at mechanical fracture were measured (Hamann, 1983; NFI, 1991). Shear stress was measured to indicate gel strength and shear strain to indicate their cohesive nature.

Different compounds affecting bonding of surimi gel

Guanidine hydrochloride (G-HCl) at 0.25, 0.5, and 1.0M; sodium dodecyl sulfate (SDS) at 5 and 10 mM; β-mercaptoethanol (β-ME) at 10 and 20 mM, were added as described above, in the gel preparation. Fracture shear stress and shear strain were measured to investigate bonding characteristics of surimi gel: G-HCl was used to break hydrogen bonds; SDS for hydrophobic interaction; β-ME for disulfide bonds.

Viscosity measurement

Each paste was divided equally into four 250 mL beakers. Viscosity changes during storatge at 5, 25, 40, and 60°C were measured using a Brookfield digital viscometer (model 5XHBTDV-I: Brookfield Engineering, Stoughton, MA) with #3 and #4 spindles for 140 hr, 40 hr, 2 hr, or 2 hr, respectively.

Statistical analysis

Six samples per treatment were tested for torsional data. Statistical analysis (t-test) was applied on the data for significant differences ($P < 0.05$) (SYSTAT, 1992). No statistical analysis was made for viscosity data.

RESULTS & DISCUSSION

Effects of settings and BPP on rheological characteristics

As a function of textural properties, fracture shear stress indicates gel strength, while fracture shear strain denotes gel deformability. Fracture stress correlates strongly with sensory hardness, and fracture strain with elasticity (Montejano et al., 1985; Hamann, 1991). As shown (Fig. 1) BPP had an effect ($P < 0.05$) on shear stress and shear strain of Pacific whiting surimi. At 60°C setting, followed by 90°C heating, no gel was formed without BPP but a strong gel resulted with BPP. This was probably due to the degradation of myofibrillar proteins by whiting protease. In the presence of BPP, there was no difference ($P > 0.05$) in shear strain between 5/90°C, 25/90°C, 60/90°C, and 90°C treatments. However, with a 25/90°C treatment, a stronger ($P < 0.05$) gel (highest fracture shear stress) was observed, suggesting that the setting effect was most pronounced in the heating. Kim (1987) reported shear stress and strain at fracture for surimi gels from two different species (Alaska pollock and Atlantic croaker) subjected to different time-temperature treatments. The strongest pollock surimi gel [failure shear stress, 94 kilo pascal (kPa)] was observed with a 4°C setting for 24 hr followed by 90°C heating for 15 min, while the strongest croaker surimi gel (96 kPa) was formed at a 40°C setting for 30 min followed by 90°C heating for 15 min. Our results and Kim's (1987) indicate a possible relationship between water temperature of the fishing grounds and the best gel-setting temperature of surimi (pollock from the Bering sea, whiting from the Pacific coast). Misima et al. (1993) also reported that Ca^{++}-activated myofibrillar Mg^{++}-ATPase, an indicator of gelation function of myosin, was highest at the habitat temperature (25.5°C) of carp.

Viscosities of Pacific whiting surimi pastes with and without BPP were compared during incubation at 5 and 25°C (Fig. 2a), and 40 and 60°C (Fig. 2b). At 5°C incubation, little change occurred in viscosity for 6 days regardless of BPP addition. Viscosity remained between 30 to 40 pascal seconds (Pa.s). Viscous character of the paste at 25°C was very similar to one at 5°C up to 20 hr incubation regardless of BPP addition (Fig. 2a). Then viscosity increased rapidly for the 25°C set sample

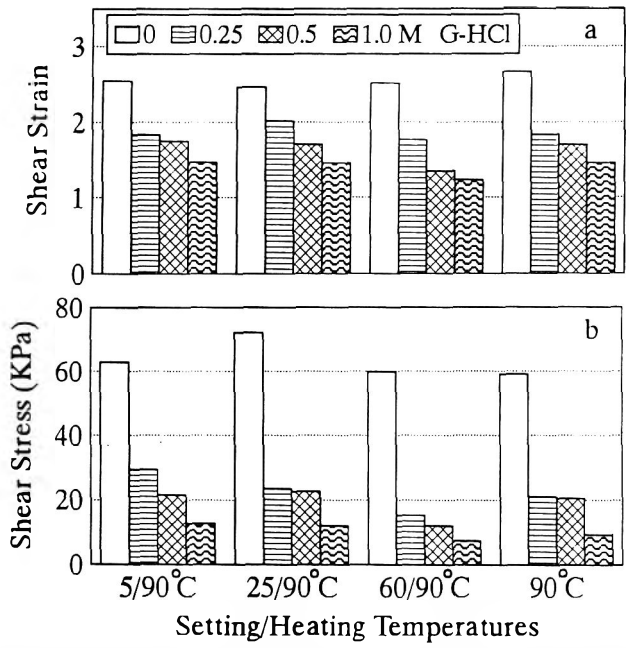

Fig. 3—Effects of guanidine hydrochloride (G-HCl) and setting/heating temperatures on gel deformability (a) and hardness (b).

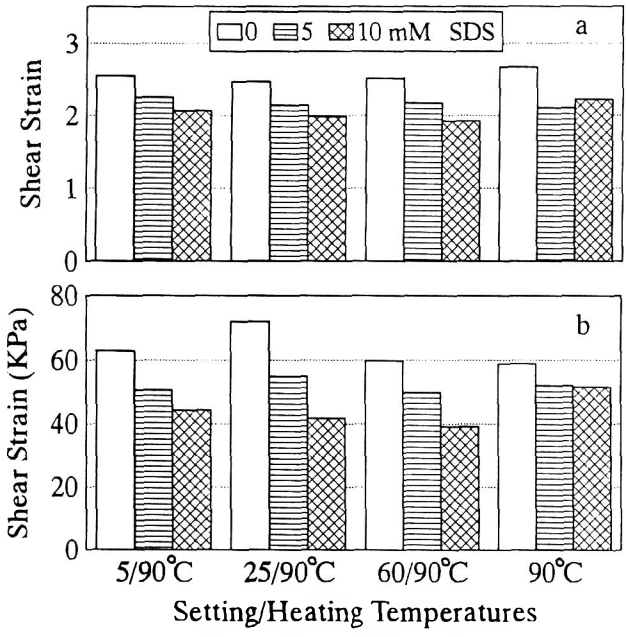

Fig. 4—Effects of sodium dodecyl sulfate (SDS) and setting/heating temperatures on gel deformability (a) and hardness (b).

suggesting that intramolecular binding of myofibrillar proteins occurred regardless of BPP addition. However, the BPP treatment showed a slightly higher viscosity. Incubation at 5°C for a longer time did not affect viscosity regardless of BPP addition. This indicated that a setting of whiting surimi at 5°C did not improve stress and strain values of gels (Fig. 1a, b). At 40°C incubation (Fig. 2b), no difference occurred in viscosity between treatments with and without BPP for the first 25 min. For the next 15 min, the viscosity of both increased; more rapidly without BPP, suggesting that gelation of myofibrillar proteins began. During further incubation (45–120 min), the viscosity of the control (without BPP) gradually decreased. Likely a protease was activated at 40°C during longer incubation. The viscosity of the BPP treatment continuously increased suggesting BPP inhibited enzymes. The rheological

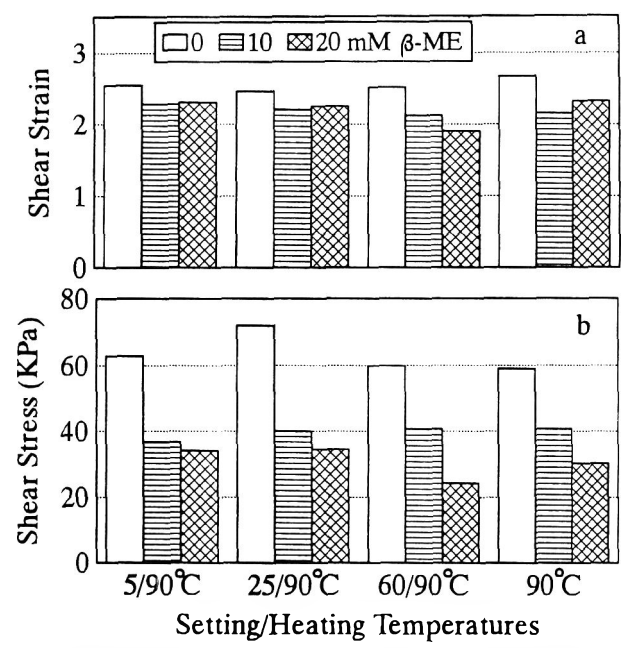

Fig. 5—Effects of β-mercaptoethanol (β-ME) and setting/heating temperatures on gel deformability (a) and hardness (b).

viscosity of the paste confirmed reports of An et al. (1994). They showed that activity of the Pacific whiting protease was observed as low as 20°C, however, the optimum temperature was around 55°C.

At a range of 55–60°C, known as an optimum temperature for fish protease activity (Lin et al., 1980; Chang-Lee et al., 1990; Wasson et al., 1992; An et al., 1994), the viscosity of the control continually decreased during 2 hr incubation (Fig. 2b). With BPP treatment, viscosity remained unchanged the first 35 min, then increased slightly due to gelation followed by a slight decrease during the last hour of incubation (Fig. 2b). Viscosity scanning during incubation at different temperatures could apparantly be a useful method for estimating effects of enzymes and inhibitors for specific fish with intrinsic enzyme problems.

Rheological characteristics of gels with added compounds

Effects of hydrogen bonds on gel strength and deformability (Fig. 3) showed shear stress (gel hardness) decreased ($P < 0.05$) with an increase in concentration of G-HCl regardless of setting/heating temperatures. Shear strain (gel deformability), also decreased ($P < 0.05$) with an increase in concentration of G-HCl in all treatments. The cleavage ability of G-HCl for hydrogen bonds was apparent in Pacific whiting surimi gels. The results for Pacific whiting were very similar to those for Jack mackerel, flatfish, and yellowtail (Niwa et al., 1982a) and Alaska pollock (Hamada, 1992). The denaturing agent guanidine likely competed for hydrogen bonds between amide nitrogen and carbonyl carbon (Rawn, 1983) and therefore, interrupted the secondary structures to form weaker gels.

A gradual reduction of gel hardness and deformability was observed with addition of sodium dodecyl sulfate (SDS) regardless of setting/heating temperature (Fig. 4). The influence of hydrophobic bonds was important ($P < 0.05$) in the gelation of Pacific whiting surimi gel as in the case of croaker myosin (Hamada, 1992) and actomyosin from mackerel, Jack mackerel, striped pigfish, and flatfish (Niwa, 1975; Niwa et al., 1982a; 1983). Cooper (1977) stated that the anionic detergent SDS bound to the hydrophobic regions of proteins and unraveled all intramolecular protein associations. Probably SDS compounds were tightly bound to hydrophobic sites of surimi proteins during the formation of gels.

Gel hardness decreased ($P < 0.05$) with addition of a reducing agent (β-ME), regardless of setting/heating temperature, while deformability decreased only slightly (Fig. 5). The reducing compound, β-ME, disrupts any disulfide bonds between cysteine residues (Rawn, 1983), which are important in covalent bonds of surimi gels. The effectiveness of disulfide bonds in Pacific whiting surimi gel was slightly less than that of other bonds. This finding was similar to the results of Niwa et al. (1982b, 1986b). Kim (1987) demonstrated the effect of another reducing compound, NEM (N-ethylmaleimide), on rigidity modulus and energy loss of croaker and pollock surimi. Fully heated gels (with or without setting) were weak or brittle when NEM was added. The influence of disulfide bonds in croaker myosin gel reported by Hamada (1992) was in contrast to our results. According to those results, the strain value of gels to which β-ME was added was somewhat larger than that without β-ME, perhaps indicating new disulfide bonds were formed between sulfhydryl groups of myosin.

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Fracture of Alaska Pollock Gels in Water: Effects of Minced Muscle Processing and Test Temperature

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ABSTRACT

The effects of temperature on instrumental textural measurements of cooked Alaska pollock gels were evaluated. Independent variables were: raw materials (minced muscle, washed minced muscle alone or with cryoprotectants), cooking schedule (90°C for 20 min with/without preconditioning at 25°C) and test temperatures. Gels tested at 5°C were more brittle than those tested at 45–55°C. Gels preset at 25°C for 1 hr prior to cooking at 90°C had higher fracture shear stress values and were influenced differently by test temperatures than those not preset suggesting cooking history influenced structure of the gels at the molecular level. Results indicated gels set at 25°C were probably stabilized by more covalent bonds.

Key Words: fish, pollock, surimi, gels, minced muscle

INTRODUCTION

MOST INSTRUMENTAL TEXTURE ANALYSES are conducted at room temperature on fully cooked gels to evaluate effects of processing variables (e.g., formulation or cooking methods). Except for a few studies emphasizing effects of test temperatures on small strain parameters (Niwa et al., 1987; Niwa et al. 1988a, b), little study has been made of the effects of test temperature on textural characteristics of cooked gels. Since fish muscle gel products (predominantly shellfish analogs of Alaska pollock in the U.S. may be served and consumed at a broad range of temperatures, the effects of serving temperature on texture is important. Measurements at room temperature may not be adequate.

Fracture shear stress is a measure of textural hardness (Montejano et al., 1985). Shear stress is sensitive to protein concentration, thermal processing effects and other modifications such as ingredients (Hamann, 1988). Fracture shear strain (textural cohesiveness) is a measure of a gel's degree of deformation before it breaks (Montejano et al., 1985). Strain is not readily changed and is predominantly influenced by protein quality (Hamann, 1988). Our objective was to establish the fundamental fracture properties of Alaska pollock gels with different cook histories at various test temperatures to better understand influences on hardness and cohesiveness of fish muscle food gels.

MATERIALS & METHODS

Meat preparation and analysis

Blocks of frozen Alaska pollock (*Theragra chalcogramma*) were obtained from the Northwest Fisheries Science Center, Kodiak, AK. Processing treatments prior to freezing yielded three distinct materials varying in number of processing steps: minced muscle (MM), washed minced muscle (WM) alone or with cryoprotectants (WMC) added (surimi). MM was prepared using a belt-type meat separator with a drum opening of 4 mm. WM was cold water washed, "refined," and "dewatered" using a refiner and screw press. WMC was prepared in the same manner as WM except for addition of 3.1% sugar, 3.7% sorbitol, and 0.2% sodium pyrophosphate. Processing of the pollock blocks was 1–2 days post-catch.

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Table 1—Comparison of tests in air or water (students t test)

	Sample size	Sample mean	Sample deviation	t-statistic	p-value
Stress					
Air	8	45.5	4.54	0.806	$p > 0.3$
Water	8	47.8	6.46		
Strain					
Air	8	3.05	0.088	1.246	$p > 0.2$
Water	8	3.15	0.20		

Moisture and protein (Kjeldahl nitrogen determination, % protein = % nitrogen × 6.25) contents were determined using AOAC (1980) methods. Frozen blocks of pollock minces were vacuum-packaged in two-layer (nylon and polyolefin) laminated plastic bags (Cryovac, Simpsonville, SC), and stored at −20°C until used.

Gel preparation

Gels of Alaska pollock and sodium chloride (NaCl) were made. NaCl content was 2.0% of the respective moisture contents for each material (0.34M NaCl assuming all water was available). The three pollock materials (MM, WM, and WMC) were tested in duplicate. To make the gels frozen pollock blocks were first tempered at 25°C for 2 hr and cut into cubes (about 5 cm cubes). The cubes were then chopped in a Stephan vertical cutter/mixer (model UMC-5, Stephan Machinery Corp., Columbus, OH) for about 15 sec to form a paste, followed by addition of NaCl. The paste was then chopped at 1750 rpm under vacuum to final temperature 5°C. It was placed into plastic bags and vacuum-sealed to further reduce air content. Stainless steel tubes (diameter = 1.87 cm and length = 17.75 cm) sprayed with a nonstick agent (Pan-Out, distributed by Pegler & Co., Lincoln, NE) were stuffed with paste using a sausage stuffer (Vogt series 9, Germany). Tubes were capped and randomly assigned to a thermal treatment in water baths: 20 min at 90°C, or 1 hr incubation (setting) at 25°C followed by cooking 20 min at 90°C. After thermal treatment, tubes were immediately removed, placed in ice-water baths and cooled 10 min. Gels were removed from tubes and stored overnight at 4°C in Whirl-Pak bags (Nasco, Fort Atkinson, WI).

Torsion test

Torsional analysis was used to determine the dependence of fracture shear stress and strain on specimen temperature. The fracture properties were determined on cooked samples reheated to 5–65°C at 10°C increments. All combinations were performed on two batches (duplicates) of each of three Alaska pollock materials. For torsional analysis, disposable notched disks were glued to the ends of cylindrical specimens (diameter=1.9 cm, length=2.87 cm). Specimens were then made into a capstan shape with minimum diameter of 1.0 cm using a modified (Kim et al., 1986) special cutter (Gel Consultants, Raleigh, NC).

Samples were twisted to fracture at 2.5 rpm (shear strain rate = 0.125 sec^{-1}) using a Torsion Gelometer (Gel Consultants, Raleigh, NC). To test at various temperatures, each specimen was placed in the gelometer, lowered into a water bath and held for 3 min prior to twisting. This time brought the temperature of the test specimen to ± 1°C of the water bath temperature. Equilibrating samples in water at 22°C before twisting did not alter stress or strain values compared to testing in air (Table 1). We assumed relative values determined in water and air would also be equivalent at other temperatures.

The torque reading and time of twist (time to failure) were recorded and converted into shear stress and shear strain at fracture. Shear stress

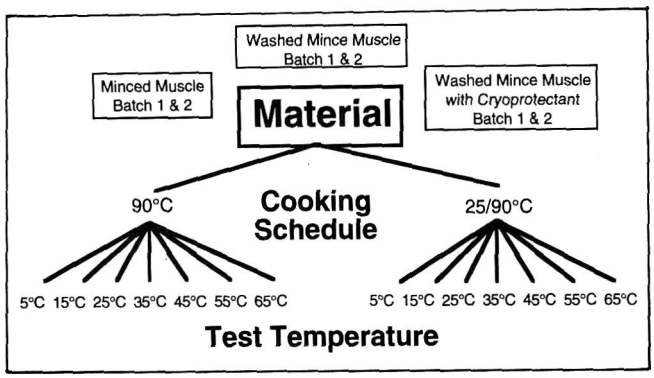

Fig. 1—Experimental design. The two cooking schedules were 90°C for 20 min or 25°C for 1 hr followed by 90°C for 20 min.

Table 2—Proximate analysis (wet weight basis)

Material	Protein (%)	Moisture (%)
Minced muscle (MM)	11.2	84.9
Washed minced muscle (WM)	16.9	81.0
Washed minced muscle with cryoprotectants (WMC)	15.9	74.2

Table 3—Sums of squares and coefficients of variability from the analysis of variance[a]

Source	df	Stress	p-value	Strain	p-value
Material	2	270700	0.0005	114.8	0.0008
Error a[b]	3	1675		0.96	
Cook	1	35470	0.0004	8.87	0.0002
Material × cook	2	7370	0.0097	0.93	0.0133
Error b[c]	3	351.9		0.06	
Temperature	6	23310	0.0001	18.10	0.0001
Material × temperature	12	8022	0.0001	1.53	0.003
Cook × temperature	6	2569	0.0006	2.33	0.0001
Error c[d]	18	1021		0.49	
Material × cook × temp.	11	935.5	0.0305	0.54	0.00079
Error d[e]	16	486.9		0.21	
Sampling error	400	6130		6.85	
Grand mean	—	44.93		2.24	
C.V. (%)	—	8.71		5.84	

[a] Analyzed as a split-split plots design.
[b] Error a is the batch (material).
[c] Error b is the cook × batch (material) interaction.
[d] Error c is the temp × batch (material) interaction.
[e] Error d is the cook × temperature × batch (material) interaction.

and shear strain were calculated using the following equations specific for specimens and apparatus (Hamann, 1991):

$$\tau\ [kPa] = \text{torque reading [instr. units]} \times 1.580\ [kPa/inst.\ units] \quad (1)$$

$$\gamma_u\ [dimensionless] = \text{chart travel/chart vel. [sec]} \times 0.150\ [rad/sec] - 0.0084\ [rad/inst.\ units] \times \text{torque}\ [inst.\ units] \quad (2)$$

$$\gamma = \ln\{1+(\gamma_u^2/2)+\gamma_u[1+(\gamma_u^2/4)]^{1/2}\} \quad (3)$$

where τ is fracture shear stress, γ is fracture shear strain and and γ_u is an intermediate quantity used in the equation for γ.

Statistical analysis

The experiment (Fig. 1) was designed as a split-split plot with whole plots assigned to a factorial of the three pollock materials and two batches. Subplots and subsubplots were assigned to two cooking treatments and seven test temperatures, respectively. The individual stress and strain values were subjected to analysis of variance using the general linear models (GLM) procedure of the Statistical Analysis System (SAS Institute, Inc., 1989). Differences within classifications were determined using contrast statements.

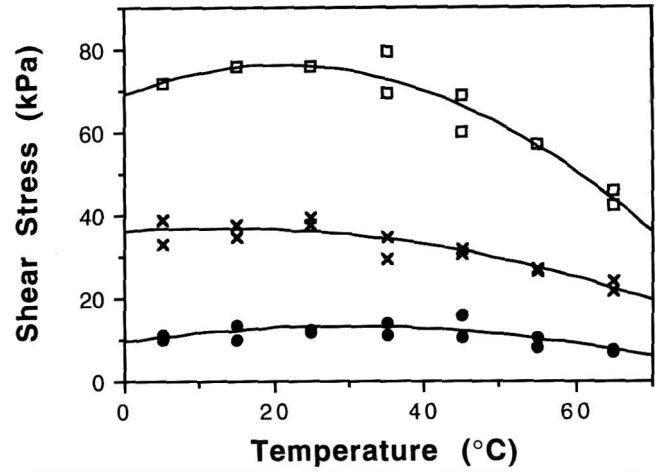

Fig. 2—Fracture shear stress relation to test temperature for 90°C/20 min gels: (● – unwashed mince, r^2=0.57; x – washed mince, r^2=0.85; and □ – washed mince with cryoprotectants, r^2=0.93). Each point is a batch average of test measurements for the respective treatment.

RESULTS

Proximate analysis

Proximate analyses of the materials were compared (Table 2). They showed compositions typical of spawning stock, flesh with increased water and decreased protein content. This may account for unexpected differences between moisture contents of unwashed and washed minces. Lanier (1986) reported similar moisture contents in such products. The moisture of WM was within previous reported levels (Lanier, 1986) and the WMC was similar to that reported by Reppond et al. (1987) and Hastings et al. (1990). Protein content was lowest in the MM. Washing and dewatering steps concentrated the proteins, predominantly salt-soluble proteins (Lee, 1984). The highest protein level was found in the WM. Protein was slightly diluted in WMC.

Torsional analysis

Shear stress and strain at fracture over 5–65°C for the three materials were compared (Fig. 2 to 5). Each point is a batch average for the respective treatment. Test temperature strongly affected fracture shear stress and strain (Table 3). Cook treatment and material also had strong influences as did interactions.

Shear stress at fracture

Gels of all materials set at 25°C for 60 min prior to cooking exhibited higher shear stress values than unset gels (Fig. 2 and 3). In several fish species set gels had greater fracture shear stress (Hamann, 1990). The set cook treatment involves a preliminary gel formation (25°C) before coagulation of protein. For MM (Fig. 3), the set gel stress values declined linearly with an increase in test temperature. Stress values of unset gels (Fig. 2) were quadratically temperature sensitive, increasing to 25–35°C.

Higher stresses were observed for WM than for the MM (Fig. 2 and 3). This difference confirmed previous results (MacDonald et al., 1990; Okada, 1964; Lee, 1984) and may be attributed primarily to two factors: protein composition and concentration. The washing step concentrates myofibrillar proteins and removes water soluble proteins. This changes protein composition, and (as in proximate analysis) WM had higher protein. The myofibrils remaining are the most active component in texture effects (Acton et al., 1983). For both

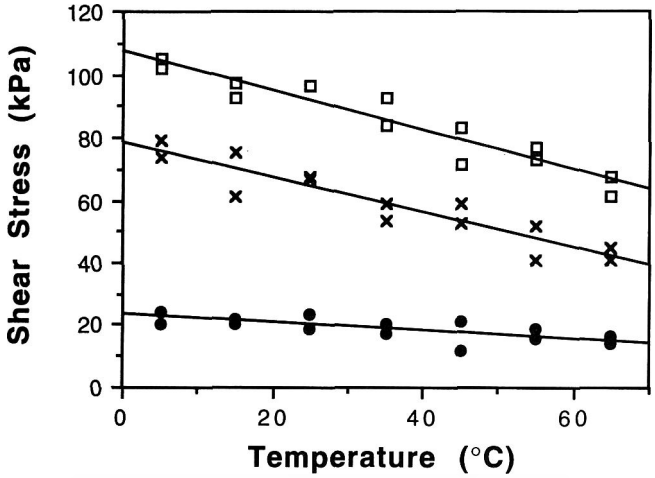

Fig. 3—Fracture shear stress relation to test temperature for 25°C/1 hr plus 90°C/20 min gels: (● – unwashed mince, r^2=0.48; x – washed mince, r^2=0.87; and □ – washed mince with cryoprotectants, r^2=0.90). Each point is a batch average of test measurements for the respective treatment.

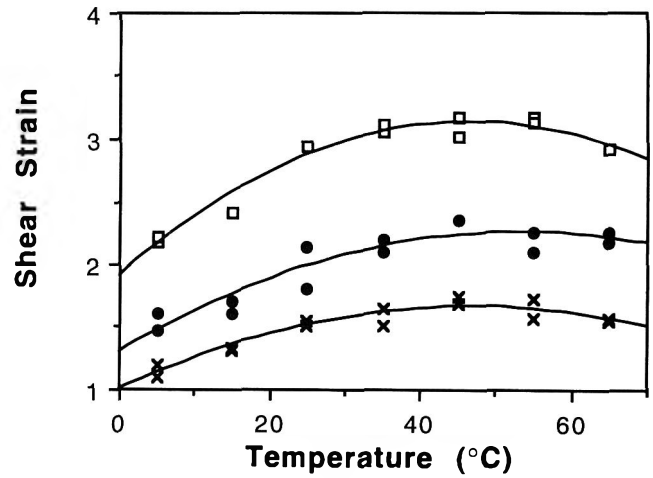

Fig. 4—Fracture shear strain relation to test temperature for 90°C/20 min gels: (● – unwashed mince, r^2=0.86; x – washed mince, r^2=0.91; and □ – washed mince with cryoprotectants, r^2=0.96). Each point is a batch average of test measurements for the respective treatment.

WM and MM set gels, stress values declined linearly with increasing temperature. Unset gels had maximum stress values at about 25°C and exhibited a quadratic relationship with temperature.

Stress values were greatest for the WMC. Test temperature effects were similar to the other materials. Set gel stress decreased linearly with increase in temperature, and unset gels had a quadratic stress relationship with test temperature. Unset surimi gels had greater quadratic curvature than those made from the other samples.

Shear strain at fracture

Strain values for both thermal treatments of minced muscle (Fig. 4 and 5) were maximum at 45–55°C. As with stress, set gels had higher strain values, but the difference varied with testing temperature. Set and unset gels had almost identical fracture strains at 45–55°C. Set gels were less test-temperature sensitive.

WM strain values were lower than those of the MM. This was in contrast to changes observed for stress. WM had lower stability to freezing due to absence of protective sarcoplasmic proteins (Shimizu and Fujita, 1985). Strain is strongly affected by protein functionality (Hamann, 1988). Fracture stress, however, is greatly affected by protein content, thus the higher stress values for WM (Table 2). For WM maximum strain values occurred at about 45–55°C.

Gels of WMC had the highest strain values, almost twice those of other materials at some test temperatures. The high strains for this material indicated that the cryoprotectants protected the functionality of the concentrated myofibrillar proteins during freezing. The trends with thermal treatment and test temperature were similar to those of WM.

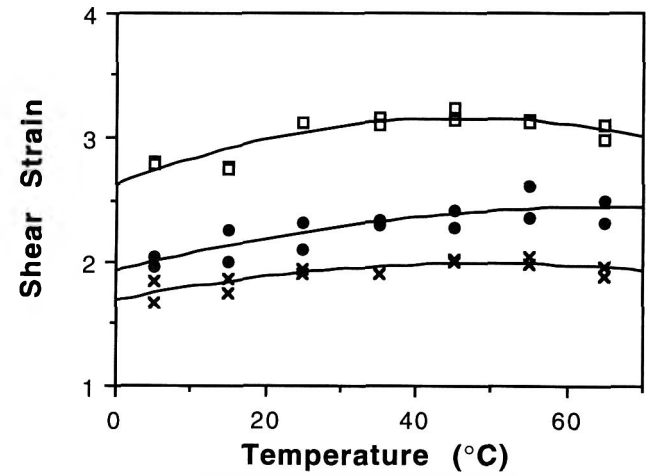

Fig. 5—Fracture shear strain relation to test temperature for 25°C/1 hr plus 90°C/20 min gels: (● – unwashed mince, r^2=0.71; x – washed mince, r^2=0.68; and □ – washed mince with cryoprotectants, r^2=0.79). Each point is a batch average of test measurements for the respective treatment.

DISCUSSION

Protein functionality

Surimi produced gels with larger fracture stresses and strains than the other gels. A question arises concerning the influence of lower moisture content (Table 2). No water was added so the WM and WMC had similar protein contents. Protein content and carbohydrate additives can influence fracture stress but do not have much influence on fracture strain (Hamann, 1988, 1991). That fracture strains for the WMC were much higher than those for the other samples is the best evidence for the effectiveness of cryoprotectants. Without cryoprotectants, the higher strains of the MM indicate proteins were more effective in gel forming than the proteins of WM. The fracture stresses for the MM gels were lower because of the lower protein content.

Test temperature effects

Minced fish gels are stabilized by several types of forces. Those considered most important are covalent bonds, hydrophobic interactions, and hydrogen bonds. Of these, covalent bonds are relatively permanent and their effects on fracture stress are considered to be independent of test temperature in the range studied (Foegeding et al., 1994). The relative strengths of the other two forces is temperature dependent. Hydrogen bonding becomes stronger as temperature decreases while hydrophobic associations tend to strengthen at higher temperatures reaching maximum near 60°C (Scheraga et al., 1962; Niwa, 1991). Maximum fracture strain values at 45–55°C were found here and by Park and Lindwall (1989) and

Niwa et al. (1987, 1988b). They observed maximum deformation and strain values, respectively, at 40–60°C before decreasing. This corresponded with the optimal temperature for hydrophobic interactions (Scheraga et al., 1962).

A 25°C setting of Alaska pollock produces nondisulfide covalent bonds in the surimi gel matrix (Seki et al., 1990; Kamath et al., 1992). Rapid heating (i.e., 90°C) produces gels with fewer such bonds. We assumed that the differences between data of Fig. 2 and 3 and between Fig. 4 and 5 reflected the greater covalent bonding in the gels of Fig. 3 and 5.

Polyacrylamide gels approach ideal elasticity (Foegeding et al., 1994), being stabilized primarily by permanent bonds (Oppermann et al., 1985). A qualitative comparison of our results with those from similar testing of a polyacrylamide gel should prove enlightening. For a polyacrylamide gel, the fracture shear stress is independent of test temperature, but fracture shear strain is inversely linearly dependent on test temperature. The polyacrylamide results should be expected for an entropy elastic material (elastic modulus is proportional to entropy constraints associated with matrix deformation) (Foegeding et al., 1994).

Polyacrylamide gel results suggest fracture strain differences between set and unset surimi gels should be greater at lower temperatures. The greater number of covalent bonds in the set gels should tend to cause the fracture strains to be higher at low temperatures. Figures 4 and 5 show that set vs unset strain differences became greater as temperature decreased.

In Fig. 3, fracture stress was inversely linearly dependent on test temperature. This probably indicates the influence of hydrogen bond strengthening was superimposed on the temperature insensitive covalent bond strength while the influence of hydrophobic associations was relatively slight at such high stress levels. Hydrogen bonds stabilize the alpha and beta structures of the gel matrix upon cooling (Niwa and Miyake, 1971; Niwa and Nakajimi, 1975; Niwa and Hamada, 1981). Possibly, the increase in covalent bonds due to setting produced a structure more favorable to hydrogen bonding.

In the case of gels cooked without the preset treatment, the influence of hydrophobic associations should be more evident since less covalent and hydrogen bonds were present. Hydrophobic associations would be quite stable from 65°C to about 40°C and weaken at lower temperatures. The flat shear stress curves below 40°C (Fig. 2) could be caused by increasing hydrogen bond strength being offset by decreasing hydrophobic association strength. At the high temperature side of the fracture stress curves, the decrease in stress as temperature increased was likely caused by weakening of hydrogen bonds.

Torsional analysis and other texture tests are usually conducted at room temperature (about 22°C). Near that temperature fracture strain is sensitive to temperature changes. It is important to control or record the temperature when performing torsional analysis to allow accurate comparisons of results.

CONCLUSIONS

TEXTURAL PROPERTIES OF FISH MUSCLE GELS were dependent upon all independent variables. Washed mince with cryoprotectants developed the highest fracture stress and fracture strain. The unwashed mince had the second highest strain (a good measure of gel quality) but was lowest in stress because of its higher moisture content. Fracture stresses of set gels were inversely linearly related to test temperature, while unset gels had a fracture stress plateau or maximum at about 25°C. All fracture strain values were maximum at about 45–55°C, and values for unset gels declined more than for set gels, at higher or lower test temperatures.

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Thermostable Proteinase in Salted Anchovy Muscle

MASAMI ISHIDA, SHOKO NIIZEKI, and FUMIO NAGAYAMA

ABSTRACT

Salted anchovy fillets produced in Japan were severely degraded above 35°C and were solubilized completely in a few hours at 55°C. In contrast, raw anchovy fillets were not solubilized, but the solubility of fillets gradually increased during the salting process. In contrast, salted and raw anchovy fillets from southern Europe were relatively stable. Proteolytic activity that digested myosin heavy chain *in vitro* was found in muscle extracts from both types of salted fillets. Enzymes with activity were high-temperature-active serine proteinases with substrate specificities similar to trypsin. Optimal pH was 7.4 (Japan) and >8.5 (Europe). Neither enzyme showed collagen-degrading activity.

Key Words: anchovy, muscle solubilization, thermostable, proteinase

INTRODUCTION

MAJOR PRODUCTS OF SALTED ANCHOVIES are processed in southern European countries where raw anchovies (*Engraulis encrasicholus*) are aged in 20–30% NaCl solution for ≥ 6 mo. They are then steeped in oil in glass jars and marketed.

Similar products are manufactured in Japan where raw anchovies (*E. japonica*) are harvested from the Pacific ocean. When salted anchovies in Japan (SAJ) are preserved under warm conditions, e.g., room temperature (≈25°C) in summer, sometimes the fillets are completely solubilized. The solubilization may be unique to SAJ, since salted anchovies in southern European countries (SAE) are not solubilized. Although such high solubilization has not been reported, a proteolytic enzyme may be involved. Prevention of such solubilization requires analysis of fundamental causes and the enzyme responsible.

Our objective was to compare two salted anchovy types—SAJ and SAE—with respect to solubility and proteolytic enzymes.

MATERIALS & METHODS

Materials

Frozen raw anchovies, *E. japonica*, commercial SAJ fillets steeped in salad oil and unfinished salted SAJ obtained during the aging process were provided by Awohata-Kewpie Co., Ltd. (Tokyo). Fresh raw anchovies, *E. encrasicholus*, were purchased from a fish market in Italy and were immediately frozen. Commercial SAE fillets steeped in olive oil (Italian product) were purchased from a department store in Tokyo. Fluorogenic peptide substrates for proteinase assay were purchased from Peptide Institute, Inc. (Osaka). Rabbit myosin heavy chain, porcine muscle type I collagen, proteinase inhibitors, and other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) or Wako Pure Chemical Industries, Ltd. (Tokyo).

Muscle tissue

Frozen raw anchovies were cut into fillets followed by defrosting on ice, then muscle was isolated from the fillet. The commercial salted fillets steeped in oil were used in the experimens after removing excess residual oil with filter paper. The unfinished-salted anchovies were defrosted on ice, briefly washed to remove salt from the body surface, cut into fillets, then muscle was isolated.

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Solubility

About 4g of muscle packed in poly-sealed film were kept for 20 hr in a water bath set at several temperatures. The degree of solubilization was determined both by appearance and by measuring the trichloroacetic acid (TCA)-soluble amino acids or nitrogen. Two to 4g of muscle or solubilized muscle tissue were transferred into a test tube on ice. One mL/g muscle of ice-cold 0.44M TCA was added to the tube, and contents were mixed by inverting rapidly 10 times. After centrifugation at 20,000 × *g* for 10 min at 4°C, the aqueous supernatant was obtained as the TCA-soluble fraction. The soluble amino acids were determined according to Lowry et al. (1951) and the soluble nitrogen by the micro-Kjeldahl method (AOAC, 1980) with the slight modification of using Se as catalyst instead of $HgO + K_2SO_4$.

Extraction of crude enzyme

About 1g of muscle tissue was transferred into a mortar on ice, 4 mL/g sample of an extraction buffer (20 mM Tris-HCl, 1 mM EDTA, 3% NaCl, pH 7.5) were added, followed by homogenation for 15 min on ice. After centrifugation at 20,000 × *g* for 15 min at 4°C, the aqueous supernatant was obtained as crude enzyme extract.

Extent of digestion of protein

Twenty μL of the crude enzyme extract, or its solution diluted with extraction buffer, were transferred into a small test tube and pre-incubated for 5 min in a water bath at the reaction temperature. The reaction was started by adding the rabbit myosin heavy chain (MHC) (16.8 μg/tube) with immediate mixing. The reaction temperatures and reaction times are described below (or in the respective Figure). The reaction was terminated by adding an equal volume of sample-preparation solution for sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) and the mixture was immediately heated in a boiling water bath for 3 min. SDS-PAGE with 7.5% gel was carried out according to Laemmli (1970), and gels were stained with Coomassie Brilliant Blue-R 250. To calculate the weight ratio of enzyme (E) to substrate (S), the weight value of muscle tissue/reaction lot (M) was used instead of E, thus the ratio was indicated as M/S. When porcine muscle type I collagen was the substrate, the reaction was conducted under the same conditions as used for MHC, except the reaction was carried out for 20 hr at 25°C. SDS-PAGE was carried out with 5% gel for collagen.

Determining cleavage of synthetic peptides

Eight fluorogenic peptides, *t*-butoxycarbonyl (Boc)-Val-Leu-Lys-MCA, Boc-Leu-Ser-Thr-Arg-MCA, Boc-Leu-Gly-Arg-MCA, carbobenzoxy (z)-Phe-Arg-MCA, benzoyl (Bz)-Arg-MCA, succinyl (Suc)-Gly-Pro-Leu-Gly-Pro-MCA, Suc-Leu-Leu-Val-Tyr-MCA, and Suc-Ala-Pro-Ala-MCA, were used for determining substrate specificity of enzymes. Each peptide was dissolved to 10 μM in dimethyl sulfoxide (DMSO). The crude enzyme extract from SAJ or SAE, 100 μL, and the extraction buffer, 600 μL, were transferred into a small test tube and pre-incubated for 5 min at 55°C. The reaction was started by adding 100 μL of substrate and was terminated by adding 1.5 mL of methanol:n-butanol:distilled water (35:30:35, v/v/v%) followed by heating for 3 min at 95°C. The increase in fluorescence was measured with an FP-770 spectrofluorometer (excitation λ = 380 nm, emission λ = 460 nm; Japan Spectroscopic Co., Ltd., Tokyo).

Effects of temperature and pH on proteinase activity

To determine the effect of temperature on MHC-degrading activity in the extracts of SAJ or SAE, the pH value of each reaction mixture was adjusted to 7.5 at various temperatures and incubations lasted 20

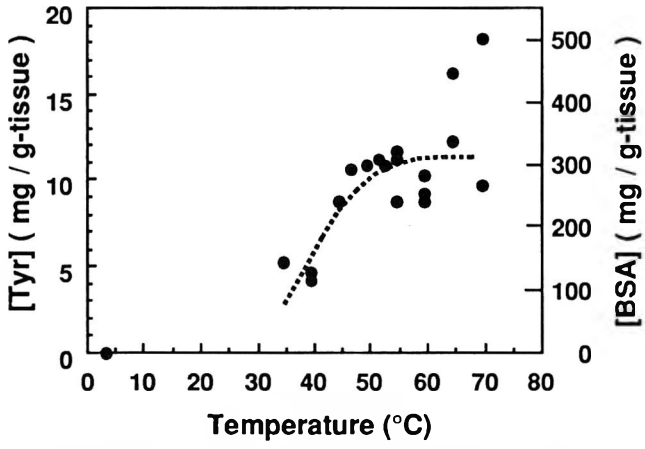

Fig. 1—Effect of temperature on solubilization of SAJ fillets: incubated 20 hr. (Solubility determined by TCA-soluble amino acids after incubation). Values of TCA-soluble amino acids were related to concentrations of tyrosine (Tyr) and bovine serum albumin (BSA).

Table 1—Relationship of temperature to the solubilization of salted anchovy fillets[a]

Temp (°C)	TCA-soluble nitrogen (mg/g-tissue)			
	SAJ[b]	SAE[c]	RAJ[d]	RAE[e]
4	2.7	2.4	NT[f]	NT[f]
35	9.6	4.5	NT[f]	NT[f]
45	17.5	6.4	NT[f]	NT[f]
55	19.0	6.8	6.2	6.5

[a] Solubilities determined by TCA-soluble nitrogen.
[b] SAJ = salted anchovies from Japan.
[c] SAE = salted anchovies from southern European countries.
[d] RAJ = raw anchovies from Japan.
[e] RAE = raw anchovies from southern European countries.
[f] NT: not tested.

hr. To determine the effects of pH on activity in extracts, the pH values of reaction mixtures were measured at 55°C and the incubations lasted 30 min at 55°C. In both cases, each M/S was 2.0 for SAJ and 20.0 for SAE.

Effect of inhibitor on proteinase activity

MHC-degrading activity in the extract of SAJ or SAE was determined with general proteinase inhibitors, i.e., soybean trypsin inhibitor (STI) (50 μM), leupeptin (100 μM), antipain (100 μM), E-64 (300 μM), EDTA (10 mM), and N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) (1 mM). Numbers in parenthesis indicate final concentration of inhibitor in reaction mixture. M/S was 2.0 for both SAJ and SAE, and each mixture was incubated for 2 hr at 55°C.

RESULTS

Solubilization of salted anchovy fillets

The SAJ fillets were highly solubilized above 35°C and were completely solubilized in 2.5–3 hr at 55°C. The level of TCA-soluble amino acids increased with temperature and reached a plateau at about 55°C (Fig. 1). In contrast, when SAJ fillets were incubated at 4°C, they were not solubilized and the level of TCA-soluble amino acids was very low. Consequently, we concluded that SAJ fillets were very soluble, depending on temperature. Since the fillets were completely solubilized within ≈1 to 3 days, even when the temperature was about 35–40°C, commercial SAJ fillets were very likely solubilized in warm summer conditions.

To compare solubilities of four kinds of anchovy fillets, i.e., SAJ, SAE, raw anchovies from Japan (RAJ), and raw anchovies from southern European countries (RAE), the liquification was compared by appearance (data not shown). Only SAJ was highly solubilized at 55°C, while no solubilization was apparent at 4°C. In contrast, SAE, RAJ,and RAE were not solubilized at either temperature. The amounts of TCA-soluble nitrogen of the SAJ and SAE fillets after incubation at various temperatures were compared (Table 1). After incubation at 55°C, the TCA-soluble nitrogen of SAJ increased about threefold compared with RAJ, although that of SAE increased little compared with RAE. When fillets were incubated at 35 or 45°C, each value of TCA-soluble nitrogen of SAJ was also higher than that of SAE.

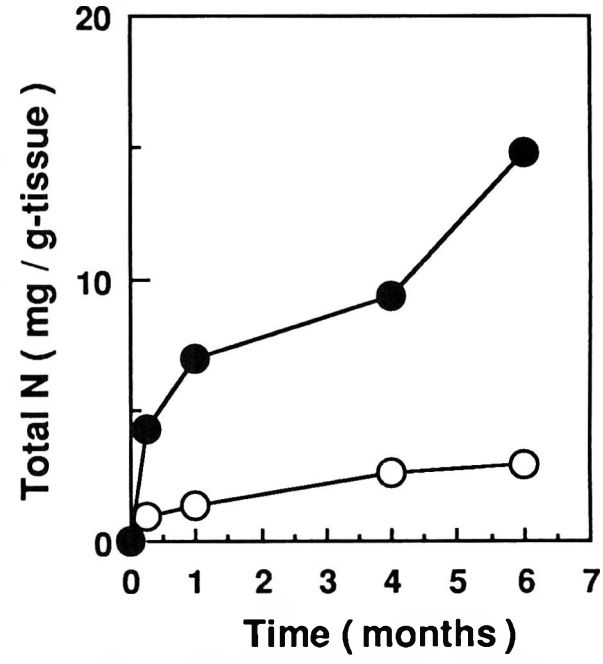

Fig. 2—Effect of length of salting period on solubilization of SAJ fillets. TCA-soluble nitrogen of the fillets before o and after • incubation for 20 hr at 55°C.

Increase in solubility after salting process

The solubilities of SAJ at four stages during the salting process were determined. The fish body after 7 days was white like that of the raw fish and it was slightly elastic. The color, flavor, taste and softness of the fish after 180 days were the same as those of salted fillets on the market, and those after 30 and 120 days were intermediate. The fillets in the four stages were incubated for 20 hr at 55°C. The fillets of 7 days were not solubilized to the same extent as raw fillets. The fillets of 180 days were severely solubilized, similar to commercial fillets. Although a small amount of liquefied tissue was observed in fillets of 30 days, most parts remained intact. The fillets of 120 days were intermediate. The TCA-soluble nitrogen also increased gradually during the salting process (Fig. 2). The TCA-soluble nitrogen of solubilized muscle tissue in stage 4 reached the same level as that of commercial fillets when solubilized (Table 1). These results indicate that solubility, which was not found in the raw fillet, increased gradually during the salting process.

Proteinase activity in anchovy fillets

Rabbit myosin heavy chain (MHC) was degraded gradually to small fragments at 55°C (Fig. 3A). The rate of MHC degradation increased with the quantity of extract: The band of MHC disappeared after 20 hr, 30 min, or 10 min, as M/S = 0.2, 2.0, or 20.0, respectively. Amounts of digested products after 20 hr or 2 hr were reduced to traces, undetectable on the gel, as M/S = 2.0 and 20.0, respectively.

The same analysis was conducted with the crude extract from SAE (Fig. 3B). Unexpectedly, degradation activity of

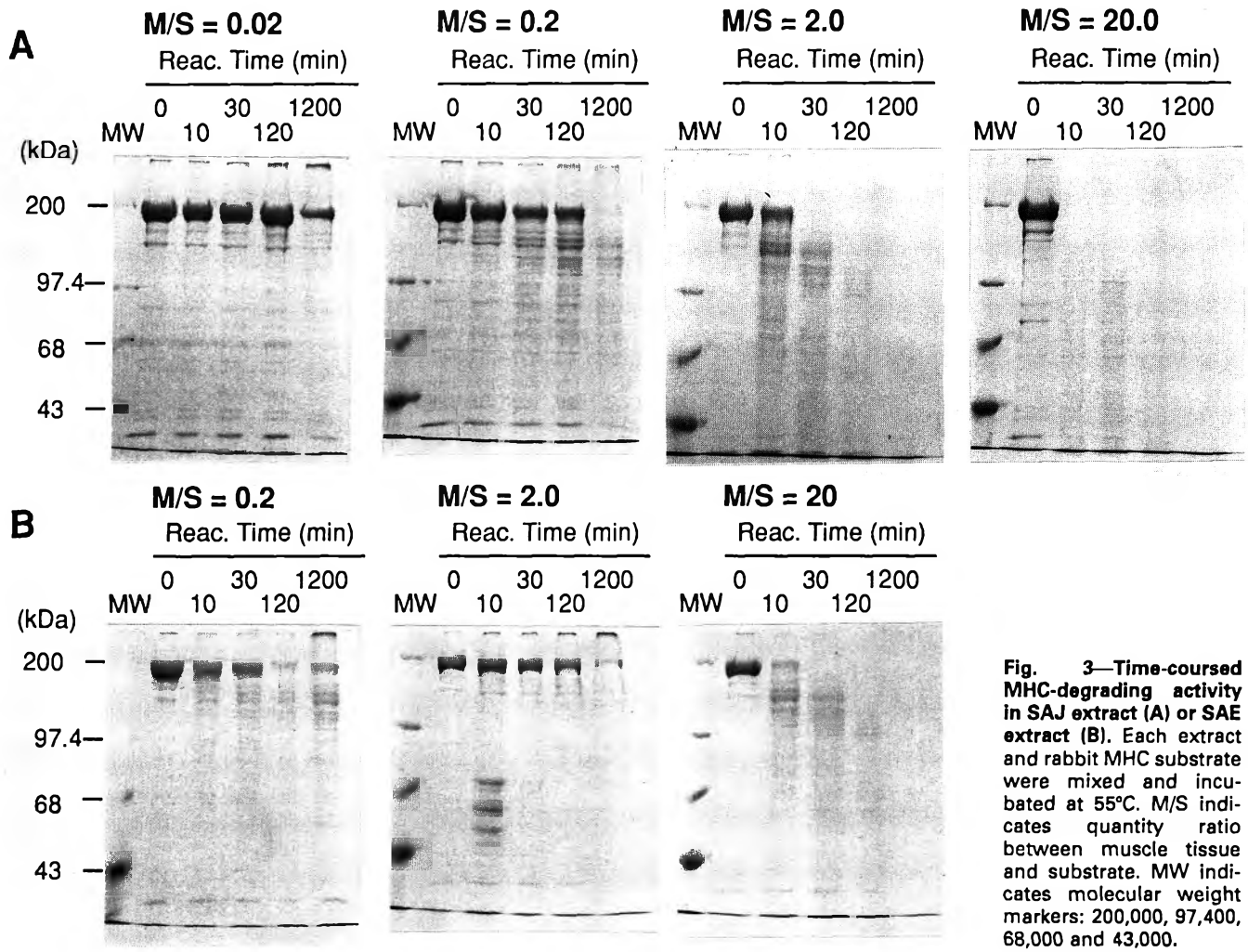

Fig. 3—Time-coursed MHC-degrading activity in SAJ extract (A) or SAE extract (B). Each extract and rabbit MHC substrate were mixed and incubated at 55°C. M/S indicates quantity ratio between muscle tissue and substrate. MW indicates molecular weight markers: 200,000, 97,400, 68,000 and 43,000.

MHC was also in SAE. The MHC-degrading activity in the muscle tissue of SAJ was higher than that of SAE. The proteolytic activities in both SAJ and SAE had similar profiles with regard to temperature-relationships (Fig. 4). Although activities were weak at 4°C, they were strongly increased at higher temperatures. The optimum pH of the activity of SAJ at 55°C was about 7.4, while that of SAE was >8.5 (Fig. 5). MHC-degrading proteinases effective at high temperatures occurred in both SAJ and SAE fillets.

To determine whether similar proteinase activity was also in raw fillets, degradation activities of MHC with crude extracts of RAJ and RAE were compared with that of the salted fillet. After incubation with a much higher quantity of extract (M/S = 20.0) for 20 hr at 55°C, bands of MHC were reduced to ≈10% with extracts of RAJ and disappeared completely with extracts of RAE. Consequently, proteinases similar to those in salted fillets may occur in raw fillets, although their activities were much weaker (see Fig. 3A and 3B).

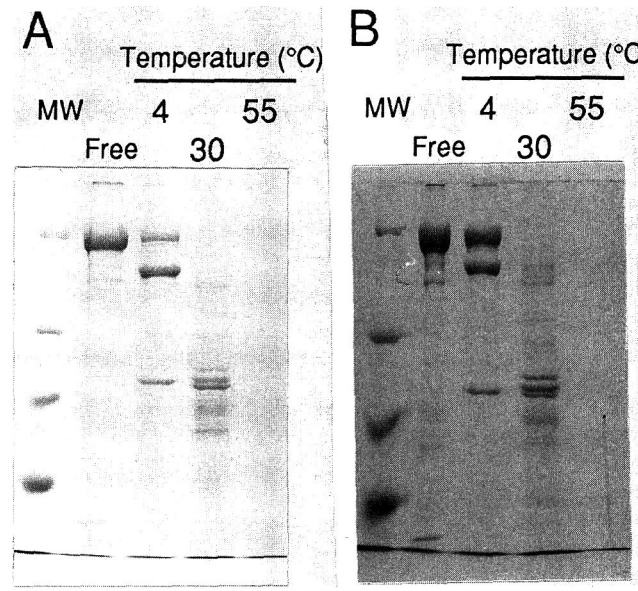

Fig. 4—Effect of temperature on MHC-degrading activity in SAJ extract (A) or SAE extract (B). Incubation without enzyme extract was performed as control. Molecular weight markers (MW) are as in Fig. 3.

Substrate specificity of the proteinases

The degradation of collagen, a major structural protein in muscle (besides myosin) by proteinase was determined (Fig. 6). To prevent thermal denaturation of collagen, the reaction was carried out at 25°C. The collagen was not degraded with the proteinases of SAJ and SAE, although the MHC was digested under the same conditions.

Activities with synthetic peptide substrates were compared (Table 2). Properties of both proteinases were similar, except that the specific activity per tissue weight of SAJ was three to five times higher than in SAE. Both proteinases specifically cleaved the peptide bond on the C-terminal side of arginine or lysine residues in modified di- to tetra-peptide substrates, such as z-Phe-Arg-MCA, Boc-Leu-Gly-Arg-MCA, Boc-Val-Leu-Lys-MCA and Boc-Leu-Ser-Thr-Arg-MCA. Activities of both

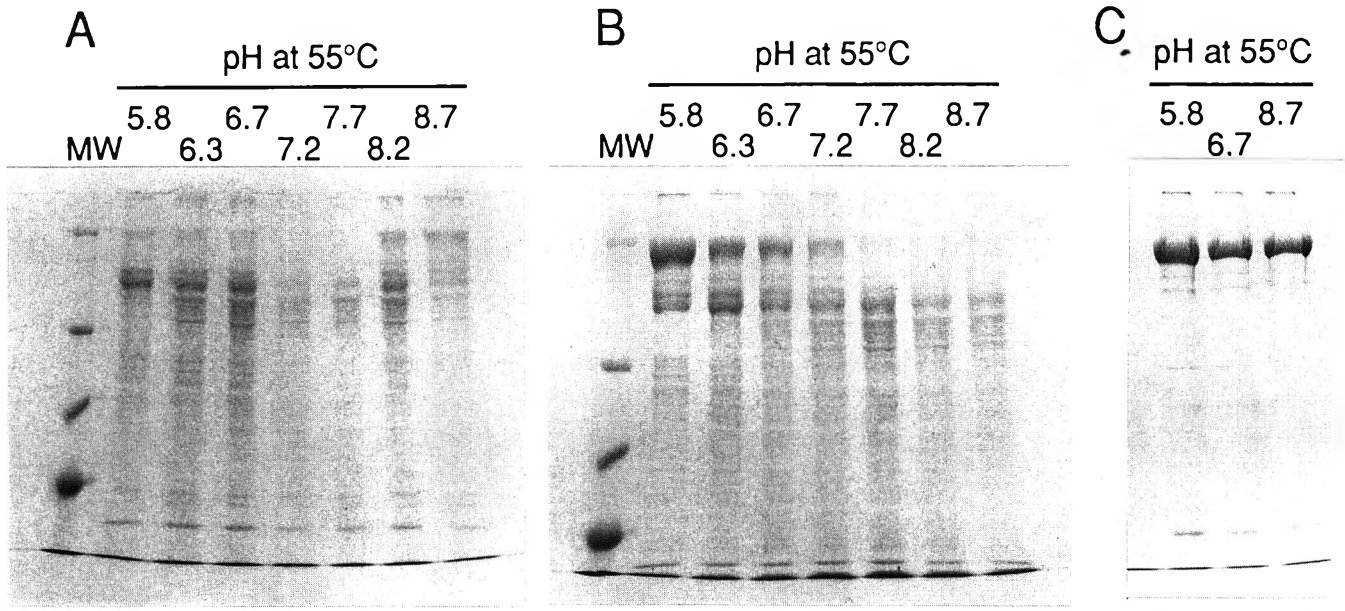

Fig. 5—Effect of pH on MHC-degrading activity in SAJ extract (A) or SAE extract (B). Incubations without enzyme extract (C) were performed as control. Molecular weight markers (MW) are as in Fig. 3.

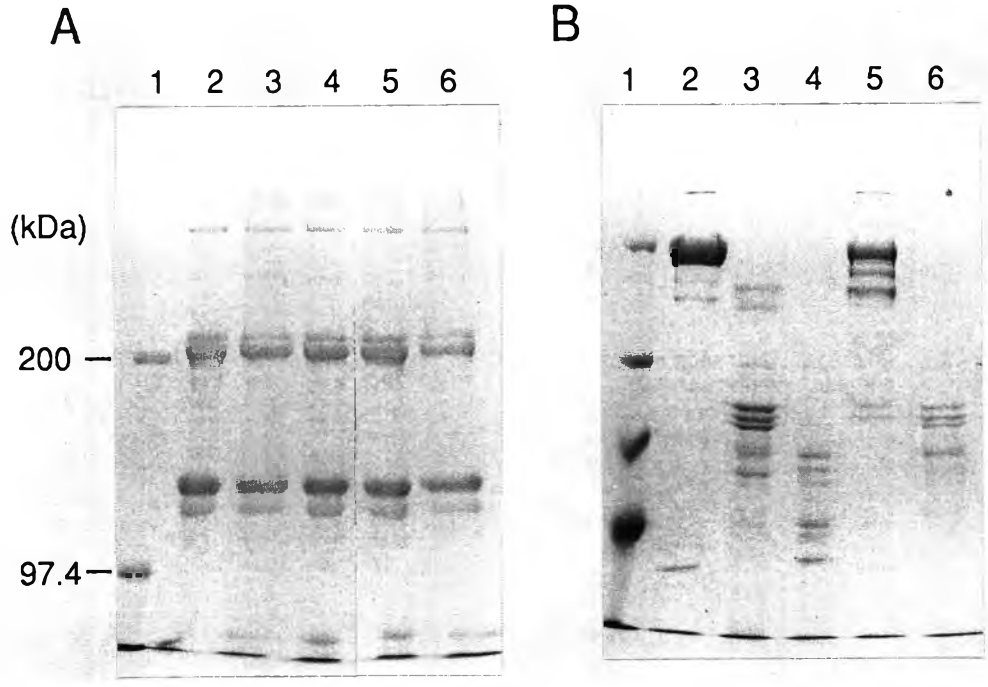

Fig. 6—Degradation activity of collagen in SAJ or SAE extract at 25°C (A). 1, molecular weight markers: 200,000 and 97,400; 2, collagen only; 3, collagen + SAJ extract (M/S = 2.0); 4, collagen + SAJ extract (M/S = 20.0); 5, collagen + SAE extract (M/S = 2.0); 6, collagen + SAE extract (M/S = 20.0). Degradation activities of MHC in extracts at 25°C (B) were measured as control. Each lane is numbered similar to those in A, except that the substrate was MHC. Molecular weight markers as in Fig. 3.

proteinases with Boc-Leu-Ser-Thr-Arg-MCA were especially high. In contrast, the proteinases did not cleave specific substrates of cathepsin H (Arg-MCA), trypsin (Bz-Arg-MCA), collagenase (Suc-Gly-Pro-Leu-Gly-Pro-MCA), chymotrypsin (Suc-Leu-Leu-Val-Tyr-MCA) or elastase (Suc-Ala-Pro-Ala-MCA).

The effect of protease inhibitors on proteinase activity (Fig. 7) showed soybean trypsin inhibitor (STI) and leupeptin interfered with activity of both proteinases. Although antipain inhibited the activity of the proteinase of SAE, its inhibition of the proteinase of SAJ fluctuated. TPCK inhibited only the activity of the proteinase of SAE, whereas E-64 and EDTA did not interfere with either proteinase.

These results indicated that proteinases of both SAJ and SAE were trypsin-like serine proteinases that were efficiently active at 55°C. Substrate activities of the proteinases may be more specific than that of trypsin because of the high selectivity of the length of the synthetic peptide substrates. These two proteinases of SAJ and SAE were not identical. They showed different pH dependence and inhibition profiles. The proteinase may be involved in the solubilization of SAJ fillet at high temperatures, since the degradation activity on MHC, *in vitro*, was very high, and the activity of SAJ was higher than that of SAE.

DISCUSSION

A TRYPSIN-LIKE SERINE PROTEINASE may cause solubilization in salted anchovy muscle. In fish muscle, many kinds of high-temperature-active latent proteinases, such as cathepsin D

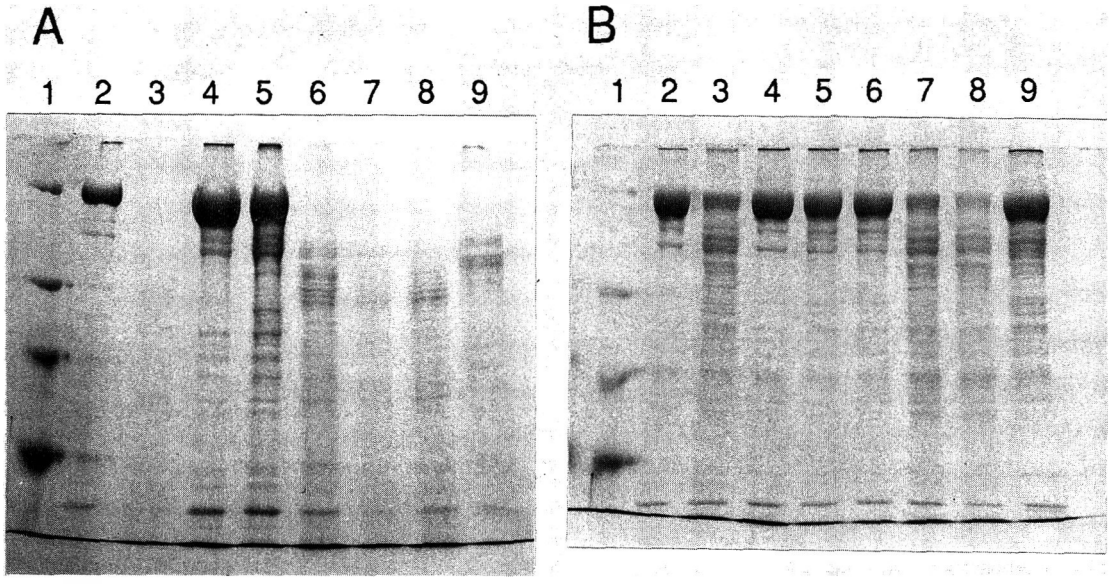

Fig. 7—Effect of various proteinase inhibitors on MHC-degrading activity in SAJ extract (A) or SAE extract (B). 1, molecular weight markers similar to those in Fig. 3; 2, MHC only; 3, MHC + enzyme extract; 4, MHC + enzyme extract + soybean trypsin inhibitor; 5, MHC + enzyme extract + leupeptin; 6, MHC + enzyme extract + antipain; 7, MHC + enzyme extract + E-64; 8, MHC + enzyme extract + EDTA; and 9, MHC + enzyme extract + TPCK.

Table 2—Substrate specificity of proteinases from SAJ and SAE on synthetic fluorogenic substrates

Substrate	Activity (pmol/min mg-tissue) SAJ[a]	SAE[b]
Boc-Val-Leu-Lys-MCA	24	5
Boc-Leu-Ser-Thr-Arg-MCA	169	32
Boc-Leu-Gly-Arg-MCA	34	8
z-Phe-Arg-MCA	24	7
Bz-Arg-MCA	0	0
Suc-Gly-Pro-Leu-Gly-Pro-MCA	0	0
Suc-Leu-Leu-Val-Tyr-MCA	0	0
Suc-Ala-Pro-Ala-MCA	0	0

[a] SAJ = salted anchovies from Japan.
[b] SAE = salted anchovies from southern European countries.

(Doke et al., 1980; Makinodan et al., 1987), neutral proteinases (Kinoshita et al., 1990) and alkaline proteinases (Makinodan and Ikeda, 1969; Iwata et al., 1973; Hase et al., 1980; Busconi et al., 1984; Doke and Minjoor, 1987; Makinodan et al., 1987; Toyohara et al., 1987; Yanagihara et al., 1991), have been reported. However, we found no reports on identification or characterization of proteinases from anchovy muscle, although chymotrypsin-like proteinases from anchovy viscera have been reported (Heu et al., 1991). Serine proteinases have been isolated from muscle of threadfin-bream (neutral proteinase) (Kinoshita et al., 1990), two species of white croaker (alkaline proteinases) (Busconi et al., 1984; Yanagihara et al., 1991) and shrimp (alkaline proteinase) (Doke and Ninjoor, 1987). The inhibition profile and optimal pH of these proteinases did not conform to those of the anchovy proteinase. Thus it is likely different from proteinases in other fish muscles.

The activity of proteinase in anchovy muscle probably increases during the salting process. The molecular mechanism of this activity increase is likely that either a potent inhibitor of the proteinase diminishes, or a latent proteinase or precursor changes to an active form. Serine proteinase inhibitors have been isolated from white croaker muscle (Hara et al., 1985; Busconi et al., 1984) and carp muscle (Hara and Ishihara, 1987). Although a similar proteinase inhibitor has not been reported in anchovy muscle, such inhibitor could possibly be used to prevent solubilization of fillets. Alternatively, the latent proteinase from threadfin-bream muscle reportedly was activated by heat treatment (Kinoshita et al., 1990). The molecular mechanism of the activation is unknown. In order to clarify the mechanism by which activity of the proteinase increases, a comparison of isolated proteinases from anchovy muscles of both SAJ and RAJ is essential. It may also be necessary to test whether an inhibitor exists in either type of anchovy muscle. Proteolysis by bacteria growing during the salting process is also possible. However, the effect of such bacterial proteolysis is not clear for preventing mechanical destruction of muscle tissue. To find the most efficient way of inactivating the proteinase, a detailed characterization of the proteinase is essential.

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—Continued on page 791

Potassium Bromate Effects on Gel-forming Ability of Pacific Whiting Surimi

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ABSTRACT

Physical and chemical analyses defined the reinforced oxidation of sulfhydryl groups on the myofibrillar proteins to disulfide bonds by bromate. Electrophoretic studies demonstrated myosin degradation during heat-setting and its protection from proteinase attack by bromate. A bromate level of 0.075% inactivated 89.9% of the proteinase activity in surimi sols. Maximum gel hardness was 0.15%. Major increases in cohesiveness and elasticity were achieved at levels ≤ 0.075%, brittleness occurred at levels ≥0.1%. Surimi gels with AA folding test grade was achieved with 0.15%. Potassium bromate probably improved surimi gelation through proteinase inactivation and reinforced disulfide formation during heat-setting.

Key Words: potassium bromate, surimi, gel forming, whiting

INTRODUCTION

PACIFIC WHITING (*Merluccius productus*) is an abundant fish resource off the west coast of the United States. However, it is underutilized due to its soft-textured flash caused by a myxosporidian-induced proteolysis that breaks down tissue (Patashnik et al., 1982; Dark, 1985). Whiting has not been generally used as food, but its abundance suggests it could be an important source of high-quality protein (Dassow et al., 1970; Erickson et al., 1983). It is considered to have potential as a protein source for analog seafood products from a surimi intermediate. Such products are prepared largely from Alaska pollock (Groninger et al., 1985).

High proteolytic activity occurs in whiting muscle and minced flesh. Most such activity continues after the flesh has been processed into surimi, which results in failure to gel by the partially hydrolyzed surimi proteins upon heat processing (Chang-Lee et al., 1989). Pacheco-Aguilar et al. (1989) reported that whiting surimi lacked strong gel-forming ability, despite the degree of washing. Additives such as dry egg white and potato starch have been used in formulation of surimi sols to achieve stronger heat-induced gelation (Chang-Lee et al., 1989). Schmidt (1981) reported that intact myosin molecule was required to produce strong and elastic gels. Such characteristics are affected by the degree of intra- and inter-strand cross-linking. Therefore, maintaining the original molecular size of the myosin heavy chains is of foremost concern.

Methods for maintaining myosin integrity throughout the heat-induced gelation appears to be a logical way to increase gel strength characteristics. Several methods to prevent textural changes in whiting flesh and surimi have been evaluated (Spinelli and Steinberg, 1978), including inactivation of proteinase activity by chemicals and enzyme inhibitors (Miller and Spinelli, 1982; Groninger et al., 1985; Chang-Lee et al., 1989). Miller and Spinelli (1982) demonstrated that potassium bromate, (an oxidizing agent) mixed into ground parasitized whiting muscle at < 0.5% caused nearly complete proteinase inactivation. Their studies using sulfhydryl-binding compounds supported a thiol proteinase as possibly response for texture deterioration of whiting flesh. Inhibition of the proteinase activity in whiting surimi, by potassium bromate, alone or mixed with dry egg white, was also documented by Groninger et al. (1985). Potassium bromate has not been approved as an additive in surimi products, but it has applications in the baking industry at a ≤50 ppm (Matz, 1972; Fitchett and Frazier, 1985; Codex Alimentarius, 1990).

Utilization of whiting on a regular basis for production of surimi-based products by chemical and/or biochemical modification of the protein system was our ultimate goal. The objective was to elucidate the mechanisms of action of potassium bromate in improvement of the gel-forming ability of Pacific whiting surimi.

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MATERIALS & METHODS

Fish and minced flesh

Fresh Pacific whiting, no more than 1 day post-catch were obtained from local seafood processing plants in Astoria, OR, during the summer of 1988. All experimental fish were held in crushed ice prior to processing.

Round fish were processed into skinless fillets by hand which were then thoroughly washed. Visceral and blood contamination was carefully avoided. Individuals with musculature visually infested with black hair-like pseudocysts and those with extremely soft or mushy texture were discarded. Fillets were ground through a 9.5-mm plate.

Washing, dewatering and refining of minced flesh

Minced flesh was washed twice with cold water, using a water-flesh ratio of 3:1. The water-flesh mixture was gently stirred for 5 min and allowed to settle another 5 min. Washing was at ≤ 10°C. Dewatering was carried out in a screwpress dehydrator Model DS-8 (Ikeuchi Tekkosho/Sato, Tokyo, Japan) with speed setting 0 6 (6 rpm) to dewater flesh after the first wash, and speed setting 0.06 (0.6 rpm) to obtain maximum dewatering following the second wash. The flesh was then introduced into a blast freezer (−30°C) for 20 min to reduce its temperature to 2–3°C before refining. Refining was carried out in an Ikeuchi flesh strainer Model S1 (Ikeuchi Tekkosho/Akashi, Tokyo, Japan). Refined flesh was introduced into a blast freezer (−30°C) for 20 min to reduce its temperature to 2–3°C prior to surimi preparation.

Surimi preparation

Refined flesh was mixed with cryoprotectants to yield the following final composition: 91.7% refined flesh, 4% sorbitol, 4% sucrose, and 0.3% Brifisol S-1 (a mixture of food-grade-quality sodium acid pyrophosphate, sodium pyrophosphate and sodium polyphosphate; BK Ladenburg Corp., N. Hollywood, CA). Mixing was carried out for 3–4 min in a Hobart silent cutter Model VCM (The Hobart Manufacturing Co., Troy, OH). Temperature during mixing was ≤10°C. Aliquots of surimi were packed into individual polypropylene (10.5 × 8.5 × 5.5 cm) trays (Kings Plastics Inc., Orange, CA) with ≈600 g surimi each, and vacuum-sealed in a moisture/vapor-proof film (Cryovac P640B with nylon base, Saran^R barrier and low-density polyethylene sealant, Cryovac Corp., Duncan, SC).

Sol preparation

Sol formulation contained 2.8% NaCl and eight different concentrations of potassium bromate (0.0, 0.025%, 0.05%, 0.075%, 0.1%, 0.15%, 0.2% and 0.25%). Potassium bromate replaced an equal amount

of NaCl in the formulation, to maintain NaCl + bromate content of 2.8%. Potassium bromate was dissolved in the cold water complement of the formulation and added after solid NaCl at the beginning of mixing. Sol moisture content was adjusted to 78% with the required amount of ice. The sum of formulation components equaled 100% and a 450-g working preparation was used. Sols were prepared by blending the working formulation in a Kitchen-Aid food processor Model KFP (Hobart Corp., Troy, OH) for 2-3 min, which allowed achieving a temperature of ≤4°C. Each sol was packed into two polypropylene (12 × 7.5 × 2.5 cm) trays (Kings Plastics Inc., Orange, CA) and vacuum-sealed in a moisture/vapor-proof film (Cryovac P640B with nylon base, Saran^R barrier and low-density polyethylene sealant, Cryovac Corp., Duncan, SC). Three sol replicates were prepared containing each of the 8 bromate levels.

Gel preparation

A sample of each sol was heat-set in a water bath under the following conditions: (a) 90°C/40 min and (b) 40°C/60 min + 90°C/20 min. Time from initiation of sol formulation to time sols were placed in the water bath was kept <15 min. Heat-set gels were immediately chilled to <10°C in an ice-water mixture and held overnight at 2–4°C prior to texture evaluation. A total of 48 gels were prepared (3 sol replications × 8 bromate levels × 2 heat-set treatments).

Texture profile analysis (TPA) and folding test (FT)

Fish gels were tempered for 30–40 min to ambient temperature (≈23°C) prior to analysis. Six cylinder-shaped samples of uniform dimensions (1.5 cm diam × 1 cm long) were cut from each gel using a sharp-edged copper tube. Texture was measured in an Instron universal testing machine Model TM-M with the 5.6 mm diam. compression plunge attached to a 22.7-kg load cell (Instron Corp., Canton, MA), following the recommendations of Breene (1975). Crosshead and chart speeds were set at 50 and 500 mm/min, respectively. Compression forces at 90% (single bite analysis) of the original gel sample length were used to compute gel hardness and brittleness. A 50% (double bite analysis) was used for cohesiveness and elasticity. Results were reported as described by Abbot (1972). For each textural parameter, 3 samples/gel/replicate were evaluated. The gel-forming capacity of surimi was also evaluated using the folding test described by Kudo et al. (1973). Ten samples/gel/replicate were evaluated.

Chemical analyses and protein extraction

Proximate composite was determined in duplicated samples using standard procedures (AOAC, 1984). The pH of samples was determined using an Orion digital pH meter Model M240 (Orion Research Inc., Cambridge, MA) on 10 g of material homogenized in a Kinematica Kriens-LU homogenizer Model CH-6010 (Brinkmann Instruments Co., Westbury, NY) with 90 mL distilled water for 30 sec.

Soluble protein in 0.6 M KCl and actomyosin (AM) were separated as described by Jiang and Lee (1985) and Jiang et al. (1985). Protein concentration was determined by the biuret method (Gornall et al., 1949), and reported as mg protein/g sample.

Ca^{++}-ATPase activity of actomyosin and proteolytic activity

ATPase activity was determined as described by Jiang et al. (1985). To achieve measurable levels of released inorganic phosphorus a 6-min incubation period was used, as recommended by Scott et al. (1988). Total activity was reported as μM Pi/min/mg AM.

Proteolytic activity in surimi sols was determined by the autolysis procedure outlined by Groninger et al. (1985), with some modifications. Four separated 3.0-g samples were weighed into 25-mL Corex tubes. To each tube, 3 mL of cold 0.1M NaCl was added while mixing thoroughly with a stirring rod. Exactly 10 min after initiation of sol preparation, three of the samples were incubated in an agitated water bath at 60°C/30 min. The remaining sample (control) was held in ice. After incubation, 6 mL of a cold 10% trichloroacetic acid (TCA) solution was added to each tube to terminate the reaction. The TCA-treated mixtures were held for 30 min at 2–4°C, then filtered through Whatman No. 1 filter paper. The amount of released tyrosine (Tyr) in each filtrate was determined by the Folin-Ciocalteau phenol reagent procedure described by Ceriotti and Spindrio (1957). Proteolytic activity was reported as μg Tyr/min/g protein.

Sulfhydryl groups

Total and reactive sulfhydryl groups were determined in actomyosin extracted from surimi sols for each level of bromate employed, as recommended by Ellman (1958) and Buttkus (1971), with some modifications. For total sulfhydryl groups (T-SH), 5 mL of actomyosin solution (1-5 mg/mL) was added to 2.4 mL of extraction solution (0.1M phosphate buffer, pH 7.0, 6 mM EDTA, 0.6 M KCL, 8M urea) and 0.1 mL of the Ellman's (Sigma Chemical Co., St. Louis, MO) reagent (0.004M 5,5'-dithiobis-2-nitrobenzoic acid in extraction solution) was incorporated. The reaction was allowed to proceed for 30 min at room temperature (≈23°C) after which the initial absorbance at 412 nm was recorded. To calculate the corrected absorbance, two blanks were prepared as recommended by Hofmann and Hamm (1978). Those were a dye blank (all reagents, but without sample) and the sample blank (all the reagents, but without Ellman's reagent). The corrected absorbance was computed by subtracting the absorbance of the two blanks from the initial absorbance. Results were reported as mols SH/1 × 10^5 g actomyosin. For determination of the reactive sulfhydryl content (R-SH), the extraction solution did not contain urea.

Viscosity

Viscosity was determined using a 40-g homogenate of surimi sol in 160 mL of a 3% NaCl solution (0-4°C). This was prepared with a Kinematica Kriens-LU homogenizer Model CH-6010 (Brinkmann Instruments Co., Westbury, NY) in a 200-mL container, chilled in an ice bath and fitted with parafilm to prevent foaming. Homogenate was transferred to a 250-mL beaker and the viscosity measured with a Brookfield viscometer Model RTV 100 (Brookfield Engineering Lab., Stoughton, MA), operated at 10 rpm and equipped with a No. 3 spindle.

SDS-PAGE electrophoresis

Polyacrylamide gel electrophoresis (PAGE), using a dissociating buffer system (sodium dodecyl sulfate, SDS) and 5% gel was performed according to Weber and Osborne (1969). A Bio-Rad Rube Gel Chamber Model 1250A (Bio-Rad Laboratories, Richmond, CA) was used. A 43-mL aliquot of extraction solution (same as used for T-SH determination) and 1 mL of 2-mercaptoethanol (ME) were added to 4g of surimi gel, followed by homogenization for 2 min with a Kinematica Kriens-LU homogenizer Model CH-6010 (Brinkman Instruments Co., Westbury, NY). This treatment completely solubilized the gels. To 1 mL of this solution (10 mg protein/mL), 3 mL of sample buffer solution (2.5 mL distilled water, 2.5 mL 1M phosphate buffer, pH 7.2, 0.8 mL glycerol, 1.6 mL 10% ME and 0.05% bromophenol blue) were added and the mixture was boiled for 3 min. Total protein concentration was adjusted to 2.25 μg/μL. Fifty μL of sample was applied to the top of the gels. Electrophoretic runs were performed at room temperature at 3 mA per tube for 20 min, increased afterwards at 8 mA per tube for ≈ 6 hr. A high-range-molecular-weight protein standard solution containing myosin, beta-galactosidase, phosphorylase b, bovine serum albumin and ovalbumin (Bio-Rad Laboratories, Richmond, CA) diluted 1:100 with sample buffer solution was used as a marker. Resulting gels were analyzed by comparing densitometric tracings of the protein bands in a Quick Scan densitometer Model 1020A (Helena Laboratories, Beaumont, TX). The approximate molecular weights of protein bands were calculated as described by Weber and Osborne (1969).

Statistical analyses

A complete randomized design was used. Analyses were carried out using ANOVA and simple regression analysis with a STAT Plus Program (Madigan and Lawrence, 1984). Duncan's multiple range test was used for multiple comparisons of means. A 2 × 8 factorial arrangement was used to evaluate interactions between heat-setting treatments and bromate levels. Significance was established at $p \leq 0.05$.

RESULTS & DISCUSSION

MANUALLY SEPARATED SKIN-FREE FILLETS yielded 31.6% minced flesh, slightly lower than reported by Crawford et al. (1979) (35.5%), Pacheco-Aguilar et al. (1989) (32.4%) and Chang-Lee (1988) (34.7%), using mechanical separation of

Table 1—Effect of varying levels of $KBrO_3$ on the protein extractability, sulfhydryl status, viscosity and Ca^{++}-ATPase activity of surimi sols at 2-4°C

$KBrO_3$(%)	SSP[a]	AM[b]	T-SH[c]	R-SH[d]	Viscosity[e]	ATPase[f]
0.000	117.4[g] (5.3)	105.2[g] (10.4)	3.7[g] (0.08)	2.6[g] (0.05)	3770[g] (42.4)	11.2 (1.34)
0.025	108.5[g] (6.9)	77.0[h] (4.4)	3.2[h] (0.09)	2.1[h] (0.06)	5415[h] (91.9)	2.5 (3.3)
0.050	109.9[g] (7.4)	77.9[h] (6.2)	3.1[hi] (0.06)	2.0[h] (0.02)	4220[i] (14.1)	0.0
0.100	86.0[h] (8.5)	38.4[i] (3.9)	2.9[i] (0.08)	2.0[h] (0.06)	3430[j] (113.1)	0.0
0.150	66.5[i] (3.5)	7.1[j] (0.35)	2.4[j] (0.06)	1.5[i] (0.02)	3395[j] (120.2)	0.0
0.200	39.7[j] (3.0)	6.2[j] (0.46)	1.9[k] (0.04)	1.3[j] (0.02)	3330[j] (42.4)	0.0

[a] Salt-soluble protein (mg/g sample), n=3.
[b] Actomyosin (mg/g sample), n=3.
[c] Total sulfhydryl (mol SH/1×10^5g actomyosin), n=2.
[d] Reactive sulfhydryl (mol SH/1×10^5g actomyosin, n=2.
[e] Viscosity (Cp), n=2.
[f] Ca^{++}-ATPase activity (μM Pi/min/mg actomyosin), n=3.
[g-k] Figures in columns with different exponent letters are significantly different ($P<0.05$). ( )=S.D.

Table 2—Effect of $KBrO_3$ addition on inhibition of total proteinase activity in surimi sols prepared from Pacific whiting surimi

$KBrO_3$ (%)	Proteinase activity (μg tyrosine/min/g protein)	Inhibition (%)
0.000	231.8 (38.5)[a]	0.0
0.025	63.8 (12.6)[b]	72.5
0.050	38.9 (10.6)[c]	83.2
0.075	23.5 (3.7)[c,d]	89.9
0.100	22.1 (8.5)[c,d]	90.5
0.150	13.1 (3.1)[d]	94.3
0.200	14.9 (4.5)[d]	93.6
0.250	14.4 (7.0)[d]	93.8

[a-d] n=3; ( )=s.d. Figures within column with same exponent letter are not significantly different ($p \geq 0.05$).

skin-on fillets. Manual separation of skin-free fillets was more labor-intensive, but allowed more effective culling of fish with mixosporidian infestation and soft texture. Water requirements amounted to only six times the weight of minced flesh, which represents a 60% reduction in water usage from the conventional surimi process described by Lee (1984). The elimination of soft and infected fish during processing was important in the high surimi yield (26.3%). It reduced losses during the washing and dewatering operations. Surimi composition was: 78.6% moisture, 12.5% protein, 0.7% lipids and 0.5% ash, confirming results reported by Chang-Lee (1988).

The surimi lacked gel-forming ability when no bromate was added during sol preparation. The short polypeptide chains resulting from the proteolytic activity further activated during heat treatment, were believed to be ineffective for a continuous three-dimensional network. Schmidt (1981) observed that the intact myosin molecule was required to produce strong and elastic gels. The effects of bromate addition during sol preparation (Table 1) indicated bromate probably induced the oxidation of sulfhydryl groups in the surimi protein system to inter-chain disulfide bonds to form larger protein aggregates. The extractable salt-soluble protein and actomyosin from sols were linearly reduced ($r^2=0.9726$ and 0.9380, respectively) as bromate concentration increased. Results did not indicate that bromate reduced surimi protein extractability. The surimi protein system was already in its sol form when bromate was added. No heat was required for onset of gelation of bromate-treated surimi. After 2–3 hr storage at 2–5°C prior to heat-setting a weak elastic gel was observed.

Total sulfhydryl content of 3.7 mols SH/1 × 10^5 g of actomyosin, determined in whiting surimi sols, although analyzed by a different procedure, was well below the level reported by Hofmann and Hamm (1978) for fish (6.7 mols/1 × 10^5 g actomyosin) (Table 1). This apparent low level of total sulfhydryl could be due to an intrinsic property of whiting muscle actomyosin or to the 6 mo frozen storage period to which surimi was subjected prior to the study. Cecil and McPhee (1959) reported that, under mild conditions, sulfhydryl groups undergo chemical oxidation to disulfides. In our study, both total ($r^2=0.9595$) and reactive ($r^2=0.8714$) sulfhydryl content of actomyosin in surimi sols decreased linearly as bromate addition increased. The probability of a significant further oxidation of newly formed disulfide bonds by bromate to sulfinic (RSO_2H) and/or sulfonic (RSO_3H) acids, was considered low, based upon results for protein extractability and viscosity.

The viscosity of surimi sols with no bromate was assumed to be an indicator of the starting linearity and length of the protein molecules in suspension. We assumed that bromate at 0.025% promoted the formation of overall linear protein aggregates due to the formation of inter-chain disulfide bonds, thus increasing the viscosity of sols. This may have been accomplished through the aggregation of short polypeptides generated by proteinase activity. We hypothesized that, at levels >0.05%, the oxidative action of bromate disrupted linearity by forming sphere-like aggregates, thus reducing sol viscosity. Results tended to confirm this hypothesis. The maximum degree of aggregation possible at 2–5°C that was reflected in a change on viscosity measurements appeared to be complete at the 0.1% bromate level (Table 1).

Sulfhydryl groups in the globular head of myosin have been reported to be involved in the ATPase activity of the actomyosin complex (Young, 1969; Taylor, 1972). According to Seki and Hasegawa (1978), the loss of Ca^{++}-ATPase activity is due to a modification of the actomyosin complex through oxidation of the sulfhydryl groups. Complete inactivation was reported when seven sulfhydryl groups per molecule were oxidized. In our study, a complete inactivation of the Ca^{++}-ATPase by 0.05% bromate suggested its involvement in the oxidation of sulfhydryl groups. Results (Table 1) were consistent with the promotion of oxidation of sulfhydryl groups of the surimi protein/polypeptide system to inter-chain disulfide bonds by potassium bromate to form larger aggregates.

Proteinase activity in different sublots of prepared surimi (Table 2) showed the initial high activity agreed with that reported by Chang-Lee (1988) for whiting surimi. The efficiency of removing proteinase from the minced flesh of whiting by washing also appeared to be almost like that of other fish species as reported by Makinodan and Ikeda (1971). In our study, incorporation of bromate up to 0.075% into surimi sols inhibited proteinase activity in a concentration-dependent manner. Higher bromate levels did not produce significant ($p > 0.05$) further inhibition. Results were similar to those reported by Groninger et al. (1985) for whiting surimi, where 40%, 77% and 86% proteinase inhibitions were observed with bromate levels of 0.02%, 0.04% and 0.05%, respectively. However, Miller and Spinelli (1982) reported, for unwashed ground whiting muscle, 47% and 63% proteinase inhibitions with 0.025% and 0.05% bromate levels, respectively.

From the data we postulated that the observed proteinase inhibition induced by bromate could be explained by the following possible mechanisms, alone or in combination. A direct proteinase inhibition may ocur through the oxidation of the cysteine residue at the active site. An indirect inhibition through covalent bond formation could occur between sulfhydryl groups on enzyme and surimi proteins. An indirect inhibition may occur through steric effects, since formed protein aggregates would be larger and more rigid and would not form enzyme-substrate complexes. Based upon levels of proteinase inhibition achieved by bromate (90-94%), a thiol proteinase appears to be the predominant proteinase system in whiting surimi. This was supported by the observation of Miller and Spinelli (1982) who stated that the enzyme producing the characteristic softening of whiting muscle was a thiol proteinase.

Under our conditions, complete proteinase inhibition was never achieved, even with the highest level of bromate. The lack of a significant ($p<0.05$) additional enzyme inhibition at bromate levels >0.075% indicated the presence of a proteinase in whiting surimi with other than thiol-active sites. Such additional proteinase could be a serine or an alkaline proteinase as suggested by Groninger et al. (1985), whose activity could be enhanced by the oxidizing conditions with bromate. This enzyme might be very active during surimi sol heat processing at <60°C.

The inhibitory effect of bromate on proteinase activity and the preservation of the myosin molecule during heat processing was clearly illustrated by SDS-PAGE of whiting surimi sols and heat-set gels treated with SDS, 2-mercaptoethanol and urea (Fig. 1a–1e). The electrophoretic separation of surimi sol proteins (Fig. 1a) showed a broad peak in the molecular range 221,000d to 161,000d in which the myosin heavy chain (mol. wt. 200,000 d) was contained. The peak with molecular weight >200,000d could be a result of covalent protein aggregation, other than disulfide bond formation (i.e., mediated by formaldehyde or malonaldehyde), occurring during surimi frozen storage, as proposed by Matthews et al. (1961) and Buttkus (1970). Peaks with molecular weight <200,000 d may be polypeptides resulting from early hydrolysis of myosin at lower temperatures. Heat treatment of the sol (40°C/60 min + 90°C/20 min) without bromate resulted in almost total disappearance of the broad peak containing myosin (Fig. 1b). In addition, several low-molecular-weight peaks in the range of 162,000d to 92,000d appeared, as the result of the intense proteolysis during heat-setting.

The addition of bromate showed an effect on recovery of proteins in the molecular range 200,000d to 100,000d. A distinct myosin peak appeared with addition of 0.025% (Fig. 1c). As bromate level increased to 0.25%, the quantity of myosin recovered from heat-set gels increased, while the six adjacent peaks (lower molecular weights) declined (Fig. 1c–e). At bromate concentration 0.15% or higher, recovered myosin clearly exceeded the concentration of the 6 adjacent peaks. This result strongly suggested that proteins comprising peaks 1 to 6 (Fig. 1c) were large protein fragments, which originated through proteolysis of myosin. We assumed that the height of the myosin peak (Fig. 1c–e) was proportional to myosin recovery after heat treatment. Based upon this assumption, a 6.3 higher fold recovery of myosin from heat-set gels was observed as bromate levels were increased from 0% to 0.25% in the sol formulation (data not shown). The inhibition of proteinase activity by the oxidative action of bromate preserved myosin during heat-setting.

The level of bromate in sols brought about more effective gel-forming ability of whiting surimi. Factorial analysis of variance revealed that varying levels of bromate improved ($p<0.001$) the texture profile analysis (TPA) parameters of hardness, brittleness, elasticity and cohesiveness (Tables 3 and 4). The heat treatment effect was significant for brittleness only (Table 3). For all the TPA parameters, the interaction of main effects was significant ($p<0.05$). Gels from the two-stage heat-set regime, with no bromate added, showed lower texture values as a result of promoted proteolysis during the incubation period of 40°C for 60 min in the heat treatment. A bromate level of 0.05% was required to overcome this disadvantage, which corresponded to an enzyme inhibition level of 83.2% (Table 2).

Optimization of elasticity and cohesiveness of gel strength (Table 4) required maintenance of a portion of the intact sol proteins away from proteinase reactions. Major increases in these functions occurred at very low levels of bromate (0.025%) that afforded only a 72.49% inactivation of proteinase (Table 2). Results suggested that proteinase inactivation was the primary mechanism for improving elasticity and cohesiveness. The strengthening of disulfide formation was relatively less important. Conversely the hardness and brittleness

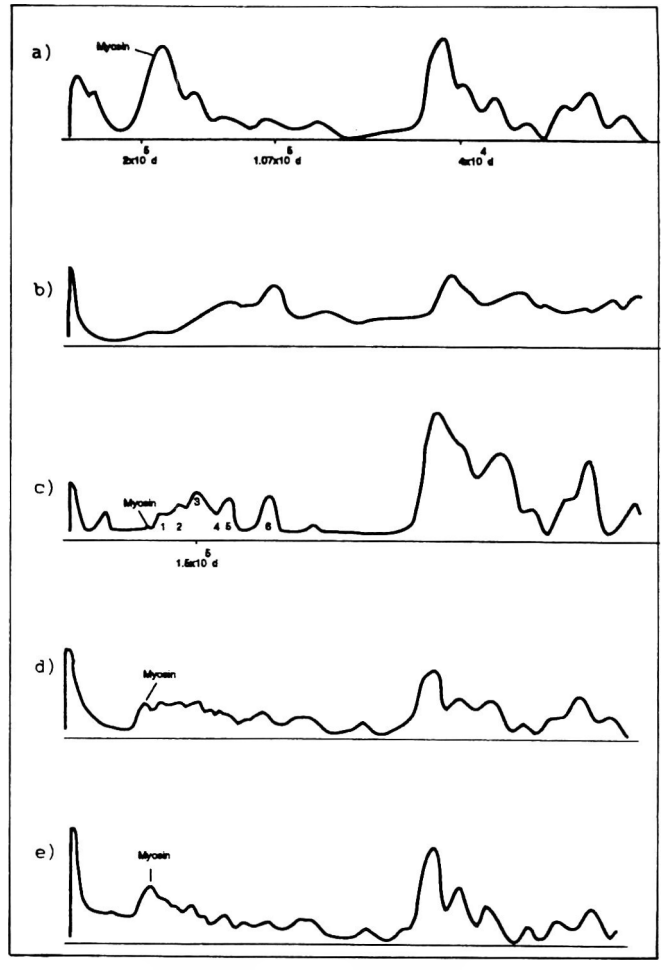

Fig. 1—SDS-PAGE of 2-mercaptoethanol- and urea-treated sols and heat-set gels (40°C/60 min + 90°C/20 min) containing varying levels of $KBrO_3$. (a) Surimi sol; (b) control gel (0% $KBrO_3$); (c) gel with 0.025% $KBrO_3$; (d) gel with 0.1% $KBrO_3$; (e) gel with 0.25% $KBrO_3$.

Table 3—Hardness and brittleness (N/g)[a] of gels heat-set under two temperature conditions as related to levels of $KBrO_3$

$KBrO_3$ (%)	Hardness		Brittleness	
	One-stage[b]	Two-stage[c]	One-stage[b]	Two-stage[c]
0.000	65.8^{i}	46.3^{h}	0.6^{h}	0.5^{h}
0.025	69.8^{i}	59.1^{hi}	2.5^{h}	2.3^{h}
0.050	67.4^{i}	72.0^{i}	3.9^{hi}	5.0^{hi}
0.075	73.3^{i}	89.4^{j}	5.2^{hi}	9.7^{hi}
0.100	92.1^{ij}	108.2^{d}	10.3^{hi}	17.5^{ij}
0.150	117.7^{de}	129.9^{ef}	26.9^{i}	62.8^{e}
0.200	128.6^{ef}	135.5^{fg}	43.5^{d}	93.3^{f}
0.250	130.8^{ef}	148.8^{g}	69.3^{e}	112.8^{g}

Analysis of variance (2×8 factorial arrangement)

	Significance	Significance
Heat treatment (H)	P > 0.050	P < 0.001
$KBrO_3$ level (%) (B)	P < 0.001	P < 0.001
Interaction (H×B)	P = 0.014	P < 0.010

[a] N/g = Newtons/g sample.
[b] 90°C for 40 min.
[c] 40°C for 60 min + 90°C for 20 min.
[d-j] Mean values in both columns for each texture parameter with same exponent letter are not significantly different ($p > 0.05$).

functions of gel strength were only optimized when sol proteins were protected from proteinase reactions and a strengthening of disulfide formation was thus accomplished by levels of bromate in excess of those required for proteinase inactivation (Table 3). A maximum level of hardness possible with bromate occurred at 0.15%. No maximum degree of brittleness was observed within the range of bromate levels tested.

Folding test grade was a function of proteinase inactivation and, to a lesser degree, reinforced disulfide formation (Table

Table 4—Elasticity (%), cohesiveness (%) and folding test grade of gels heat-set under two temperature conditions as related levels of $KBrO_3$

$KBrO_3$ (%)	Elasticity		Cohesiveness		Folding test	
	One-stage[a]	Two-stage[b]	One-stage[a]	Two-stage[b]	One-stage[a]	Two-stage[b]
0.000	49.1[l]	39.7[k]	32.7[l]	23.3[k]	1.0[k]	1.0[k]
0.025	84.9[cd]	83.5[c]	60.5[c]	57.8[c]	1.5[l]	1.5[l]
0.050	85.2[cd]	86.2[cde]	77.8[d]	78.5[de]	2.1[c]	2.2[c]
0.075	85.0[cd]	88.2[ef]	78.3[de]	81.0[def]	2.5[c]	3.3[d]
0.100	86.7[de]	89.2[ef]	81.8[efg]	84.2[g]	3.7[de]	4.1[e]
0.150	92.1[gh]	93.3[ghi]	88.5[hi]	91.7[ij]	4.6[f]	4.9[f]
0.200	90.7[fg]	94.2[hi]	85.5[gh]	90.3[ij]	4.8[f]	5.0[f]
0.250	95.0[i]	96.5[i]	91.9[ij]	93.2[j]	5.0[f]	5.0[f]
	Analysis of variance (2×8 factorial arrangement)					
	Significance		Significance		Significance	
Heat treatment (H)	$P > 0.050$		$P > 0.050$		$P = 0.019$	
$KBrO_3$ level (%) (B)	$P < 0.010$		$P < 0.010$		$P < 0.001$	
Interaction (H×B)	$P < 0.010$		$P < 0.010$		$P > 0.050$	

[a] 90°C for 40 min.
[b] 40°C for 60 min + 90°C for 20 min.
[c-l] Mean values in both columns for each texture parameter with same exponent letter are not significantly different ($p > 0.05$).

Table 5—Coefficients of determination (R^2) for the regression of texture profile analysis parameters, folding test scores, proteinase activity[a] and myosin recovery[b] on $KBrO_3$ levels and other variables

	Factor (x)				
	$KBrO_3$ level[c]		Proteinase activity		Myosin recovery
Factor (y)	One-stage[h]	Two-Stage[i]	One-Stage[h]	Two-stage[i]	Two-stage[i]
Hardness (N/g)	0.8656[d]	0.8939[d]	0.5267[e]	0.8153[f]	0.9472[f]
Brittleness (N/g)	0.9310[f]	0.9111[f]	0.7995[f]	0.8480[f]	0.9320[f]
Cohesiveness (%)	0.8199[e]	0.8514[e]	0.9384[g]	0.9524[g]	0.7970[e]
Elasticity (%)	0.9534[e]	0.9771[e]	0.9361[g]	0.9501[g]	0.6157[e]
Folding test	0.8787[d]	0.8384[d]	0.7858[f]	0.8575[f]	0.9353[f]
Myosin recover		0.9260[d]			
Proteinase activity					0.8546[f]

[a] Activity in sol (μg tyrosine/min/g protein).
[b] Peak height of myosin recovered from $KBrO_3$-treated gels/peak height of myosin recovered from gels containing no $KBrO_3$ (control).
[c] $n = 24$.
[d] Best fit: linear ($y=mx+b$).
[e] Best fit: logarithmic ($y=a+b\ln x$).
[f] Best fit: power ($y=ax^b$).
[g] Best fit: exponential ($y=ae^{bx}$).
[h] 90°C for 40 min.
[i] 40°C for 60 min + 90°C for 20 min.

4). Optimization was achieved with 0.15% bromate, where 94.34% proteinase inactivation and a 3.8-fold recovery of myosin from heat-set gels were observed. This opimization did not require either hardness or brittleness values for the gels containing bromate levels ≥ 0.2%. This suggested that the degree of disulfide formation afforded by those bromate levels was in excess of that required for optimum whiting surimi gel grade.

Overall, the two-stage heat-setting process produced gels yielding a higher ($p < 0.05$) folding test grade than the one-stage method. Improvement in folding test grade observed for both heat-set procedures was incremented ($p < 0.05$) with respect to bromate up to 0.15%. Higher bromate levels did not alter ($p > 0.05$) folding test grades. Unlike TPA parameters, heat-setting conditions and bromate level did not interact to affect folding test grades. This subjective test was not sufficiently sensitive to detect differences between the two heat-setting procedures, as was observed for brittleness of gels containing bromate levels ≥ 0.15%

A correlation matrix compared the regression of TPA parameters, folding test scores, proteinase activity and myosin recovery on bromate levels and other variables (Table 5). A negative power function correlation of proteinase activity with myosin recovery from the two-stage heat-set gels indicated the protection afforded on myosin by bromate. The relationship of myosin recovery to gel characteristics supported the positive action of bromate on improvement of protein functionality from whiting surimi. Gel hardness, brittleness and folding test scores were a power function of myosin recovery and cohesiveness and elasticity were a logarithmic function.

CONCLUSIONS

POTASSIUM BROMATE improved the gel characteristics of whiting surimi sols probably through proteinase inactivation and strengthening of disulfide formation. Enhancemnent of gel strength characteristics by bromate levels, in excess of those required for proteinase inactivation (>0.075%), indicated the strong effect of the oxidative action of bromate on strengthening disulfide formation. The relative importance of these two mechanisms varied with respect to bromate concentration and, to a lesser extent, to heat-setting conditions. Results for interactions of bromate with surimi proteins indicated a level of 0.15% was required to achieve biochemical and chemical modifications necessary for strong heat-set gels. This was substantially higher than the 0.005% currently approved for use in bakery products, but was necessary to overcome the lack of functionality of protein systems. Higher levels produced stronger gels, as measured by texture profile analysis, but the additional strenghtening of gels did not appear necessary for optimum folding test grade. Levels of bromate >0.15% may induce sufficient disulfide cross-linking to impart strength to some gels.

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*THERMOSTABLE PROTEINASE IN SALTED ANCHOVY MUSCLE. . .*From page 785

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Microstructural and Rheological Properties of Cooked Squid Mantle

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ABSTRACT

Changes in tissue structure and rheological properties of squid were studied as related to cooking time using the mantle of squid just after instant killing. Collagen fibers of integument were tautly shrunk and voids were formed among the fibers by cooking. Muscle fibrils were considerably dehydrated and disconnected. Rupture energy (RE) for breaking raw squid parallel to the circular musculature was greater than that for breaking across the circular musculature. Cooking softened squid flesh and reduced RE both in parallel and transverse breaking of circular musculature. Reduction in RE was considerable in parallel breaking but not notable in transverse breaking.

Key Words: squid, texture, collagen, rheology

INTRODUCTION

UNLIKE OTHER FISHERY PRODUCTS, the sensory perception value of squid flesh is generally determined by its feel and touch to the teeth, i.e. its rheological properties rather than taste (Takahashi and Takei, 1955; Otwell and Hamann, 1979a). Such properties of squid flesh have been commonly measured by determining tensile strength which is comparatively easy. However, several researchers have reported that the *most* comparable method for human sensory evaluation of squid flesh during mastication would be a series of compressive rupture tests at constant speed (Akabane et al., 1981; Kuragano et al., 1984; Kugino et al., 1993). A common characteristic of squid is that it becomes hard and has gum-like properties after cooking (Ampola, 1974). Some experimental reports with *Ommastrephes sloani pacificus* showed that cooked squid flesh became dehydrated, shrunk and had increased breaking strength when it became hard (Migita, 1953; Takahashi and Takei, 1955). However, other reports with *Loligo pealei* (Otwell and Hamann, 1979b), *Illex illecebroses* (Stanley and Hultin, 1982), and *Illex argentinus* (Kolodziejska et al., 1987) showed that the shear force of cooked squid flesh decreased and the flesh softened. Whether such differences were due to experimental conditions, sample species, freshness or other factors is unclear. Rheological properties of squid flesh during cooking have not been fully elucidated. Furthermore, changes in rheological properties of fish and squid flesh have been hypothesized as less dependent on decomposition on a molecular level of constituent proteins than on macro-scale changes in tissue structure (e.g., connective tissue, muscle fibers, elastic protein in cells, membrane structure, various matrix structures) Hatae et al. (1985, 1990) and Kugino et al. (1993). It is important to know how processing squid flesh, especially cooking, affects such properties. Reports on such changes are few and much remains unknown. Our objective was to examine changes in the microstructural and rheological properties of squid flesh upon cooking using the mantle of fresh squid.

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MATERIALS & METHODS

Squid sample preparation

Live squid (*Sepioteuthis lessoniana*) were caught off the coast of Nagasaki, Japan, and the mantles were used for all experiments. The live squid were decapitated and immediately cut open; the mantle on the abdominal side was incised along the body axis, then the viscera, head, arms and skin (1st and 2nd tunic of the outer layer) were removed. The squid weighed about 1000g and measured about 10 mm in thickness in the middle of the mantle. Mantles were cooked in boiling water for 5 or 30 sec, 1, 3, 6, 10, 20 or 30 min. After cooking, mantles were cooled to room temperature (≈23°C) by rinsing with running tap water.

Light microscopy

For light microscopy, tissues were fixed in 10% formaldehyde (Wako Pure Chemicals Co., Tokyo, Japan) and embedded in paraffin (Wako Pure Chemicals Co., Tokyo, Japan). Tissue slices (7 mm thick) were stained with hematoxylin and eosin (Muto Pure Chemicals Co., Tokyo, Japan) and observed using TMS-F light microscope (Nikon Co., Tokyo, Japan).

Electron microscopy

Electron microscopic observations of muscle structure of raw and cooked squid were as reported by Kugino and Kishino (1991). Muscle tissues of squid mantle cooked in boiling water were divided into samples immediatey after cooking. Small sections of muscle fiber, as samples for electron microscopic examination, were prefixed for 2 hr in 6% glutaraldehyde (Nisshin EM Co., Tokyo, Japan) in 0.1M cacody-

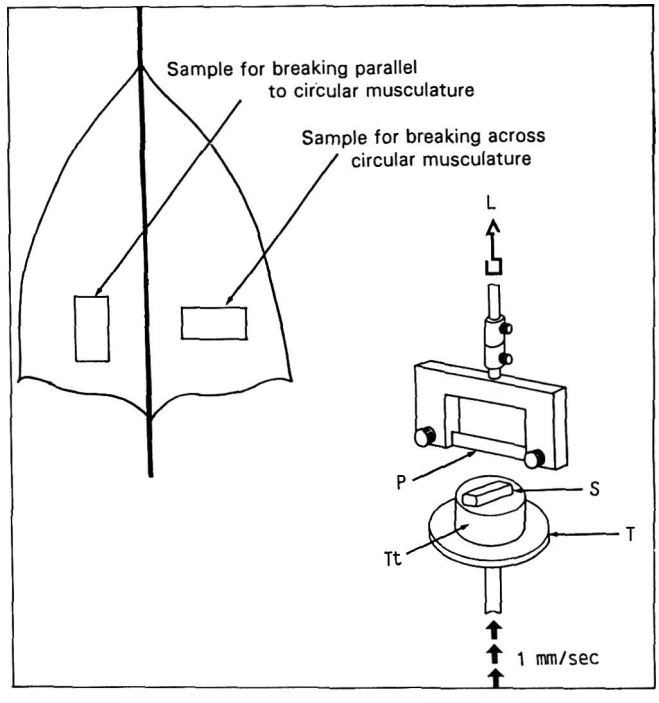

Fig. 1—Sample cutting method and equipment for determination of rheological properties. L, load cell; P, stainless steel plate plunger; S, specimen; Tt, teflon stage; T, stage of rheometer.

late buffer (pH=7.4) and then post-fixed for 2 hr in 1% osmium tetroxide (Nisshin EM Co., Tokyo, Japan) in the same buffer at 4°C. Tissues were dehydrated in a series of increasing ethanol concentrations, embedded in Epon 812 (Nisshin EM Co., Tokyo, Japan), and sectioned with a glass knife on an LKB Ultramicrotome (LKB Instruments, Rockville MD). Sections for electron microscopy were stained with uranyl acetate (TAAB Laboratories, Reading, MA) and lead citrate (Nakarai Chemicals Co., Kyoto) and observed at 75 kv using an HU-12 electron miroscope (Hitachi Co., Tokyo).

Rheological properties

Rheological properties of the squid mantle were measured according to the method of Kugino et al. (1993). Parts of raw squid were cut with a razor blade to provide test pieces for rupture properties. The 15 mm wide test pieces were cut from the uniform thickness portion in the middle of the mantle parallel and perpendicular to the body axis (see Fig. 1). Rheological properties were measured using RE-3305 rheometer (Yamaden Co., Tokyo) with a 0–20 kg load cell. The specimen was placed on the stage of the rheometer with the outer tunic up, and rupture stress, rupture strain, and rupture energy were measured using the constant speed compressive rupture test (compression rate: 1 mm/sec; clearance: 0.05 mm; temperature: 25°C) with a 0.3-mm-thick stainless steel plate plunger (Fig. 1).

RESULTS

LIGHT MICROSCOPIC OBSERVATIONS of the mantle tissue of cooked squids were compared (Fig. 2-3). After cooking for 6 min, the third layer of integument of the mantle fell off and only the fourth layer was connected with muscle layer (Fig. 3a,b). Collagen fibers, a main component of the fourth layer, appeared to shrink tautly due to thermal denaturation, and voids were also observed among collagen fibers (Fig. 3b). Light micrographs of the middle part of muscle layer showed that muscle fibers, which constitute the circular musculature, seemed to shrink slightly and to turn slightly wavy due to thermal denaturation (Fig. 3c). However, the individual muscle fiber had no notable change in structure like rupture and the structure of the muscle layer remained generally intact (Fig. 3c,d). After cooking for 30 min, the changes observed on the mantle appeared to proceed further (Fig. 4). The transmission electron microscope indicated changes in the muscle layer (Fig. 5) where no notable change in structure could be observed with the light microscope. In the circular musculature after cooking for 6 min, muscle fibrils had undergone considerable shrinkage by thermal denaturation and voids appeared scattered among the fibrils. In addition, muscle fibers also were conspicuously shrunken and showed marked disconnections. In the circular musculature after cooking for 30 min, shrinkage of the muscle fibrils advanced further, most voids among fibrils disappeared, and disconnection of the fibers proceeded further.

In fresh raw squids, rupture energy needed to break them across the direction of the body axis (along the direction of the circular musculature) was greater (Fig. 6) than that in breaking them in the same direction as the body axis (across the circular musculature). Thus anisotropic mechanical features in the rheological properties of squid flesh were recognized. After cooking the rupture energy of the squid flesh in both directions decreased and the squid flesh became soft. The reduction of rupture energy by cooking was notable in breaking along the circular musculature but was only slight across the circular musculature. After cooking for 6 min, the rupture energy was almost identical irrespective of breaking direction and after a longer cooking time, rupture energy in breaking across the circular musculature increased more compared to the raw squid. Stress-strain curves of raw, 6 min-cooked and 30 min-cooked squid flesh (Fig. 7) showed the squid flesh was broken parallel to the circular musculature (Fig. 7a), cooking significantly extended the distance between yield and break points (plastic deformation region), indicating texture became mushy. When the squid flesh was broken across the circular musculature (Fig. 7b), the plastic deformation region decreased with cooking time. However, prolonged cooking for 30 min increased the stress, strain and rupture energy values slightly compared to cooking for 6 min.

DISCUSSION

THE MANTLE OF SQUID IS QUITE UNIQUE, and very different from other marine species in structure and chemical composition (Matsumoto, 1959; Katsumi and Matsumoto, 1969; Ward and Wainwright, 1972; Moon and Hulbert, 1975; Gosline and Shadwick, 1983). The integument of the squid mantle consists of 4 layers and only the first and second layer are removed when peeled off in the usual way. The third layer is removable by cooking treatment. The fourth layer, which has collagen fibers along the direction of the body axis as a major component, is tough though very thin and adheres closely to the muscle layer, so its removal by peeling is difficult. The fourth layer is greatly distorted by cooking and this is due to excessive shrinkage of collagen fibers which are the main component of the fourth layer (Suyama, 1980). This muscle layer occupies the largest part of the squid mantle and almost all of the fibers in the muscle layer are circular musculature which encircle the body axis. Besides, a very few of the muscle fibers are radial muscle extending radially from the body axis. When squid mantle with such structures is cooked, several factors are expected to act in a complex way to change the tissue structure and its rheological properties. Muscle tissue of squid lacks connective tissue which connects muscle fibers with one another and into bundles and firmly binds the muscle fiber layer (Moon and Hulbert, 1975; Suyama, 1980). When the muscle tissue of squid is cooked in hot water, the network of connective tissue is severely damaged and disappears by solubilization and gelatinization. About 10% of all the proteins are removed from muscle tissue by coagulation and decomposition of sarcoplasmic proteins followed by dehydration (Otwell and Hamann, 1979a; Stanley and Smith, 1984; Sikorski and Kolodziejska, 1986; Kolodziejska et al., 1987). Muscle fibrils of squid consist of proteins which are fairly stable against denaturation and are insoluble in water (Migita, 1953). Some investigations using scanning electron microscopy showed that by cooking, proteins of the muscle fibrils were denatured, coagulated, shrunken, and the density of tissue increased but the structure of the fibers remained mostly intact (Stanley and Smith, 1984; Otwell and Giddings, 1980). Our light microscopic observations also revealed that collagen fibers of cooked mantle integument shrunk and the individual fibers partially ruptured followed by disconnection of fibers. However, in the muscle layer, although several voids were among the muscle fibers, individual fibers were not ruptured and their structure was relatively intact. However, from observations with the transmission electron microscope, the muscle fiber structure was notably obscured because of thermal denaturation of muscle fibril proteins. Dehydration and disconnection of muscle fibrils were thought to result from shrinkage and denaturation decomposition of connective tissue among the muscle fibers. These microstructural changes in the fibers resulting from cocking suggest not only shrinkage and hardening of muscle fiber proteins but also brittleness of muscle which may occur through collapse of the muscle layer structure. In the rheological property measurements, the rupture energy of the raw squid mantle was greater in breaking parallel to the circular musculature than in breaking across the musculature and both rupture energies decreased when squid had been cooked. The decrease of rupture energy was most notable in breaking parallel to the circular musculature. After cooking 6 min or longer the rupture energy in breaking across the circular musculature became greater than that in breaking parallel to the musculature compared to raw squid flesh. Otwell and Hamann (1979b) also reported that shear force of frozen and thawed squid flesh (*Loligo pealei*) decreased by cooking and the force in shearing cooked squid flesh across the circular

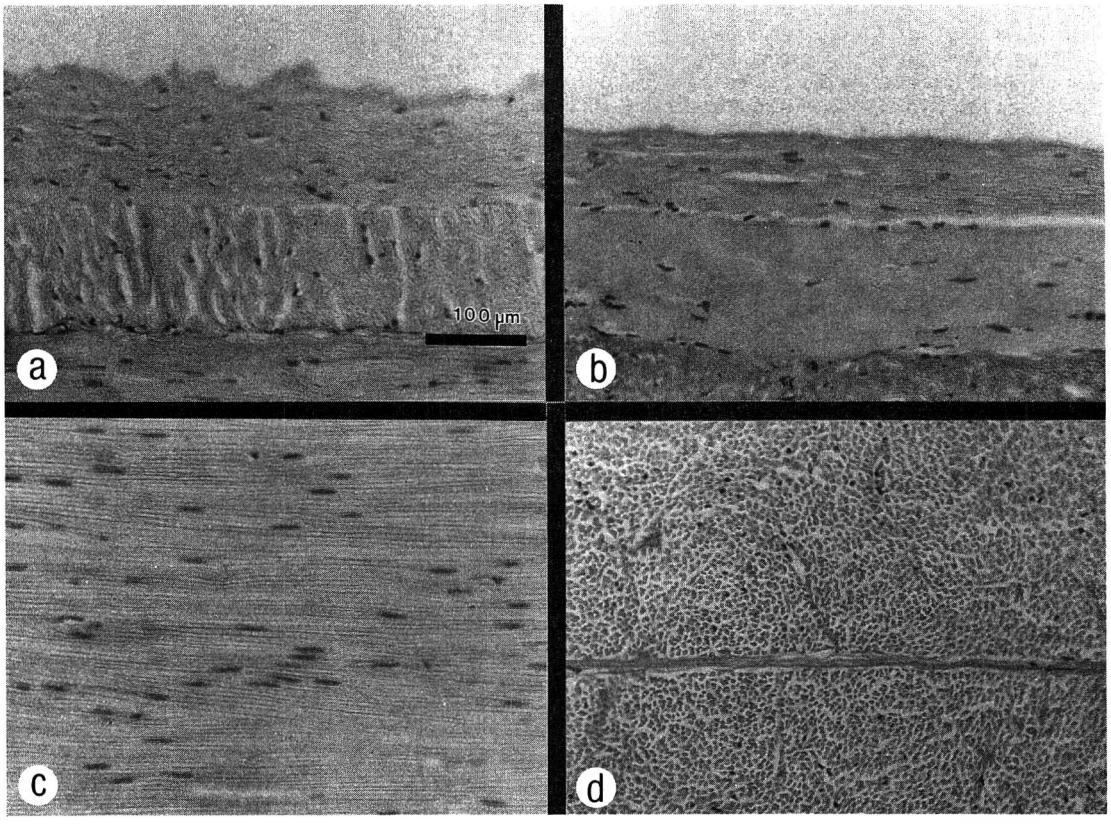

Fig. 2—Light micrographs of mantle of raw squid. (a) Outer layers of mantle broken parallel to circular musculature; (b) Outer layers of mantle broken across circular musculature; (c) Muscular tunics of mantle broken parallel to circular musculature; (d) Muscular tunics of mantle broken across circular musculature.

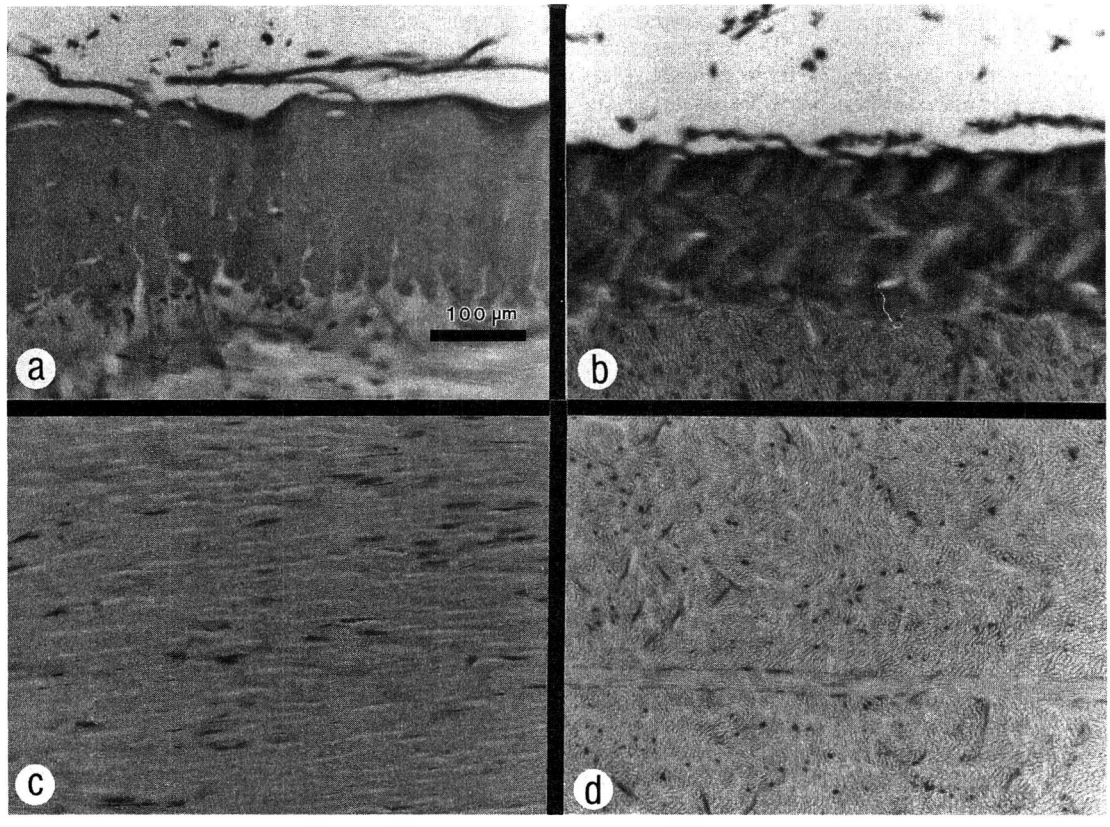

Fig. 3—Light micrographs of mantle of squid cooked for 6 min. (a) Outer layers of mantle broken parallel to circular musculature; (b) Outer layers of mantle broken across circular musculature; (c) Muscular tunics of mantle broken parallel to circular musculature; (d) Muscular tunics of mantle broken across circuclar musculature.

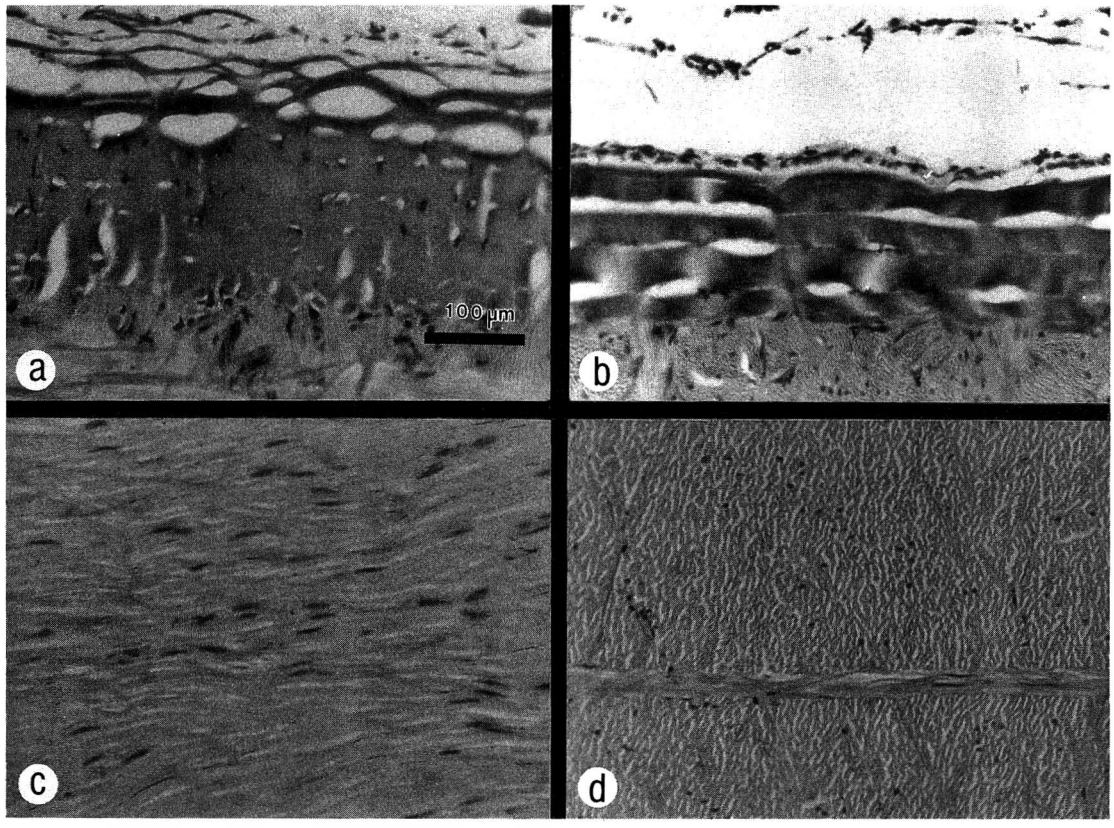

Fig. 4—Light micrographs of mantle of squid cooked for 30 min. (a) Outer layers of mantle broken parallel to circular musculature; (b) Outer layers of mantle broken across circular musculature; (c) Muscular tunics of mantle broken parallel to circular musculature; (d) Muscular tunics of mantle broken across circular musculature.

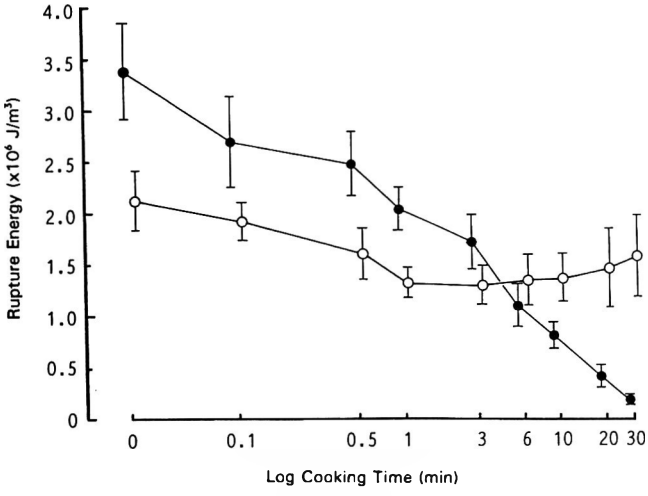

Fig. 6—Changes of rupture energy of squid mantle by cooking. Means and standard deviations for 10 separate experiments. (●) Sample broken parallel to circular musculature; (○) Sample broken across circular musculature.

musculature was greater than in shearing it parallel to the circular musculature. Such anisotropic mechanical features were present during changes in the rheological properties of squid flesh. They surmised that such features would come from gelatinization of integument connective tissue built along the direction of body axis and thermal hardening of muscle fibers. Kuo et al. (1990) also reported on an experiment in which frozen squid flesh (*Illex illecebroses, Loligo pealei*) was similarly thawed and then cooked. The decrease in tensile strength of cooked squid flesh was more conspicuous in shearing it parallel to the circular musculature than in shearing it across the musculature. Kuo et al. (1990) proposed that such aniso-

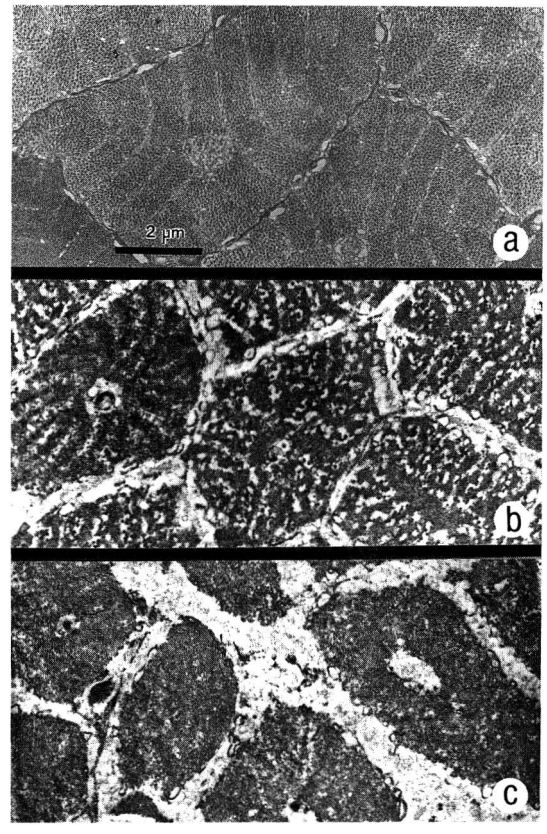

Fig. 5—Electron micrographs of raw and cooked squid mantle muscle. (a) Circular musculature of raw squid mantle; (b) Circular musculature of squid mantle cooked for 6 min; (c) Circular musculature of squid mantle cooked for 30 min.

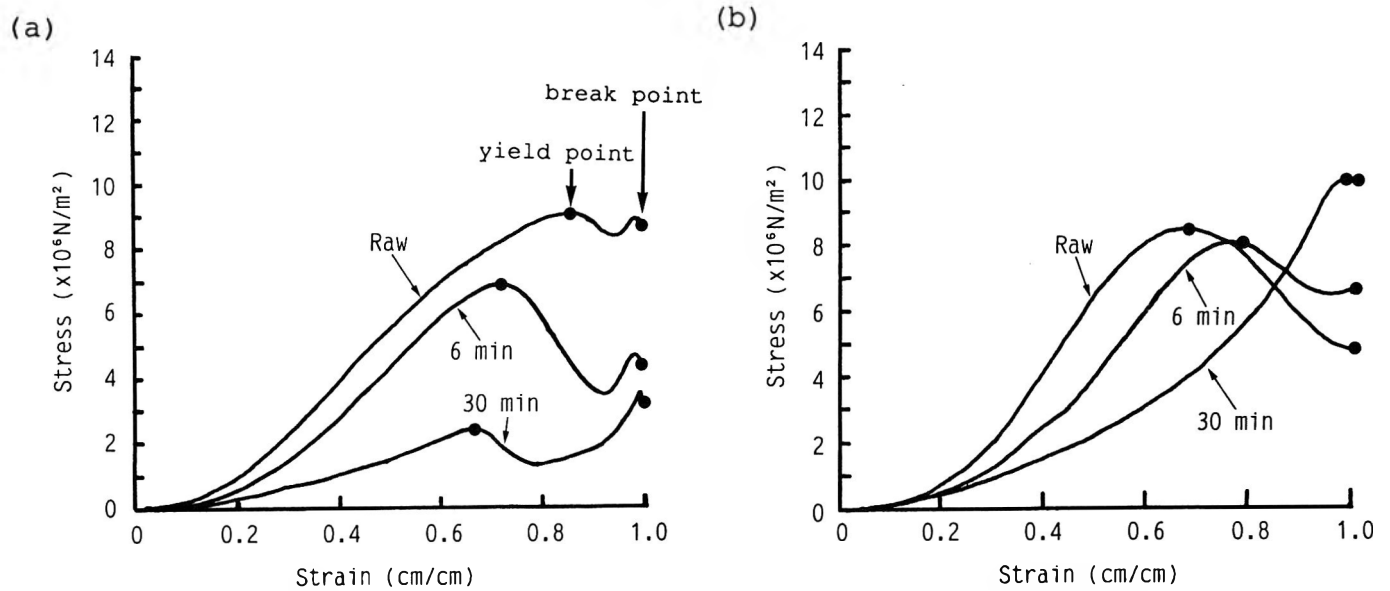

Fig. 7—Stress-strain curves of raw and cooked squid mantle muscle. (a) Sample broken parallel to circular musculature; (b) Sample broken across circular musculature.

tropic features might be related to thermal solubilization and gelatinization of the integument collagen fibers built parallel to the body axis. Subsequently, they examined such changes with decomposition treatment using enzymes. From their results such anisotropic features were not only attributable to solubilization and gelatinization of collagen but also were influenced by changes in other tissue constituents including the muscle fibers (Kuo et al., 1991). In our experiment in which *Sepia officinalis* (Kugino, 1994) was cooked in 60°C water (at which solubilization and gelatinization take place) and 100°C water, the anisotropic features were definite with cooking at 100°C but hardly notable with cooking at 60°C. We cannot explain the anisotropic mechanical features of squid flesh based only on solubilization and gelatinization of integument collagen fibers.

Based on the results of this investigation and another experimental report (Kugino, 1994), the influence of integument collagen fibers over such anisotropic features is not perceived as important. The main cause of softening and the anisotropic features is likely brittleness of muscle due to collapse of muscle layer structure as dehydration of muscle fiber proteins occurs by thermal denaturation and disconnection by shrinkage.

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Refreezing Rate after Glazing Affects Cod and Rainbow Trout Muscle Tissue

KATARINA NILSSON and BO EKSTRAND

ABSTRACT

Fillets of cod and cutlets of rainbow trout were frozen, glazed and then either rapidly or slowly refrozen for storage. Spontaneous and forced thaw drip and liquid holding capacity were measured in all samples, and a sensory test was used to differentiate groups. In rainbow trout marker enzyme activity was also determined. For both species fast refreezing after glazing resulted in less thaw drip, either spontaneous or forced. Liquid holding capacity was increased with a longer refreezing time for cod, but not for rainbow trout. Leakage of intracellular marker enzyme activity was highest in slowly refrozen rainbow trout samples.

Key Words: cod, trout, glazing, freezing, sensory quality, refreezing rate

INTRODUCTION

GLAZING PROTECTS FISH and shellfish from desiccation and lipid oxidation during frozen storage. It is usually done by immersing the frozen product in water or by spraying it with water. The amount of water bound in the ice layer depends on such factors as product size and temperature and water temperature (Graham, 1981; Santos and Regenstein, 1990). The protective effect of glazing has been reported in many studies (Jadhav and Madgar, 1970 a,b,c; Bruenner, 1983;). Attempts have been reported to increase the protection against oxidation by adding different anti-oxidants to glazing water (Colakoglu and Kundakci, 1983; Stodolnik and Matyjasczcyk, 1990).

Due to the glazing process and treatments directly following glazing, the product temperature may rise to about −10°C. The time required to establish frozen storage temperature again might be very long and depends on the refreezing treatment.

Our objective was to study the effect of two treatments to decrease fish temperature after glazing (refreezing) on muscle tissue of cod and rainbow trout.

MATERIALS & METHODS

Fish

Cod (*Gadus morhua*) were obtained fresh as fillets, each weighing 250–450g, at the local fish market, frozen on the same day, and kept on ice until use. Rainbow trout (*Oncorhynchus mykiss*) (average weight 1.3 kg) were from the Björkö Trout Hatchery (Göteborg) and kept on ice for 3 days until resolution of rigor mortis. Six cutlets, weighing 125–145g, were cut from the mid-part of each fish and kept on ice until freezing.

Freezing and glazing

Freezing was done in a spiral-belt freezer at −35°C. Freezing time was 35 min for both cod and rainbow trout. All samples were placed in a −20°C environment for 60 min to adjust temperature before glazing. A nonglazed group of both cod and rainbow trout samples was directly sealed in plastic and kept at −30°C until use. Glazing was done by dipping fish twice for 15 sec in +1°C ice-water, with a 30-sec interval between dips. The applied glazing constituted between 6.5% and 8.5% of fish sample weight. Total handling time of the glazing process was 5 min and during glazing the fish sample temperature increased from −20°C to −6°C (Fig. 1).

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Refreezing

Glazed samples of both species were divided into two groups for different treatments to lower the fish temperature, from −6°C to −20°C (refreezing). The first group was placed in the spiral-belt freezer so that a fast return to frozen storage temperature was achieved (12 min to −30°C). The samples of the second group were placed in a cardboard box, then into a tempered freezer at −10°C. Temperature of the freezer was gradually lowered to −20°C over 116 hr (5 days). All fish samples were sealed in plastic bags and stored at −30°C for 3 wk.

Thawing and drip

Fish were thawed at room temperature (+22°C). It was considered complete when the fish core temperature had reached +3°C. This resulted after a thawing time of 4 hr. All fish samples were placed on and covered with paper tissues to collect spontaneous thaw drip, defined as the difference in paper weight before and after thawing. The "fish drip" was then calculated as total spontaneous drip minus weight of the glaze.

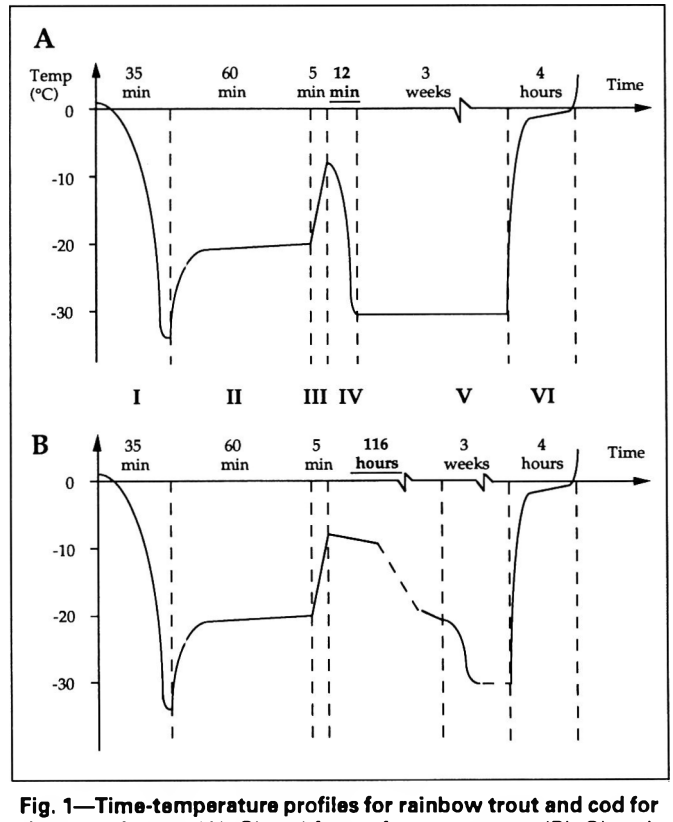

Fig. 1—Time-temperature profiles for rainbow trout and cod for the experiment. (A) Glazed-fast refrozen groups; (B) Glazed-slowly refrozen group. I= freezing; II= adjustment of temperature; III= glazing; IV= refreezing; V= frozen storage; VI= thawing.

Table 1—Liquid loss in cod muscle

	Nonglazed	Glazed	
		Fast frozen	Slow frozen
Total spontaneous thaw drip[a]	6.2±0.4 (8)	11.2±0.3 (17)	16.0±0.6 (10)
"Fish" drip[b]	6.2±0.4 (8)	4.7±0.3 (17)	6.3±0.4 (10)*
Forced liquid loss[a c]	16.9±0.6 (3)	17.2±0.5 (3)	18.5±0.6 (3)[ns]
Liquid holding capacity[a]	15.8±0.8 (8)	11.8±0.4 (10)	9.2±0.3 (10)**

[a] Liquid released (g) as % of total muscle sample weight (g). Means ± standard error (n).
[b] Fish drip = Total spontaneous thaw drip (g) − Glaze water (g), % of total muscle sample weight (g). Means ± standard error (n).
[c] Triplicate determinations of each n.
Statistical significance of differences between fast and slow: ns = not significant, * = $p< 0.05$, ** = $p< 0.01$

Table 2—Liquid loss and enzyme activity in rainbow trout muscle

	Nonglazed	Glazed	
		Fast frozen	Slow frozen
Total spontaneous thaw drip[a]	1.4±0.1 (22)	6.5±0.2 (39)	8.7±0.2 (23)
"Fish" drip[b]	1.4±0.1 (22)	0.2±0.1 (39)	0.4±0.1 (23)*
Forced liquid loss[a c]	6.7±0.6 (3)	8.4±0.5 (3)	9.9±0.3 (3)*
Liquid holding capacity[a]	3.6±0.4 (10)	2.9±0.1 (10)	4.1±0.2 (10)**
Marker enzyme[d c]	16.5±0.6 (3)	18.5±1.3 (3)	27.8±2.0 (3)*

[a] Liquid released (g) as % of total muscle sample weight (g). Means ± standard error (n).
[b] Fish drip = Total spontaneous thaw drip (g) − Glaze water (g), % of total muscle sample weight (g). Means ± standard error (n).
[c] Triplicate determinations of each n.
[d] The enzyme activity of NAG is expressed as the activity in the forced liquid loss related to in % the total activity in a homogenate from the same individual. Means ± standard error (n).
Statistical significance of differences between fast and slow: * = $p< 0.05$, ** = $p< 0.01$

Forced thaw drip

Thawed skinless muscle tissue (10g) was cut out and centrifuged at 28000 × *g* for 30 min. The forced out liquid was collected with a Pasteur pipette and weighed. In the thaw drip from rainbow trout samples, the activity of lysosomal β-N-glucosaminidase (NAG) was measured as an indication of effects of freezing-thawing on membrane integrity.

Liquid holding capacity

Muscle tissue (150 g) from five individual samples in each test group, (cod and trout) was coarsely chopped in a mixer for 3 × 5 sec. The liquid loss, (i.e. the amount of fluid released per 15-g sample) was determined according to the net test described by Hermansson (1986). Samples were placed into a metallic cylinder, sealed and incubated in a water bath (65°C, 30 min). Samples were cooled under running tap water for 15 min to +15°C. In accordance with the net test, liquid loss was determined after centrifugation at 210 × *g*, 10 min.

Enzyme activity

The enzyme activity of β-N-glucosaminidase (NAG) (E.C. 3.2.1.30) was measured both in forced thaw drip and in the supernatant of a homogenate from the same individual sample. The enzyme activity in the forced thaw drip is a specific indicator of lysosomal leakage and it is directly related to the total enzyme activity determined in the supernatant from the homogenate. For a detailed description of the enzyme activity assay see Nilsson and Ekstrand (1993). Enzyme activity was calculated as katal · g^{-1} protein. The total activity was determined and related to tissue sample weight. The protein concentration was determined spectrophotometrically, using the Coomassie blue staining method according to Bradford (1976) (Bio-Rad, Richmond, CA).

Sensory difference test

A sensory difference test was used both for cod and trout. It was performed as a triangle test, in which judges picked the odd sample of three. The fast refrozen groups were tested vs the nonglazed and the slowly refrozen groups, respectively. A trained test panel of nine members performed the tests in two replicate series. Samples of 50g were cut from the thawed cod and rainbow trout, vacuum-sealed, labeled and heat-treated with steam (+90°C) until the center temperature reached 65°C. Samples were then placed on preheated stainless Petri dishes and kept warm on a hot plate until tested.

Table 3—Sensory difference tests for rainbow trout and cod

	Rainbow trout		Cod	
Replicate	GF-NG[b]	GF-GS[c]	GF-NG[b]	GF-GS[c]
I[a]	7**	7**	5[ns]	7**
II[a]	5[ns]	6*	2[ns]	6*
Σ	12***	13***	7[ns]	13***

[a] The number of odd samples picked from nine possible triangles.
[b] Glazed-fast refrozen vs nonglazed.
[c] Glazed-fast refrozen vs glazed-slowly refrozen.
ns= not significant, * = $p< 0.05$, ** = $p< 0.01$, *** = $p< 0.001$

Statistical analysis

A statistical analysis of all experimental data was performed using the two-tailed Student's T-test, except for results from the triangle tests. In those the number of correct responses was analyzed for statistical significance according to binomial distribution.

RESULTS & DISCUSSION

THIS INVESTIGATION CONCERNED two refreezing treatments for restoring fish to the desired storage temperature after an increase in temperature caused by glazing. One group chilled to the storage temperature within 12 min, could be regarded as an ideal treatment because of short fluctuation in temperature. The other group was slowly restored to frozen storage temperature under controlled conditions, over 116 hr. This was done to simulate possible delays in glazed fish and shellfish during normal commercial handling. The temperature profiles for the experiments were as recorded (Fig. 1).

Fast refreezing of cod resulted in less spontaneous thaw drip, as well as less forced thaw drip, compared to slowly refrozen samples (Table 1). The "fish drip" gave a higher value for the slowly refrozen group than for the fast refrozen group ($p<0.05$). For liquid-holding capacity total liquid loss was measured on coarsely chopped heat-treated samples. The nonglazed samples that were not subjected to temperature fluctuations after freezing released most liquid/unit weight. The samples from the slowly refrozen group had better liquid-holding capacity than the fast refrozen samples.

The different refreezing treatments resulted in different liquid-holding properties in thawed whole muscle tissue and coarsely chopped heat-treated muscle from cod (Table 1). The slow refreezing treatment allowed recrystallization, which affected the muscle protein and membrane structure. In whole muscle tissue, this might lead to release of liquid, formerly entrapped within the cells or bound to intact protein structures. It may also affect protein solubility, which would increase gel formation during heating. This combined with the formation of new protein aggregates, could explain the increased liquid-holding capacity and different functional properties of slowly refrozen cod after heat treatment. Studying muscle structure by light microscopy in heat-treated (+50°C to +70°C) samples of coarsely chopped cod and salmon filets, Ofstad et al. (1993) reported granulated material in intercellular spaces between muscle fibers. This may have been a mixed phase of collagen and sarcoplasmic proteins.

Results for rainbow trout (Table 2) showed that both spontaneous and forced thaw drip were greater in the slowly refrozen group than in the fast refrozen samples. These results confirmed earlier findings for Pacific salmon, in which slow freezing resulted in higher thaw drip than from fast-frozen salmon (Bilinski et al., 1977).

—Continued on page 838

Solids Extraction of Cod Frame and Effects on Ultrafiltration of the Aqueous Extract

M.T. RODRIGUEZ-ESTRADA, S. CHUNG, and P. CHINACHOTI

ABSTRACT

Grinding, pH adjustment, heat treatment and centrifugation were studied to recover solids from ground cod frames and to apply ultrafiltration (UF) to the resulting aqueous phase. Early heat treatment (90°C/20 min) dissolved collagen into aqueous phase resulting in a very low UF flux. A pH adjustment to 4.5 after grinding (≈0.32 cm die), with no heat resulted in efficient separation after centrifuging. UF of the aqueous phase showed an increase in initial flux (23 L/hr m²). However, when the same aqueous material was heated and then centrifuged (1–2% solids removed), UF flux was further increased to 46 L/hr m² and this process gave highest solids recovery (90%).

Key Words: cod, fish frame, ultrafiltration, solids extraction

INTRODUCTION

LARGE AMOUNTS OF FISH WASTE generated each day, unless properly treated, can create ecological hazards (Danish Ministry of Fisheries, 1983). Fish frames contain considerable proteins and minerals and recovery of solids would reduce pollution and provide good sources of protein and other organics. Methods for fish protein recovery from stickwater of fish meal and surimi factories have included evaporation and drying, precipitation, centrifugation and filtration (Del Valle and Aguilera, 1990).

Fish frame refers to the entire residue after filleting, including head, bone and tail with ≈22% solids (mostly proteins). In order to achieve a concentrate (minimizes transportation and storage cost), fish frame must be ground into a slurry and concentrated. Thus, the solids yield would be the key monitoring factor among products and the waste water quality would be the key factor among discharges.

Concentration by evaporation of fish frame to 55% solids has been shown to be adequate. The process used in some plants, includes papain hydrolysis, grinding and heat prior to concentration by evaporation. However, even with marked reduced viscosity by such treatments, fouling on the heat exchanger surfaces of evaporators is adverse when the product is > 45% solids. At that point, a scraped surface type evaporation appears necessary. Thus, evaporation might become more costly and alternative means of solid recovery are needed. Change in solids recovery and dewatering would affect not only energy and capital costs, but also eliminate enzyme needs as well as lowering transportation and storage costs.

Precipitation of protein has usually been by adjusting pH to isoelectric point (pI) range (3.8–4.9) for fish proteins (Maeda and Ozawa, 1975; Del Valle and Aguilera, 1990; Emelyanova et al., 1977; Welch and Zall, 1979; Hang et al., 1980; Vega and Brennan, 1987). Heat treatment can also decrease protein solubility by denaturation, enhancing hydrophibic interactions between nonpolar segments of polypeptides (Morrisey et al., 1987). Additive effects between pH precipitation and heat denaturation could improve the dewatering process (Cheftel et al., 1985; Motomura and Kawazoe, 1976; Niki et al., 1985), resulting in upto 95% protein removal and 80% decrease in chemical oxygen demand (COD) of the waste stream. Centrifugation recovers suspended fish solids in fish meal bloodwater (Del Valle and Aguilera, 1990), and cod fish processing waste water (Bisera, 1980). Combined pH, heat and centrifugation effects have been common to effectively separate proteins from aqueous suspensions.

Membrane processing also has been widely used (e.g., Cheryan, 1986; Koseoglu et al., 1991). Chao and Davis (1981) and Green et al. (1984) ultrafiltered wastewater of minced fish using hollow fiber polysulfone membrane with 50,000 molecular weight cut-off (MWCO). Wasteater was concentrated up to 10-fold by volume. UF has been attractive because of less energy demand than evaporation (Almas, 1985; Del Valle and Aguilera, 1990). However, concentration polarization and fouling often result in low flux, higher membrane treatment costs and clean up. The concentration of fish waste by UF was highly impaired by membrane fouling (Chung, 1991). Proteins are major cause of fouling in such processes (Velicangil and Howell, 1977; Cheryan, 1986). One way to improve membrane performance and dewater fish solids may be by heat and acid precipitation, followed by centrifugation. The resulting aqueous phase could be further treated by UF, depending on amount, and chemical and physical nature of suspended solids.

Our objective was to study dewatering and solids recovery techniques and their effects on UF performance with resulting aqueous extracts. Such procedures and evaluation of extracted fractions could lead to better ways to eliminate specific foulants from ground fish frames.

MATERIALS & METHODS

Materials

Fresh cod fish frames were obtained from Steve Connolly Sea Food Inc. (Goucester, MA) and kept on ice during transportation to Amherst, MA (≈3 hr). They were immediately ground (1.27 cm die diam) using a heavy duty grinder (John E. Smith & Sons, Buffalo, NY) and then stored in a freezer at −20° until used. The ground fish was thawed overnight before experiments. Phosphric acid 85% (w/w) and sodium hydroxide solution 50% (w/w) both analytical grade (Fisher Scientific Co., Fair Lawn, NJ), were used for pH adjustment.

Methods

The experiment was designed to determine the influence of the following factors on solids recovery.

Grinding. Coarse ground fish frame was further ground (0.32 cm die diam) using a meat grinder (model 2822, U.S. Slicing Machine Co. Inc., Laporte, IN). Finely and coarsely ground fish frames were compared for solids recovery after adjusted to pH of 2.0 to 7.0 an centrifuged at 23,500 × *g* for 20 min (Sorvall Superspeed RC2-B model Ivan Sorvall, Inc., Newtown, CT). The resulting precipitate and supernatant were analyzed for solids content.

pH. Finely ground cod frames (50g), were mixed with varying amounts of phosphoric acid and sodium hydroxide to obtain pH 2.0–7.0. These samples were centrifuged at 23,500 × *g* for 20 min and analyzed for solids content.

Heat treatment. Finely ground cod frames (150g) were adjusted to ph 4.5, held at 90°C for 20 min with continuous stirring, and then cooled in an ice bath to room temperature (≈23°C) before being centrifuged as above. The resulting supernatant was ultrafiltered (stirred

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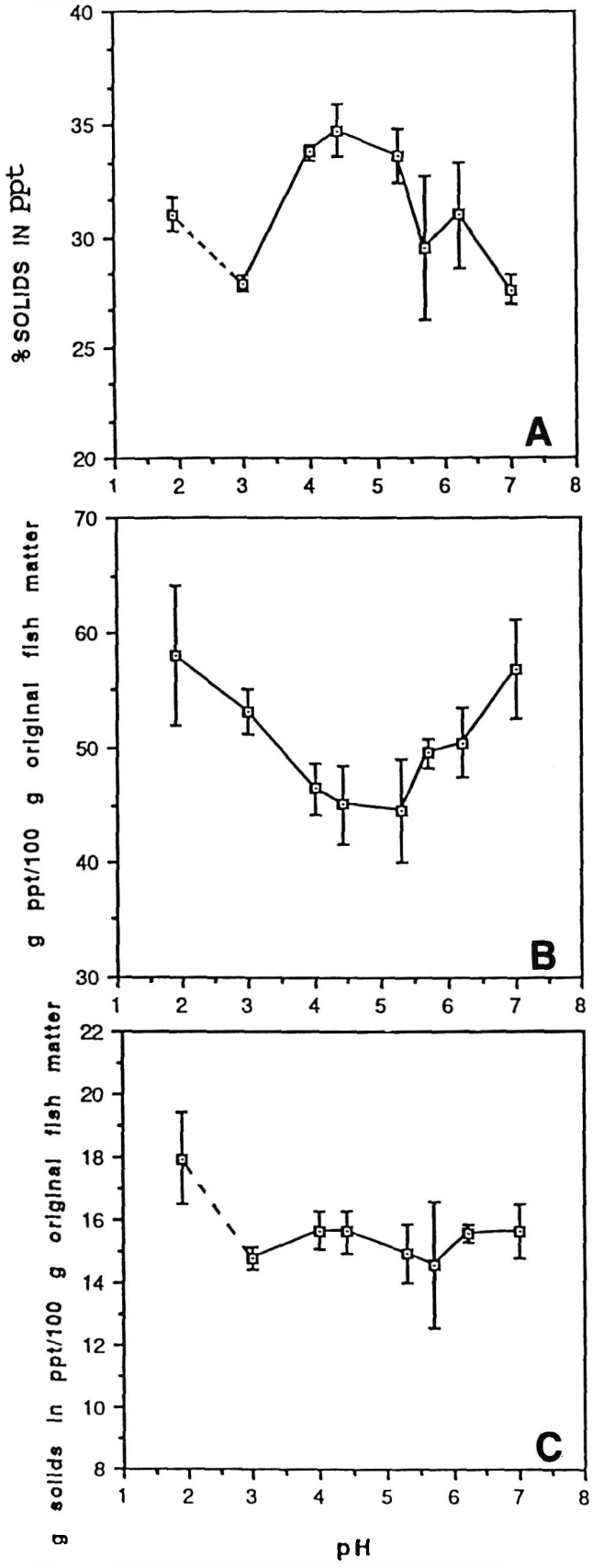

Fig. 1—Effect of pH on percent solids in the precipitate, precipitate yield, and solid yield. (Avg of two batches.)

cell model UHP-76, Amicon Div., W.R. Grace and Co., Danvers, MA) with a 10,000 MWCO cellulose regenerated membrane (YM 10, Amicon), at 483 KPa. Flux and solid content of permeates were determined.

G-force during centrifugation. Finely ground cod frames (200g) adjusted to pH 4.5, were centrifuged in the range of 1,020–30,629 × g for 20 min. Precipitates and supernatants were analyzed for solids.

Heating supernatant after centrifugation. Finely ground cod frames (50g) were adjusted to pH 4.5, and centrifuged (23,500 × g for 20 min). The resulting supernatants used then heated to 90°C for 20 min (water bath), cooled to room temperature (using ice bath) and further centrifuged at 23,500 × g for 20 min. The supernatants before and after heat treatment were then ultrafiltered to compare effects of heat on flux and solids content of permeates.

UF membrane. Three experiments were done to dewater ground fish waste. Main differences were pretreatments of incoming UF streams and thus their compositions. In the first ("heat" experiment) coarsely ground fish frame (pH 7.0) was heated to 65°C/15 min, then to 90°C/20 min (steam-injected cooker, from Custom Stainless Equipment Co. Inc., Rosa, CA) and pressed through a 100 mesh cider bag (10,550 KPa, hydraulic press, Wabash Metal Products Company, Inc., Wabash, IN). In a second ("pH" experiment) the sample was finely ground, adjusted to 4.5 (no heat) and similarly pressed. In a third ("pH-heat" experiment) the sample was finely ground, pH adjusted to 4.5, pressed, and the aqueous phase then heated to 90°C/20 min, before centrifuged (basket centrifuge model SBR, International Equipment Co., Boston, MA).

Aqueous extracts from these three experiments were ultrafiltered by a HF-lab-5 ultrafiltration unit (Romicon, Inc., Woburn, MA) with a 10,000 MWCO polysulfone membrane. The UF unit was connected to a feed tank (≈35L capacity), with immersed cooling coil to keep a relatively constant retentate temperature of 22°C. The permeate was collected over time or VCR (volume concentration ratio) the ratio of initial feed volume to volume of retentate. This experiment was carried out at 25°C and 103 KPa transmembrane pressure (ΔP_T). All experiments were done at least twice and variation among batches was within 7.0–8.5%.

Sample analyses

Total solids were determined by drying 24 hr in a vacuum oven at 60°C and 75.9 cm Hg (Method 934.01, AOAC 1990). Ash content was measured by incineration at 550°C to constant weight (Method 900.02A, AOAC, 1990). Nitrogen content was obtained by Kjeldahl (Method 928.08, AOAC, 1990). The level of BOD (Biological Oxygen Demand) of the UF permeate was determined according to APHA et al. (1985) after 5 days incubation at 20°C with inoculated microorganisms. All analyses were done at least in duplicate and experimental errors were within 2%.

RESULTS & DISCUSSION

Effect of grinding

Coarse and finely ground fish frames were compared for solids content in the precipitate (ppt), ppt yield, and solids yield (after centrifugation). Solids content in the ppt (Table 1) was similar at pH 4.5 and slightly different at pH 7.0. The ppt yield (weight/100g original matter) was significantly higher with coarse grinding than with fine grinding and higher at pH 7.0 than at pH 4.5 due to water trapped. However, the solids yielded in the ppt did not significantly vary regardless of grinding and pH.

Effect of pH

The ppt of solids content (Fig. 1A) was significantly higher at pH 4.5 than any other pH, resulting in 34% in the ppt. Thus, the solids were extracted best at pH 4.5. The amount of ppt (Fig. 1B) was lowest at that pH because of relatively high dewatering efficiency. With respect to solids yield (Fig. 1C) in the ppt, no significant variation was observed at most pH levels. Adjusting pH to 4.5 was effective in increasing ppt solids content and this was consistent with pI of fish proteins (Cheftel et al., 1985; Del Valle and Aguilera, 1990). Solids in the supernatant (data not shown) at pH 4.5 (6.4% solids) were also lowest, indicating that the precipitate lost most of its water to the supernatant, diluting the solids.

Effect of gravitational force

Solids content in the ppt increased sharply (Fig. 2) as g force was raised from 1,020 × g to 9,150 × g, reaching a value

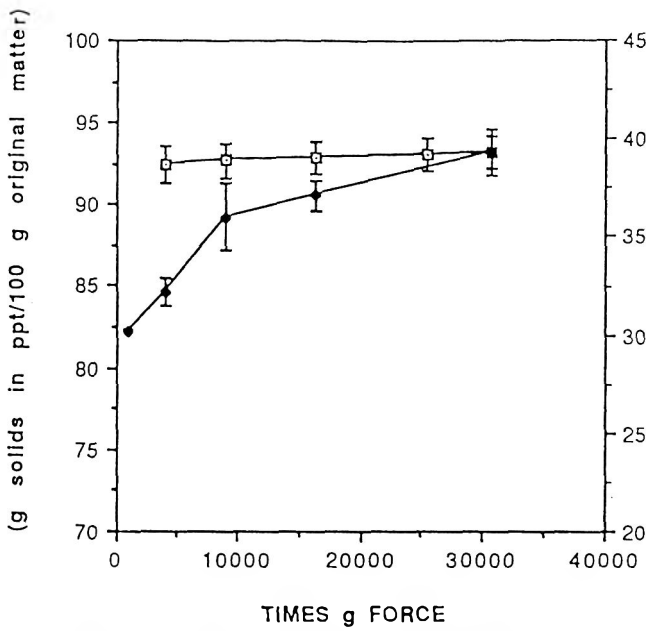

Fig. 2—Effect of gravitational force on solids yield (□–□ left axis) and percent solids (●–● right axis) in precipitate after centrifugal separations (20 mins) of ground fish frame (pH 4.5). (Avg. of two batches.)

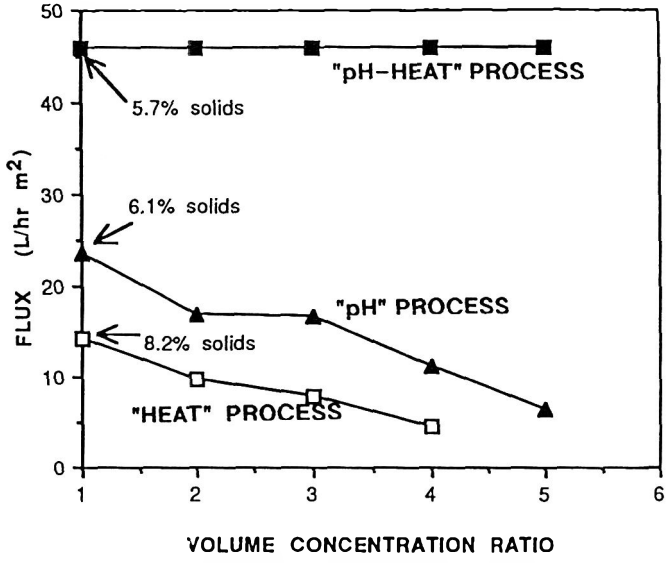

Fig. 3—Changes in flux during pilot scale ultrafiltration of fish wastes, using three different processes. (Avg. of two batches.) Ultrafiltration wes performed using a Romicon Lab-5, hollow fiber polysulfone membrane PM-10 (10,000 MWCO) at 86 KPa of initial transmembrane pressure and 25°C.

close to 36% solids. A further increase in g force resulted in a slower rise in solids. However, the solids yield in the precipitte were consistently high (92.8%), regardless of g force (Fig. 2). No significant effect of g force on supernatant solids was observed (6.1 ± 0.6%). Therefore, centrifuging at 9,150 × *g* was effective for obtaining a high solids content in the precipitate (36%). However, to achieve maximum dewatering, centrifugation at 30,000 × *g* would give the highest solids in the precipitate.

Effect of heat before centrifugation

The application of heat (90°C, 20 min) right after pH adjustment had a very noticeable liquiefying effect on the slurry making it much easier to centrifuge. However, it did not improve solids yield in the ppt but slightly increased solids content in the supernatant from 7.1% to 9.4%. If the aqueous phase was further ultrafiltered, it would cause major membrane fouling (no heat). An increase in supernatant solids content and membrane fouling were attirubted to collagen that was solubilized upon heating (Gildberg, 1982; Tarrant, 1982; Hultin, 1985). The supernatant gave a strong gel upon refrigeration overnight, confirming the presence of solubilized collagen (Morrisey et al., 1987).

Heating of supernatant after centrifugation

Heating of supernatant after centrifugation did not significantly reduce solids content of the liquid phase but when subjected to UF resulted in very significant improvement (1.5 times) in flux. This was probably due to heat precipitation of water soluble fouling proteins. A cross-flow hollow fiber pilot plant UF experiment also gave similar results indicating that the heat treatment was effective in precipitating water soluble proteins that fouled the membrane.

Ultrafiltration

The flux of the permeate from the "pH" process was 1.5 times higher (Fig. 3) than that from the "heat" process. This was probably mainly due to dissolved collagen and the higher solids in initial feed material in the "heat" process. Nevertheless, membrane fouling occurred in both processes (decreasing flux and ΔP_T) over time. Apparently, membrane fouling created back pressure in the system.

The decrease in flux in the "heat" process could result from several possibilities including increased solids in the retentate, increased fouling and decreased volumetric flow rate. The transmembrane pressure (ΔP_T), remained relatively constant over 1–3 VCR and then decreased. Considerable pressure drop (VCR 1–3), decrease in flux was probably due to increase in retentate solids which influenced greatly the viscosity and Reynolds number of retentate.

On the other hand, flux in the "pH-heat" process was consistently high at 45 L/hr m² (three times in the "heat" process, or double that of the "pH" process) and very little decrease in ΔP_T. This indicated a significant improvement in UF membrane performance. Although application of heat in this last process did not significantly reduce solids content of the UF feed, it apparently precipitated fouling proteins.

General mass balance

An adjustment of pH to 4.5 improved solids extraction (Fig. 4) resulting in relatively lower solids content in the aqueous phase as found in the "pH" and the "pH-heat" processes. After UF, although the final recombined product increased in

Table 1—Precipitate yields from coarse and fine grinding at pH 4.5 and 7.0 (avg. of two batches)

	Fine grinding (0.32 cm)		Coarse grinding (1.27 cm)	
	pH 4.5	pH 7.0	pH 4.5	pH 7.0
% Solids in ppt	34.7 ± 1.3[a]	27.5 ± 1.0[b]	32.0 ± 3.3[a]	24.8 ± 0.9[c]
ppt yield (g ppt/100g original matter)	45.0 ± 3.4[d]	56.9 ± 4.4[e]	54.6 ± 3.9[e]	65.6 ± 3.0[f]
Solids yield (g solids in ppt/100g original matter)	15.6 ± 0.7[g]	15.7 ± 0.8[g]	17.4 ± 0.9[g]	16.3 ± 0.8[g]

[a-g] Superscripts are results of significant test at 95% confidence level.

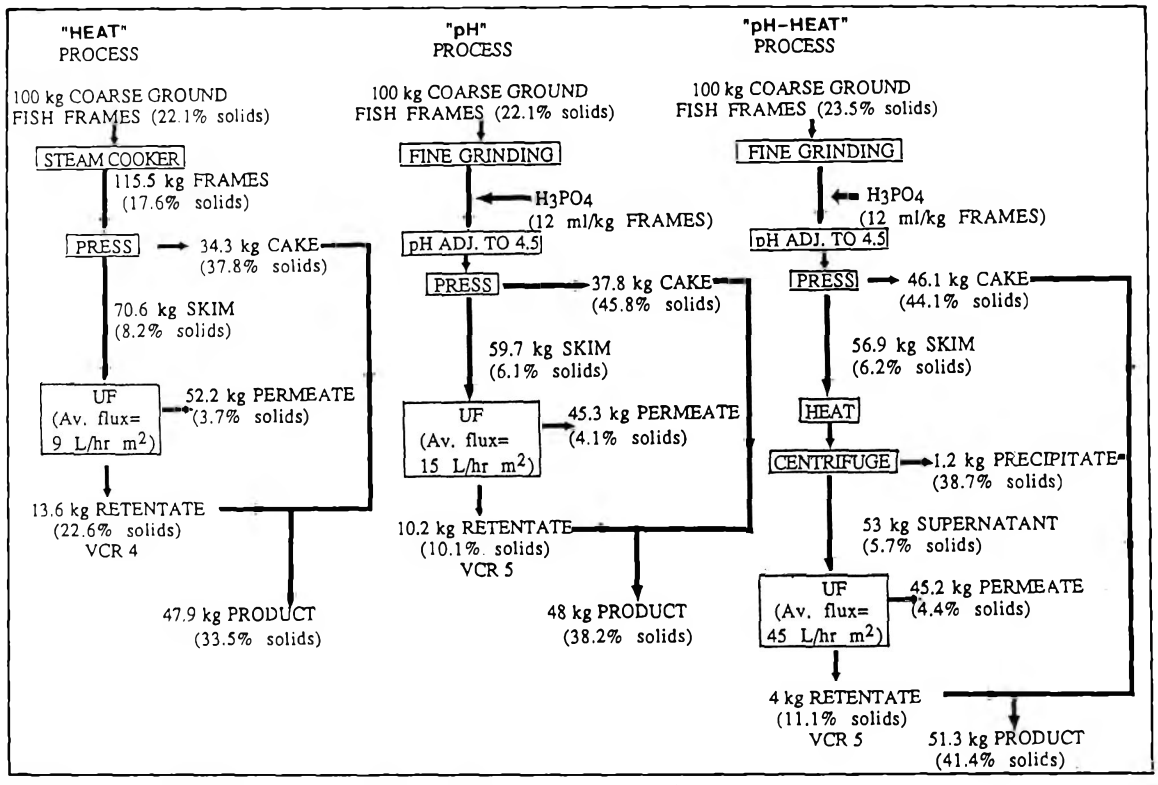

Fig. 4—General mass balance of the three processes. (Avg. of two batches.)

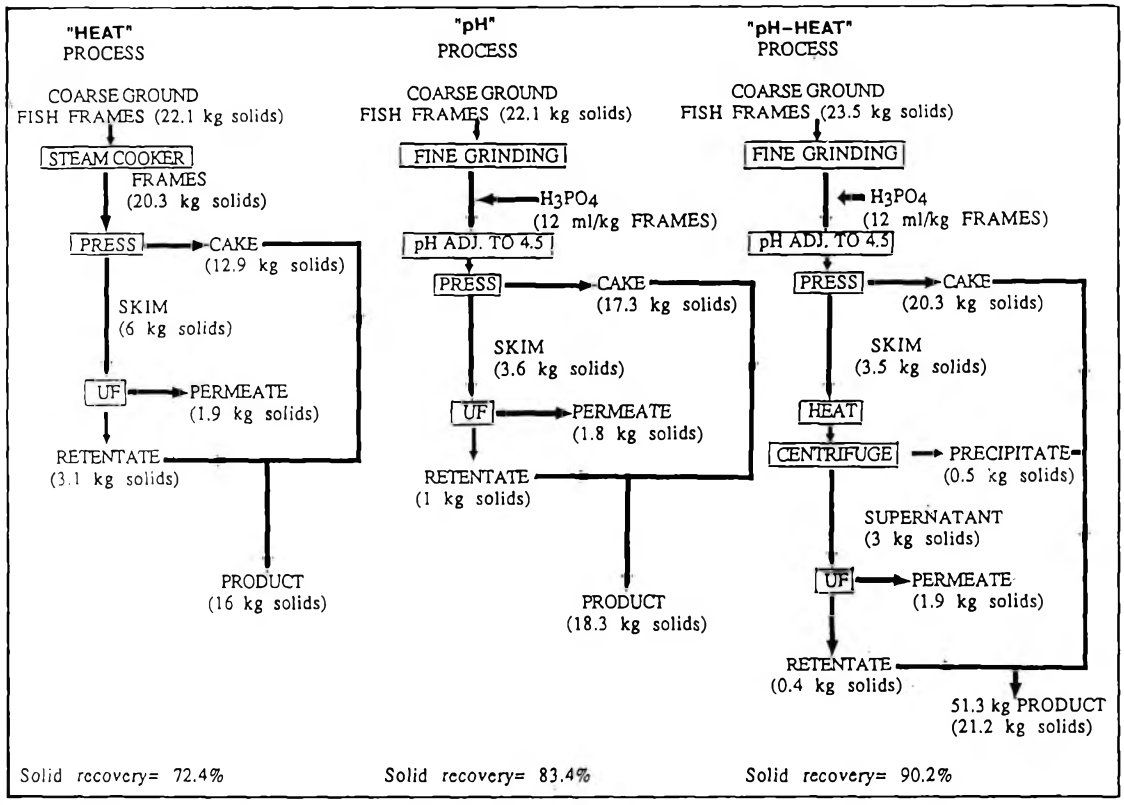

Fig. 5—General solid balance of the three processes. (Avg. of two batches.)

Table 2—Some physical and chemical values of the UF permeate using three different processes (avg. of two batches)

	% Total Solids	% Ash	% N (nitrogen)	BOD (mg/L)
"Heat" Process	3.7	0.8	0.5	6,480
"pH" Process (VCR 5)	4.1 ± 0.2	2.2 ± 0.1	0.2 ± 0.0	10,300 ± 212
"pH-heat" Process (VCR 5)	4.4 ± 0.5	1.1 ± 0.2	0.3 ± 0.0	11,000 ± 238

Table 3—Contents of different molecular weight fractions in the permeate from the "pH" and "pH-heat" process (avg. of two batches)

Molecular wt (daltons)	"pH" Process		"pH-heat" Process	
	% Solids	% Nitrogen	% Solids	% Nitrogen
< 5,000	0.3 ± 0.0	0.01 ± 0.0	0.3 ± 0.2	0.01 ± 0.0
1,000 <x< 5,000	0.1 ± 0.0	0.04 ± 0.0	0.3 ± 0.0	0.01 ± 0.0
<1,000	3.7 ± 0.4	0.17 ± 0.0	3.8 ± 0.2	0.25 ± 0.0

solids, the premeate of the "pH" and the "pH-heat" did not show any improvement (lowering) in percent solids compared to the "heat" process. Further treatment of the permeate therefore would still be required. The "pH-heat" process would be the most economical, both in terms of membrane area requirements and membrane clean-up cost due to high flux. A permeate flux of 14–25 (L/hr m^2) is suggested as minimum for UF processes to be economically viable (Chao and Davis, 1981). The "pH-heat" process, gave the highest flux of 45 L/hr m^2, and is therefore most recommended. The "pH-heat" gave the highest solid recovery (90.2%, Fig. 5) compared to the "heat" process (72.4%) and the "pH" process (83.4%).

Permeate composition

The "pH" and "pH-heat" processes gave permeates with higher total solids (Table 2) than the "heat" process. Although their nitrogen contents were lower than that of the "heat" process, their BOD levels were relatively high, 10,300-11,000 mg/L (estimated at 10.9-13.8 kg BOD/ton of seafood processed). This range of BOD was only slightly above the limit established by Environmental Protection Agency for mechanized processes for non-Alaskan conventional bottom fish (9.1 kg BOD/ton of seafood processed). If the "pH-heat" process were to be considered, use of UF membrane with <10,000 MWCO or different membrane type may help reduce BOD of the permeate to the acceptable level. Ash content of the permeate was higher in the "pH" and "pH-heat" than in the "heat" process (Table 2) because of the phosphoric acid used to adjust pH.

Note that the analytical values of ash and proteins (calculated from N×6.25) did not sum up to the total percent solids in the permeate from the "pH" and "pH-heat" processes. This was perhaps due to the use of value 6.25, based on average estimated values for proteins. It might not be suitable for fish frame systems with notable amounts of low molecular weight (small peptides, free amino acids, ammonia salts, nucleotides, etc.). Permeates of the "pH" and "pH-heat" processes also consisted of nitrogen components (Table 3) mostly <1,00 daltons. Another factor that might have influenced these results was the lipid fraction, that was not measured. However, lipid is usually present in a relatively small concentration in cod (less than 1%, EPA, 1975; Spinelli and Dassow, 1982).

CONCLUSION

pH ADJUSTMENT to 4.5 and centrifugation (9,000–13,000 × g) can effectively dewater ground cod frames. The aqueous phase (≈6.4% solids) needs further treatment before being discharged. For ultrafiltration, heating the slurry would result in dissolved collagen in the aqueous phase and badly fouled membranes. However heating the aqueous phase (after most suspended solids removed) helped extract foulants resulting in increased UF flux rates. UF, operated with polysulfouled membrane (10,000 MWCO), led to two to three times improvement in flux as compared to other processes. The permeate, slightly too high in BOD, could probably be further improved by changing membrane type and MWCO.

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Initial Interfacial Tension and Oil Uptake by Deep-fat Fried Foods

ELI J. PINTHUS and ISRAEL SAM SAGUY

ABSTRACT

Fundamental theory of surface chemistry was utilized to develop an equation for calculating initial interfacial tension between a product and liquid. The relationship provides a practical approach to overcome the complexity of quantitatively determining interfacial tension of foods. The range of interfacial tension between a restructured potato product and the frying medium was 0.01 to $5.23 \cdot 10^{-3}$ N/m. This range was determined using various frying media or additives. A power relationship was found between the initial interfacial tension and uptake of oil or other frying media. A model of capillary rise in porous media was implemented to explain the linear relationship between uptake and contact angle.

Key Words: oils, fats, interfacial tension, surface tension, fat absorption

INTRODUCTION

DEEP-FAT FRYING is a complex and important operation in industrial preparation of foods (Varela, 1988). Vast amounts of foods are produced by this method, but frying remains more an art than a science or technology (Blumenthal, 1991). Many factors affect oil uptake, including oil quality, frying temperature and duration, product shape, its content (e.g., moisture, solids, fat, protein), porosity, pre-frying treatments (e.g., drying, blanching) and coating (Selman and Hopkins, 1989). Gel-strength is another important factor which affects oil uptake (Pinthus et al., 1992). Surface chemistry has been applied to help understand various effects (e.g. wetting adhesion, capillary penetration and displacement) in relation to detergency, printing, textile, membrane technology and immunology (Adamson, 1990). The effectiveness of many scientific or commercial applications of multi-phase systems depends on the ability to control phase boundaries or interfacial interactions (Meyers, 1992).

Oil uptake during deep-fat frying of products initially containing little or no fat, takes place at the surface of the product. The absorbed oil is located near the surface and the crust (Farkas et al., 1992; Keller et al., 1986; Varela, 1988). Extensive research has focussed on surface chemistry and its application to food systems (e.g. ice cream, margarine, whipped toppings), concentrating on emulsification, solubilization and foaming (Dickinson and Stainsby, 1988; Friberg, 1976). Evaluation of the quality of frying oils based on the relative interfacial tensions of oil solutions in cyclohexane was suggested by several researchers (Hau et al., 1986; Yoshikawa et al., 1981; 1980). However, no published reports have been found on analyzing oil uptake during deep-fat frying by applying interfacial chemistry data.

Our objective was to elucidate and quantify the interfacial tension between oil or other frying media and a restructured potato product and its effect on uptake during deep frying.

THEORY

Interfacial tension determination

Deep-fat fried foods initially containing little or no fat, consist of two liquid phases (water and oil), in a porous solid medium. In such a system, the contact angle effects determine which pores will be filled by which liquid. The interfacial tension governs whether external oil would overcome the energy barrier to penetrate a porous structure, due to pressure gradients (Miller and Neogi, 1985). Hence, determination of the contact angle and interfacial tension are very important.

A fundamental equation relating contact angle to interfacial tension for a system in equilibrium is Young's (1805) equation:

$$\gamma_{la} \cos(\theta) = \gamma_{sa} - \gamma_{sl} \quad (1)$$

Where: θ = contact angle; γ = surface tension (N/m); s = solid; l = liquid; a = air (gas phase which may also contain liquid vapor).

In Eq. (1), $\cos(\theta)$ is derived from interfacial tension at equilibrium conditions. For practical purposes, it is defined that if the contact angle is $>90°$, the liquid does not wet the solid and tends to move about on the surface and not enter capillary pores. A liquid completely wets a solid only when the contact angle is zero (Adamson, 1990).

When Young's equation is applied to a frying system, γ_{sa} may be replaced by γ_s. This assumes that the absorbed oil film pressure, π_e, ($= \gamma_s - \gamma_{sa}$) for frying is zero. This assumption is justified, when θ is measured at room temperature, where vapor pressure of both oil and water are quite low. Therefore, the air on top of a liquid droplet is practically dry. Also, in a deep-fat frying process, the influence of vapors in the air is negligible because for most of the frying duration the product is immersed.

Interfacial tension

Young's equation contains two variables (γ_l and θ) which may be measured directly. However, quantifying of the other two (i.e., γ_s and γ_{sl}) is difficult. Deriving the interfacial tension (γ_{sl}) without the surface tension γ_s (not known for most solids) is not simple. To overcome this, Girifalco and Good's (1957) equation indicates the interfacial free energy should follow the relationship:

$$\gamma_{sl} = \gamma_s + \gamma_l - 2\Phi_{sl} (\gamma_s \gamma_l)^{0.5} \quad (2)$$

Where: Φ_{sl} = interaction parameter. Practically, Φ_{sl} expresses the partial contributions of intermolecular forces (dispersion, dipole-dipole, hydrogen bonding and repulsion; Adamson, 1990). It could be expressed:

$$\Phi_{sl} = (D_s^d D_l^d)^{0.5} + (D_s^i D_l^i)^{0.5} + (D_s^p D_l^p)^{0.5} \quad (3)$$

Where: D = relative contribution; D_s^d = solid contribution due to dispersion forces; i = induction forces; p = polar forces.

Combining Eq. (1) and (2) (assuming $\pi_e=0$), yields:

$$\gamma_{sl} = \gamma_l \left[\frac{[1+\cos(\theta_e)]^2}{4\,\Phi_{sl}^2} - \cos(\theta_e) \right] \quad (4)$$

where: θ_e = contact angle at equilibrium. Equation (4) allows calculation of interfacial tensions of liquid/solid and alleviates the need for γ_s. To use this equation the values of θ_e and γ_l at equilibrium should be measured, while Φ_{sl} is usually approximated.

Practical equation

As a first approximation, Φ_{sl} is defined as the molar volume of phase A and B (Girifalco and Good, 1957):

$$\Phi_{sl} = \frac{4\,V_A\,V_B}{[V_A^{0.33} + V_B^{0.33}]^2} \quad (5)$$

Where: V_A = molar volume of phase A; V_B = molar volume of phase B.

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Table 1—Frying media

	Generic name	Source
Sorbitan mono-oleate	Span 80	SMAZ 80 (PPG, Chicago, IL)
Polyoxyethylene sorbitan mono-oleate	Tween 80	TMAZ 80 (PPG, Chicago, IL)
Polyglycerol polyricinoleate	PGPR	Crester PR (Croda, UK)

Table 2—Interfacial tension between restructured potato product and frying media

Frying medium	Density (g/mL)	Contact angle (o)	Surface tension γ_l(N/m*10^3)	Interfacial tension γ_{sl} (N/m*10^3)
Soy oil	0.91	38	30.0	0.34
Soy oil (used)	0.91	33	29.6	0.19
Tween 80	1.08	77	34.8	5.23
Span 80	1.00	21	30.9	0.03
PGPR	0.97	59	31.7	1.94
Tween 80/oil[a]		~0	—	0.01
Span-80/oil[b]		~0	—	0.01

[a] Immersion in Tween 80 prior to frying in oil
[b] Immersion in Span 80 prior to frying in oil.

Equation (5) is an approximation which applies to a "regular interface." This approximation is not very applicable for food undergoing frying as the calculation of molar volume is not possible.

Another approximation for Φ_{sl} is more suitable for the frying medium. When either phase A or B is not polar, the dominating interaction between phases is through dispersion forces. A similar effect is observed between a polar and solid phase. Usually, foods are fried in nonpolar medium (e.g., soy oil), and the polar contribution is negligible. Thus, assuming $\Phi_{sl}=1$ is justified. Furthermore, this assumption has often been applied to other systems (Adamson. 1990). This approximation yields:

$$\gamma_{sl} \cong \gamma_l \left[\frac{[1 + \text{Cos}\ (\theta_e)]^2}{4} - \text{Cos}\ (\theta_e) \right] \quad (6)$$

or:

$$\gamma_{sl} = \frac{\gamma_l}{4} [1 - \text{Cos}\ (\theta_e)]^2 \quad (7)$$

Equation (7) provides an instrumental means for calculation of interfacial tension between food products and frying media. The two necessary values, namely θ_e and γ_l, could be measured directly.

Dependence of oil uptake on capillary rise

A model for overall capillary rise in a "microscopically uniform" (i.e., cylindrical capillary) porous medium including two fluids was suggested by White (1982), and further developed by Marmur (1989):

$$h_0 = \frac{(1 - \varepsilon_o)\ S_o\ \gamma_{lf}\ \text{Cos}(\theta_e)}{\rho\ g\ \varepsilon_o} \quad (8)$$

where: h_0 = capillary rise; g = gravitational acceleration; ρ = density difference between rising liquid and fluid which originally occupied the capillary; γ_{lf} = interfacial tension liquid/fluid (e.g., oil/water); θ_e = contact angle; ε_o = overall porosity; S_o = overall specific area of the solid.

Equation (8) is useful to assess h_0. Assuming an effective capillary radius, the total capillary volume derived from the utilization of h_0 would represent the quantity of the uptake medium. We further assumed that both S_o and ε_o were constant for the restructured potato product we studied and that γ_{lf} was proportional to γ_l. Thus Eq. (8) could be utilized to estimate the amount of medium uptake which is proportional to $\gamma_l \text{cos}(\theta)_e)/p$. Obviously, this model was based on the assumption of a uniform capillary structure. This assumption is a first estimation (Marmur, 1988), and should be further developed to account for local structure variations, and possible hysteresis.

MATERIALS & METHODS

Restructured potato

Restructured potato products shaped as cylinders (0.02m diameter × 0.03 m length) were prepared according to Pinthus et al. (1992). In one experimental series additional ingredients were added to the product mass at levels of 1–3%, replacing the identical amounts of potato flakes. The ingredients were: soy protein (Purina 590, Purina, Brussels, Belgium), vital wheat gluten (Amylum, Aalst, Belgium) and a natural ingredient (subject to a patent to be filed). The gel strengths of the restructured potato products were 1700 ± 30 and 1530 ± 25 Pa/s (Pinthus et al., 1992) for the first and second experimental series, respectively.

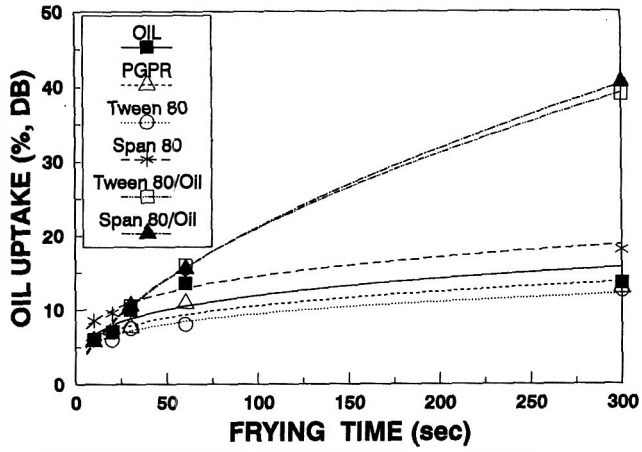

Fig. 1—Effect of frying medium on oil uptake during deep frying of a restructured potato product.

Samples were fried at 170°C in a bench-top deep-fat fryer (0.12m depth) containing 2.5L soy oil or other frying media (Table 1). When oil was used as frying medium, it was preheated for 2 hr prior to frying (Blumenthal, 1991), and discarded after 6 hr. Some samples were immersed prior to frying for 20 sec in either SMAZ 80 or TMAZ 80, and wiped gently with a paper towel to remove excess emulsifier. Net mass gain due to this immersion was < 0.1% of initial mass. Samples were fried for different periods of time from 30 to 300 sec, removed from the frier and allowed to cool to room temperature (23 ± 2°C) on a paper towel.

Surface tension and contact angle

Liquid surface tension was determined at room temperature (23 ± 2°C) on a Tensiometer (Lauda, Konigshofen, Germany), using the De Nouy method (DIN #53914, 1980). The equilibrium contact angles between frying media and sample were measured on a Goniometer (NRL C.A. Model 100; Rame-Hart Inc., Mountain Lakes, NJ). The restructured potato product was extruded into a synthetic casing sleeve and kept frozen (−20 ± 1°C) until evaluated (Pinthus et al., 1992). The sleeves were peeled and the restructured potato was allowed to equilibrate to room temperature (23 ± 2°C) for 15 min prior to measurement. Note that shorter equilibration periods resulted in inconsistent results. The equilibration period allowed the sample external surface to become dry which ensured reproducible results. Droplets of 0.1 mL liquid were deposited with great care from a pipette. The equilibrium contact angle was measured several times on both sides of three or more drops. The values reported are mean values with a standard deviation of ≈ ± 3°. The contact angle did not vary for about 120 sec during the measurement. Gel-strength, moisture content, oil or frying medium uptake and statistical analyses were carried out as previously described (Pinthus et al., 1992).

RESULTS & DISCUSSION

THE EFFECT OF FRYING MEDIUM on surface tension and interfacial tension (derived from Eq. (7) (Table 2) showed contact angle of the frying medium varied from ~21 to 77°, while the surface tension γ_l, was within a more narrow range, 29.6 to 34.8 * 10^{-3} N/m. Used oil (exposed to 10 frying cycles of 2 hr at 170°c during a period of 1 month), had a lower interfacial tension than fresh oil (0.19 vs 0.34 * 10^{-3} N/m, respectively). This was related to the formation of surfactant products during the frying process (Blumenthal, 1991). Dipping the potato product in an emulsifier prior to frying practically reduced the contact angle to zero (complete wetting), resulting in a very

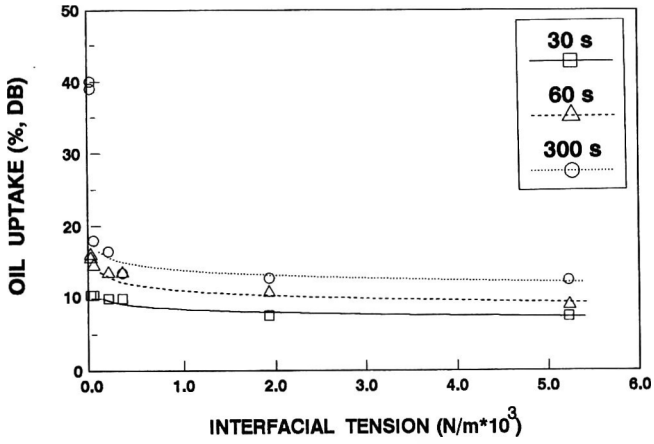

Fig. 2—Effect of initial interfacial tension between a restructured potato product and various frying media on oil uptake during deep-fat frying for 30, 60 and 300 sec.

Table 3—Interfacial tension between restructured potato products containing some natural ingredients and oil

Additive	Interfacial tension (N/m *10^{-3})
Restructured potato (control)	0.33
Natural ingredient 1%	0.69
Natural ingredient 2%	1.27
Gluten 1%	1.66
Soy protein 1%	1.66
Natural ingredient 3%	1.88

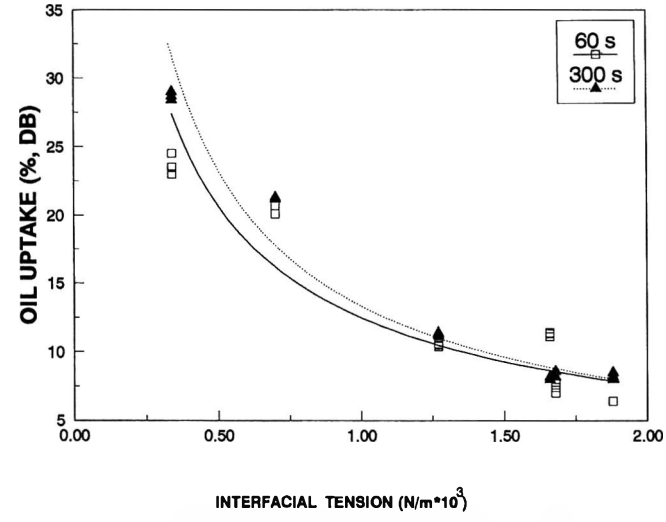

Fig. 3—Effect of initial interfacial tension between soy oil and a restructured potato product containing various natural ingredients on oil uptake during deep-fat frying for 60 and 300 sec.

low interfacial tension. Also Tween 80, a hydrophilic emulsifier, had a much larger interfacial tension compared to oil. However, Span 80 (hydrophobic emulsifier) had a significantly lower interfacial tension. The interfacial tension between frying medium and product, was not affected by the restructured potato product gel-strength (between 850 to 1860 Pa/s utilized by Pinthus et al., 1992). Hence, interfacial tension and gel-strength were independent and their effects on uptake should be evaluated separately.

The hydrophilic Tween 80 was absorbed by the product to an apparent lower extent than oil (Fig. 1) throughout frying. Note the marked effect of dipping the product in an emulsifier prior to frying. The existence of surface active agent (either hydrophilic or hydrophobic) on the product/oil interface, markedly reduced the interfacial tension. A similar observation was

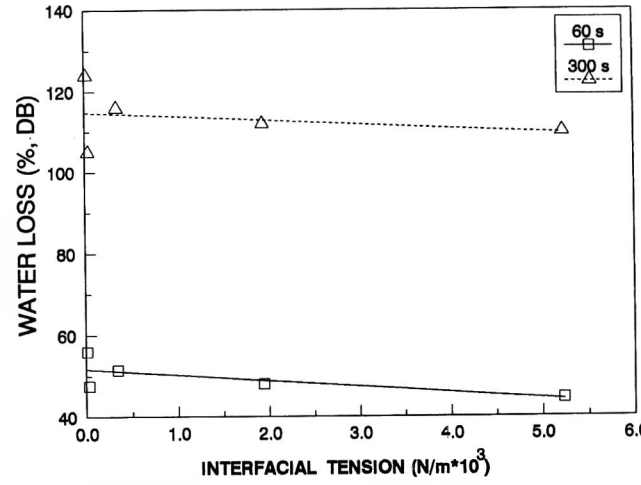

Fig. 4—Effect of initial interfacial tension between various frying media and a restructured potato product on water loss during deep frying for 60 and 300 sec.

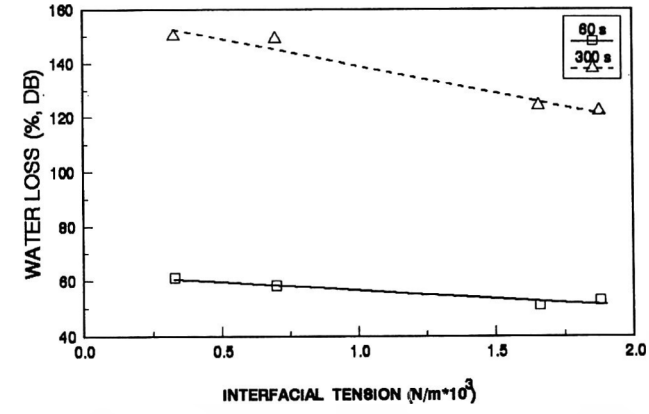

Fig. 5—Effect of initial interfacial tension between soy oil and a restructured potato product containing various added natural ingredients on water loss during deep-fat frying for 60 and 300 sec.

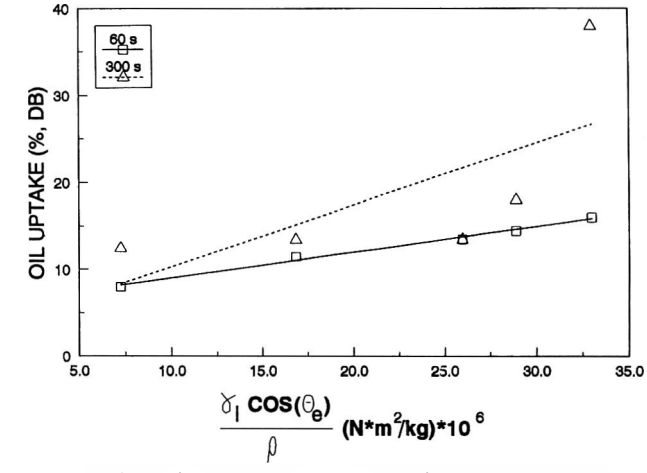

Fig. 6—Effect of $\gamma_l \cos(\theta_e)/\rho$ on uptake of a restructured potato product fried for 60 and 300 sec in various frying media.

reported for emulsification processes (Dickinson and Stainsby, 1988; Friberg, 1976).

Interfacial tension between product and frying medium also affected (Fig. 2) uptake for three frying times (30, 60 and 300 sec, representing short and medium range times). Each data point is an average of three measurements with ±2% coefficient of variation. To describe the effect of interfacial tension

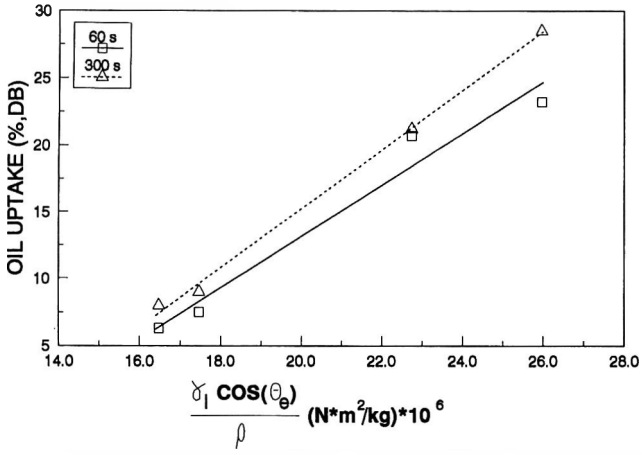

Fig. 7—Effect of $\gamma_l\cos(\theta_e)/\rho$ on oil uptake during deep-fat frying of a restructured potato product containing various added natural ingredients fried for 60 and 300 sec.

on liquid uptake (oil or other frying medium), several models were attempted. Best-fit was obtained when a power relation was implemented within the range of interfacial tension of zero to 2.0 ($N/m*10^{-3}$) and frying time ≥60 sec, ($p \leq 0.01$):

$$\text{Oil uptake}(\%, \text{DB}) = a\,(\gamma_{sl})^{-b} \qquad (9)$$

Medium uptake increased markedly when initial interfacial tension was lower. This may suggest that a hydrophobic surface would increase oil uptake during deep-fat frying. However, treatments of additives which would increase the interfacial tension would reduce oil uptake. The effect of product hydrophobicity has been suggested (e.g., Blumenthal, 1991).

The relationship between frying medium uptake and interfacial tension was shown for cases where interfacial tension was modified by changing frying media. In another experimental series several additives were incorporated in the product mass. Additives which had different interfacial tensions were chosen for further evaluation and the interfacial tensions (oil/product) were developed (Table 3).

When oil uptake was related to interfacial tension (Fig. 3) a power relation was again found (R^2 was 0.85, 0.96 for 60 and 300 s frying time, respectively; $p \leq 0.01$). In the relation between water removal from fried product, and interfacial tension for various frying media (Fig. 4) the decline in water evaporation with increased interfacial tension was insignificant at both 60 and 300 s frying time. On the other hand, when the formulation of the fried product was modified, the linear effect of initial interfacial tension on water loss (Fig. 5) was significant (R^2 of 0.96 and 0.91; at 60 and 300 s frying time, respectively; $p \leq 0.01$). Further research is needed to elucidate effects of a possible structural change that may have modified surface characteristics (e.g., roughness, pore size).

Note that the initial interfacial tension affected uptake throughout the process (60 through 300 sec). This effect was not restricted to very short frying periods and could be extended with increased confidence up to 300 sec. However, immediately after initiation of frying, oil was absorbed on the surface of the product, and consequently the interfacial tension product/oil was reduced to zero. This change indicated that relating oil uptake to initial interfacial tension may be justified, if oil absorption is considered as taking place at a moving oil "front" interface. The relation between uptake and capillary rise and $\cos(\theta_e)$ was expressed in Eq. (8). We assumed that S_o and ε_o and other coefficients resulting from deviation from the theoretical model were the same for all restructured potato product cylinders, and that γ_{lf} was proportional to γ_l. Thus, the capillary rise, h_o and uptake should be directly proportional to $\gamma_l\cos(\theta_e)/\rho$.

The mechanism of penetration may be considered by the model of capillary displacement in porous media (Marmur, 1989). In that model, a liquid-vapor interface was continuously created by "re-exposing" the liquid to vapor inside the porous medium and its boundaries. The re-exposing effect would be absent in the case of penetration into capillaries where the liquid-vapor interfacial area may fluctuate but not continuously increase (Marmur, 1988). To elucidate this mechanism, further studies are needed on the effects of capillary rise, surface characteristics (e.g. roughness) and porosity on oil uptake during deep-fat frying.

When effects of $\gamma_l\cos(\theta_e)/p$ (Eq. 8) and frying time on oil or frying media uptake (Fig. 6 and 7) were compared, high linear correlation coefficients were obtained ($R^2 > 0.97$, except for 300 sec). This suggests that capillary displacement could be very important in the mechanism of oil uptake. The results of 300 sec frying time deviated from the expected dependency due to one data point that was obtained when the product was immersed in an emulsifier prior to frying. This outlier point reflects a case where penetrating frying oil had to pass first through the surface covered by a film of an emulsifier. Then the interface changed to a "regular" restructured potato product surface, while the penetrating liquid changed its characteristics by accumulating the emulsifier. Under such conditions, the dependency of uptake on $\gamma_l\cos(\theta_e)/p$ would undoubtedly be more complex.

Note that oil uptake affected the amount of water the product lost during frying. Several mathematical relationships between moisture loss and oil uptake have been suggested (e.g., Mittelman et al., 1982; Rice and Gamble, 1989). However, the effect of oil uptake on moisture loss has not been reported. A possible explanation may be related to the crust formed on the surface of food during the process. The crust may act as a diffusion barrier that limits mass transfer. Inner moisture converted to steam may find selective channels in the structure and escape through open capillaries, pores and/or crevasses. Oil that enters the voids left by the water may be important in keeping the structure from collapsing (Lozano et al., 1980), and may contribute to keeping capillaries open. Hence, moisture loss could be affected by the amount of oil or frying medium uptake.

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Quantitative Determination of Water and Lipid in Sunflower Oil and Water/Meat/Fat Emulsions by Nuclear Magnetic Resonance Imaging

S. L. DUCE, S. ABLETT, T. M. GUIHENEUF, M. A. HORSFIELD and L. D. HALL

ABSTRACT

A Dixon NMR imaging experiment was used to map the spatial distribution of either the water or lipid protons in sunflower oil and water and meat and fat emulsions. A phase correction algorithm was applied to the experimental image data compensate for regional inhomogeneities of the magnetic field and allowed the water and lipid concentrations from any region in the image to be measured quantitatively.

Key Words: lipids, oils, meat emulsions, NMR imaging

INTRODUCTION

NUCLEAR MAGNETIC RESONANCE (NMR) IMAGING (MRI) provides two and three dimensional images from large, heterogeneous opaque systems without damaging the sample. It provides advantages as an imaging technique in food research (Wang et al., 1988; Chen et al. 1989; Ishida et al., 1989; HeilGerman and McCarthy 1989; Duce et al. 1990a,b; Heil et al., 1990). The image signal rises from protons on mobile molecules, typically water and lipids. The contrast in an image depends on factors such as concentration of protons, their ^1H longitudinal (T_1) and transverse (T_2) relaxation times, type of NMR pulse sequence used and timings within the sequence. Image intensity is normally a combination of all those variables, so NMR imaging usually has been only used for qualitative information. However, considerable effort has been directed toward image protocols that give quantitative data (Duce et al., 1990a; Attard et al., 1991; Doran et al., 1992), where the signal in the image is sensitive to only one of the parameters.

Our objective was to determine whether it was possible to measure water and lipid content of water and lipid emulsions directly from the NMR image. Several emulsion systems have been studied (Heil et al., 1990; Kauten et al., 1991; Winkler et al., 1991; Simoneau et al., 1991) but in most cases only partial discrimination of water- and lipid-enhanced images were presented. Several NMR imaging pulse sequences can discriminate between lipid and water protons on the basis of the differences in NMR properties (Pykett and Rosen, 1983; Dixon, 1984; Bottomley et al., 1984; Hall and Sukumar, 1984; Haase et al., 1985; Dumoulin and Vatis, 1985). We used a modified Dixon experiment (Dixon, 1984; Horsfield et al., 1990) to produce chemical shift resolved images of stable sunflower oil and water and meat/fat emulsions, which would allow the water or liquid lipid contents of these biphasic samples to be determined quantitatively from any region in the image.

MATERIALS & METHODS

Chemical shift resolved imaging

The NMR imaging experiments were carried out on an Oxford Research Systems Biospec I spectrometer that operates at 84.7 MHz for protons, connected to an Oxford Instruments 31 cm horizontal bore, 2 T superconducting magnet. Linear magnetic field gradients of 8 kHz/cm were generated using 20 cm diameter home built gradient coils (Carpenter et al., 1989). The samples were studied in a split-ring solenoid resonator with an inner diameter of 6 cm (Hall, 1985). All experiments were performed at ambient temperature (294 K).

The Dixon NMR imaging sequence differentiates between lipid and water signals on the basis of their chemical shifts. It involves the acquisition of two image data sets. The first is produced by a spin warp sequence (Edelstein et al. 1980) in which the water and lipid signals at the center of the gradient echo are inphase. The second image is produced from a modified spin warp sequence, which incorporates an additional time delay ($\Delta\tau$) before the 180° refocusing pulse. Generally $\Delta\tau$ is chosen so that at the center of the gradient echo the phase angle between the water and the lipid resonances differs by 180°. Thus, $\Delta\tau$ is typically equal to $1/(2\Delta f)$, when the lipid signal is regarded as a single resonance, and Δf is the difference between the water and lipid chemical shift frequencies. When the transmitter reference is set at the water precessional frequency, the addition of the two phase sensitive image data-sets produces water-only and subtraction produces lipid-only images.

Data sets (128 × 128) were acquired using a 180° slice-selection Dixon imaging sequence (Dixon, 1984). The 90° pulse length was 50 μs and the 180° slice-selective pulse excited a horizontal plane 3 mm thick through the sample. At $\Delta\tau$ delay of 1.67 ms was used, the echo time and recycle time of each experiment were as stated in figure legends. The Dixon images were Fourier transformed in phase-sensitive mode. A post-acquisition processing scheme was applied to compensate for phase errors caused by inhomogeneities in the external magnetic field (Yeung and Kormus, 1986; Bordello et al. 1987; Horsfield et al., 1990). The two data sets were co-added and -subtracted to produce water- and lipid-resolved images, respectively.

Sample preparation

Stable sunflower oil emulsions were prepared using a Silverson high speed mixer to disperse the sodium caseinate solution and oil, and a Crepaco homogeniser to develop the emulsion. Nine emulsions were prepared with water contents from 50% to 95% w/w using a 2% aqueous sodium caseinate solution, and seven other emulsions were prepared with water contents from 30% to 90% w/w using a 10% aqueous sodium caseinate solution. All emulsions were imaged in 1 cm diameter vials along with vial of sodium caseinate solution and a vial of sunflower oil which served as reference samples.

Seventeen meat and fat emulsions were prepared by homogenising chicken meat with pork fat; the water contents (% w/w) of the meat emulsions were determined by oven drying on sand at 373 K for 16 hr, and the lipid contents (% w/w) were determined by acid hydrolysis followed by Soxhlet solvent extraction. Four emulsions were imaged simultaneously in 2 cm diameter vials along with a 1 cm diameter vial of 0.1 molar manganese chloride solution which served as the water reference.

RESULTS & DISCUSSION

THE WATER- AND LIPID-RESOLVED IMAGES of the 2% sodium caseinate, sunflower oil emulsions (Fig. 1) showed that the image intensity in the water-resolved images increased as a function of increasing water content in the emulsions. Also, the image intensity in the lipid-resolved images increased as a function of increasing lipid content. The signal intensity "I" in a chemical shift resolved spin echo image is dependent on

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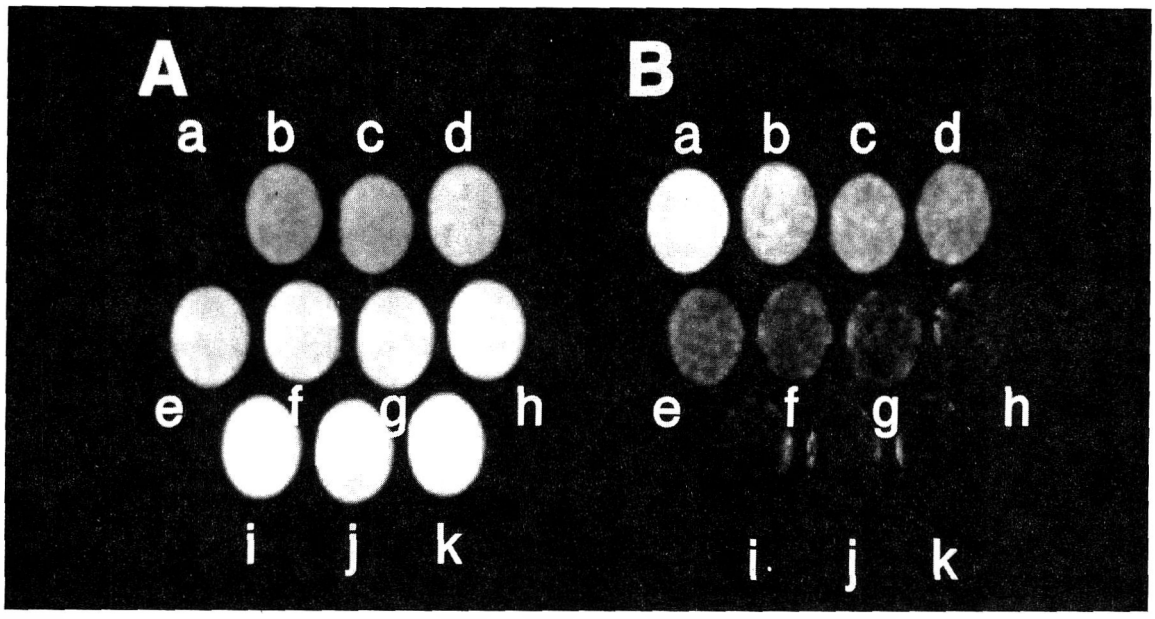

Fig. 1—128×128 chemical shift resolved images of sunflower oil/water emulsions with 2% sodium caseinate emulsifier: (A) Water image; (B) Lipid image. TE = 8 ms, TR = 10s, slice thickness = 3 mm. (a) Sunflower oil reference, (b) 50% water emulsion, (c) 58.4% water emulsion, (d) 68.2% water emulsion, (e) 75% water emulsion, (f) 81% water emulsion, (g) 86% water emulsion, (h) 89.7% water emulsion, (i) 93% water emulsion, (j) 95.2% water emulsion, (k) water reference.

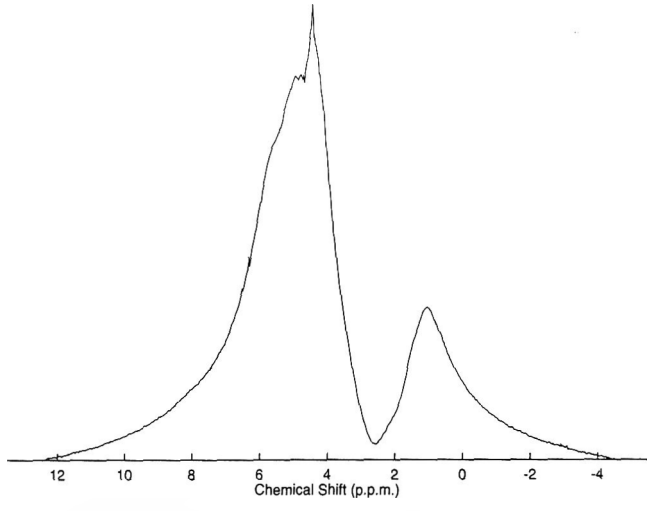

Fig. 2—84.7 MHz ^{1}H spectrum of 60:40 w/w meat and fat sample acquired at 294 K.

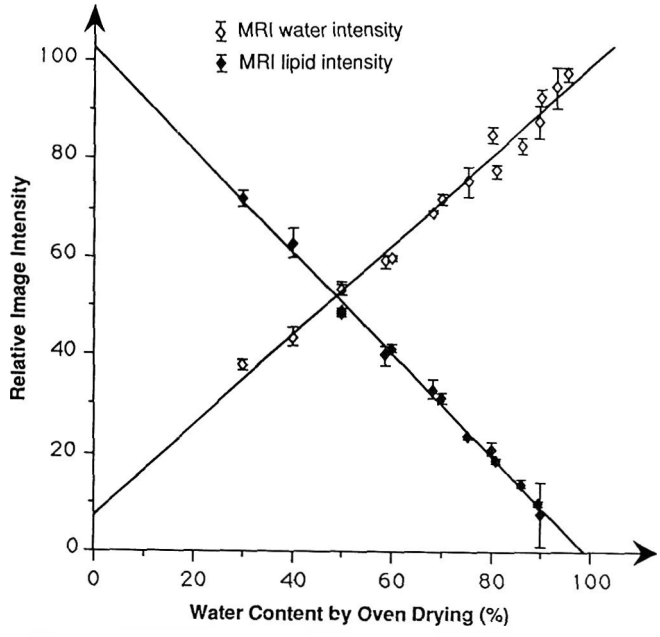

Fig. 3—Normalized water- and lipid-resolved image intensity of sunflower oil/water emulsions vs water content of emulsions. Pooled standard deviation of water image data was 1.46 and of the lipid image data was 1.35.

several parameters, but principally on the density of either the water or lipid protons (ρ) in the region, their longitudinal (T_1) and transverse (T_2) relaxation times, the echo time (TE) and recycle time (TR) in the pulse sequence, such that:

$$I = k \cdot \rho \cdot \exp(-TE/T_2) \cdot [1-\exp(-TR/T_1)] \qquad (1)$$

where k is a constant, and assuming that TR>>TE, and that mass transport of the nuclear spins during the echo period is negligible. For all our imaging experiments the recycle time was at least five times longer than the longest longitudinal relaxation time of the water and liquid lipid protons in the sample ($T_1 < 0.8$ s), and the echo time (TE) was significantly shorter than the transverse relaxation time of the water and liquid lipid protons ($T_2 > 40$ ms). Consequently the T_1 and T_2 terms in Eq. (1) could be neglected as they were both effectively, unity. Thus under these conditions only the concentration of either the water or lipid protons would affect the intensity of signal "I" in each pixel of the images.

A ^{1}H spectrum of 60:40 w/w meat and fat sample (Fig. 2) showed the water protons resonate at 4.7 ppm, and the aliphatic lipid resonances overlap and can be regarded as a single peak at 1.1 ppm. Thus using a 2 T external magnetic field (B_o) the water and lipid resonances were 300 Hz apart. The water resonance linewidth at half height is 150 Hz and the majority of this line broadening arises from inhomogeneities in the external B_o magnetic field. Local magnetic field inhomogeneities are also present at the liquid-solid or liquid-air interfaces. These gradients are produced by abrupt changes in the magnetic susceptibility at these interfaces (Callaghan, 1990). In our study, the local field gradients were strongest at the edges of the vials and could cause distortions at the edges of the images. However, the modified Dixon imaging sequence is quite tolerant to line broadening resulting from magnetic field inhomogeneities because it is based on a spin echo pulse sequence

Table 1—Water and lipid content of sunflower oil emulsions and the mean normalized water- and lipid-resolved image intensity[a]

Water content of emulsion (% w/w)	Lipid content of emulsion (% w/w)	Mean water-resolved image intensity	Mean lipid-resolved image intensity
30	70	38	72
40	60	43	63
50	50	53	49
58.4	41.6	59	40
60	40	60	41
68.2	31.8	69	32
70	30	72	31
75	25	76	24
80	20	85	21
81	19	78	19
86	14	83	14
89.7	10.3	88	10
90	10	93	11
93	7	95	5
95.2	4.8	98	—

[a] Pooled standard deviation of water image data was 1.46 and of lipid image data was 1.35.

Table 2—Water content of meat/fat emulsions and mean normalized water-resolved image intensity[a]

Water content of emulsion (% w/w)	Mean water-resolved image intensity
21.4	24
26	29
34.6	35
42.4	48
44.4	44
50.7	51
51.4	53
56.5	59
57.1	57
62.2	62
63.1	61
66.7	65
69.8	67
72.0	71
74.8	75
74.8	77

[a] Pooled standard deviation of water image data as 2.55.

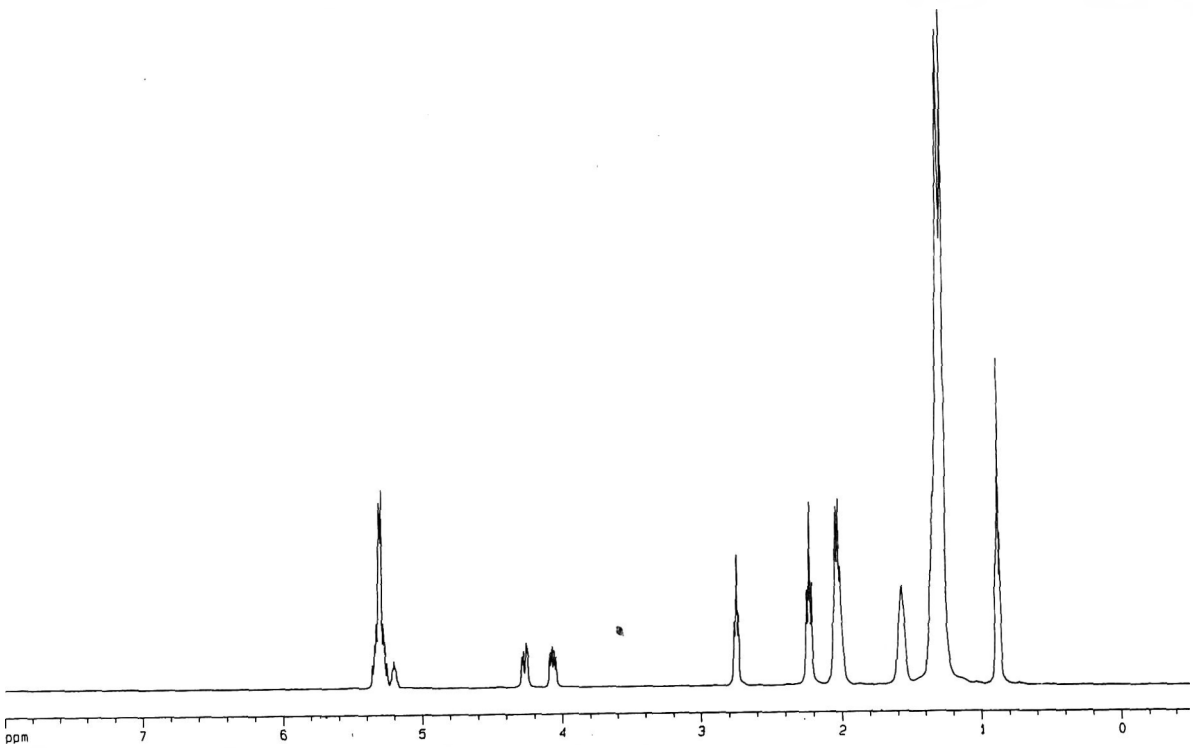

Fig. 4—High resolution 300 MHz ^{1}H spectrum of sunflower oil acquired at 294 K.

which refocuses the coherent dephasing of the nuclear spins. Also a post acquisition processing scheme is applied which corrects for phase errors produced by magnetic field inhomogeneities (Horsfield et al., 1990). The program is based on an algorithm developed by Borrello et al. (1987).

The 16 sunflower oil emulsions were imaged 3 times each and the average pixel intensities from the water- and lipid-resolved images were measured; the average noise per pixel in the images was also estimated. The water or lipid concentration of the emulsion was determined by subtracting the mean noise value from the image intensity and then normalising the signal with respect to water or lipid reference signal as appropriate, which was assigned as 100. The normalised water- and lipid-resolved image intensity for the different emulsions was plotted vs the gravimetric water content of the emulsion (Fig. 3) and the results were summarised (Table 1). A linear least square fitting algorithm (Press et al., 1988) was used to analyze the data. A linear correlation occurred between the water-resolved image intensity and water content, (correlation coefficient 0.98 and standard deviation 1.46). The χ^2 value was 7.71 and the goodness-of-fit coefficient Q was 0.91 accounting for 2% systematic noise error in the image signal intensity data. However, although the value of the image intensity from this linear correlation at 100% water content was 99.6 (Fig. 3), the image intensity at 0% water content was not zero as expected, but 7.5. The normalized water-resolved image intensity from all the emulsions with low water content was higher than their gravimetric water contents (Table 1). That was because the sunflower oil is a mono-unsaturated lipid. A high resolution 300 MHz ^{1}H spectrum of sunflower oil is displayed (Fig. 4) in the olefinic proton resonances between 4-5.5 ppm and thus they would contribute to the signal in the water-resolved image. The relative intensity of the olefinic protons compared to the cluster of proton resonances with chemical shifts between 1–3 ppm is 15:100, which is higher than observed in the Dixon image of pure sunflower oil. This contribution would be significant for emulsions with water contents < 50%. It is possible to improve the accuracy of this experiment for low water content samples that contain molecules with protons that have a similar chemical shift as water. Either a calibration curve could be produced using samples of known water content or alternatively a simpler method would

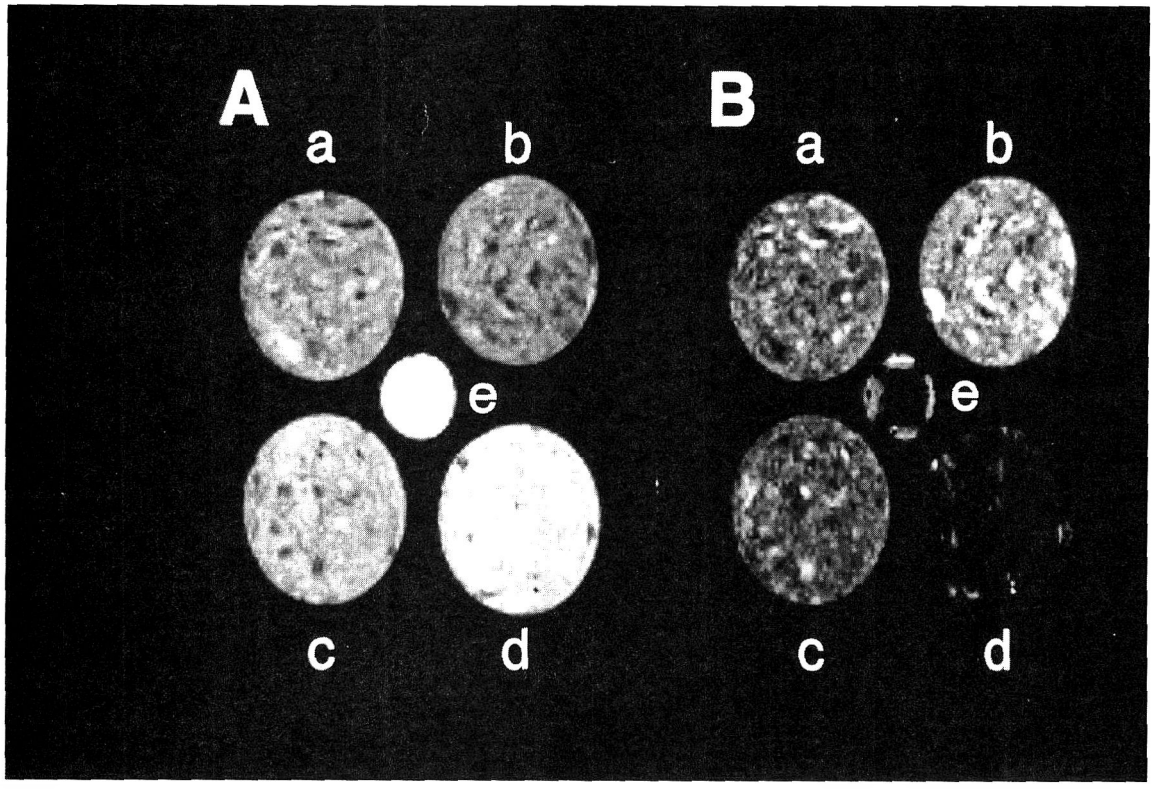

Fig. 5—128×128 chemical shift resolved images of meat/fat emulsions: (A) Water image; (B) Lipid image. TE = 8 ms, TR = 5 s, slice thickness = 2.5 mm. (a) 51.4% moisture content emulsion, (b) 44.4% moisture content emulsion, (c) 57.1% moisture content emulsion, (d) 74.8% moisture content emulsion, (e) Water reference.

be to correct the data using an internal calibration sample. In that case a vial of pure lipid could be used. If the normalised water-resolved signal of the unknown sample is I_W and that of the 100% lipid calibration sample is I_L; then it is possible to determine the water content (w_C) of the sample to a first order compensation as $w_C = I_w - \{(100 - I_w)/100\} I_L$ (%). There is also a linear relationship between the lipid-resolved image intensity normalised to the 100% lipid reference and the water content of the emulsions. This has a correlation coefficient of 0.99 and a standard deviation of 1.35. The χ^2 value was 2.00 and the goodness-of-fit coefficient Q was 0.99 accounting for 2% systematic noise error in the image signal intensity data. In this linear correlation the normalised image intensity is as expected, at 0% water content the image signal is 101.5 and at 100% water content is zero (Fig. 3). Thus the lipid content of these sunflower oil emulsions could be measured directly from the normalised image intensity of the lipid resolved image. NMR is an inherently insensitive technique and much of the scatter in the data is due to radio-frequency noise arising from the sample, the probe and electronic circuits. Because this noise is random, results can be improved by increasing the signal-to-noise of the data either by signal averaging or preferably studying the same sample a number of times and averaging results. The averages of three experiments are reported in Tables 1 and 2. Also, the contribution of noise in the image can be determined and it is worthwhile to correct for this systematic error during data analysis.

The water- and lipid-resolved images of four meat emulsions and the water reference are displayed (Fig. 5). As with sunflower oil emulsions, the image intensity in the water-resolved images increased as a function of increasing water content in the emulsions. The image intensity in the lipid-resolved images increased as a function of increasing lipid content. Seventeen meat emulsions were imaged at least three times each; the average pixel intensity from the water images was measured and the average noise per pixel in the water-resolved images was estimated. The water content of the emulsions was

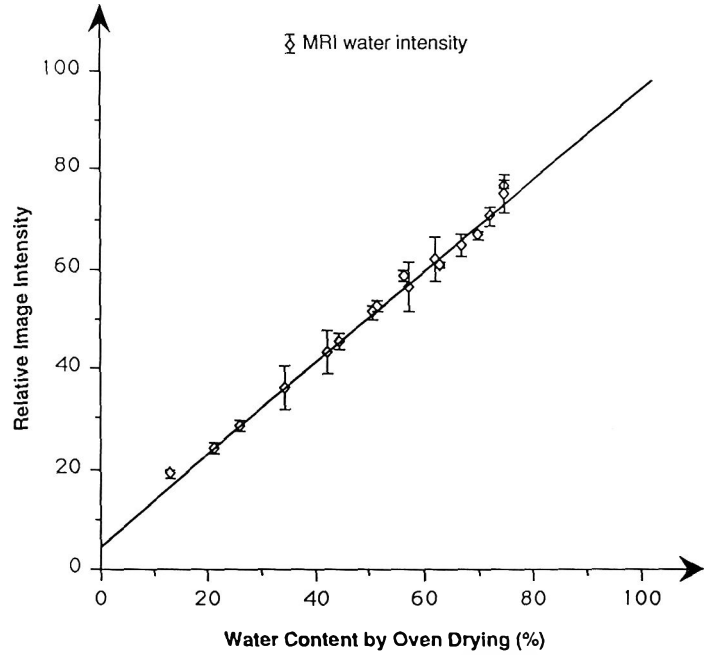

Fig. 6—Normalized water-resolved image intensity of meat/fat emulsions vs moisture content of emulsion. Pooled standard deviation of the water image data was 2.55.

determined by subtracting the mean noise value from the image intensity and then normalising the signal with respect to the water reference sample, which was assigned as 100. The normalized water-resolved image intensity for the different meat emulsions was plotted vs the gravimetric water content of the emulsions (Fig. 6) and the results were summarised (Table 2). The high signal intensity regions at the edges of the lipid-resolved image of the water reference sample (Fig. 5Be)

are magnetic susceptibility artifacts. These artifacts arise from local magnetic field gradients at the air-glass-liquid interface and are produced by abrupt changes in magnetic susceptibility at this interface (Callaghan, 1990). The lipid-resolved images were not analyzed because the lipids in the pork fat which are in the solid domains do not contribute to the image signal because they have very rapid transverse relaxation rates. Consequently the image signal intensity is dependent on both the lipid concentration and its solid-to-liquid ratio, and thus it was not possible to measure the lipid concentration from a single room temperature image. A linear correlation occurred between the normalized water-resolved image intensity of the meat emulsions and water content (correlation coefficient 0.98, standard deviation 2.55). The χ^2 value was 3.83 and the goodness-of-fit coefficient Q was 0.99 accounting for 2% systematic noise error in the image signal intensity data. The image intensity from the linear correlation for a sample with 80% water content was 79 (Fig. 6). However, as with the sunflower emulsions the image intensity at 0% water content was not zero as expected but 5.6. There are two possible reasons for this offset: either the lipids contain unsaturated olefinic protons which have a chemical shift similar to water and which consequently contribute to the signal in the water-resolved image. Alternatively the homogeneity of the magnet is not sufficient to completely resolve the water and aliphatic lipid protons and as a result the lipid protons have a small component which contributes to the water-resolved signal. The contribution from the lipid protons in the water resolved image was quite small and would only be significant when emulsions had high lipid content.

CONCLUSIONS

IT IS POSSIBLE TO MEASURE WATER and liquid lipid concentration of biphasic emulsions directly by chemical shift resolved NMR imaging. However, the solid-to-liquid ratio of the lipid must be known if the total lipid content is to be determined from a single, room-temperature measurement. The ability to differentiate water from lipid protons and distinguish between solid- and liquid-lipids has great promise. For example by acquiring variable temperature chemical shift resolved images the melting and crystallisation of lipids or emulsions could be studied.

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Flavor Interactions with Fat Replacers: Effect of Oil Level

J.P. SCHIRLE-KELLER, G.A. REINECCIUS, and L.C. HATCHWELL

ABSTRACT

The influence of fat and various fat replacers on headspace concentration of flavor compounds was studied. Fat had a predictable influence on vapor pressure of flavor compounds relating to their fat solubility. Protein-based fat replacers behaved more like fat than did carbohydrate-based products which had little interaction. Protein-based products had substantial effects on saturated and unsaturated aldehydes.

Key Words: flavors, fat substitutes, flavor oils, protein/carbohydrate-based products

INTRODUCTION

FAT CANNOT BE REMOVED from some foods and produce an acceptable product since fat makes important contributions in several ways (Forss 1969). It is a very important and major part of some foods (e.g., Hollandaise sauce or premium ice creams). Fats also function as flavor precursors (Nawar 1989; Ho et al. 1989). This is noted in fermented products such as cheese or deep fried foods like potato chips or french fries. The food develops a different aroma profile without the flavor compounds contributed by fat. Fat also exerts a "balancing" effect on aroma profile. Fats dissolve many flavor components and reduce their vapor pressures (Forss 1969, 1972; Buttery et al., 1971, 1973). The degree of reduction depends on the nature of the compound and its chemical properties. Removing fat from foods results in the lack of a flavor "medium" and thus fat soluble flavor constituents normally in a lipid phase may stand out of the profile. The flavor becomes imbalanced and the product loses some degree of acceptability. To make acceptable reduced fat or nonfat foods, processors need to understand how flavor compounds interact with fat and fat replacers.

Our objective was to determine how decreasing the fat content of a model system influenced the vapor pressure (aroma contribution) of flavor compounds. This would help in understanding how flavor perception changes with fat content and what level of fat reduction results in a significant flavor change. As a complement to previous work (Schirle-Keller et al., 1992) evaluating the interactions of a few flavor compounds with various fat substitutes we also studied the interactions of different homologous series of flavor compounds with selected fat replacers.

MATERIALS & METHODS

Flavor compounds

Acetaldehyde, diacetyl, propanol, pentanone, pentanol, ethyl sulfide, hexanal, t-2-hexenal, ethyl benzene, styrene, octanone, ethyl caproate, limonene and ethyl heptanoate were used. The flavor compounds were made up into one stock solution containing 0.5% of each in propylene glycol. For the study on homologous series flavor compounds interactions with selected fat replacers, the following groups of flavor compounds were used: **aldehydes**—acetaldehyde, propanal, butanal, pentanal, hexanal, heptanal, octanal, nonanal and decanal; **ketones**—acetone, butanone, pentanone, hexanone, heptanone, octanone, nonanone, decanone and acetophenone; **sulfur compounds**—thiopropanol, ethyl sulfide, allyl disulfide and Me-thio-Me-pyrazine; and **unsaturated carbonyls**—t-2-hexenal, t-2-nonenal and benzaldehyde. Each group of compounds was made up as a single stock solution at 0.5% each in propylene glycol. This solvent was used because of its low vapor pressure, i.e., it would not interfere with analysis of highly volatile compounds like acetaldehyde and diacetyl.

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Table 1—Composition of model systems used in flavor/fat replacer interaction studies

System[a]	Component (g) Water	Tween 80	Substitute	Oil
S-100	233.75	1.25	12.5	—
S-300	221.5	1.25	25.0	—
Oil	233.75	1.25	—	12.5
Water	250	—	—	—
Slendid®	241.25	1.25	5.0[b]	—
Stellar®	233.75	1.25	12.5	—

[a] All systems contained 2.5g of flavor solution.
[b] Composed of 4.27g Slendid®, 0.27g Genu pectin and 0.46g calcium chloride.

Fat replacers

The following fat replacers were studied: Simplesse® S-100 and S-300 (The NutraSweet Co, Mt. Prospect, IL), Stellar® (A.E. Staley Co., Decatur, IL), and Slendid® (Hercules Inc., Wilmington, DE). Crisco oil (Procter and Gamble, Cincinnati, OH) was used as a lipid control. Tween 80 (Fischer Scientific, Fairlawn, NJ) was used as an emulsifier in all systems.

Model systems

The model systems used to determine flavor/fat interactions were made of variable proportions of water and oil (0, 0.1, 1, 2, 5, 10, and 20%) plus 0.5% Tween 80. For experiments considering interactions of a broad range of flavor compounds with selected fat replacers, model systems were as presented in Table 1. The quantity of Simplesse used in each system was based on its protein content; S-100 was used at 5% while the S-300 was used at 10% resulting in equivalent protein concentrations.

Sample preparation

A Waring Blendor was used to disperse each model system. In the flavor/fat interaction study, the model flavor solution (1g) was added to the model system (99 g including 0.5 g Tween 80) and mixed by hand to disperse the flavor system. In the other study, the model system base was divided into four lots of ca. 49.5g each to which were added 0.5 g of one of the four flavor solutions. The flavor system was readily dispersible and thus was easily blended into the base by hand mixing.

Flavor analysis

Two headspace vials were filled with 15 mL of each of the flavored base systems, capped, sealed and stored at 4°C for 24 hr. Concentrations of volatile flavor compounds in the headspace above each sample were determined using a static headspace method combined with gas chromatographic (GC) analysis. A Hewlett-Packard Headspace Autosampler (HSS 19375A) was used. The sample was tempered at 60°C for 40 min prior to injection. Sampling conditions were: 30 sec pressurization, 30 sec of loop filling and 30 sec of injection. No cryofocusing was used and the split ratio in the injection port of the GC was 30 to 1. A Hewlett-Packard model 5880 gas chromatograph equipped with a flame ionization detector was used. The column was a 30 m ×

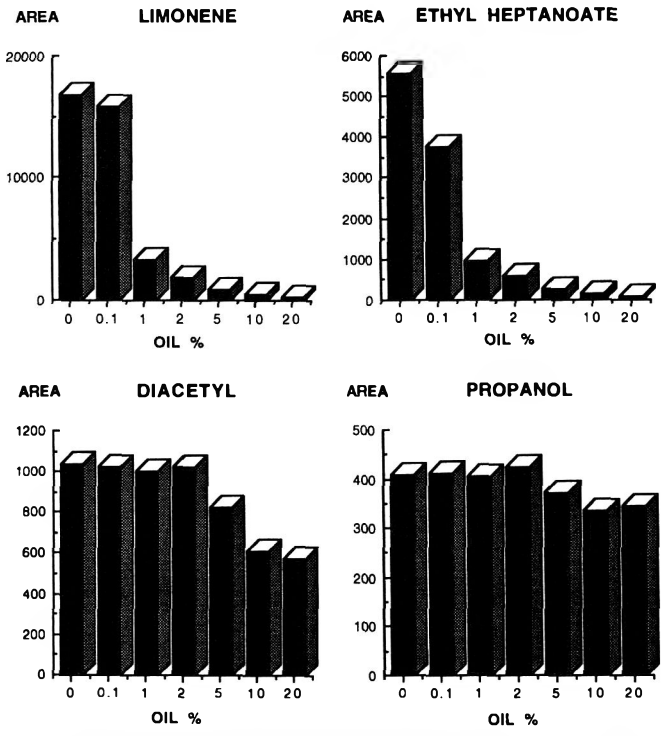

Fig. 1—Influence of oil content on the gas chromatographic peak areas of some specific flavor components.

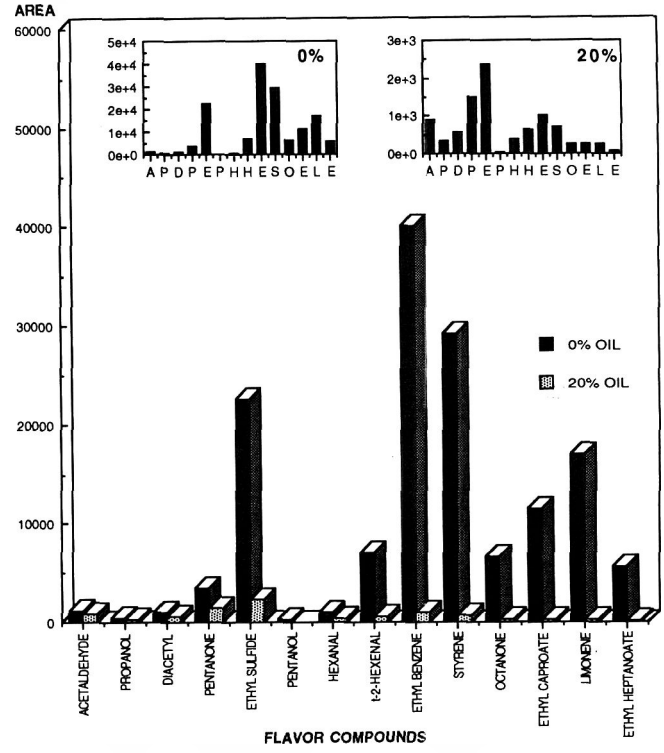

Fig. 2—Influence of oil content on gas chromatographic peak areas of several volatile compounds in the headspace above a 0% oil vs a 20% oil model system. Inserts show headspace profiles of same compounds on different Y axis for comparison.

0.32 mm (1μm film thickness) DB5 (J & W Scientific, Folsom, CA). The GC column conditions were: initial temperature 40°C, initial time 1 min, program rate 5°C/min and final temperature 160°C.

Data analysis and presentation

In the flavor/fat interaction study, the absolute GC peak areas of each flavor compound are presented. In the flavor/fat replacer study, the sample containing only water, Tween 80 and flavor was used as a control for analysis. The gas chromatographic peak area of each volatile flavor compound in the headspace above each sample was divided by the corresponding gas chromatographic peak area of the same compound above water for data presentation. This gives a range of possible results of 0 to 1; a value of 1 represents no interaction different from water and a value of 0 would represent complete interaction (i.e. no measurable amount in the headspace). These values are referred to as "relative vapor pressure" (RVP) in the text.

RESULTS & DISCUSSION

Flavor/fat interactions

Our primary goal was to determine how much of an effect reducing the fat or oil content of a food product had on the vapor pressure (sensory intensity) of flavor compounds. This would help determine the effects of reducing the fat content by 20 or 50%, for example, on the flavor of a food product. It may also help determine any benefit from including 1, 2, 5 or 10% fat on flavor quality. Results on a few representative flavor compounds are reported here since the behavior of many of them was similar depending on fat solubility.

Small changes in oil content had very significant effects (Fig. 1) on the vapor pressures of fat soluble flavor compounds (limonene and ethyl heptanoate). Reducing the oil content from 20% to 10% increased the vapor pressure (GC peak area) of both compounds by ≈2. This was expected since these compounds are only very slightly water soluble and thus their concentration in the oil phase increased by about 2-fold. As the oil phase was further decreased, the vapor pressure of the two compounds continued to increase proportionally. The amount of flavor compound in the headspace was strongly influenced by the amount of oil. Such change in vapor pressure would be evident in sensory perception: the higher vapor pressure would probably increase the sensory intensity.

The vapor pressure of water soluble flavor compounds was influenced much less than that of oil soluble compounds by the presence of oil. Oil content had much less effect on vapor pressure of diacetyl (Fig. 1) than on that of either limonene or ethyl heptanoate. The vapor pressure of diacetyl changed about 50% over the entire oil range studied. Propanol, which is much more water soluble showed even less change decreasing about 20% over the oil range. This would result in a change in balance of flavor compounds in the headspace above a food, i.e. a change in aroma character, depending on fat content. This is illustrated by comparing the headspace concentrations of all aroma compounds studied in 0% oil vs 20% oil system (Fig. 2—inserts). The aroma profiles of the two systems were quite different and the oil-containing system had much less of most aroma compounds in the headspace (Fig. 2—main fig). This would comprise a weaker aroma for a given concentration of flavor in the food.

Flavor/fat replacer interactions

The RVP of all flavor compounds decreased in oil-containing systems as fat solubility increased. Compounds such as acetaldehyde are water soluble and were largely unaffected by the presence or absence of oil. As carbon chain length increased and fat solubility increased, the RVP decreased approaching zero for long-chain compounds. This confirmed previous reports (Forss, 1969, 1972; Buttery 1971, 1973).

The protein-based fat replacers (S-100 and S-300) exhibited some fat-like properties showing an interaction (reduced RVP) with longer carbon chain aldehydes (Fig. 3). This reduction in RVP would probably be desirable since it would result in a flavor balance similar to that in a fat-containing food. Aldehydes added to carbohydrate-based fat replacer systems (Stellar® and Slendid®) had RVPs of ≈ 1 indicating no flavor interactions. The increases in RVP for nonanal were not valid,

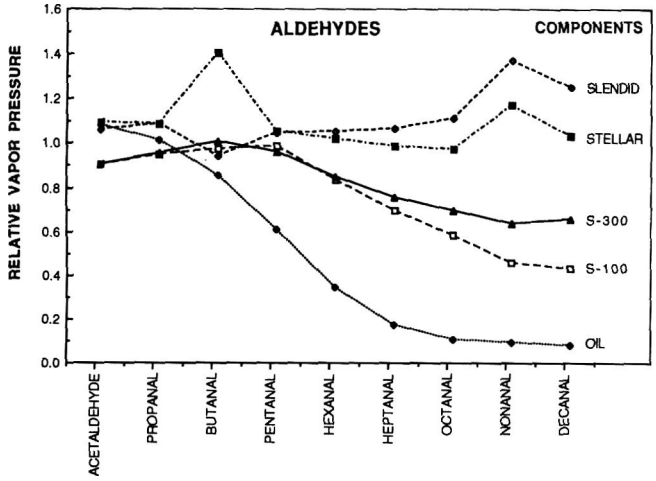

Fig. 3—Influence of carbon chain length of aldehydes on concentrations in headspace above oil-containing model systems.

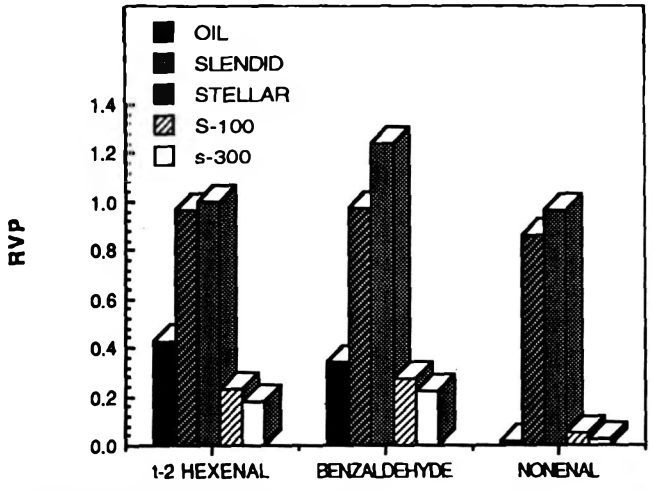

Fig. 4—Gas chromatographic peak areas of unsaturated aldehydes in headspace above oil-containing model systems.

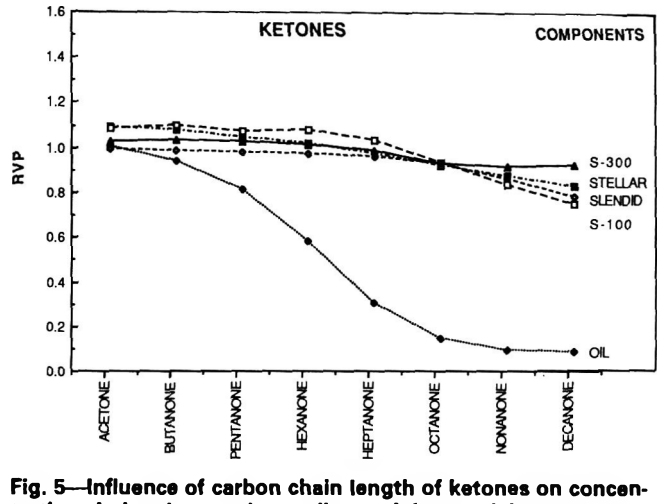

Fig. 5—Influence of carbon chain length of ketones on concentrations in headspace above oil-containing model systems.

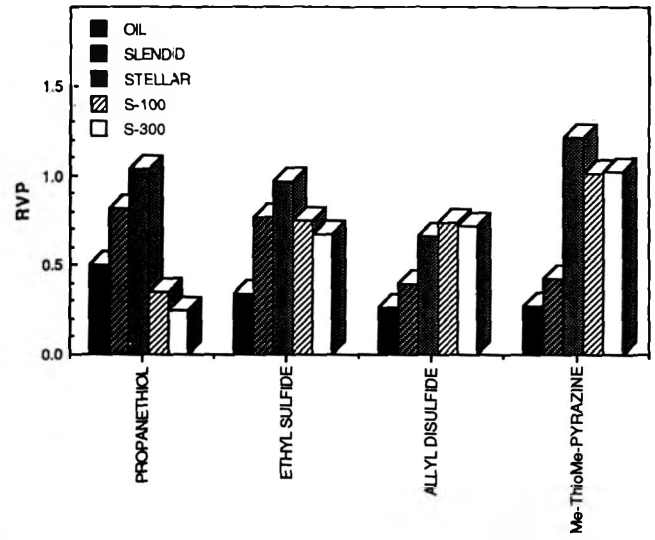

Fig. 6—Gas chromatographic peak areas of sulfur compounds in headspace above oil-containing model systems.

but resulted from variations in results (GC area counts were very low and small errors appear larger).

The unsaturated aldehydes showed substantial interactions with the protein-based fat replacers (S-100 and S-300) but no interaction with carbohydrate-based fat replacers (Stellar® and Slendid®) (Fig. 4). We did not determine the mechanism of interaction with the protein-based replacers. However, Schiff's base formation is likely. The similar vapor phase concentrations between fat-containing and protein-based fat replacer-containing systems suggested that the aroma profile of unsaturated aldehydes in S-100 and S-300 systems would likely be indistinguishable from comparable fat-containing systems. This interaction would be highly desirable and would result in a characteristic aroma profile.

Little interaction was noted between any of the fat replacers and the ketones studied (Fig. 5). Some interaction appeared to occur between the S-100 and the longer chain ketones but the effect was very slight.

All of the sulfur compounds had some fat solubility, and thus had reduced RVPs in the fat-containing model system (Fig. 6). However, interactions which occurred between fat replacers and sulfur compounds was difficult to interpret. We did not determine the mechanism of interactions noted. However, note that propanethiol interacted substantially with the two protein-based fat replacers. This may have been due to disulfide bond formation with the cysteine side chains of the proteins. The vapor pressures of the ethyl sulfide and allyl disulfide could have been reduced by the presence of hydrophobic locations in the protein. Alternatively, a reducing environment would readily convert them to the corresponding thiol. The vapor pressure of the thiopyrazine would be strongly influenced by the pH of the system. Lower pHs would decrease its vapor pressure because it would be ionized.

The presence of Slendid® reduced the headspace concentration of allyl disulfide and the thiopyrazine. Since Slendid® is based on pectin (some proportion of free carboxylic acid groups), we would expect a pH effect. The effects of Slendid®, and Stellar® to a lesser extent, on the vapor pressure of allyl disulfide was unexplainable. The reasons for reduced vapor pressure can only be determined through further research.

CONCLUSIONS

THE BEHAVIOR OF FLAVORS (i.e., interactions and potential sensory contribution) in oil systems was directly related to their oil solubility. Also the larger the molecular weights, the lower the RVP. Little observable flavor interactions occurred with fats other than predictable vapor pressure lowering due to solubility effects. The protein-based fat replacers exhibited more flavor interactions than did carbohydrate-based products. Foods with protein-based fat replacers should be more characteristic of fat-containing products in flavor profile than would those with carbohydrate-based replacers. However, ei-

—Continued on page 875

Unhydrogenated Palm Oil as a Stabilizer for Peanut Butter

M. J. HINDS, M. S. CHINNAN, and L. R. BEUCHAT

ABSTRACT

Response surface methodology was used to investigate the potential of unhydrogenated palm oil (PO), with and without peanut shell flour (PSF), to prevent oil separation in peanut butter. Percent oil separation, texture and color attributes were measured after 0, 1 and 2 wk storage at 15, 25 and 35°C. Computer-generated contour plots indicated that 2.0–2.5% PO should effectively stabilize peanut butter stored at 21–24°C for ≥ 1 year without affecting color. PSF (≥ 0.8%) decreased L value of color by ≥ 5% but did not increase firmness of experimental products containing PO. Samples stabilized with PO were softer than those containing a commercial stabilizer.

Kew Words: peanut butter, palm oil, stabilizer, oil separation.

INTRODUCTION

STABILIZERS IN PEANUT BUTTER prevent gravitational separation of less dense oil from solid particles during storage at ambient temperatures (Freeman and Singleton, 1952). Traditionally, hydrogenated vegetable oils, mono-, di- or tri-glyceride fractions of vegetable oils, or combinations of these, with melting points > average ambient temperatures, (i.e. > 25°C) have been used. At a temperature above its melting point, the stabilizer is mixed with other ingredients in a peanut butter formulation. Thus, on subsequent cooling crystallization of the stabilizer occurs throughout the product, preventing separation of the oil-meal mixture (Woodroof, 1983).

During hydrogenation of vegetable oils, unsaturated fatty acids are converted to their *trans* isomers. Dietary *trans* fatty acids have similar properties to long-chain saturated fatty acids (Anon., 1992; Borenstein and Beck, 1991; Davignon et al., 1980). Human consumption of *trans* fatty acids adversely affects health (Wood et al., 1993; Davignon et al., 1980) and correlates positively with increased risk of coronary heart disease (Willett et al., 1993) and increases in total and low-density-lipoprotein serum cholesterol levels (Troisi et al., 1992; Zock and Katan, 1992; Anon., 1991; Crapo, 1991; Mensink and Katan, 1990). These findings and the high per capita consumption of peanut butter in the U.S. (Woodroof, 1983) indicate the need for peanut butter stabilizers that are low both in long-chain saturated fatty acids and *trans* fatty acids.

Unhydrogenated, refined, bleached and deodorized (RBD) palm oil, from the mesocarp of the fruit of *Elaeis guineensis*, was investigated as an alternative stabilizer for peanut butter because it satisfies the above criteria. RBD palm oil (palm oil) has other properties which may prove advantageous. Its fatty acid composition is: C16:0 (palmitic) 43.1-46.3%, C18:0 (stearic) 4.0-5.5%, C18:1 (oleic) 36.7-40.5%, C18:2 (linoleic) 9.4-11.9%, with traces of C12:0, C14:0, C18:3, and C20:2 totalling less than 2% (Rossell et al., 1985). Both palmitic and oleic acids appear to be neutral in influencing cholesterol levels in normal individuals (Ng et al., 1992). Palm oil does not significantly elevate serum cholesterol levels (Wood, 1992) and may reduce lipoprotein- and apolipoprotein-associated cardiovascular problems (Sundram et al., 1992). Its slip melting point is 36–40°C (Kheiri, 1985; Timms, 1985). Due to the high proportion of symmetrical triglycerides, on rapid cooling palm oil produces fine *beta* prime crystals (Kheiri, 1985; Young, 1985) which stabilize oil dispersion (Kheiri, 1985) and impart smooth texture to products (Kheiri, 1985; Young, 1985). Its trisaturated glycerides [~10%] can function as accelerators during solidification (Okawachi et al., 1985) while its diglycerides [~6.9%] (Goh and Timms, 1985) should prevent hardening (brittleness) on cooling (Okawachi et al., 1985) and enhance stability of the β' crystals (DeMan et al., 1989; Hernqvist et al., 1981). Compared to vegetable oils, RBD palm oil contains relatively high concentrations of the natural antioxidants α, β and δ tocotrienols [totalling 400–750 ppm] (Goh et al., 1990) and 356–630 ppm tocopherols (Goh et al., 1985).

The melting point of RBD palm oil is relatively low compared with those (≥ 60°C) of commercial stabilizers (Woodroof, 1983; Tressler and Woodroof, 1976). Thus, we postulated that peanut butter stabilized with palm oil may be softer in texture than commercial brands and might therefore require incorporation of a firming component. Collins and Sanchez (1979) reported briefly on the potential of peanut shell flour (at levels of 0.33–1.00%) to increase firmness of peanut butter.

Our objective was to determine the effectiveness of unhydrogenated, refined, bleached and deodorized palm oil to stabilize peanut butter, and to predict the optimum palm oil level, as influenced by storage temperature, for stable, smooth peanut butter. The effect of peanut shell flour on texture was also included. Stabilizing effectiveness was defined as the ability to prevent oil separation in peanut butter.

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MATERIALS & METHODS

Peanut butter composition

Peanuts were obtained from 3 sources. Raw Florunner peanut seeds (certified by the Georgia Seed Development Commission and containing 49% fat, dry basis) were purchased from the University of Georgia Southwest Branch Experiment Station, Plains, GA. Roasted, blanched, and electronically sorted Florunner peanut seeds (49% fat, dry basis) were donated by Specialty Brands, Dromedary Plant, Woodbury, GA. Raw Virginia-type hand-picked peanuts (Gillam Bros. Peanut Sheller Inc., Windsor, NC) were purchased from J.J. Jardina Co., Inc., Forest Park, GA.

A commercial stabilizer (Fix-X™) was obtained from Proctor & Gamble, Cincinnati, OH. Fix-X (mp = 65.5°C) is a fully hydrogenated blend of rapeseed and cottonseed oils containing 33–37% C22:0 (behenic acid). Unhydrogenated palm oil (Malaysian RBD, slip mp = 37.5°C) was donated by Premier Edible Oils, Portland, OR. Granulated cane sugar, non-iodized table salt, and commercial peanut butters [Peter Pan© Creamy (340-g bottles) and Kroger© Creamy (510-g bottles)] were purchased from a local supermarket.

Physical properties of peanut butter samples containing palm oil were compared with those of reference samples stabilized with Fix-X. According to the manufacturer specifications, peanut butter containing 2.25% Fix-X shows no oil separation when stored at 50°C (Proctor & Gamble, Cincinnati, OH). Fix-X was selected because experimental peanut butters stabilized with 1.5% Fix-X and stored at 35°C for 2 wk had less oil separation than two commercial peanut butters under similar conditions.

Preparation of peanut butter

Peanut shell flour was prepared from the shells of Virginia-type peanuts according to the method of Collins and Sanchez (1979). Flour was analyzed for aflatoxin using the Aflatest Mycotoxin System (Vicam, Somerville, MA). Peanut butter was prepared using essentially

Table 1—Fractional factorial design for peanut butter composition and storage temperatures

Palm oil (%)	Peanut shell flour (%)	Storage temp (°C)
3.0	1.5	25
3.0	0	25
2.0	1.5	25
2.0	0	25
3.0	0.75	35
3.0	0.75	15
2.0	0.75	35
2.0	0.75	15
2.5	1.5	35
2.5	1.5	15
2.5	0	35
2.5	0	15
2.5	0.75	25
2.5	0.75	25
2.5	0.75	25

the procedure outlined by Woodroof (1983). Roasted, blanched and sorted Florunner seeds were ground coarsely in a Morehouse vertical mill (Morehouse Industries, Los Angeles, CA; with 0.25 mm clearance between stones) at 74°C, and the paste was held at 66–74°C. Other ingredients were blended into the paste (60–66°C) and the resulting mixture was ground finely (0.13 mm clearance between stones) at 74–80° C to produce peanut butter.

Stabilizing effects of palm oil

Preliminary tests were conducted to evaluate the stabilizing effects of palm oil. Peanut butter was prepared from Florunner seeds. They were roasted in 13.6-kg batches at 177°C for 21 min in a Preedit Electric Roaster (Model 37, Erie, PA), air-cooled for 10 min in a perforated metal box attached to a blower, blanched (Laboratory Blancher Model EX, Ashton Food Machinery, Newark, NJ) and sorted manually.

To determine an appropriate range of palm oil for subsequent trials, a factorial arrangement of five formulations of peanut butter containing palm oil (2%, 2.5%, 3%, 3.5% and 4%) and four storage temperatures (30, 35, 50 and 60°C) was applied. Peanut butter stabilized with 1.5% Fix-X served as control. Using a 50-mL syringe, triplicate 25-mL graduated cylinders were filled with 20-mL aliquots of peanut butter from each formulation. Percent oil separation was measured after 24 and 48 hr storage.

Two batcches of peanut butter (one with 2.5% palm oil and one with 1.5% Fix-X) were prepared. Cylindrical glass bottles (2.4 cm i.d., to monitor oil separation) and jars (6.2 cm i.d., for other physical tests) were filled. Samples were stored in triplicate at room temperature (21°C) and four other temperatures (25, 30, 35 and 65°C). Percent oil separation was measured periodically during storage. Other physical properties were assessed after 1 wk storage. Apparent viscosity was determined using a Brookfield Digital Viscometer (Brookfield Engineering Laboratories, Stoughton, MA) equipped with a TD spindle (cross bar = 20.4 mm) at a speed of 0.5 rpm. To measure color attributes, peanut butter was thoroughly stirred in the jar and then transferred to a polystyrene petri dish (100 mm diameter). The lid of the dish was pressed onto the surface of the peanut butter to remove air bubbles. The covered dish was then turned upside down to provide a uniform surface. A Minolta Chroma Meter II Reflectance System (Minolta, Japan) using a D65 light source at 0° viewing angle was used to make six measurements/sample (five near the circumference and one in center). Calibration was based on a standard tile with color space coordinates: $L^* = 69.82$, $a^* = +19.75$ and $b^* = +31.75$.

Optimizing oil stabilization

Experimental design. Response Surface Methodology (RSM) was used to determine optimum conbinations of unhydrogenated, refined, bleached and deodorized palm oil (2.0, 2.5 and 3.0%), peanut shell flour (0, 0.75 and 1.5%) and storage temperature (15, 25 and 35°C) to produce a stable product. According to Box and Draper (1987), this fractional factorial design necessitated 15 combinations containing palm oil (Table 1). Selection of levels for independent variables was based on results from preliminary tests and observations of Collins and Sanchez (1979).

Roasted Florunner seeds (from Specialty Brands) were used for RSM studies. Ingredients in the combinations, in addition to those listed in Table 1, were 4.71% granulated cane sugar, 0.79% salt and roasted peanut seeds to total 100% by weight. Peanut butter containing 1.25% Fix-X, 93.25% roasted seeds, 4.71% sugar and 0.79% salt was used as a standard. The level of Fix-X in the formulation was reduced to 1.25% because preliminary studies revealed that peanut butter containing 1.5% Fix-X was too firm and had a crusty, cracked surface appearance.

Hot (74–80°C) peanut butter was cooled to 60°C, deposited into jars (200-mL Ball), cooled in water (10°C) for 30 min and then held undisturbed at 38 ± 3°C for 48 hr prior to storing at 15, 25 or 35°C. To facilitate quantification of oil separation, 20-mL aliquots of peanut butter (52–60°C) were drawn by vacuum into graduated 25-mL pipets. They were plugged at their bases and stored in a vertical position at 15, 25 or 35°C.

Physical tests. Percent oil separation, texture and color of all formulations were measured after 0, 1 and 2 wk storage. Triplicate determinations/treatment were carried out for texture and color. Six replicates were used for percent oil separation, and mean values from pairs of adjacent pipets were analyzed. Data collected after 2 wk storage were considered representative for reliable information on the stabilizing effects of palm oil.

The graduated pipets facilitated accurate measurement of small volumes of oil on the surface of the peanut butter formulations. Percent oil separation was calculated by dividing the volume of oil that separated by the original volume of peanut butter before oil separation.

Samples containing palm oil were softer than those made in preliminary trials. Thus, for meaningful comparisons of texture of samples with palm oil to texture of those containing Fix-X, firmness of samples (instead of viscosity) was determined. Both viscosity and force of penetration measurements have been reported to evaluate texture of food systems similar tc peanut butter (Descamps et al., 1986; Schmidt et al., 1985). Firmness of peanut butter formulations was interpreted (Collins and Sanchez, 1979; Friedman et al., 1963) from the force required for a cylindrical probe (1.27 cm diameter) to penetrate the peanut butter to a distance of 3.5 cm below the surface. Force was measured using an Instron Universal Testing Machine (Model 1122, Instron Corporation, Canton, MA) fitted with a 50-kg load cell descending at a crosshead speed of 20 mm/min. Color attributes were measured using procedures similar to those in the preliminary studies. CIELAB values obtained were used to calculate hue angle (arctangent of b^*/a^*).

Table 2—ANOVA: Overall effect of independent variables on response variables[a]

Independent variable	dF	Sums of squares: Oil (%)	Texture	L value	Hue angle
Palm oil	4	0.6**	3.2	0.8**	69.9×10^{-6}*
Peanut shell flour	4	0.2**	5.8	106.5**	47.6×10^{-4}**
Storage temperature	4	3.0**	395.3**	0.2	7.0×10^{-6}

[a] Significance level: **$p \leq 0.01$, *$p \leq 0.05$

Table 3—ANOVA and model fitting for the response variables[a]

Source	dF	Sums of squares: Oil (%)	Texture	L value	Hue angle
Model	9	3.31**	404.0**	107.5**	4.84×10^{-3}**
Linear	3	2.71**	255.7**	106.1**	4.73×10^{-3}**
Quadratic	3	0.05	146.2**	1.4**	0.10×10^{-3}**
Cross product	3	0.54**	2.1	0.03	6.90×10^{-6}
Residual	35	0.25	55.2	0.79	19.8×10^{-5}
Lack of fit	3	0.11**	5.7	0.58**	5.6×10^{-5}*
Pure error	32	0.14	49.5	0.21	14.2×10^{-5}
% Variability explained (R^2)		93.1	88.0	99.3	96.1

[a] Significance level: **$p \leq 0.01$, *$p \leq 0.05$

Statistical analyses

The RSREG procedure of SAS Institute, Inc. (1985) was used to determine the effects of independent variables (palm oil, peanut shell flour and storage temperature) on physical quality characteristics of peanut butter samples after 2 wk storage. Percent oil separation, texture, hue angle and L value (lightness) of color were analyzed. RSREG is based on a second order polynomial equation to perform regression:

$$Y = b_0 + b_1{*}x_1 + b_2{*}x_2 + b_3{*}x_3 + b_4{*}x_1^2 + b_5{*}x_2^2 + b_6{*}x_3^2 + b_7{*}(x_1{*}x_2) + b_8{*}(x_1{*}x_3){*} + b_9{*}(x_2{*}x_3) \quad [1]$$

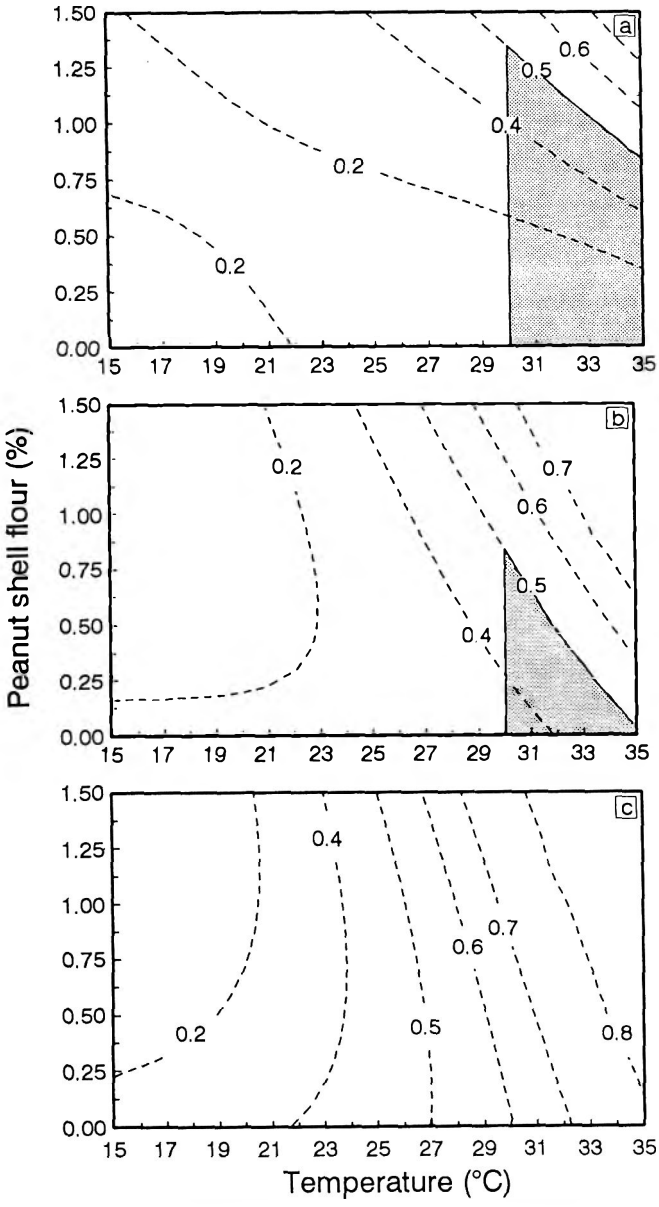

Fig. 1—Oil separation (%) as related to peanut shell flour (%) and temperature (°C). (a) 2.0% palm oil; (b) 2.5% palm oil; (c) 3.0% palm oil. Shaded regions represent combinations with ≤ 0.5% oil separation.

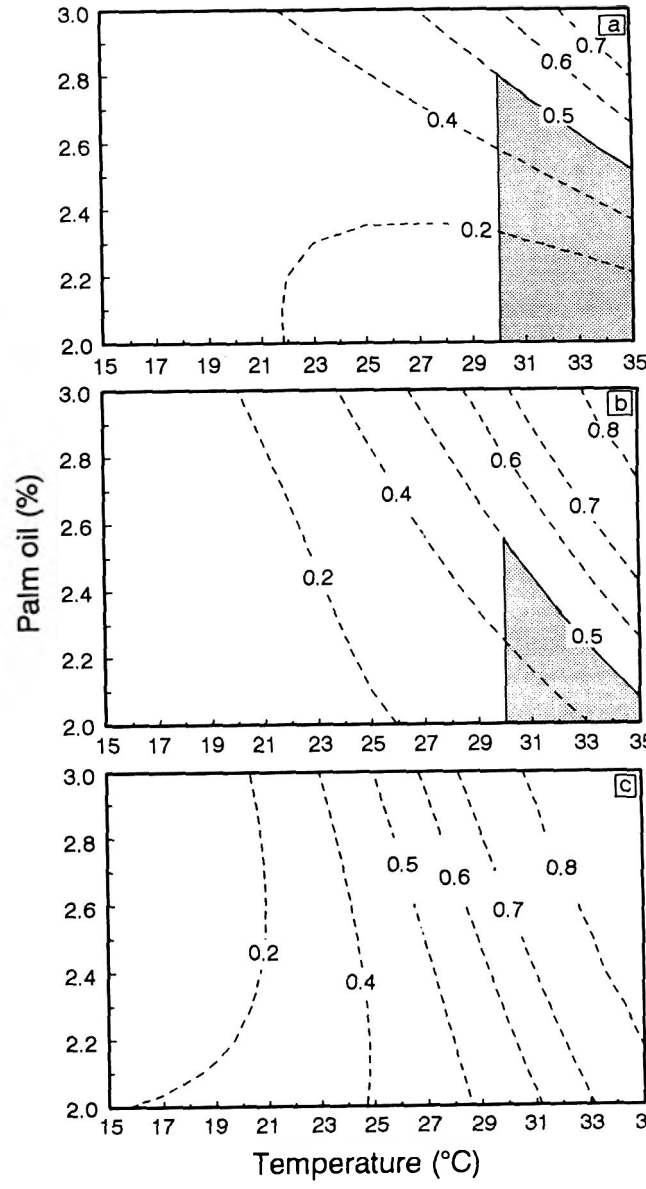

Fig. 2—Oil separation (%) as related to palm oil (%) and temperature (°C). (a) 0% peanut shell flour; (b) 0.75% peanut shell flour; (c) 1.5% peanut shell flour. Shaded regions represent formulations showing ≤ 0.5% oil separation.

where Y = response variable; and x_1, x_2, x_3 = independent variables. If the lack of fit was not significant, regression coefficients ($b_0 \ldots b_9$) were used to generate contour plots for response variables. If the regression model from RSREG showed a significant lack of fit, simple mathematical transformations were performed on independent and response variables to improve the fit (Box and Draper, 1987) before contours were generated.

RESULTS & DISCUSSION

Preliminary observations

After 24 hr, none of the samples stored at room temperature (21°C), 30 or 35° exhibited oil separation. The separation in those stored at 50°C was 0.42% for samples stabilized with 1.5% Fix-X, and 0.49, 0.49, 0.60, 0.62 and 0.62% for those containing 2.0, 2.5, 3.0, 3.5 and 4.0% palm oil, respectively. After 48 hr, no oil separation occurred in samples stored at 21 or 30°C or in those containing 1.5% Fix-X or 2.0 and 2.5% palm oil stored at 35°C. Oil bubbles were observed at the surface of peanut butters containing 3.0–4.0% palm oil and stored at 30 and 35°C. All samples stored at 60°C contained a thin layer of oil on the surface after 24 hr.

A comparison of the control containing 1.5% Fix-X with samples containing 2.5% palm oil after 1 wk storage revealed that at 21°C the control was more viscous (4.12×10^6 cps) than samples containing palm oil (1.6×10^6 cps) and its surface was crusty and cracked. All samples stored at 21, 25 and 30°C showed no oil separation. However, oil separation in those stored at higher temperatures was 0 and 0.5% (at 35°C) and 7.3 and 3.1% (at 65°C) for Fix-X and palm oil formulations, respectively. No differences occurred in CIELAB values due to stabilizer or storage temperature.

ANOVA and model fitting from RSM

Analysis of variance determined the overall effects of independent variables on response variables of samples stored for 2 wk. Percent oil separation was affected by all three independent variables, texture was affected by storage temper-

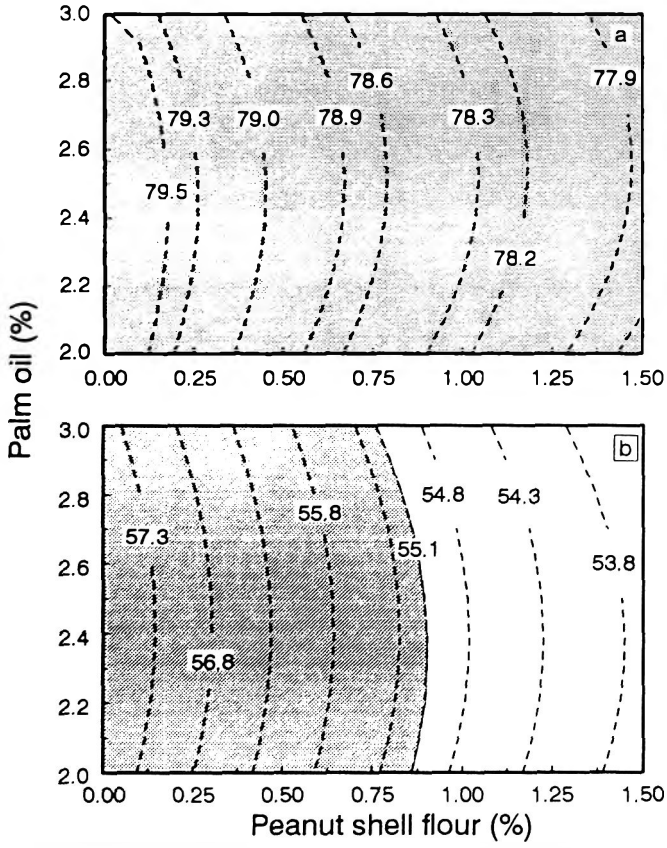

Fig. 3—Color attributes (as related to palm oil and peanut shell flour) of samples stored at 35°C. (a) hue angle; (b) L value. Shaded regions represent combinations with hue angles and L values that show ≤ 2° deviation and ≤ 5% variation, respectively, from corresponding attributes of the standard (Fix-X) formulation.

ature only, and peanut shell flour was the main component affecting color (Table 2).

Analysis of variance for response variables (Table 3) indicated that the model developed for texture was adequate, and had no significant lack of fit. However, regarding percent oil separation and L value, there was significant lack of fit. Because the ranges between maximum and minimum values for these response variables were small, several expansive transformations were evaluated in attempts to improve the fit of these models (Box and Draper, 1987). Equation 2 was the most appropriate for calculating percent oil separation, giving a statistically non-significant lack of fit and explaining 90.2% of the variability:

$$(\%\ \text{oil})^2 = 1.0 - 6.9\cdot10^{-1}\cdot PO - 4.9\cdot10^{-2}\cdot PSF - 4.8\cdot10^{-5}\cdot ST^3 + 1.2\cdot10^{-1}\cdot(PO^2) - 6.8\cdot10^{-2}\cdot(PSF\cdot PO) + 6.4\cdot10^{-2}\cdot(PSF^2) + 1.8\cdot10^{-5}\cdot(ST^3\cdot PO) + 1.2\cdot10^{-5}\cdot(ST^3\cdot PSF) + 1.6\cdot10^{-10}\cdot(ST^4) \quad [2]$$

where PO = palm oil, PSF = peanut shell flour, ST = storage temperature. Transformations on the RSREG model for L value were not utilized to plot contours because exponential powers of 7 for both palm oil and peanut shell flour were required for a non-significant lack of fit. Contractive transformations (Box and Draper, 1987) improved the fit of the model for hue angle (h°). Equation 3 provided a statistically nonsignificant lack of fit and explained 96.1% of the variability:

$$(h^\circ)^{1/2} = 1.1 + 1.8\cdot10^{-2}\cdot PO + 2.5\cdot10^{-3}\cdot(PSF)^{1/3} - 1.0\cdot10^{-4}\cdot ST - 4.1\cdot10^{-3}\cdot(PO^2) + 2.0\cdot10^{-3}\cdot(PSF)^{1/3}\cdot PO - 1.6\cdot10^{-2}\cdot(PSF)^{2/3} + 1.8\cdot10^{-5}\cdot(ST\cdot PO) - 1.8\cdot10^{-5}\cdot ST\cdot(PSF)^{1/3} + 9.9\cdot10^{-7}\cdot(ST^2) \quad [3]$$

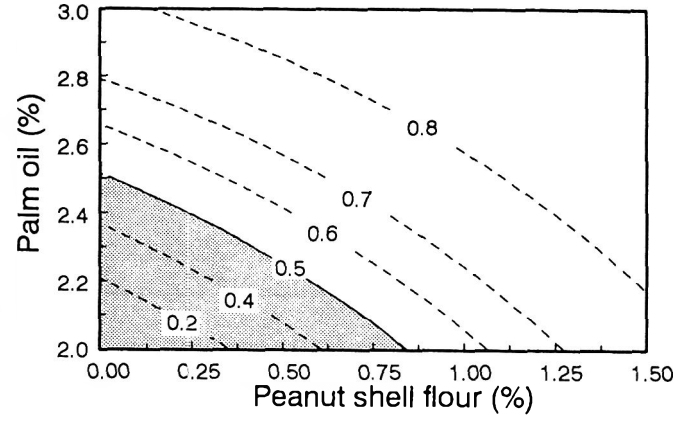

Fig. 4—Oil separation (%) for peanut butter samples at 35°C. The shaded region represents composition for optimum oil stability.

Physical properties

Percent oil separation. The experimental storage temperatures facilitated differentiation of samples in terms of oil stability. The establishment of a limit to denote optimum stability in combination stabilized with palm oil was based on several criteria. USDA regulations specify a maximum of 0.5 mL free oil/jar (jar size not specified) of freshly-manufactured product after 24 hr storage at 30°C (Woodroof, 1983). This is equivalent to 0.17% and 0.10% for 340-g and 510-g jars, respectively. The manufacturers of Fix-X affirmed that 1.25% Fix-X would effectively stabilize peanut butter stored at 26.6°C. We observed samples with commercial stabilizers and held at 35°C for 2 wk. Oil droplets occurred in air bubbles within the body of the standard formulation stabilized with 1.25% Fix-X. The 340-g and 510-g jars of commercial peanut butters made from ingredients similar to the standard samples but stabilized with partially hydrogenated vegetable oils both showed 1% oil separation. These commercial products had been purchased from the supermarket (21–24°C) and were tested 1 yr before their labeled expiration dates. Based on specifications and observations of peanut butters with commercial stabilizers, a maximum of 0.5% oil separation after 2 wk storage at 30–35°C was established as an indicator of stability for the experimental combinations stabilized with palm oil. Also, because commercial peanut butters (which should have remained stable for 1 year at 21–24°C) showed 1% oil separation after 2 wk at 35°C, this implied that the palm oil peanut butters, based on projected stability of commercial products, should remain stable for ≥1 year at ≈21–24°C. Only those products meeting the limit of ≤ 0.5% oil separation at 30–35°C are discussed. Thus 30–35°C represents the region of interest on the contour plots.

Plots were generated from data on samples with palm oil fixed at constant levels (Fig. 1). Products containing 2% palm oil and 0–1.3% peanut shell flour should be stable (Fig. 1a). Those with 2.5% palm oil should contain ≤ 0.8% peanut shell flour, but optimum stability should be achieved with 0% peanut shell flour (Fig. 1b). Peanut butter containing 3% palm oil would be unstable (Fig. 1c).

Plots were generated from combinations with three levels of peanut shell flour (Fig. 2). Those containing no peanut shell flour and 2–2.8% palm oil should be stable (Fig. 2a). If 0.75% peanut shell flour is used, the maximum palm oil should be 2.5%, but the most stable products would contain 2.0–2.1% palm oil (Fig. 2b). If the level of peanut shell flour was increased to 1.5%, all combinations with 2.0–3.0% palm oil would be unstable (Fig. 2c).

Color. Limits were arbitrarily set to accept stable experimental samples with hue angles and L values that showed ≤

2° deviation and ≤ 5% variation, respectively, from corresponding attributes of the Fix-X (control) formulations. Plots generated for hue angle of samples containing palm oil at 35°C (Fig. 3a) showed a slight decrease in hue angle of peanut butter samples as the level of peanut shell flour was increased. Products with ≈2.5% palm oil and 1.5% peanut shell flour would have the smallest hue angle (77.9°). This value would be within the proposed 2° deviation from that of the Fix-X control which was 79.6°. Therefore, hue angles of all experimental samples containing palm oil should be within the limit for color. Limits for acceptable hue angle need to be confirmed with consumer acceptance research.

The L value of the products containing palm oil was significantly affected by both peanut shell flour and palm oil. The L value plot (Fig. 3b) was constructed using a regression model in which the effect of the nonsignificant interaction between storage temperature and palm oil was ignored. Results showed that the peanut butter became darker (lower L value) as the level of peanut shell flour was increased. However, at a constant level of peanut shell flour, 2.3–2.5% palm oil should produce lighter samples. For products which would be ≤ 5% darker than standards, ≤ 0.8% peanut shell flour would be required. Collins and Sanchez (1979) reported a significant decrease in L value when ≥ 0.67% peanut shell flour was used in peanut butter.

Texture. Storage temperature was the most significant factor affecting texture of peanut butters containing either Fix-X or palm oil. Products stored at 15°C were the firmest. However, all samples containing palm oil were ≥ 5% softer than those with Fix-X when stored at similar temperatures. The soft texture may have been caused by the temperature (35–41°C) at which samples were held for 48 hr after initial cooling (30 min at 10°C) of the jars. This high temperature could have either prevented solidification or induced melting of low-melting point and some intermediate-melting point glycerides in the palm oil (Kheiri, 1985). The first 48 hr after production is the important setting period for peanut butter when containers should be stored undisturbed at 10°C (Woodroof, 1983). In our experiment, incomplete setting of samples probably occurred and thus prevented maximum firmness even when samples were subsequently transferred to cooler environments.

Collins and Sanchez (1979) evaluated addition of peanut shell flour to peanut butter with 2 levels of a commercial stabilizer. Products were stored for 4 wk at 22°C. They reported that formulations with 1% peanut shell flour were firmer ($p \leq 0.05$) when means for the 2 levels of stabilizer were combined but not when the levels were analyzed separately. However, experimental combinations containing palm oil and peanut shell flour which were stored at 25°C behaved differently. Generally, samples with 1% peanut shell flour were softer than those with less flour; 0.5–0.8% peanut shell flour improved firmness only when ≥ 2.8% palm oil had been used. Plots for combinations with 2.0–2.5% palm oil held at 35°C indicated that the firmest products would contain 0.0–0.4% peanut shell flour. However, samples containing 0.4% peanut shell flour may be similar in firmness to those without flour. Conclusions about the effectiveness of peanut shell flour to increase firmness of peanut butter stabilized with palm oil were not definite because experimental samples may not have been effectively set. Further research is needed to determine product composition for optimum firmness and stability.

The plot of effects at 35°C shows combinations of palm oil (2.0–2.5%) and peanut shell flour (0–0.8%) that should result in optimum oil stability (Fig. 4). For a given stability value, an inverse relationship occurs between palm oil and peanut shell flour. Comparison of the region for optimum oil stability (Fig. 4) with color attributes (Fig. 3) indicates that combinations of 2.0–2.5% palm oil with 0.0–0.8% peanut shell flour should produce peanut butter within our constraints for acceptable color. Thus, 2.0–2.5% palm oil should adequately stabilize peanut butter. Note that peanut shell products were included for experimental data, but they are not permitted in the U.S. for inclusion in commercial peanut butter.

CONCLUSIONS

COMPARISON OF PEANUT BUTTER compositions containing unhydrogenated palm oil with samples containing commercial stabilizers (after accelerated shelf-life storage) demonstrated the potential of palm oil to effectively stabilize peanut butter. RSM predicted that 2.0–2.5% palm oil would avoid oil separation in peanut butter for ≥ 1 yr at 21–24°C. Such levels of palm oil should not affect color of the product. Samples stabilized with palm oil were softer than those containing commercial stabilizer. Peanut shell flour did not effectively increase firmness of products stabilized with 2.0–2.5% palm oil. For incorporation of peanut shell flour ≤ 0.8% would provide stable combinations that are ≤ 5% darker than those without peanut shell flour.

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—Continued on page 832

Blanching, Freezing and Frozen Storage Influence Texture of White Asparagus

M.T. SANCHEZ-PINEDA-INFANTAS, G. CANO-MUÑOZ, and J.R. HERMIDA-BUN

ABSTRACT

We studied effects of three methods of blanching in conjunction with freezing, on texture of white asparagus as defined by three measures: maximum shear force, cutting energy, and total fiber content. We also assessed shelf life of asparagus kept in frozen storage at −22°C. Methods of blanching were total immersion in hot water, progressive immersion in hot water and steam. An increase in total fiber content was found throughout frozen storage. This increase correlated with lignification of vascular bundles in the basal segment of spears, even during frozen storage. This was reflected in an increase in maximum shear force and cutting energy required. The shelf life of frozen asparagus was 12 mo using total fiber content as a criterion.

Key Words: asparagus, blanching, freezing, texture, storage

INTRODUCTION

LIKE OTHER actively growing plants, newly harvested asparagus continued metabolic activity leading to histological changes which may affect marketing of fresh or processed products. Fibrousness may be the most important feature of such changes, and directly affects consumer acceptance. Increased fibrousness is largely due to tissue lignification as a result of enzyme activity (Isherwood, 1963). Lignin deposition is undesirable with respect to eating quality (Van Buren, 1979). Maturation of asparagus (in the field or during storage) results in undesirable increases in toughness (Sharma et al., 1975). The chemical nature of such toughening is complex and obscure, but essentially consists of deposition of hydrophobic lignin on the surface of cellulose fibers and inside hydrophilic gels. The physical/chemical association of lignin with polysaccharides in the stalk cell wall provides a balanced structure. Maximum shear force and cutting energy are related to the degree of lignification (Smith et al., 1987).

Fibrousness is influenced by the cultivar, climate conditions before and during harvesting, stalk length and location of cut, and processing conditions (Backinger et al., 1957; Werner et al., 1963; Haard et al., 1974; Sharma et al., 1975; Clore et al., 1976; Sosa-Coronel et al., 1976; Powers and Drake, 1980, Simón, 1983, Testoni and Eccher, 1985). Peeling reduces the maximum force to cut asparagus, and also reduces fiber content by elimination of the outermost layers of stalk which are the most heavily lignified (Mac-Gillivray, 1933). Kramer et al., (1960) recommended use of total fiber content as the best criterion for assessing shelf-life of fresh asparagus. Sharma et al. (1975) judged shelf-life in terms of maximum shear force required.

Our objective was to assess the influence of peeling, blanching, and freezing on texture and total fiber content, and to test storage times and conditions over a long enough time to assess the shelf-life of the frozen product. Shelf-life was defined as the time from harvesting to the time when the consumer would consider the product unacceptable.

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Table 1—Blanching conditions

	Temp (°C)	Time (sec)	
		> 14 mm	< 14 mm
Total immersion	90	180	150
Progressive immersion			
Base	90	180	150
Center		120	100
Bud		60	50
Steam, vertical position, bud down	90	180	150

MATERIALS & METHODS

Material

White asparagus spears (*Asparagus officinalis*, L., var. Desto) were harvested manually in Palma del Río (Córdoba, Spain) and rapidly transported for processing to a pilot freezing plant. Spears were washed, drained and cut to 16 cm lengths (measured from the bud). Broken, twisted or discolored stalks were discarded. Spears were divided into two batches according to diameter, measured 14 cm from the bud (large > 14 mm; medium < 14 mm) and peeled manually. Peeled spears were blanched by immersion in hot water and by steam (Table 1). Two types of water blanching were used: total immersion and progressive immersion. For the latter the upper third of the spear (the bud area) was immersed for one-third of the total duration of treatment, the central third for two thirds of the duration and the basal segment for the entire duration of blanching. Both immersion processes involved placing spears butt end down in metal baskets which were lowered totally or progressively into boiling water. Spears for steam treatment were placed bud down in metal baskets and transported by conveyor belt through a steaming unit.

Samples were hydrocooled at 18°C, drained to eliminate excess moisture during freezing, and placed in high-quality hermetically sealed plastic bags to prevent dehydration. Bags were placed on perforated trays in a forced-air ventilation unit inside a cold storage chamber and frozen at −30°C for 40 min. They were subsequently stored up to 12 mo at −22°C. Samples were removed at 0, 1, 2, 4, 8, and 12 mo.

Samples for texture determination using an Instron Universal Texturometer were thawed at room temperature for about an hour. Samples for fiber content assessment by the maceration method were thawed as part of the blending process to prevent possible enzyme regeneration during thawing.

Measurement of texture

Texture was measured as outlined by Wiley et al., (1956). A shear curve was obtained using a Warner-Bratzler shear cell fitted to an Instron 1140 Universal Texturometer. Blade speed was set at 20 mm/min. Force-time curves were recorded using an Instron Series IX Automated Testing System fitted to an IBM AT computer. Unlike previously reported methods, samples in our study were cut individually. The stalk was placed in the shear cell below the triangular blade area, so that the blade would pass through the stalk before entering the bottom groove, thus severing it completely. Each test was replicated 6 times.

Fiber content

Fiber content was measured by mechanical separation of fibrous material, as described by Smith and Kramer (1947), with modifications. A 100-g sample of asparagus was macerated in 400 mL water for 150 sec in a Waring Blendor. Modification of the Smith and Kramer

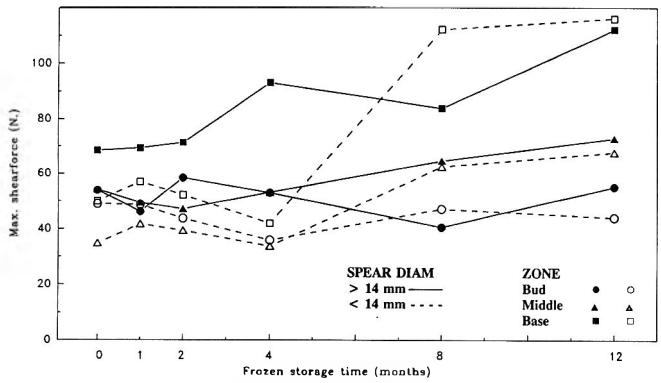

Fig. 1—Maximum shear force changes during frozen storage for product blanched by steam.

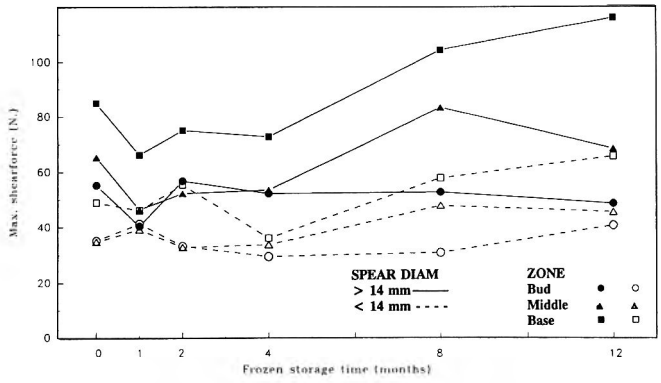

Fig. 2—Maximum shear force changes during frozen storage for product blanched by progressive immersion in hot water.

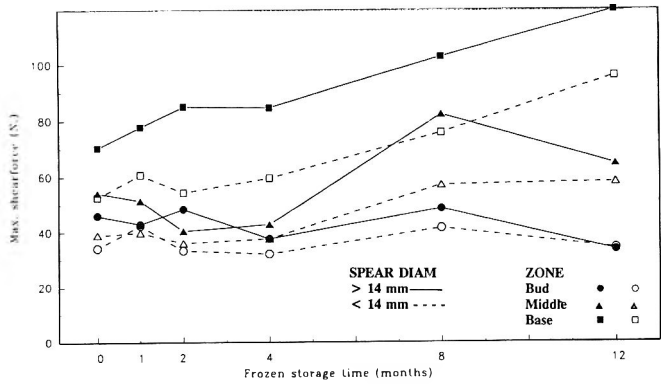

Fig. 3—Fiber content relation to frozen storage time under different process conditions.

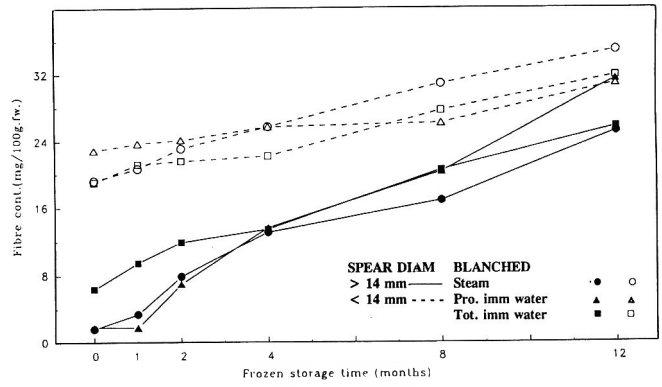

Fig. 4—Fiber content relation to frozen storage time under different process conditions.

method was essential to determine fiber content in peeled asparagus. Fibrous material was separated from parenchymatous tissue by transferring pulp to a 30 mesh screen and washing with water. Screens containing fibrous material were oven-dried for 2 hr at 100°C, then cooled and weighed on an analytical balance ± 0.1 mg. Each test was replicated three times. The number of repetitions was decided based on the negligible variation in results and the large quantity of asparagus required for each test.

Statistical analysis

All data were analyzed using ANOVA and Duncan's multiple range test, by means of SAS statistical software (SAS Institute Inc., 1987).

RESULTS & DISCUSSION

IN THE TIP AREA of the spear, no significant differences (α=0.01 and α=0.05) were detected in maximum shear force values throughout frozen storage. In the central portion of the spear, also were no significant differences (α=0.01 and α=0.05), except for vapor-blanched asparagus (both diameters) and spears blanched by total immersion (medium diameter). In those samples a significant increase in maximum shear force (α=0.05) was recorded from the fourth month onwards. Significant differences (α=0.05) were found in maximum cutting force values in the basal portion of the spear during frozen storage (Fig. 1 to 3).

Total fiber content of blanched and frozen asparagus differed (α=0.05) (regardless of blanching method or spear diameter) during frozen storage (Fig. 4). Determination coefficients ranged from 92% to 99%. In all tests, fiber content was higher in stalks of medium diameter than in those of large diameter. A liner correlation was found between maximum shear force and total fiber content in the basal segment, regardless of blanching method or stalk diameter. This confirmed findings of others (Kramer et al., 1949; Wiley et al., 1956; Backinger et al., 1957; Werner et al., 1963; Simón, 1983) for other varieties and storage conditions. Determination coefficients ranged from 73% to 95%, (mean = 82%).

Maximum shear force values in the upper and central segments remained constant throughout frozen storage (with exceptions indicated). These findings suggested that the increased corresponded largely to the basal segment of the stalk, where lignification of the vascular tissue continued during frozen storage (Smith et al., 1987). This also accounted for the increase in maximum shear force values in basal segments throughout the storage period.

No significant differences (α=0.01 and α=0.05) in cuting energy (area below the curve) were recorded for the upper 2 segments of the spears during frozen storage. The only exception was vapor-blanched stalks of medium diameter, where an increase (α=0.05) in required cutting energy was found from the fourth month of storage. Significant differences occurred, however, (α=0.05) in the basal segment during storage.

A linear correlation was found between required cutting energy in the basal portion and total fiber content, regardless of stalk diameter and blanching method. This confirmed results reported by Backinger et al., (1957) for other varieties and cultivation conditions. Determination coefficients ranged from 50% to 84% (mean ≈ 69%). Results therefore confirmed that the increase in total fiber content of stored asparagus was due to lignification of the basal segment. This was evident in the increased cutting energy required over the storage period.

A linear correlation occurred between maximum shear force and cutting energy, regardless of blanching method, area of cut and stalk diameter; this had also been reported by Backinger et al., (1957). Determination coefficients ranged from 68% for asparagus blanched in steam to 92% for asparagus blanched by total immersion (large diameter), (mean 82%).

Maximum shear force and cutting energy could be used industrially as measures of fibrousness in asparagus.

In assessing shelf life of blanched asparagus kept in frozen storage, two criteria were used: Kramer et al. (1960) recommended a limit for total fiber content. Asparagus with a total fiber content of <0.2% was considered very tender. According to this criterion, blanched frozen asparagus had a shelf life of 12 mo. All samples we tested had a total fiber content of <0.2%.

Sharma's criterion for assessing shelf life was based on maximum cutting force required. If the shear force required for 20 cm from the tip were >6.4 kg, the asparagus was considered unacceptable. We used this criterion solely for purposes of comparison, since the stalk length, variety, and cultivation conditions were totally different. By this criterion, large-diameter raw spears were unacceptable, since that figure was exceeded in the basal segment of all samples. Identical results were also obtained for all samples and those blanched stalks kept in frozen storage.

The basal segment of raw medium-diameter spears did not exceed 6.4 kg. Once blanched and frozen, shelf-life was determined as 8 mo for asparagus blanched by total immersion or by steam, and 12 mo for that blanched by progressive immersion. The time-temperature ratio was the same for all three. Based on the fiber content limit both the time-temperature ratio for blanching and the frozen storage temperature of −22°C would ensure shelf life of 12 mo. This would enable asparagus to be consumed throughout the time from one harvest to the next.

CONCLUSIONS

BLANCHING by progressive immersion in boiling water ensured an almost uniform texture along the length of the spear. A progressive increase in total fiber content throughout storage was due to continuing lignification even in frozen asparagus. This mainly affected the basal portion of the spear, as indicated by increase in maximum shear force and cutting energy. Maximum shear force and cutting energy could be used industrially as a measure of fibrousness in asparagus, since both measures provide similar information.

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Tyrosinase, Laccase, and Peroxidase in Mushrooms (Agaricus, Crimini, Oyster, and Shiitake)

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ABSTRACT

Enzyme assays and electrophoresis were used to monitor the activity of tyrosinase, laccase, and peroxidase in *Agaricus bisporus* (common cultivated button mushrooms and Crimini mushrooms), Oyster, and Shiitake mushrooms. The three enzymes could be differentiated using specific substrates and inhibitors. Tyrosinase seemed to be the major phenol oxidase in the *Agaricus* strains, while Oyster and Shiitake mushrooms had much lower levels. Peroxidase activity was low or undetectable in all types examined. Control of enzymatic browning in different mushroom types may depend upon the distribution of different oxidases within any given type.

Key Words: mushrooms, tyrosinase, laccase, peroxidase, enzymatic browning

INTRODUCTION

TYROSINASE, LACCASE, AND PEROXIDASE are phenolic oxidative enzymes which cause browning in many fruits, vegetables, and other foods (Mayer and Harel, 1979, 1991; Mayer, 1987; Vamos-Vigyazo, 1981; Robinson, 1991). Each has been reported present in mushrooms (Kumar and Flurkey, 1992; Perry et al., 1993; Turner, 1974; Wood, 1980a; Vamos-Vigyazo, 1981). Determining amounts of each and differentiating between them has been difficult because of similar substrate utilization, variations in distribution among mushroom species, tissues and developmental stages, and lack of specific inhibitors.

Selective substrates have been used to differentiate these enzymes with moderate success. Tyrosine and dopa are relatively specific substrates for tyrosinase (Marr, 1984; Mayer and Harel, 1979; Vamos-Vigyazo, 1981) while syringaldazine and toluquinol are relatively specific for laccase (Harkin and Obst, 1973; Allan and Walker, 1988). Guaiacol has been used as selective substrate for peroxidase and to differentiate it from tyrosinase (Vamos-Vigyazo, 1981). However, depending on the mushroom species, laccase can use guaiacol and dopa as substrates, and in the presence of hydrogen peroxide, peroxidase can use syringaldazine and toluquinol as substrates (Kumar and Flurkey, 1991; Wood, 1980a; Allan and Walker, 1988; Harkin and Obst, 1973).

Selective inhibitors of tyrosinase include salicylhydroxamic acid (SHAM) (Allan and Walker, 1988), tropolone (Kahn and Andrawis, 1985), 4-hexylresorcinol (4HR) (Dawley and Flurkey, 1993a; Dawley and Flurkey, 1993b), cinnamic acids (Walker and McCallion, 1980), and 2,3-naphthalenediol (Mayer et al., 1964; Kumar and Flurkey, 1991). SHAM, tropolone, and 4HR are quite effective at low concentrations while cinnamic acids and naphthalenediol required much higher concentrations for similar levels of inhibition. Cetyltrimethylammonium bromide (CTAB) has been a relatively specific inhibitor for laccase in some species of mushrooms (Walker and McCallion, 1980). Its general effectiveness as a laccase inhibitor has not been reported in great detail.

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Our objective was to apply a combination of selective substrates and inhibitors to determine the distribution of three phenol oxidases (tyrosinase, laccase, and peroxidase) in four types of commercial mushrooms.

MATERIALS & METHODS

Materials

Common cultivated mushrooms (*Agaricus bisporus*), Crimini (a brown *Agaricus bisporus*), Enoki, Oyster, and Shiitake mushrooms were purchased from local stores as packaged produce. Samples were either (1) lyophilized for 72 hr, ground into a fine powder with liquid nitrogen, resuspended in 10 volumes of 50 mM sodium phosphate (pH 6.0) containing 1 mM ascorbic acid and homogenized in a Waring Blendor for 1 min or (2) homogenized directly in 10 volumes of buffer for 1 min (Dawley and Flurkey, 1993b). Suspensions were centrifuged and filtered through Whatman #1 paper. Filtrates were stored at −80°C in small aliquots.

Enzyme assays

Tyrosinase. Tyrosinase activities were assayed at 25°C in 0.1M sodium phosphate buffer (pH 6.0) using either 5 mM p-cresol, 5 mM dopa, or 1 mM tyrosine as substrates. The increase in absorbance at 475 nm was monitored for dopa, 400 nm for p-cresol, and 280 nm for tyrosine. All slopes were calculated from the linear portion of the absorbance vs time curve. One unit of activity was defined as an increase in 1 absorbance unit/min. Specific activity was defined as units of enzyme/mg protein. When needed, SHAM (100 μM) or CTAB (1 mM), respectively, was added to assays to inhibit tyrosinase and/or laccase.

Laccase. Laccase activities were determined at 25°C in 0.1 M sodium acetate buffer (pH 5.0) using either 5 mM p-cresol, 1 mM diaminobenzidine (DAB), 5 mM dopa, 5 mM p-phenylenediamine, 2 mM toluquinol or 20 μM syringaldazine as substrates. The increase in absorbance was monitored at 400 nm for p-cresol, 400 nm for diaminobenzidine, 475 nm for dopa, 400 nm for p-phenylenediamine, 250 nm for toluquinol, and 525 nm for syringaldazine.

Peroxidase. Peroxidase activities were measured at 25°C in 0.1M sodium phosphate buffer (pH 6.0) using either 1 mM diaminobenzidine, 5 mM o-dianisidine, or 1 mM tropolone as substrates in the presence or absence of 0.01% hydrogen peroxide. The increase in absorbance at 400 nm was monitored for diaminobenzidine and o-dianisidine and at 408 nm for tropolone.

Protein content was determined by the method of Lowry et al. (1951).

Electrophoresis

Native electrophoresis was carried out as reported (Kumar and Flurkey, 1992; Dawley and Flurkey, 1993a) using preparative 8% polyacrylamide mini-gels. Crude extracts were layered along the stacking gel surface. After the electrophoretic run was terminated, gels were soaked 10 min in 0.1M sodium phosphate (pH 6.0) for tyrosinase or 100 mM sodium acetate (pH 5.0) for laccase. Strips (1 cm wide) were cut out of the gel and soaked in the appropriate buffer with or without inhibitor (100 μM SHAM or 10 mM CTAB) for 10 min. Strips were then soaked in buffer containing substrate, 2 mM dopa to detect tyrosinase or 1 mM DAB to detect laccase (with or without inhibitors SHAM or CTAB). Another strip was incubated in either dopa or DAB containing 0.01% hydrogen peroxide to detect peroxidase isoforms. Crude extracts from *Agaricus* gave patterns that could be recorded within 30 min using Kodak EDF duplicating film with dopa or DAB as substrates. Crude extracts from Oyster, Enoki and Shiitake mush-

rooms did not provide patterns that could readily be recorded even after several hours in the staining solution. Thus, all reported patterns are computer drawn representations from relative mobility data of individual isozymes found in crude extracts. Comparisons of isozyme band intensities are only valid between samples from the same extracts and same types of mushrooms.

RESULTS & DISCUSSION

COMMON CULTIVATED MUSHROOMS (*Agaricus bisporus*), Crimini, Enoki, Oyster, and Shiitake were used because of commercial use and availability. A few substrates, reported to be specific for each enzyme, were initially selected for differentiating tyrosinase, laccase, and peroxidase. The inhibitors, SHAM and CTAB, were also chosen because of reported selectivity in distinguishing ortho- and para-diphenol oxidases. In addition, latent tyrosinase activity was examined in each type mushroom by activation with SDS (Moore and Flurkey, 1989). In general, hydroxylation of tyrosine was inhibited by SDS and oxidation of dopa was increased by SDS (data not shown). However, conditions for maximal activation or inhibition have not been reported in any of these mushroom types. Therefore, we studied only unactivated tyrosinase activities. Similar studies were also carried out on Enoki mushrooms, but we did not detect tyrosinase, laccase, or peroxidase with confidence.

Agaricus bisporus mushrooms

In *Agaricus bisporus* the major phenol oxidase appeared to be tyrosinase. This enzyme used tyrosine and p-cresol in cresolase reactions and dopa in catecholase reactions (Table 1). Based on specific activity, dopa oxidation was higher than that of p-cresol or tyrosine. All three activities were completely inhibited by SHAM, a potent inhibitor of mushroom tyrosinase. Although none of the activities were inhibited by CTAB, some appeared to increase in the presence of CTAB.

Both extracellular and intracellular laccase have been reported in *Agaricus* (Turner, 1974; Kumar and Flurkey, 1991; Wood, 1980a,b) with levels much lower in intracellular enzymes. Apparent laccase activity in *Agaricus bisporus* was detected based on a variety of substrates (Table 1). Oxidation of p-cresol showed the highest specific activity and was inhibited by SHAM indicating that activity may be due to tyrosinase rather than laccase. CTAB appeared to inhibit laccase when toluquinol and syringaldazine were used as substrates but not when p-cresol or phenylendiamine were substrates. Based on specific activity as a relative indicator, laccase activity seemed to be much lower than tyrosinase activity.

Oxidation of DAB and o-dianisidine occurred in the absence of hydrogen peroxide, suggesting presence of a laccase activity or some other phenol oxidase. A small to moderate increase in these activities was noted in the presence of hydrogen peroxide. Tropolone, a reported inhibitor of tyrosinase and substrate for horseradish peroxidase, showed no oxidation in the presence or absence of hydrogen peroxide, indicating very low levels of peroxidase activity (or inability to utilize tropolone as a substrate). This would contrast with Kahn (1985) who reported oxidation of tropolone by horseradish peroxidase, and Kahn and Andrawis (1985) who reported that mushrooms showed no peroxidase activity.

Native PAGE indicated two dopa oxidase and four DAB oxidase isoforms. Both of the dopa oxidase isoforms were inhibited by SHAM, 4-hexylresorcinol (4HR) and tropolone (Fig. 1, data not shown). Similar results were reported by Kumar and Flurkey (1992) and Dawley and Flurkey (1993b). CTAB had no effect on one of the two dopa oxidase forms, but inhibited a more mobile isoform. Hydrogen peroxide completely inhibited enzyme staining with dopa as a substrate. Inhibition of *Agaricus* tyrosinase by hydrogen peroxide was reported by Andrawis and Kahn (1985). Most of the enzyme forms of higher Rf detected using DAB were not inhibited by SHAM, indicating they were laccase types. Also they were not inhibited by CTAB, which suggests that CTAB may not be a specific inhibitor of *Agaricus* laccase. Addition of hydrogen peroxide and DAB resulted in the appearance of several new bands, indicating they were from a peroxidase-like origin. Some of these co-migrated with apparent laccase isoforms.

Crimini mushrooms (*Agaricus bisporus*, brown colored)

Oxidation of p-cresol, dopa, and tyrosine occurred in Crimini extracts with dopa oxidase activity showing the greatest activity (Table 1). Its specific activity was higher than that in common mushrooms. All activities were strongly inhibited by SHAM. CTAB showed slight inhibition of dopa and tyrosine oxidase activity but not when p-cresol was substrate.

The presence of laccase in Crimini extracts was suggested not only by the oxidation of p-phenylendiamine, toluquinol, syringaldazine, and DAB, but also by the relatively low degree of inhibition by SHAM. Oxidation of p-cresol was inhibited strongly by SHAM, suggesting this oxidation may be carried out by a tyrosinase activity in Crimini extracts. CTAB inhibition was variable and CTAB caused activation with some substrates.

Oxidation of DAB and *o*-dianisidine occurred in the absence of hydrogen peroxide and no increases occurred in the presence of hydrogen peroxide. Very little activity was observed using tropolone as a substrate. This suggests that very little peroxidase-like activity was in Crimini mushrooms.

Table 1—Substrate and inhibitor specificity of oxidases in *Agaricus bisporus* and Crimini mushrooms[a]

	AGARICUS			CRIMINI		
	CONTROL	SHAM[c]	CTAB[c]	CONTROL	SHAM[c]	CTAB[c]
TYROSINASE						
p-cresol	100(0.044)	0	148	100(0.023)	2	150
dopa	100(0.556)	0	112	100(2.604)	3	86
tyrosine	100(0.002)	0	150	100(0.018)	1	81
LACCASE						
p-cresol	100(0.084)	0	101	100(0.123)	7	232
diaminobenzidine	100(0.001)	93	nd[b]	100(0.001)	80	nd
p-phenylenediamine	100(0.008)	75	100	100(0.161)	105	107
syringaldazine	100(0.002)	100	50	100(0.008)	130	69
toluquinol	100(0.004)	91	25	100(0.002)	86	210
	−hydrogen peroxide	+hydrogen peroxide		−hydrogen peroxide	+hydrogen peroxide	
PEROXIDASE						
diaminobenzidine	100(0.030)		133	100(0.291)		66
0-dianisidine	100(0.090)		115	100(0.266)		89
tropolone	0		0	(<0.001)		—

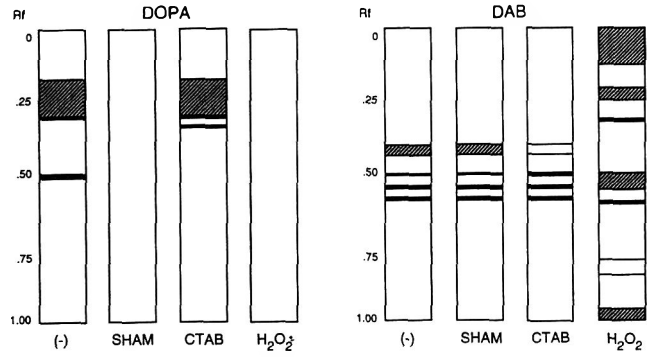
[a] Numbers in parentheses represent original specific activities (units/mg protein). Other numbers represent percent of control values.
[b] nd = not determined; (—) = value too low to measure.
[c] SHAM and CTAB final concentrations in the assay were 100 μM and 1 mM, respectively.

Table 2—Substrate and inhibitor specificity of oxidases in Oyster and Shiitake mushrooms[a]

	OYSTER			SHIITAKE		
	CONTROL	SHAM	CTAB	CONTROL	SHAM	CTAB
TYROSINASE						
p-cresol	100(0.010)	0	0	(<0.001)	—	—
dopa	(<0.001)	—	—	(<0.001)	—	—
tyrosine	100(0.018)	0	0	(<0.001)	—	—
LACCASE						
p-cresol	100(0.002)	123	18	100(0.001)	100	200
diaminobenzidine	100(0.001)	80	nd	100(0.072)	90	nd
p-phenylenediamine	100(0.008)	105	100	100(0.022)	86	77
syringaldazine	100(0.001)	123	50	100(<0.001)	—	—
toluquinol	100(0.006)	42	27	100(0.001)	100	200
	−hydrogen peroxide	+hydrogen peroxide		−hydrogen peroxide	+hydrogen peroxide	
PEROXIDASE						
diaminobenzidine	100(0.010)		110	(<0.001)		—
0-dianisidine	100(0.009)		98	(<0.001)		—
tropolone	(<0.001)		—	0		—

[a] Footnotes same as in Table 1.

Agaricus



Crimini

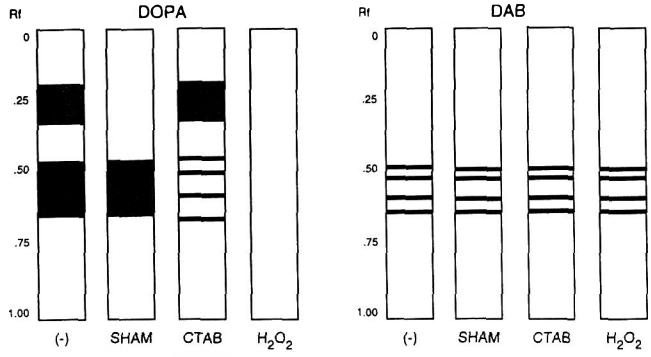


Fig. 1—Native electrophoresis of oxidases in *Agaricus bisporus* and Crimini mushrooms. Gel strips incubated with either dopa or DAB, in presence or absence of SHAM (100 μM), CTAB (1 mM) or hydrogen peroxide. Intensities represent degree of staining within that particular extract.

Native PAGE showed 2 major dopa oxidase isoforms and four DAB oxidase isoforms in Crimini extracts. These were similar to patterns observed from the other *Agaricus bisporus* strain. One of the dopa oxidase isoforms was completely inhibited by SHAM, 4HR, and tropolone (Fig. 1, data not shown). The other broad isoform was resolved into four distinct isoforms in the presence of CTAB. The 4 forms were similar in Rf to those localized with DAB. None of the DAB staining bands were inhibited by SHAM or CTAB, suggesting they were probably due to laccase and that laccase was not inhibited by CTAB. No new bands appeared when DAB was present in conjunction with hydrogen peroxide, which confirmed results with enzyme assays for peroxidase activity.

Oyster mushrooms (*Pleurotus ostreatus*)

Oyster extracts showed low levels of tyrosinase activity using dopa, p-cresol, and tyrosine as substrates. Dopa oxidase activity in Oyster extracts was much lower than that in *Agaricus* and Crimini extracts. The little activity present was inhibited by both SHAM and CTAB (Table 2). These results were similar to those of Oddson and Jelen (1981) and Bano and Rajarathnam (1988) who reported polyphenoloxidase activity was lower in *Plerotus florida* than *Agaricus bisporus*. Both investigators used Hunter color values as an indicator of polyphenoloxidase activity in Oyster mushrooms.

Laccase activity was also low in Oyster mushroom extracts. This agreed with classification of phenol oxidases in oyster mushrooms into a high laccase, low tyrosinase group by Marr et al. (1986). An extracellular catechol oxidase was noted by Tsuruta and Kawai (1983) and by Kim et al. (1986) in oyster mushrooms but was not examined by us. SHAM inhibited oxidation of toluquinol and DAB but not p-cresol, p-phenylenediamine, or syringaldazine. The inhibition by CTAB was again variable depending upon substrate.

DAB, o-dianisidine, and tropolone were apparently oxidized in the absence of hydrogen peroxide. No important increase in oxidation was noted in the presence of hydrogen peroxide, suggesting the absence or low levels of peroxidase activity.

Two dopa oxidase isoforms were indicated by native PAGE. SHAM inhibited the slower moving form and resolved the broader moving form into 3 or 4 separate forms (Fig. 2). Similar results were observed using 4HR and tropolone (data not shown). No inhibition of the slower band (R_f 0.25) by CTAB was noted, suggesting that form was a tyrosinase-like activity. CTAB increased the staining and clarity of the faster moving isoforms. No bands were found when hydrogen peroxide was added to dopa solutions. Four DAB isoforms were observed. None of those were inhibited by SHAM or by CTAB although the banding pattern, in presence of CTAB, did not appear exactly identical to patterns in the absence of CTAB. This may have been due to errors in determining or estimating R_f values rather than presence of new isoforms. No new bands were noted when hydrogen peroxide was present, again indicating low or nonexistent levels of peroxidase.

Shiitake mushrooms (*Lentinula edodes*)

Very low levels of tyrosinase or peroxidase-like activity were present in Shiitake extracts with a variety of substrates (Table 2). Low levels of laccase activity were observed using p-cresol, toluquinol, and syringaldazine as substrates. Higher activity was found with p-phenylenediamine and DAB as substrates. None of these activities was inhibited by SHAM. The effect of CTAB on laccase activity was variable depending on substrate.

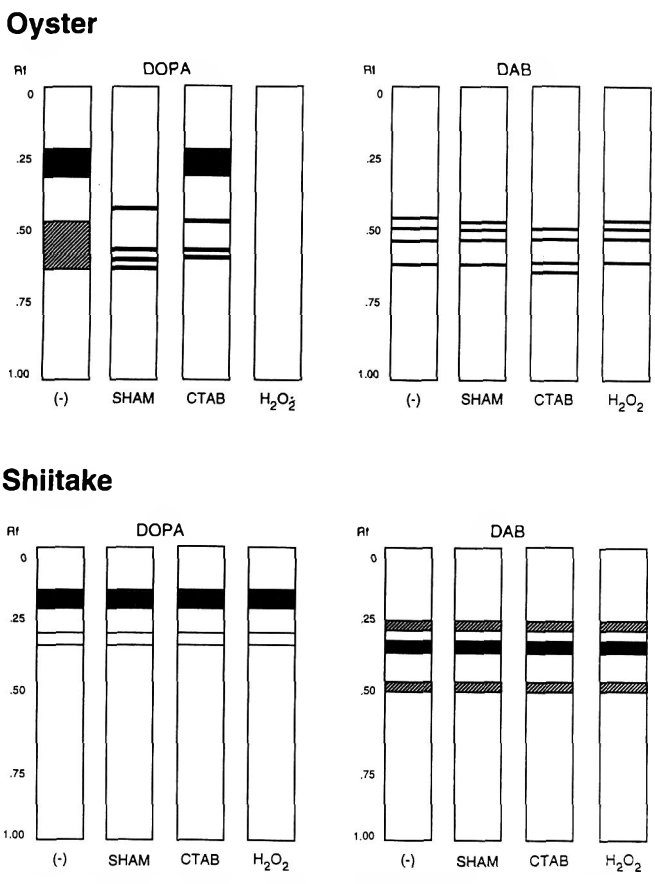


Fig. 2—Native electrophoresis of oxidases in Oyster and Shiitake mushrooms. See Fig. 1 for descriptions.

Two dopa oxidase isoforms were noted after native PAGE. Neither were inhibited by SHAM, 4HR, tropolone, or CTAB (Fig. 2, data not shown), indicating they may be of a laccase-like origin. No new bands resulted when hydrogen peroxide was included in the staining reaction. Three DAB isoforms were observed using DAB as a stain for laccase activity. None of these activities were inhibited by SHAM or hydrogen peroxide. CTAB decreased the intensity in each band slightly, and no new bands were formed in the presence of hydrogen peroxide. The three DAB staining bands were probably due to a laccase, while the slower moving dopa stained band (R_f 0.2) may have been SHAM resistant tyrosinase or some other oxidase.

CONCLUSIONS

FUNGAL TYROSINASE, LACCASE, AND PEROXIDASE activities were differentiated using selective substrates and inhibitors. SHAM, 4HR, and tropolone were selective inhibitors of tyrosinase and distinguished tyrosinase from laccase using *p*-cresol and dopa as substrates. CTAB does not seem to be a selective inhibitor of all fungal laccases and its use appears to be problematical and variable when trying to differentiate tyrosinase from laccase. Peroxidase activity was apparently very low or nonexistent in all mushrooms types we examined. Peroxidase was distinguished from laccase using diaminobenzidine or p-phenylenediamine as substrates in the presence or absence of hydrogen peroxide and from tyrosinase using tropolone as a tyrosinase inhibitor. The distribution of these phenol oxidases was varied in different types of mushrooms and would be important in developing methods to control enzymatic browning as related to specific enzymes.

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Optimizing Thermal Process for Canned White Beans in Water Cascading Retorts

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ABSTRACT

Based on thermal degradation kinetics and heat transfer expressed as the Ball formula method, a simplified approach was used to optimize sterilization processes for thermal softening of white beans (*Phaseolus vulgaris*, subsp. *nanus* Metz., variety *Manteca de Leon*). Constant retort profiles in a still and end-over-end rotary water cascading retort (Barriquand Steriflow) were used. Quality attributes of beans processed at the optimum were evaluated by a trained taste panel and by a tenderometer. Both approaches could distinguish ($P<0.01$) between attributes of products from optimal rotary and still processes. End-over-end rotation resulted in faster heat penetration and better quality retention of beans. Texture of white beans processed at 4° or 8°C from the optimal temperature could be distinguished ($P<0.01$) by the sensory panel and by the tenderometer.

Key Words: canned beans, softening, thermal processing, optimization

INTRODUCTION

SINCE THERMAL INACTIVATION OF MICROORGANISMS is much more temperature dependent than quality and nutrient degradation, optimization of a thermal process in terms of quality is possible. Differences in temperature dependence suggest that high temperature-short time (HTST) processes are favorable (Lund, 1977). The slow heat transfer rate in solid and high viscous foods limits the applicability of HTST processes. Several optimization studies have been reported (Teixeira et al., 1969, 1975; Thijssen et al., 1978; Saguy and Karel, 1979; Ohlsson, 1980a, b, c; Nadkarni and Hatton, 1985; Tucker and Holdsworth, 1990; Hendrickx et al., 1992; Banga et al., 1991; Silva et al., 1992a). All studies however focused on conduction heating foods only and were limited to theoretical considerations. For convection and mixed mode heating (e.g. packaged particulated foods) no optimization studies have been reported. Experimental validation of optimum conditions defined using mathematical procedures has not been reported. The usefulness of any mathematical procedure should therefore be assessed as a priority (Silva et al., 1993a). The objective of our study was to design an optimal process for a particulated in-pack food, (thermal softening of white beans in brine) considering still and end-over-end rotary processes in a water cascading retort. Computed optimal processes were validated using a trained taste panel and a tenderometer.

MATERIALS & METHODS

Materials

White beans (*Phaseolus vulgaris*, subsp. *nanus* Metz., variety *Manteca de leon*) were harvested in the summer of 1991 in Spain. They were stored dry (0.19 g moisture/g dry weight) at 15°C. Before heat treatment, they were soaked in distiled and demineralized water at 15°C for at least 16 hr. Preliminary experiments showed beans reached maximal moisture content after 16 hr (1.22 g moisture/g dry weight). The mean sizes of the dry beans were 15.14mm ± 1.30mm length, 10.58mm ± 0.80mm width and 8.71 ± 0.80mm height. Beans were processed in glass jars (Carnaud-Giralt Laporta S.A., Spain) of 172 mm height, 40.5mm diameter and a thickness of 2.6mm. Each jar contained 440g of soaked beans and was filled with distiled and demineralized water until the required gross headspace was reached.

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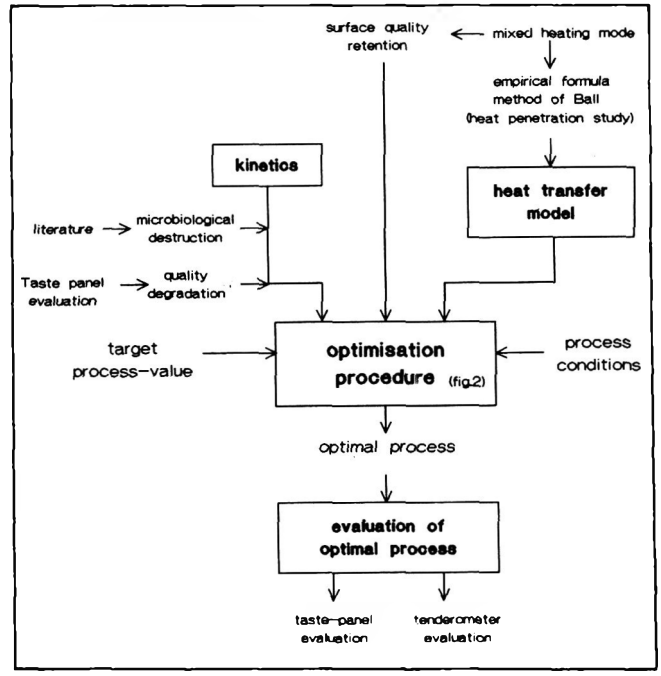

Fig. 1—Schematic flowsheet of optimization approach for minimizing thermal softening of white beans in brine processed in a water cascading pilot retort considering still and end-over-end rotary conditions.

Still and end-over-end rotary processes were simulated in a Steriflow simulator (single-door unit with one cage), microflow type 911R n°877 of Barriquand (France). The datalogger (MDP 8250 analog input-relay output system from Mess + System Technik GmbH) was installed with a thermocouple box containing the reference junction and connected to a personal computer. The μV signal ws converted to °C with a maximum error of 0,01°C in a temperature range 0 to 170°C. The datalogger was calibrated using the PVG77 thermocouple voltage calibrator from Ellab (Denmark). The output was recorded with an accuracy of 0.1°C. For rotational processes a slipring contact (DCS85-12, Ellab, Denmark) was needed. All temperature sensors were copper-constantan thermocouples (type T) of Ellab (Denmark). For heat penetration measurements needle type thermocouples with rounded tip (160×1.2mm, SSA-12xxx-G700-SF, where xxx stands for the length of the needle) were used. For the coldest spot determinations temperatures were registered using four point probes (ST4-11120-G700-SL). Thermocouples were calibrated in icewater (0°C) and at the temperature used during the heat penetration tests (121°C). The entire length of the lead wire was in the retort during the test. All thermocouples measured temperatures without 0.2°C and could be used for the heat penetration study.

Methods

Strategy. The optimization approach is shown in Fig. 1. Optimization of sensory quality deterioration must be based on heat transfer in

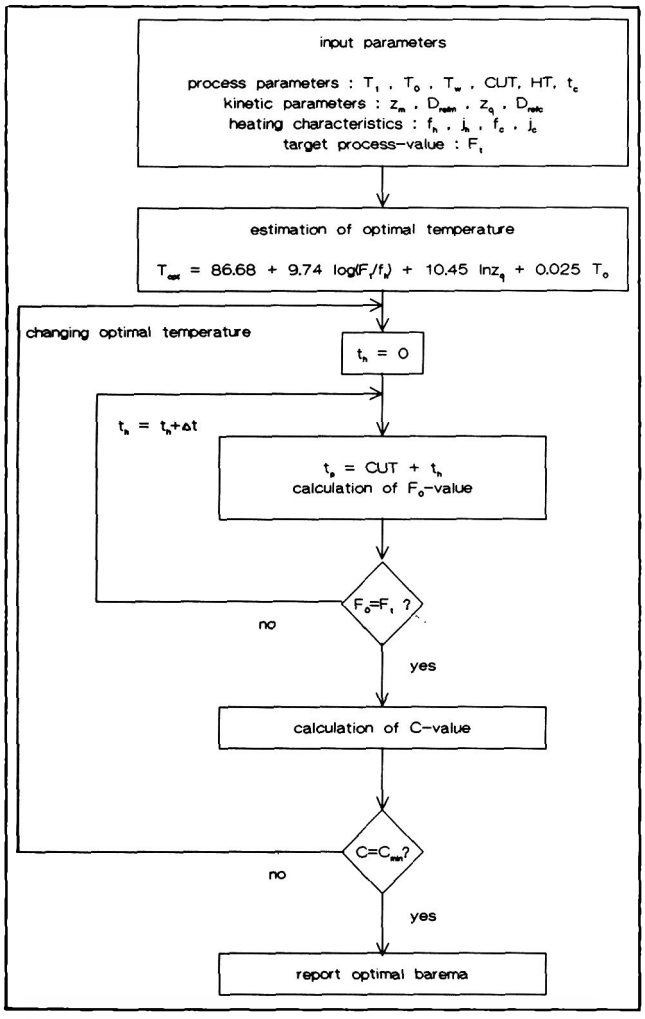

Fig. 2—Schematic flowsheet of the computer program to calculate optimal processes.

the food and also on the kinetics of microbiological inactivation and quality degradation (Ohlsson, 1980c). Available analytical and numerical solutions refer to the Fourier's equation (conduction). Because of the complexity of solving the coupled mass and heat transfer equations for a mixed mode heating food, heat transfer in a mixed mode food (i.e. white beans in brine) was modelled using an empirical formula method. The great advantage of the empirical formula method of Ball is the simplicity and wide applicability independent of the mode of heat transfer. Optimization can be considered in terms of maximizing surface quality, when e.g. appearance or aroma is considered or in terms of volume average quality as an indicator for consistency, taste or nutrient retention (Ohlsson, 1980a; Silva et al., 1992b). Although hardness degradation should be optimized in terms of overall quality retention, surface cook-value was chosen as the optimization parameter. For the optimization of volume average quality retention the time-temperature profile as a function of position was needed, which could hardly be predicted for a mixed type heating food. The use of optimal sterilization conditions for surface quality does not imply a decrease in volume average retention > 8% and in some cases no reduction at all was verified. The use of optimal conditions that maximize surface quality can be suggested when the aim is to obtain a final product with a well-balanced maximum quality (Silva et al., 1993b). For sterilization of low-acid foods, processed in the range 110 to 130°C, the heat resistance of *Clostridium botulinum* spores was chosen as a reference, characterised by 10C° z-value and D-value at reference temperature (121.15°C) of 0.21 min. Kinetic parameters for thermal softening of white beans were previously reported using sensory analysis (Van Loey et al., 1994).

Heat penetration. For the coldest spot determination 4-point probes measured temperatures at different depths along the central axis of 3 glass jars. The coldest spot in a container was defined according to Zechmann and Pflug (1989) as the location in the container which received the lowest sterilization value (F_o-value) from the process. The F_o-value was calculated using the general method of Bigelow (1921).

Heating characteristics (f_h and j) were determined by experiments on five containers using needle type thermocouples with a rounded tip (160×1.2mm, SSA-12xxx-G700-SF) placed in a bean at previously determined coldest spots. The containers of interest were placed along the central axis of the cage to avoid variations due to rotation angles. Simultaneously, two thermocouples (SSR-60020-G700-SF) registered the heating medium temperature every 15 sec.

For coldest spot determinations as well as for the heat penetration study the following retort heating profile was used: An equilibration phase to an initial product temperature of 40°C preceded all processes that consisted of a linear increase in retort temperature up to 121°C during 16 min followed by a holding phase (13 min) at 121°C. The degree of agitation depends mainly on the rotational speed on the size of the headspace bubble and on the consistency of the product (Berry and Bradshaw, 1980). Thus the influence of headspace (10 and 20mm) was studied as well as rotational speed (0, 5, 10, 15 and 20 rpm) on the heat penetration characteristics, determined according to the Ball formula method (Ball and Olson, 1957).

Optimization procedure. The optimal processing barema was defined as the temperature-time combination which results in a food product with minimum surface cook-value after achieving the desired degree of sterility. It was estimated using a computer program developed at the Unit of Food Preservation of the KUL. In development of the program some simplifying assumptions included: There is no heat transfer resistance at the product surface (infinite Biot number), the time-temperature profile consists of a come-up-time with a linear increase of temperature from initial to constant holding temperature, the food product is homogeneous and isotropic, initial temperature is uniform and kinetic parameters are described by the empirical D-z-model. The flow diagram of this optimization program is shown in Fig. 2. Process parameters were T_0=40°C, T_w=15°C, CUT_{reton}=16 min both for still and rotary processes, HT=13 min and t_c=17 min. The kinetic parameters for *Cl. botulinum* spore inactivation (z_m=10°C, D_{ref}=0.21 min, T_{ref}=121.15°C) were selected while for thermal softening of white beans kinetic parameters were determined in a previous study (Van Loey et al., 1994), namely z_q=21°C, D_{ref}=1416 min at T_{ref}=100°C. Based on published values for low acid canned foods in general (Pflug, 1987a, b) 6 min was chosen as the target process-value. The heating characteristics of white beans were determined in a heat penetration study. Berry and Bush (1989) found the extrapolation of data to other retort temperatures a safe practice for conduction or induced convection heated products.

A target process-value, F_t, specified at the coldest spot and calculated by the Ball formula method was the optimization constraint. As starting value for iterative estimation of the optimal temperature the generalized (semi)-empirical formula for conduction heated foods with infinite surface heat transfer coefficient (Eq. 1) developed by Hendrickx et al. (1993) was applied.

$$T_{opt} = 86.68 + 9.74 \log(F_t/f_h) + 10.45 \ln z_q + 0.025\, T_0 \quad (1)$$

Secondly, the holding time, initialized at zero, was incremented iteratively by a Δt_h (Eq. 2) until the desired degree of sterility (target process-value F_t, calculated by the Ball formula method (Ball and Olson, 1957) using the previously determined heat penetration characteristics f_h, j_h, f_c and j_c) was achieved at the coldest spot of the product. The cooling period lethality included all temperatures with significant contribution to total lethality. The convergence criterion was (F_t-F_0<0.01).

$$\Delta t_h = \frac{F_t - F}{10^{(T_1 - T_{ref})/z_m}} \quad (2)$$

For the generated time temperature profile to reach the target process-value the resulting surface cook-value could be calculated using Eq. (3), (4) (contribution of respectively come-up-time and holding time to the surface cook-value) and (5). Cook-values were calculated using 100°C as reference temperature. Since an infinite surface heat transfer coefficient was assumed the temperature profile of the product surface equaled the retort temperature profile. To optimize for the surface cook-value the computer program was integrated in an optimization routine, using the Davis, Swann and Campey method (Saguy, 1983).

$$C_1 = \frac{z_q \cdot CUT}{2.303.(T_1 - T_0)} \cdot \left(\exp\left(2.303.\frac{T_1 - T_{ref}}{z_q}\right) - \exp\left(2.303.\frac{T_0 - T_{ref}}{z_q}\right)\right) \quad (3)$$

Table 1—Influence of rotational speed and headspace on location of coldest spot for white beans in brine (mm from bottom of glass jar)

	Coldest spot (mm)	
rpm	HS = 10 mm	HS = 20 mm
0	10	10
5	20	30
10	40	50
15	50	70
20	60	70

Table 2—Influence of rotational speed and headspace on heating characteristics and process-values measured at the coldest spot of a container filled with soaked white beans in brine processed in a water cascading retort

Rpm	Headspace (mm)	f_h (min)	j_h	F_0 (min)
0	10	13.4±0.6	6.3±0.6	3.5±0.3
	20	12.9±0.5	6.6±0.5	4.0±0.3
5	10	9.5±0.4	12±2	8.2±0.3
	20	8.5±0.3	19±3	9.3±0.4
10	10	8.1±0.3	21±3	9.5±0.5
	20	7.4±0.3	31±3	10.6±0.4
15	10	7.5±0.3	29±5	10.4±0.5
	20	6.7±0.3	41±6	11.3±0.4
20	10	7.0±0.3	40±8	11.0±0.4
	20	6.5±0.4	56±24	11.6±0.5

$$C_7 = \exp\left(2.303.\frac{T_1 - T_{ref}}{z_q}\right). HT \quad (4)$$

$$C_{tot} = C_1 + C_2 \quad (5)$$

Evaluation procedure of optimal processes. In the optimization procedure a few simplifying assumptions were made. A trained taste panel and an industrial tenderometer checked the theoretically computed optimal processes. The first aim of the evaluation was to check whether the taste panel could distinguish between the optimal process and equivalent processes at temperatures deviating 4°C and 8°C from the optimal temperature. A rotational speed >15 rpm did not improve heat penetration and caused more mechanical damage (more broken beans). Thus, the optimal and deviating still processes with headspace 10 mm and the optimal and deviating processes at 15 rpm and headspace 20 mm were executed in the pilot retort. A second purpose was to indicate an in-batch variation in the optimal processes. The third aim of the evaluation, was to compare the optimal still process with the optimal rotating process for distinguishable quality levels of thermal softening.

Prior to sensory evaluation, glass jars which had the same heat treatment were mixed to obtain homogeneous samples. Individual samples (± 20g of white beans) were served in plastic cups (King disposables, 72 mm diameter and 22 mm height), coded with three random digits. Hardness of white beans was examined under red light to mask differences in appearance. For hardness testing, a dinner-plate, a fork and a spoon were placed at the disposal of the judges. In order to eliminate distraction and prevent communication among panelists, evaluations took place in individual booths. Each booth was provided with a service-hatch connected to the preparation room. The sensory evaluation technique was a pairwise ranking test with Friedman analysis (Meilgaard et al., 1987). Samples were presented using a randomized balanced block design with replication (Cochran and Cox, 1957). Four or five pairs of white beans were presented simultaneously and the twelve trained judges were asked to indicate the hardest sample of each pair.

Hardness of 200g heat-treated beans was measured by use of an industrial tenderometer (FMC, model 4011). An FMC tenderometer measured the resistance of the product to compression and shear between two sets of blades. The equipment was calibrated. Each heat treatment was evaluated five times with intermediate cleaning of the blades. Results were expressed in tenderometer units.

RESULTS & DISCUSSION

Heat penetration

An increase in rotational speed or headspace moved the coldest spot from the bottom towards the center of the jar (Table 1). The heat penetration characteristics at the coldest spot (Table 2) were specific for a given product, fill weight, headspace, type of container, dimensions of the container, rotational speed, come-up-time of the retort, heating medium and initial temperature. Mean f_h-values and process-values were plotted (Fig. 3) as a function of rotational speed and headspace. Influence of headspace on the heat penetration rate for different speeds indicated no differences in f_h-values ($p<0.05$) for still processes due to headspace whereas for rotary processes (rpm=5, 10, 15 or 20) f_h-values were distinguished ($p<0.05$). Comparison of mean f_h-values for different rotational speeds at a given headspace indicated differences ($P<0.05$) except for 20 mm headspace the difference in heat penetration characteristics between 10 and 15 rpm and between 15 and 20 rpm was not significant ($P<0.05$). Berry and Dickerson (1981) studied whole kernel corn in brine processed in a Steritort and found headspace had no effect on still processes. For agitating retorts however an increase in headspace within normal limits tended to increase the sterilization value probably because of the increased ability of the brine to move effectively through the agitated can. Increasing rotational speed (Table 2) resulted in a faster heat penetration (lower f_h-value). Several researchers studying axial or end-over-end rotation came to similar conclusions (Conley et al., 1951; Berry et al., 1979; Berry and Bradshaw, 1980; Naveh and Kopelman, 1980; Berry and Dickerson, 1981; Berry and Bradshaw, 1982; Berry and Kohnhorst, 1985). However the influence of rotational speed on heat penetration rate is limited (Fig. 3). Besides as rotational speed was increased to 20 rpm, more broken beans and consequently leakage of starch appeared. Therefore an optimal heat penetration with acceptable mechanical damage occurred at a rotational speed of 15 rpm.

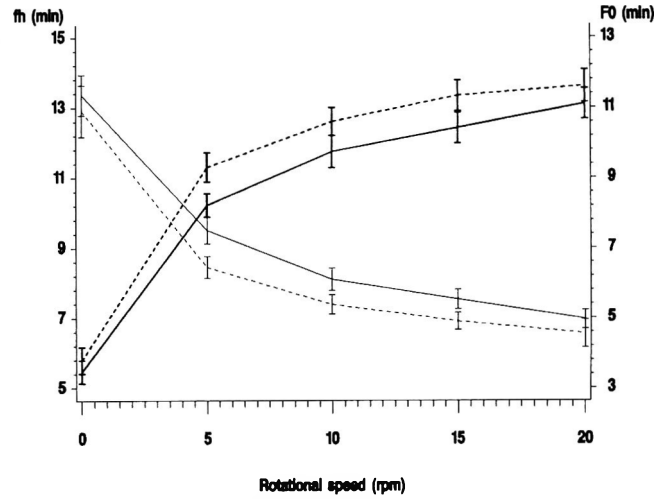

Fig. 3—Mean f_h-values and mean process-values with indication of standard deviation of white beans in brine treated in a Barriquand Steriflow simulator as a function of rotational speed and headspace. (CUT=16 min, HT=13 min, T_1=121°C, T_o=40°C). Bold curves represent process-values while normal curves stand for f_h-values. (----- 20 mm HS; ——— 10 mm HS)

Optimization

For different headspaces and for still as well as rotary conditions a minimum (Fig. 4) and thus an optimal process could be found. Optimal processes were computed (Table 3) for different processing conditions. Both for still and rotary processes an increase of headspace had little effect on optimal temperature, which was expected since headspace had a main effect on j-value and little effect on f_h-value (Table 2). The optimal temperature correlated loglinearly to the f_h-value while the heat penetration parameter j had a minor impact on optimal temperature (Hendrickx et al., 1993). Agitation resulted in higher

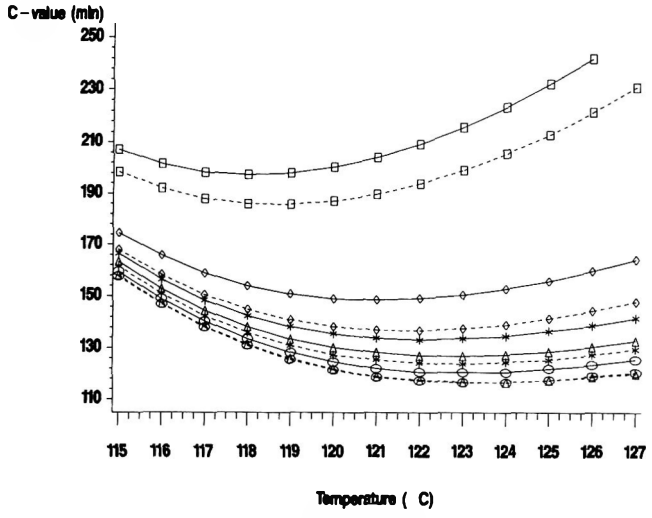

Fig. 4—Calculated cook-values for white beans in brine processed in glass jars as a function of sterilisation temperature, rotational speed and headspace. Straight curves represent a headspace of 10 mm while dotted curves a headspace of 20 mm. (CUT=16 min, F_0=6 min, T_o=40°C). □=0 rpm; ◇=5 rpm; *=10 rpm; △=15 rpm; ○=20 rpm).

optimal temperatures and better quality retention in terms of thermal softening as compared to still processes. However, the advantage of processing at higher rotational speed was limited (Table 3) when the calculated optimal cook-values (a measure of quality retention) were reported for different rotational speeds. Above 15 rpm, increasing rotational speed had a limited influence on calculated optimal cook-value. Ohlsson (1980a, b) and Hendrickx et al. (1993) plotted optimal temperatures as a function of f-slope values for different z_q and F_0-values. A change of f-slope value from 5 min to 10 min, the range of agitated white beans, corresponded to a change of ± 3°C in optimal temperature.

Taste panel and tenderometer evaluations of optimal processes

To validate the computed processes, optimal as well as deviating processes, (Table 4) were executed in the pilot retort and evaluated by trained taste panel and a tenderometer. Deviations from optimal temperatures were distinguished on the 1% level except for still processed beans at 122°C which showed better quality than the computed optimal process at 118°C. The beans processed at 122°C were judged harder ($P<0.05$) than those processed at the calculated optimal temperature. Samples from the process deviating 8°C were softer ($P<0.01$) than those from the process at the calculated optimal temperature. The taste panel rejected the calculated optimal process and accepted the still process at 122°C (t_h=15 min, C=188 min) as optimal. Results of the tenderometer evaluation confirmed the ranking of the processes. A still process at 122°C was also judged harder than the calculated optimal process at 118°C. This could be explained by assumptions made in the optimization procedure. Surface resistance to heat transfer was considered negligible. This was only valid for condensing steam sterilization and processing of metal containers (Tucker and Holdsworth, 1990) but white beans were processed in glass jars. The influence of a finite surface heat transfer coefficient on optimal temperature has not been systematically studied. It is expected that for low Biot numbers, when thermal gradients occur only during a very short time period compared to total holding time, the optimal temperature would increase. A second reason is the optimization of the surface cook value whereas for hardness degradation the volume average quality should be considered. Optimal temperature to maximize overall quality is higher than that to maximize surface quality because with higher temperatures, higher overprocessing at the surface is compensated by higher overall quality (Silva et al., 1993a). Ohlsson (1980c) stated that the optimal temperature for maximizing overall quality of an infinite slab was about 7.5 to 10°C higher than the correspondent optimum to maximize surface quality. For rotary processes the taste panel distinguished between deviations of 4°C and deviations of 8°C. Data were not sufficient to indicate in-batch variation. Thermal softening was definitely less by agitation based on the taste panel. Agitated beans were harder ($P<0.01$) than still processed beans.

Table 3—Theoretically calculated optimal processes and resulting cook-values for white beans processed in a water cascading retort as a function of rotational speed and headspace

Rotational speed (rpm)	Headspace (mm)	Temp (°C)	HT (min)	Cook-value (min)
0	10	118	25.5	196
	20	118	23.9	192
5	10	121	13.1	148
	20	122	10.5	137
10	10	122	9.6	133
	20	123	8.1	125
15	10	123	7.9	128
	20	123	7.6	117
20	10	123	7.9	123
	20	123	7.6	117

(F_0=6 min; T_0=40°C; T_c=15°C; z_q=21.3°C; h=∞)

Table 4—Optimal and deviating processes executed in a pilot retort to test optimisation results

rpm/HS	Process	Temp (°C)	HT (calculated) (min)	HT (requested) (min)
0/10	opt.	118	25.5	25.2
	dev.1	122	16.9	15.1
	dev.2	110	90.3	85.4
15/20	opt.	123	7.6	7.0
	dev.1	119	13.8	12.9
	dev.2	115	28.6	25.6

CONCLUSION

OPTIMAL PROCESSES were developed which are valid for the considered containers, products, fill weights, retorts, location in the retort, processing medium and processing conditions. Results confirm the usefulness of the surface cook-value as an optimization parameter and the efficiency of this simulation approach to design optimal processes for a mixed type heating food. Scaling up of optimal processes designed at pilot scale to a commericial food processing plant requires the study of several critical factors (e.g. loading pattern, steam supply, container position,...) which may affect temperature and heat transfer distributions and thus quality distribution.

NOTATION

C	Cook - value or process value of quality retention (min)
CUT	time required for the heating medium to reach the sterilization temperature (min)
D_{ref}	decimal reduction time at reference temperature (min)
F_o	sterilising value at reference temperature 121.15°C and z_m=10°C (min)
F_t	target sterilising value at reference temperature 121.15°C and z_m=10°C (min)
f_h	slope factor of a heating curve (Ball terminology) (min)
HS	headspace (mm)

HT	holding time (min)
j_h	heating lag factor (dimensionless)
P	probability level
rpm	rotations per minute
T	temperature (°C)
T_{ref}	reference temperature (°C) T_{ref}=121.1°C for the calculation of the process-value T_{ref}=100°C for the calculation of the cook-value
T_o	initial product temperature (°C)
T_l	processing temperature (°C)
T_w	cool water temperature (°C)
T_{opt}	optimal processing temperature (°C)
temp	temperature (°C)
t	time (min)
t_c	cooling time (min)
z_m	temperature dependence of the decimal reduction time of index microorganism (°C)
z_q	temperature dependence of the decimal reduction time of quality index (°C)

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Oligosaccharides, Antinutritional Factors, and Protein Digestibility of Dry Beans as Affected by Processing

ZACHARIE BARAMPAMA and RONALD E. SIMARD

ABSTRACT

Dry beans (*Phaseolus vulgaris*) were subjected to soaking, cooking or a combination of both prior to fermentation, and then assessed for oligosaccharides, antinutritional factors and *in-vitro* protein digestibility. Results showed an important decrease in raffinose oligosaccharides and antinutritional factors. However, an increase of trypsin inhibitor and tannin contents occurred respectively in cooked or soaked-cooked fermented beans and in raw or soaked fermented beans. Appreciable improvement in *in-vitro* protein digestibility was only observed in cooked or soaked-cooked beans. After fermentation, the largest decreases were observed in soaked-cooked beans (92.75%) for raffinose, in cooked beans (31.57%) for phytic acid, in soaked beans (90.86%) for stachyose, and in raw beans for trypsin inhibitor (38.77%). The highest increase due to fermentation was observed in raw beans for *in-vitro* protein digestibility (1.73%).

Key Words: dry beans, raffinose oligosaccharides, antinutrients, digestibility.

INTRODUCTION

AMONG LEGUMES FOR HUMAN FOOD, dry beans (*Phaseolus vulgaris*) are most consumed as whole beans (Doughty and Walker, 1982). They constitute an important source of protein, vitamins (especially B) and mineral elements throughout the world (Koehler et al., 1987). In Burundi dry beans are the main source of protein and the basis of the diet, with a daily per capita consumption of about 123g (vs 6.6g in USA) in 1980-1990 (Hosfield, 1991). Dry beans have beneficial effects on human health, being very low in sodium (Cheftel et al., 1986), cholesterol (Luc et al., 1991), and saturated fatty acids but rich in unsaturated fatty acids such as linoleic acid (Besançon, 1978). Also dry beans are high in fiber content which may have hypoglycemic and hypocholesterolemic effects and may reduce risks of colon cancer (Anderson et al., 1990).

Despite such advantages, dry beans present some undesirable characteristics which limit their acceptability. These include the hard-to-cook phenomenon (Salunkhe and Kadam, 1989), the presence of oligosaccharides causing flatulence (Rackis, 1975) antinutritional factors (Liener, 1975) and low protein digestibility (Salunkhe and Kadam, 1989). In order to improve the nutritional quality of dry beans, treatments such as soaking, cooking, germination, irradiation or supplementation have been applied (Vishalakshi et al., 1980; El Nahry et al., 1977). The effects varied with the cultivar and the treatments. In general, all treatments, except supplementation, reduced oligosaccharides and antinutritional factors (Kaul and Bajwa, 1987; Sathe et al., 1983; Deshpande and Cheryan, 1983). Soaking, cooking, and irradiation improved protein digestibility but not biological value (El Nahry et al., 1977; Reddy et al., 1979). Germination improved protein digestibility (Sathe et al., 1983) as well as biological value (Everson et al., 1944; Chattopadhyay and Banerjee, 1953). Supplementation which has shown positive effects on biological value is considered to have no effect on protein digestibility (Hernandez-Infante et al., 1979).

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Fermentation, which has appeared to reduce oligosaccharides and antinutritional factors and increase protein digestibility and biological value in other legumes (Ejiofor and Oti, 1987), has not been extensively applied for dry beans. Data are also lacking on the effects of such treatment when preceded by soaking and/or cooking, even for fermented legumes. Fermentation may cause additional improvements in nutritional quality of beans previously soaked and/or cooked. Little is published about the nutritive quality of dry beans grown in Burundi and the first data have been published only recently (Barampama and Simard, 1993) on composition, protein quality and antinutritional factors of some dry bean varieties. That data is somewhat incomplete as it covers raw beans, while the legume is usually consumed as cooked or soaked-cooked beans. Information is thus needed about the nutritional quality of soaked and/or soaked-cooked beans.

Our objective was therefore to determine whether soaking or cooking, and/or fermentation could reduce raffinose oligosaccharides, antinutritional factors and improve protein digestibility of dry beans.

MATERIALS & METHODS

Dry beans and preparation

The dry bean variety *Phaseolus vulgaris,* var. Dore de Kirundo, grown in Kirimiro (Burundi) and selected for its high content of flatulent factors and low digestibility (Barampama and Simard, 1993), was used. *Lactobacillus fermentum* ATCC 14931 was used throughout for fermentation of dry beans.

Three batches were made from a sack of beans (from Burundi). From each batch, four samples of 500g of dry bean seeds were taken (Fig. 1) and randomly distributed according to four treatments (raw, soaked, cooked, and soaked-cooked beans). Beans were then rinsed 3× in distilled water and dried in a ventilated oven at 55°C for 24 hr. After drying, one sample of each batch was ground directly in a cyclotec (Tecator, Hoganas, Sweden). The other beans were first given appropriate treatments (soaking for 12 hr at 20°C at a 1:10 dry beans: water ratio, cooking on a gas burner for 1 hr 30 min at 100°C at a 1: 5 dry beans:water ratio, and soaking followed by cooking for 1 hr), redried at 55°C for 48 hr, and finally ground.

For fermentation, 200g of flour were taken from raw, soaked, cooked, and soaked-cooked beans and placed in 800 mL glass containers. It was then wet with deionized water at a flour:water ratio of 1:2 (raw and soaked beans) or 1:3 (cooked or soaked-cooked beans), and fermented with 8 mL of *Lactobacillus fermentum* suspension (10^8 cells/mL) reactivated in MRS (Difco) at 37°C for 16 hr. Fermentation was carried out aseptically (in containers filled with distilled water and containing 10 mL of 10% potassium sorbate as antifungal) at 37°C for 72 hr. Chemical and microbiological analyses (pH, titrable acidity, total bacteria) were done at regular intervals (0; 12; 24; 48; 72 hr) on fermented samples. After fermentation, dry bean pastes were dried at 55°C for 48 hr before grinding in a small coffee mill. The dry bean powders were placed into plastic containers and maintained at 4°C during the period of analyses.

Subsamples of powders were then taken from raw, soaked, cooked, soaked-cooked beans, fermented or not, and analyzed. Three replicate analyses were conducted for each of the eight treatments. Concentrations of oligosaccharides and antinutritional factors were calculated on a dry weight basis.

Chemical and microbiological analyses

pH was measured with a Fisher pH meter, model Accumet 930, on a suspension of 20g of sample in 100 mL demineralized water. Ti-

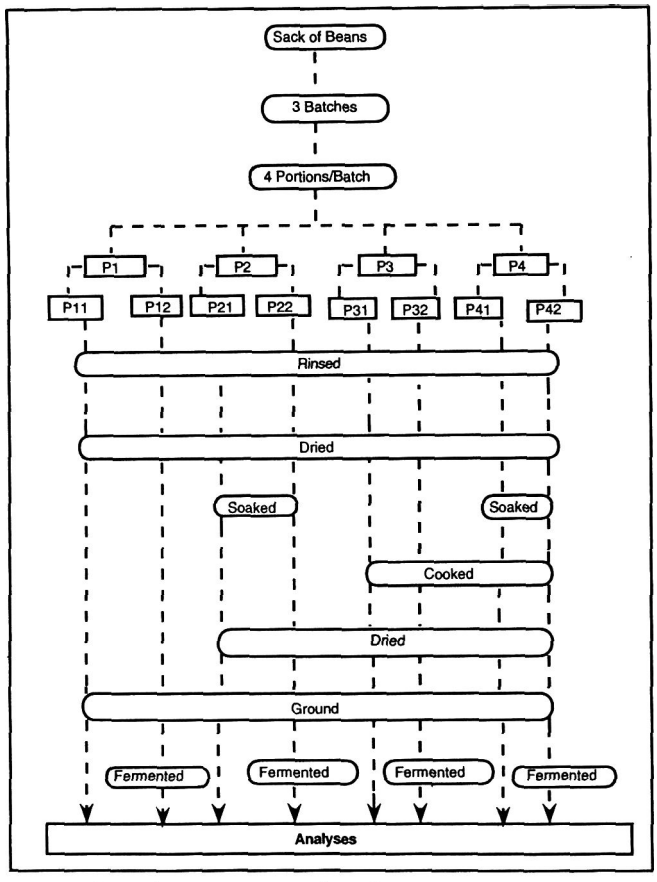

Fig. 1—Steps in preparation of dry beans.

trable acidity was estimated by titrating a suspension (20g in 100 mL deionized water) of the sample with 0.1N NaOH using phenolphthalein as indicator. **Microbiological analysis** of dry bean paste was done by sampling (11 g) aseptically and blending in 99 mL sterile water. Then, appropriate dilutions were pour plated with PC agar (Difco) for total bacteria count. After incubation at 30°C for 3 days, colonies were counted with a Quebec Colony Counter.

Raffinose oligosaccharides

Extraction (method of Agbo et al., 1985 slightly modified). To about 1g of sample in a 50 mL polyethylene centrifuge tube, 10 mL of 80% ethanol : water (V/V) was added for the first extraction. The sample was thoroughly mixed using a vortex mixer, and then shaken in a water-bath at 80°C for 15 min. The mixture was centrifuged for 5 min at 653 × g using a Sorvall RC-5B centrifuge (Du Pont, Newton, CT), and the supernatant transferred to another 50 mL polyethylene tube collector. To the sample residue in the first centrifuge tube, 5 mL of 80% ethanol:water (V/V) was added and the extraction repeated as previously described. Finally, 10 mL of 80% ethanol:water (V/V) was added to the same sample residue and the extraction repeated as in the previous step. The three supernatants were combined in a second polyethylene centrifuge tube, and 2 mL of 10% lead acetate:water (W/V) was added to deproteinize the solution. The mixture was shaken using a Vortex mixer until homogeneous, and then centrifuged for 10 min at 653 × *g*. The extract was transferred to another centrifuge tube, then 0.5 mL of 10% oxalic acid:water (W/V) was added. This extract was centrifuged for 20 min at 653 × *g* to remove lead oxalate, the clear extract then quantitatively transferred to a 25 mL volumetric flask, and brought to volume with deionized water.

Estimation by HPLC (method of Doyon et al., 1991). The liquid chromatograph used was "Waters Millipore" (Ville St. Laurent, Québec, Canada), equipped with a UV detector (model 481; 210 nm) and refractometer (model 410). The prepared extracts were purified by passage through a Sep-pak C18 (Waters Associates, Milford, MA) and injected into the HPLC column after filtration through 0.45 µm SM111 cellulose acetate filters (Sartorius). Samples were eluted with 0.0065N sulfuric acid at a pump rate of 0.4 mL/min. Then, a standard solution of raffinose oligosaccharides (raffinose, stachyose; Sigma Chemical, St. Louis, MO) was prepared at 1 mg/mL each. Fifteen microliters of standard solution was injected into a separation column Ion-300 (Mandel Scientific, Rockwood, Ontario, Canada) using a 100-µL microsyringe. Sugars were quantified by comparison with the standard, and expressed as mg/g.

Antinutritional factors

Trypsin inhibitor activity was assayed according to AACC method 71-10 (1980). Duplicate samples (1g) were extracted with 50 mL of 0.005 M NaOH at room temperature (≈23°C) for 1 hr. After appropriate dilution, extract was incubated with trypsin and benzoyl-DL-arginine-p-nitroanilide hydrochloride for 10 min at 37°C in a water bath shaker. Trypsin inhibitor activity, expressed as trypsin inhibitor units/g sample (TIU/g), was calculated from absorbance read vs a blank in a spectrophotometer. One trypsin unit (TU) was defined as the increase by 0.01 absorbance unit at 410 nm for 10 mL of reaction mixture.

Tannins were assayed according to the modified vanillin-HCl method of Price et al. (1978). A 2 g sample (previously dried in a ventilated oven at 55°C for 24 h) was extracted with 50 mL 99.9% methanol for 20 min at room temperature with constant agitation. After centrifugation for 10 min at 653 × *g*, 5 mL of vanillin-HCl (2% vanillin 1% HCl) reagent was added to 1 mL aliquots and the color developed after 20 min at room temperature was read at 500 nm. Correction for interference from natural pigments in dry bean was achieved by subjecting the extract to the conditions of the reaction, but without vanillin reagent. A standard curve was prepared using catechin (Sigma Chemical, St. Louis, MO) after correcting for blank, and tannin concentration was expressed in mg catechin equivalents.

Phytic acid was evaluated by the method of Eskin et al. (1980). Duplicate samples weighing 5g were extracted with 100 mL 2.4% HCl for 15 min at room temperature. After centrifugation and filtration on Whatman No. 1, 2mL were diluted to 25 mL in deionized water and 10 mL of the diluted extract was evaluated for phytic acid content. Phytic acid was first concentrated on anion exchange resin. After inorganic phosphate elution with 15 mL of deionized water followed by 15 mL of 0.05M NaCl, phytate was eluted with 15 mL of 0.7M NaCl. Phytate concentration in the eluate (3 mL) was then determined by reaction with 1 mL of the Wade reagent (0.03% $FeCl_3 \cdot 6H_2O$ and 0.3% sulfosalicylic acid in distilled water). Final color development was measured at 500 nm by spectrophotometer after vortexing 5 sec and centrifuging 10 min at 653 × *g*.

In-vitro protein digestibility (IVPD)

IVPD was evaluated according to the method of Hsu et al. (1977). Fifty milliliters of aqueous protein (6.25 mg protein/mL) in distilled water were adjusted to pH 8.0 with 0.1N HCl and/or NaOH while stirring in a 37°C water bath. The enzyme mixture (1.6 mg trypsin; 3.1 mg chymotrypsin and 1.3 mg peptidase/mL) was maintained in an ice bath and adjusted to pH 8.0 with 0.1N HCl and/or NaOH. Five milliliters of the multienzyme solution (this solution was freshly prepared before each series of tests, and its activity was determined using the ANRC casein) were then added to the protein suspension which was being stirred at 37°C. A pH drop was observed, and recorded over a 10 min period using a pH meter. Percent protein digestibility (Y) was then calculated from the equation Y = 210.464-18.10X, where X is the pH change after 10 min.

All enzymes [trypsin (porcine pancreatic trypsin type IX, with 12,700 BAEE units/mg), chymotrypsin (bovine pancreatic chymotrypsin type II, 52 units/mg protein) and peptidase (porcine intestinal peptidase grade III, 102 unit/g)] were purchased from Sigma Chemical.

Statistical analyses

A completely random design was used and data obtained subjected to analysis of variance and the Duncan - Waller Multiple Range Test (Steel and Torrie, 1980).

RESULTS & DISCUSSION

Fermentation

For all the fermented beans, pH decreased (Fig. 2a). The decrease was more rapid for raw or soaked beans during the first 12 hr. Then pH decreased slowly, becoming practically constant after 72 hr. In cooked or soaked-cooked beans, the

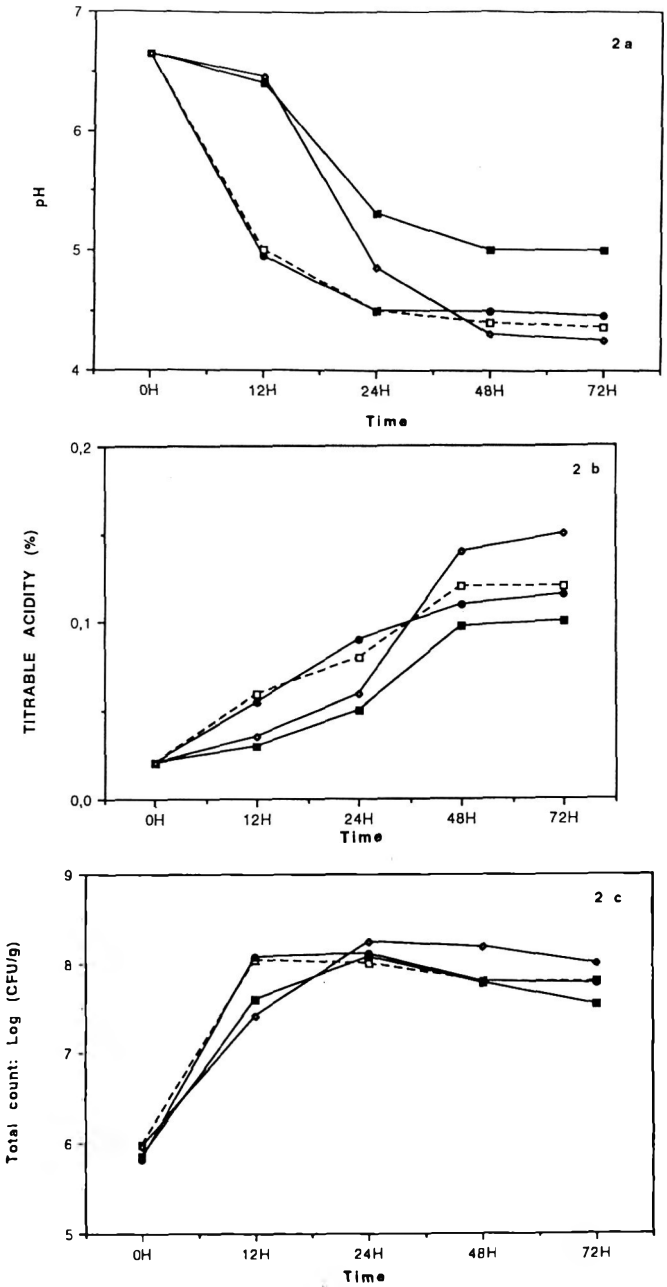

Fig. 2—Evolution of chemical and microbiological parameters during fermentation of dry beans. (-□-) raw; (-●-) soaked; (-■-) cooked; (-○-) soaked-cooked.

pH decreased after 24 hr fermentation, then became almost constant in cooked beans, while it continued to decrease steadily until 72 hr of fermentation for soaked-cooked beans. The greatest pH decrease was observed for soaked-cooked beans. Titrable acidity in raw, soaked, cooked or soaked-cooked fermented beans (Fig. 2b) showed an increase for all samples. This increase was more rapid during the first 12 hr in raw or soaked beans and only after 24 hr in cooked or soaked-cooked beans. The greatest amount of lactic acid was produced from soaked-cooked beans.

Bacterial growth during fermentation (Fig. 2c) showed a maximum number of bacteria after 12 hr for raw or soaked beans and after 24 hr for cooked or soaked-cooked beans. This low bacterial count in cooked or soaked-cooked beans during the first hours of fermentation was probably due to a lack of available essential amino acids or to a loss of soluble sugars after cooking. Buckle and Sambudi (1990) and van der Poel et al. (1990) also reported a loss of amino acids and soluble sugars after soaking and/or cooking of some legume seeds. Afterwards, bacterial numbers were constant (except in cooked unsoaked beans), before decreasing progressively. The increase of bacterial growth corresponded to the decrease in pH and the increase in titrable acidity.

Effect on raffinose oligosaccharides

Raffinose oligosaccharides (stachyose, raffinose) (Fig. 3) varied from 1.18 (soaked-cooked fermented) to 24.88 mg/g (raw unfermented) for stachyose, and from 0.05 (soaked-cooked fermented) to 1.84 mg/g (raw unfermented) for raffinose, and treatment means were different. All treatments reduced raffinose and stachyose (Table 1) and fermentation produced a sharp reduction in raffinose (97.28%) and stachyose (95.26%) in soaked-cooked beans. This decrease was caused by a loss of sugar to water by diffusion and/or by enzymatic degradation in fermented beans. Sugar reduction has also been reported by Kosson and Bakowski (1986) for soaked or cooked beans, and by Ibrahim and Antai (1986), for fermented legumes. The greatest decrease of flatulent factors was observed for soaked-cooked and fermented beans but fermentation effects seemed more marked in soaked beans, at least for stachyose (90.86%). Among treatments applied prior to fermentation, none inhibited the decrease in sugars during fermentation.

Effect on antinutritional factors

Antinutritional factors (trypsin inhibitor, phytic acid and tannin) of treated and untrated beans (Fig. 4) varied from 0.65 (soaked-cooked) to 29.30 × 10^{-3} TIU/g (raw unfermented) for trypsin inhibitor, from 8.34 (soaked-cooked) to 15.79 mg/g (raw unfermented) for phytic acid, and from 4.64 (cooked-fermented) to 22.72 mg catechin equivalents/g (soaked-cooked) for tannins. Significant differences between treatments were noted for all three. All treatments decreased trypsin inhibitor (Table 1). The largest decrease compared to raw beans was observed for soaked-cooked beans (97.78%). This reduction was due to thermal inactivation of trypsin inhibitor. A similar observation was reported by Salunkhe and Kadam (1989), Doughty and Walker (1982), and Kaul and Bajwa (1987) in legumes subjected to soaking or cooking treatments. Fermentation also decreased trypsin inhibitor except when applied to cooked or soaked-cooked beans. The largest decreases were observed for raw beans (38.77%) or soaked beans (34.35%). Similar changes were reported by Padhye and Salunkhe (1978), who studied biochemical changes in fermented mungo beans. Fermentation caused an increase in trypsin inhibitor when beans were previously cooked (28.00%) or soaked-cooked (112.31%). That increase was probably due to the trypsin inhibitor retention in tannin-protein complexes which may occur during cooking of beans. Formation of such complexes in cooked beans has been reported by Kaur and Kappour (1990) and Salunkhe and Kadam (1989). Another explanation for the increase in trypsin inhibitor may be the conversion of tannins in proteins (Salunkhe and Kadam, 1989) or regeneration of the inactivated trypsin inhibitor which would be favored by the fermentation treatment in cooked beans. Deshpande and Nielson (1987) also reported regeneration of inhibitory activity in dry bean samples held at 4°C for 20 hr after heating.

For phytic acid, a significant decrease occurred for soaked-cooked beans (due probably to diffusion of the antinutrient in water) and for fermented beans (Table 1). The greatest reduction was noted for soaked-cooked beans (47.18%) compared with raw beans. Soaking or cooking did not reduce phytic acid content. This disagreed with reports of Salunkhe and Kadam (1989) for other legumes. Fermentation decreased phytic acid with the help of phytases in raw (20.13%) and cooked beans (31.57%), while it had no effect on soaked beans, in confirmation of results of Salunkhe and Kadam (1989). Cooking

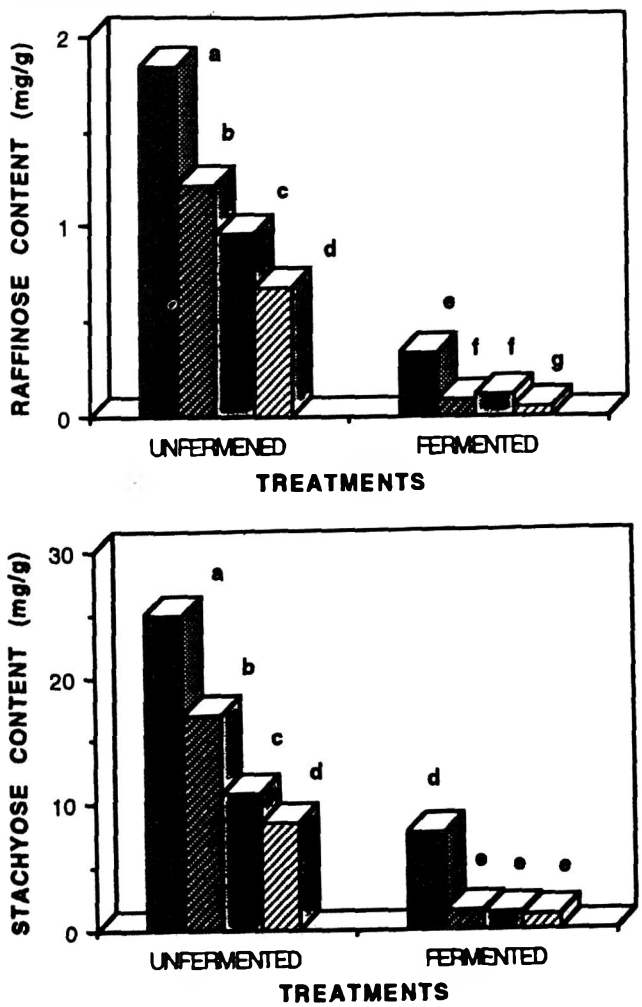

Fig. 3—Raffinose oligosaccharides content in dry beans subjected to various treatments. Raffinose oligosaccharides content (means of three replicate analyses) is expressed as mg/g (DWB). Means with different letters are significantly different ($P < 0.05$). (■) raw; (▨) soaked; (□) cooked; (▨) soaked-cooked.

Table 1—Variation[a] (%) in oligosaccharide, antinutritional factors, and *in vitro* protein digestibility of dry bean

Treatment	Stachyose	Raffinose	Trypsin inhibitor	Phytic acid	Tannin	Digestibility
Unfermented[c]						
R	0.00	0.00	0.00	0.00	0.00	0.00
S	−31.35	−33.15	−25.29	−7.02	−28.75	1.43
C	−56.51	−47.28	−97.44	−8.73	−59.81	12.31
SC	−65.71	−62.50	−97.78	−47.18	−84.64	13.32
Fermented[c]						
R	(−69.01)[b]	(−82.06)	(−38.77)	(−20.13)	(34.64)	(1.73)
S	−93.72 (−90.86)	−95.10 (−92.68)	−50.95 (−34.35)	−10.70 (−3.95)	5.01 (47.38)	1.73 (0.30)
C	−94.21 (−86.69)	−94.02 (−88.65)	−96.72 (28.00)	−37.55 (−31.57)	−68.60 (−21.88)	13.32 (1.14)
SC	−95.25 (−86.16)	−97.28 (−92.75)	−95.29 (112.31)	−41.48 (0.00)	−89.17 (−29.52)	13.97 (0.75)

[a] Variation in reference to raw beans.
[b] Values in parentheses represent variation occasioned by fermentation.
[c] R = raw; S = soaked, C = cooked; SC = soaked-cooked.

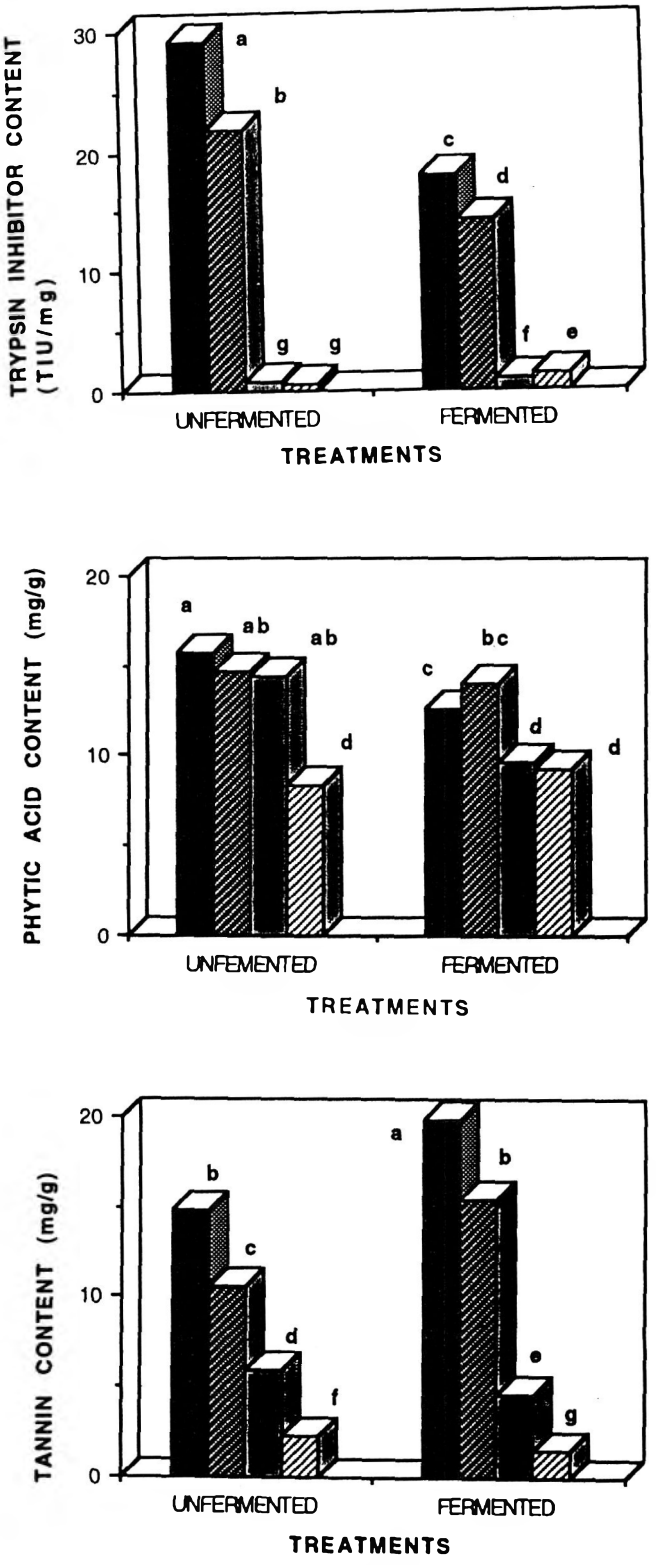

Fig. 4—Antinutritional factor level in dry beans subjected to various treatments. Antinutritional factor level (means of three replicate analyses) is expressed as mg/g (DWB) for tannin and phytic acid; TIU/mg (DWB) for trypsin inhibitor. Means with different letters are significantly different ($P < 0.05$). (■) raw; (▨) soaked; (□) cooked; (▨) soaked-cooked.

intensified fermentation effects on phytic acid decrease while soaking diminished them.

A decrease in tannin content (Table 1), was observed after soaking (28.75%), cooking (59.81%), soaking-cooking (84.64%), cooking-fermentation (68.60%) and soaking-cooking-fermentation (89.17%). This reduction was probably due to diffusion of this antinutrient in water or to the formation of insoluble tannin-protein complexes not extractable from beans. Salunkhe and Kadam (1989) also reported a tannin decrease in soaked beans. Kaur and Kapoor (1990) reported a decrease in tannins in rice beans during soaking and cooking. However, an increase in tannin content (Table 1) was observed in raw (34.64%) and soaked

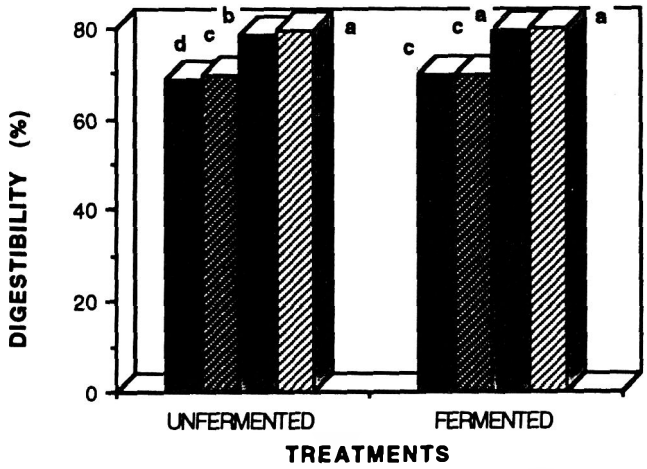

Fig. 5—*In-vitro* protein digestibility of dry beans subjected to various treatments. Dibestibility (means of three replicate analyses) is expressed as %. Means with different letters are significantly different ($P < 0.05$). (■) raw; (□) soaked; (□) cooked; (□) soaked-cooked.

(5.01%) fermented beans. A decrease of tannin content was observed in cooked (21.88%) or soaked-cooked (29.52%) fermented beans while tannins increased in raw (34.66%) or soaked (47.38%) fermented beans. In general, fermentation tended to substantially increase tannins, perhaps due to microbial activity, and cooking favored tannin reduction during subsequent fermentation. These observations confirmed those by Khetherpaul and Chauhan (1991) on fermented pearl millet.

Effects on in-vitro protein digestibility

In-vitro dry bean protein digestibility (Fig. 5) varied from 68.68 (raw unfermented) to 79.84% (soaked-cooked) and means of treatments were different. Compared with raw beans, all treatments improved protein digestibility (Table 1). A slight improvement in protein digestibility was found for fermented soaked beans. A similar observation was made by Padhye and Salunkhe (1978), who studied protein digestibility in soaked soybeans. Protein digestibility was also slightly improved for fermented raw beans also in agreement with Salunkhe and Kadam (1989). According to Kaul and Bajwa (1987), fermentation may improve protein digestibility by reducing protease inhibitor and/or by modifying the conformation of dry bean proteins. Soaking may also decrease antinutritional factors in dry beans (Deshpande and Cheryan, 1983), and thus improve protein digestibility. Cooking with or without fermentation significantly improved protein digestibility.

Similar observations have been reported by Gabrial et al. (1974). According to Salunkhe and Kadam (1989), improvement of protein digesitibility may result from protein denaturation and inactivation of trypsin inhibitor by heat treatment. We have observed that the highest values for protein digestibility were obtained when beans were cooked after soaking. This agreed with El Nahry et al. (1977), who noted that protein digestibility was more improved in soaked-cooked beans than in cooked beans. The greatest effects of fermentation on protein digestibility were found on raw beans. However, increase (1.76%) was slight compared with that from (15.38%) soaking-cooking the beans. Fermentation effects were also slight in cooked (1.16%) or soaked-cooked (0.76%) beans and slight in soaked beans. Improvement in dry bean protein digestibility was due to pre-fermentation treatments (cooking or soaking-cooking) but not fermentation.

CONCLUSION

Soaking decreased oligosaccharides and antinutritional factors except phytic acid. Cooking reduced antinutritional factors and considerably increased dry bean protein digestibility, especially when preceded by soaking. Fermentation reduced raffinose oligosaccharides and trypsin inhibitors, whereas its effects on other antinutritional factors were widely influenced by pretreatment. It also improved protein digestibility, except for beans subjected to soaking only, but the improvement was very slight. Highest decrease in oligosaccharides and antinutritional factors and the increase in protein digestibility were observed in soaked-cooked fermented beans. Thus, soaking, cooking and fermentation is an appropriate combination to improve nutritional quality. The best effects of fermentation were often noted in raw beans but not in soaked, cooked or soaked-cooked beans so raw beans may be more useful for lactic fermentations.

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 From page 798

higher activity in the forced thaw drip, indicating a modified membrane structure. Slightly increased enzyme activity occurred in the fast refrozen group notably less than from the nonglazed group. The slow refreezing treatment after glazing seemed to influence the muscle membrane integrity in a way similar to that shown for slow freezing and slow thawing (Nilsson and Ekstrand, 1993,1994). Only slight differences between groups occurred in liquid holding capacity test of rainbow trout. In contrast to cod, a tendency toward higher liquid holding capacity occurred in the fast refrozen group.

The sensory difference test on cod indicated differences between the two glazed groups (Table 3). The nonglazed group was also tested vs the fast refrozen group, but no differences occurred. Results from the sensory difference tests for rainbow trout (Table 3) revealed significant differences between nonglazed and fast refrozen groups. The fast refrozen and slowly refrozen group were also different.

CONCLUSION

THE FASTER THE FISH WERE REFROZEN to the storage temperature the less the leakage of marker enzymes and the less thaw drip. Liquid-holding capacity in cod needs further study in relation to changes in protein and tissue structures.

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Gamma-Irradiated Dry Bean (*Phaseolus vulgaris*) Starch: Physicochemical Properties

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ABSTRACT

Great Northern bean starch, irradiated at doses of 2.5–20 kGy, had increased free and total acidity, concomitant with a pH decrease from 6.9 to 3.9 and a rapid increase of reducing value. The swelling power of the bean starch decreased with irradiation dose. A rapid increase of water and 80% ethanol solubility at 25°C was observed at doses ≥10 kGy. No differences were detected on damaged starch or changes in X-ray diffraction pattern. ESR spectra of the irradiated bean starch after 9 and 11 mo storage (−40°C) showed the presence of long life radicals, which could be stabilized after adding water.

Key Words: bean, starch, swelling power, X-ray diffraction, free radicals

INTRODUCTION

THE DEPOLYMERIZING EFFECT of gamma-irradiation treatment on starch, oligomers (e.g., cyclohepta-amylose), and degradation of glucose in the solid state has been reported (Phillips and Young, 1966a, b; Baugh et al., 1976; Michel et al., 1980). Although many reports of irradiated wheat, corn, and potato starches have been published (Samec, 1958, 1960; Radley, 1960; Greenwood and MacKenzie, 1963), information is limited on irradiation treatment of legumes, particularly, *Phaseolus* starches. Rayas-Duarte and Rupnow (1993) reported that irradiated bean starch had two molecular weight species smaller than the amylose fraction of untreated/control starch and an increase in gelatinization temperature.

Irradiation of corn, potato, and sweet potato starches produced increases in water solubility, reducing power, and acidity, accompanied with decreased iodine-binding and viscosity (Radley, 1960; Samec, 1960; El Saadany et al., 1974). Raffi et al. (1981e) studying bean starches found that the total acidity of haricot beans was significantly lower than in other starches (normal corn, high amylose and waxy corn, and wheat). When a corn starch model was used to estimate results of bean starch irradiation, higher free acidity values were obtained (Raffi et al., 1981e). However, the formic acid content in irradiated potato, rice, and haricot bean starches analyzed by gas chromatography was greater than the free acidity determined by pH (Raffi et al., 1981e). Raffi et al. (1981b) proposed relatively labile linkages between formic acid and macromolecular chains of starch, (stable formic esters of starch) that may influence acidity values which had been found at higher doses.

A major concern of food safety agencies is the need for quantitative methods to determine the dose irradiated food or its components received. International efforts have been focused on the effectiveness of different techniques to quantify irradiation exposure of food (Crawford and Rehe, 1991; Glidewell et al., 1933). Electron spin resonance (ESR) has been reported to detect directly irradiated foods (unpaired electrons) and, potentially, the mechanisms involved in food irradiation (Glidewell et al., 1993). ESR or electron paramagnetic resonance (EPR) spectroscopy uses the absorption of microwave energy from free radicals which display characteristic properties. The detection is limited to materials with hard matrices but is nondestructive and requires small samples (Crawford and Rehe, 1991; Goodman et al., 1989). Helle et al. (1992) used low microwave power to detect two signals in the ESR spectra of irradiated spices, but the complexity of the spectral analyses makes it unlikely for a quick and easy detection method (Glidewell et al., 1993). The transformation induced by radiation of a solute in dilute solution occurs mainly from interactions with primary radicals from water hydrolysis, which can react with sample components (Goodman et al., 1989). However, the primary radicals of water could cause chemical changes, such as hydrolysis and oxidation. Identifying products after irradiation of carbohydrates has shown that oxidation was a predominant transformation (Raffi et al., 1981a-c; Kochetkov et al., 1979). Colonna et al. (1987) suggested that in the dry state (less than 12% moisture), water hydrolysis was negligible. Earlier ESR studies on dried food, irradiated up to 10 kGy, included rye bread, egg powder, beef, milk powder, and starch (O'Meara and Shaw, 1957). After 5 min and 6–22 days of irradiation, using the amplitude of the ESR signal to estimate spin concentration, a gradual disappearance of free radicals was reported. Diehl and Hofmann (1968) with irradiated (10 kGy) dried whole egg and potato starch reported a rapid decrease of spin concentration during the first few hours followed by a slower decrease.

Researchers have reported the susceptibility of C-H bonds of polysaccharides to hydroxyl radical reaction with subsequent hydrogen loss (Kochetkov et al., 1979; Colonna et al., 1987). Adding water may increase the number of interactions. ERS can detect secondary radicals in solids, frozen samples or polymeric crystalline structures, such as bone, cuticle, protein, or cellulose (Glidewell et al., 1993). Diehl (1982) indicated that most of the irradiation-induced chemical changes occur rapidly, i.e., oxidation and hydrolysis. However, research reports often do not report the time after irradiation at which measurements or analyses were performed. The presence of radicals with long existence allowed detection of ESR signals for up to 8 mo after irradiation (Diehl and Hofmann, 1968).

Studies on irradiated starches reported different free radical spectra (spin concentration) kinetic parameters, depending on starch origin (Raffi et al., 1981d, e). During the first 1 or 2 hr, different spectra were obtained; but after several days, only slight variations were observed with a first-order rate decrease (intensity of signal). Final spectra seemed to be similar for all investigated starches. Raffi et al. (1981d, e) observed that initial kinetics may reflect differences in molecular arrangements, but the most abundant radicals were essentially the same for different starches. Though radiolysis products are varied, oxidized products with unchanged or shorter carbon chains have been reported (Tollier and Guilbot, 1972; Raffi et al., 1980; 1981a, b). Dauphin et al. (1974) reported that formic acid was the main contributor of free acidity in irradiated (up to 20 kGy) corn starch with the concentration proportional to irradiation.

Our objective was to determine changes in physicochemical properties induced by gamma irradiation of isolated bean starch.

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MATERIALS & METHODS

DRY BEAN (*PHASEOLUS VULGARIS* c.v. Great Northern UI 59) foundation seed was stored at −40°C until needed. Isolation of bean starch was based on the procedures of Hoover and Sosulski (1985) and Abbas and Berry (1986) as previously described (Rayas-Duarte and Rupnow, 1993). Starch samples (15 g) were packed in paper and polyethylene bags and irradiated with a Co^{60} source at ≈25°C and atmospheric air. The doses applied were 2.5 and 5 (low doses), 10 (medium), and 20 (high) kGy.

Reducing value

The reducing value of starch was analyzed, using the alkaline 3,5-dinitrosalicylic acid (DNS) reagent (Bernfeld, 1955; Bruner, 1964). Irradiated bean starch (2 g) was dissolved in 20 mL deionized water and stirred for 2 hr at room temperature (≈23°C). Aliquots of extract were centrifuged (Eppendorf Centrifuge 5412, Brinkman Instruments, Inc., Westbury, NY) for 3 min (8000×*g*). Aliquots (0.1–1 mL) of supernatant, which had absorbance equivalent to 40–90 μg of glucose, were diluted to 1 mL with water if needed, combined with 1 mL DNS solution, and mixed thoroughly. Tubes were placed in a boiling water bath for 10 min and diluted with 5 mL deionized water. The absorbance at 540 nm was recorded. Reducing sugar amount was calculated by linear regression, using a glucose standard curve.

Swelling power and solubility in water and 80% ethanol

The gravimetric method of Leach et al. (1959) was used. Other analyses were as described by Tollier and Guilbot (1970). Starch samples (0.5–0.6 g) were weighed and stirred in 15 mL of deionized water for 1 hr. The starch suspension was centrifuged for 5 min at 2300×*g*. The supernatant was saved and the residue resuspended in 15 mL deionized water. The 1 hr stirring and centrifugation steps were repeated four times. Supernatants were pooled and evaporated to dryness in a vacuum oven at 60°C. The dried water-soluble starch fractions were resuspended in 5 mL water and analyzed for total sugar content with a modified phenol-sulfuric method (Rayas-Duarte and Rupnow, 1993).

The carbohydrate fraction soluble in ethanol was analyzed as for water solubility, substituting 80% aqueous ethanol (v/v) for water. Water and ethanol solubility tests were conducted at 25°C.

Free and total acidity

Analyses followed the procedures of Dauphin et al. (1974) and Berger et al. (1977). Free acidity was determined on an aqueous extract of 1g bean starch in 10 mL boiled and degassed deionized water. Suspensions were flushed with nitrogen, tightly capped with rubber stoppers, and stirred for 1.5 hr. Starch was allowed to settle, and the decanted supernatant was titrated to pH 7, using standardized NaOH (2.3×10^{-3} N). Samples for measurement of total acidity were extracted as described for free acidity. After 1.5-hr extraction, the suspension was adjusted to pH 11 with 2 N NaOH and heated in a 50°C water bath for 30 min. After cooling, the starch was allowed to settle and the supernatant recovered and titrated to pH 7 with standardized HCl (6×10^{-3} N). Milliequivalents of [H^+] were calculated for free and total acidity.

X-Ray diffraction

Bean starch samples were pressed into pellets, and the diffraction pattern was obtained with a Diano diffraction generator (Diano Corp., Woburn, MA), equipped with a Ni-filtered copper tube. The scanning regions of the diffraction angle of 2θ were 5° to 30° in increments of 0.1°, counting 10 sec at each increment. The diffractometer was controlled with a computer, using Nicolet Inc. (Madison, WI) software for graphic output.

Electron spin resonance

ESR spectra of irradiated bean starch were recorded at 25°C on an ER 200 D10 Brüker spectrometer, using quartz tubes (4 mm i.d. × 250 mm; Wilmad Glass Co., Buena, NJ), with the sample at one-fourth capacity. Field intensity for the reference and samples was 3485 Gauss (G), scan range 100 G, and scan time 100 sec. Gain used for the DPPH reference {2,2-bis[4-(1,1,3,3-tetramethylbutyl)phenyl]-1-(2,4,6-trinitrophenyl)-hydrazyl} was 8×10^3. The gain for bean starch samples stored 9 days and 11 mo (−40°C) was 5×10^5 and 10×10^5.

The following parameters were used in all cases: microwave frequency 9.8 GHz, microwave power 10 dB and 21.8 mW, and modulator/receiver frequency 12.5 Hz. Samples at 9 days storage after irradiation had strong ESR signal spectra. Samples were hydrated, using an excess of deionized water, freeze-dried, and analyzed again to obtain information about the stability of radicals after their exposure to aqueous media.

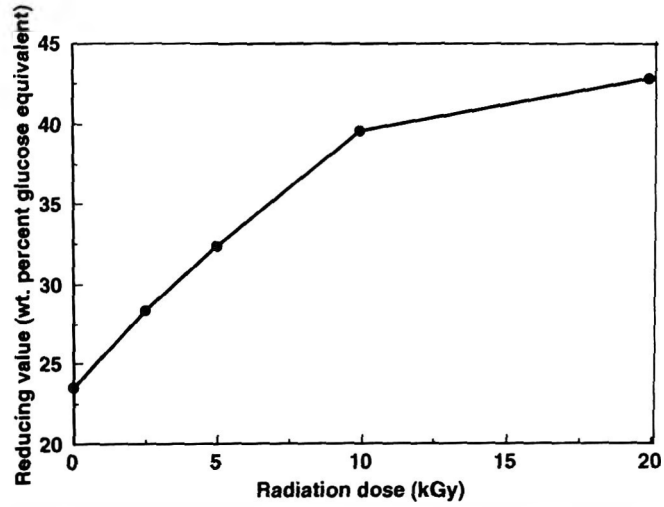

Fig. 1—Reducing value of gamma-irradiated Great Northern bean starch as assayed with 3,5-dinitrosalicylic acid.

Damaged starch

AACC method 76-30A (1983) was used to determine damaged starch. A 15-min hydrolysis at 30°C was performed in 1g of starch, using Enzeco fungal α-amylase (30,000 SKB units/g, Enzeco Corp., New York). Enzymatic hydrolysis was stopped by adding 3.7N H_2SO_4, and reducing sugars were determined with the AACC ferricyanide method 80–60 (1983). Table 22-18 (AACC, 1983) was used to determine the amount of maltose/10 g sample, which was converted to milligrams of starch hydrolyzed by multiplication with the empirical factor (0.082).

RESULTS & DISCUSSION

THE REDUCING VALUE OF IRRADIATED Great Northern bean starch, as determined with 3,5-dinitrosalicylic acid reagent, increased as irradiation dose was increased. A linear relationship between reducing power and radiation dose was observed (Fig. 1). Radley (1960) and Hofreiter and Russell (1974) reported a linear relationship of radiation dose and carbonyl groups. Whistler and Ingle (1965) reported that aqueous solutions of amylose, irradiated at 3 kGy, increased reducing values. The new reducing end groups resulted from the hydrolysis of starch molecules (Whistler and Ingle, 1965).

The swelling power determined as weight of hydrated granules was reduced in all irradiated samples (Fig. 2a). This was more pronounced at >70°C. At 2.5 kGy, the swelling power was reduced 25% (90°C). Nearly flat swelling patterns were observed for bean starch irradiated at doses of 5 through 20 kGy (Fig. 2a). Radley (1960) reported a total loss of swelling power of corn starch, irradiated at high doses (1000 kGy). The swelling and solubility patterns were similar for all doses (Fig. 2ab).

Several researchers reported restricted swelling of legume starches (Correa et al., 1965; Doublier, 1987). Leach et al. (1959) suggested that bean starch granules might possess strong binding forces that restrict swelling. The water-soluble compounds induced by 2.5 to 20 kGy (Fig. 3) further reduced overall swelling power of the bean starch granule. Lai and Varriano-Marston (1979) reported that black bean starch sam-

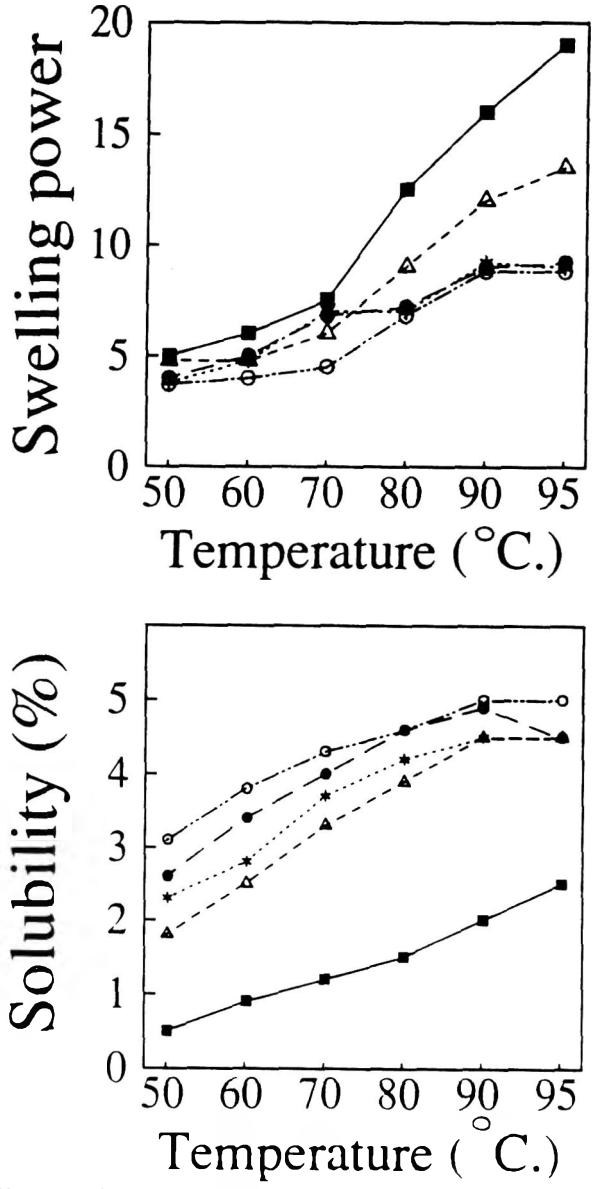

Fig. 2—Relation of swelling power and percent solubility to gamma-irradiation dose of Great Northern bean starch. Samples stored at 25°C for 4 mo after irradiation. Control; △ 2.5 kGy; * 5 kGy; • 10 kGy; o 20 kGy.

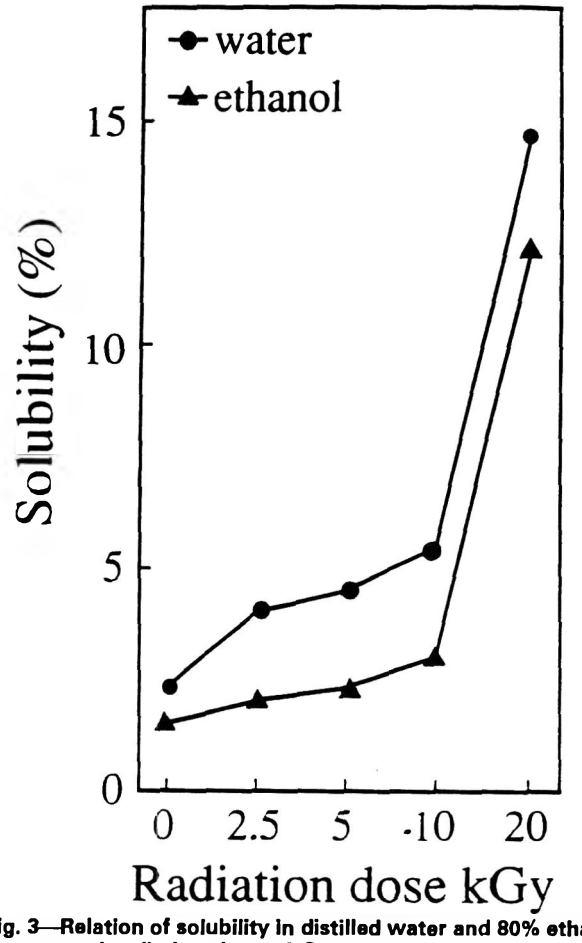

Fig. 3—Relation of solubility in distilled water and 80% ethanol to gamma-irradiation dose of Great Northern bean starch at 25°C. Samples stored at 25°C for 3 mo after irradiation.

ples taken from the Visco-Amylograph appeared deformed but granule integrity was preserved during heating. The amount of amylose diffused from the starch bean granules might be limited (Lai and Varriano-Marston, 1979). Such reduced swelling power should be considered when irradiated bean starch is to be used as a food ingredient.

The bean starch solubility in water and ethanol at 25°C (Fig. 3) increased as irradiation dose was increased. Both solubilities increased slightly at doses up to 5 kGy, and a sharp increase was observed at doses ≥10 kGy. As expected, the data (Fig. 3) indicate that at the highest irradiation dose (20 kGy), degradation of the bean starch molecules was more pronounced, with an increased yield of soluble lower molecular weight compounds. Several researchers reported increases in material soluble in water and ethanol as evidence of molecular degradation of starch by irradiation (Radley, 1960; Whistler and Ingle, 1965; Tollier and Guilbot, 1972). Acidity and pH values (Fig. 4) showed free acidity increased at doses >5 kGy; however, the total acidity increased at lower doses (2.5 kGy).

Athanassiadis and Berger (1973) postulated that the opening of lactone-type compounds at pH 10 contributed to total acidity. The existence of lactone products in irradiated cycloheptaamylose has been confirmed (Baugh et al., 1976; Phillips and Young, 1966a, b). Reported total and free acidity values of irradiated corn starch were lower than those we found. Susceptibility to irradiation-induced acidity and variable time elapsed from irradiation to analysis could account for the differences (Diehl, 1982). The content of lipids, especially unsaturated lipids (Andrianarison et al., 1992), could also affect susceptibility of starch to irradiation. The importance of observed increases in bean starch acidity in food systems and their effects on functional properties of seeds and starch need to be evaluated.

Similar starch damage values (<0.5%) were obtained for control and irradiated Great Northern bean starch samples. No published reports of damaged starch determined enzymatically in irradiated starches were found. A few reports on digestibility of irradiated starch are available (Watanabe et al., 1977; Kume and Tamura, 1987). A slight increase in the *in vitro* digestibility of irradiated (10 kGy) starches was reported when pancreatic α-amylase was used in both high amylose and regular corn starch (Watanabe et al., 1977). However, *in vivo* digestibility of irradiated starches was lower than the nonirradiated high amylose and regular corn starch. Glucoamylase digestibilities of irradiated (wheat, corn, sweet potato, and potato) starches were unchanged, but decreased in raw tapioca starch (Kume and Tamura, 1987).

X-ray diffraction analysis of Great Northern bean irradiated starch showed no change in pattern compared to controls (nonirradiated) (Fig. 5). X-ray diffraction, an averaging method, would not indicate small changes in crystallinity. No notable changes in structure were produced by the gamma-irradiation treatment up to 20 kGy dose (Fig. 5). Though both amylose

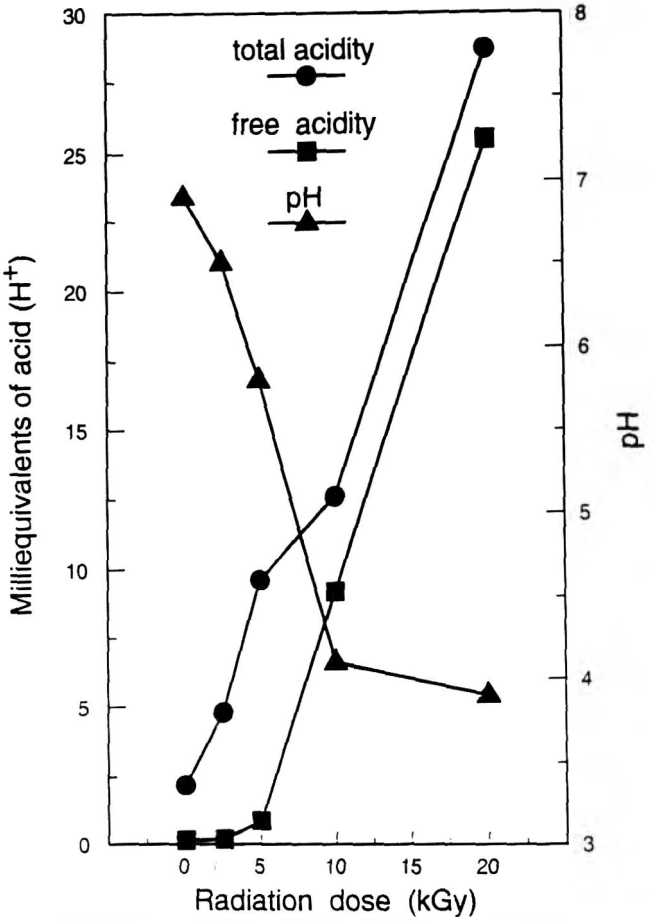

Fig. 4—Relation of total and free acidity to gamma-irradiation dosage of Great Northern bean starch. Samples analyzed after 5 mo storage at 25°C.

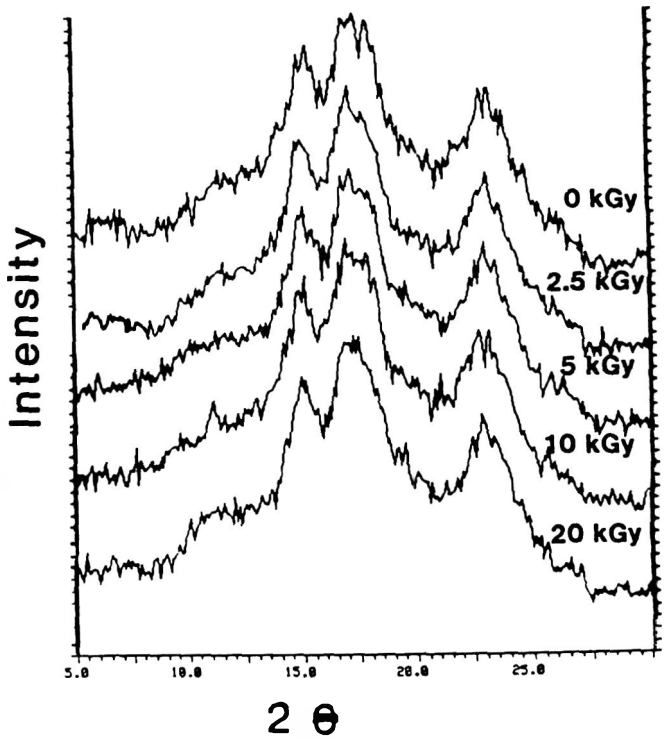

Fig. 5—X-ray diffraction patterns of Great Northern bean starch. Samples were pressed into pellets and analyzed with a Diano diffraction generator, equipped with a copper tube. Samples were stored 1 mo at 25°C after irradiation.

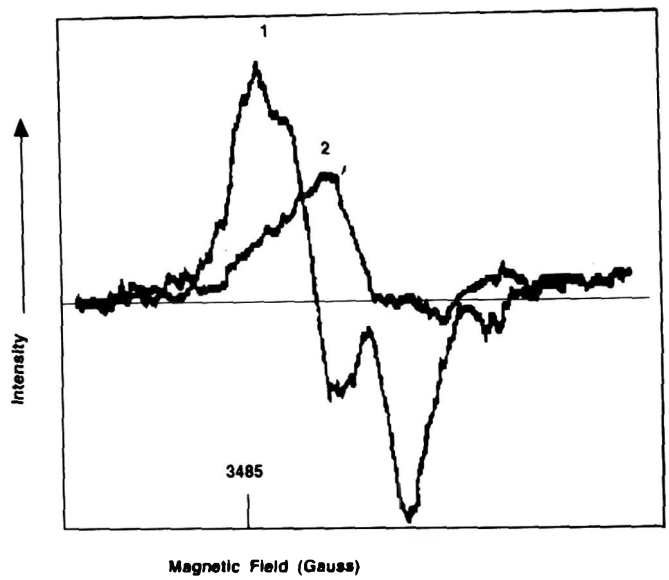

Fig. 6—Election spin resonance spectra of gamma-irradiated Great Northern bean starch. Samples exposed to 20-kGy dose and stored at −40°C for (1) 9 days, Gain 5 × 10^5; and (2) 11 months, Gain 10 × 10^5.

and amylopectin might have been partly degraded to smaller molecules (Rayas-Duarte and Rupnow, 1993), the crystallinity pattern was essentially not altered. Ciesla et al. (1991) reported a significant decrease in intensity of X-ray reflection, using small-angle X-ray scattering, when water suspensions of irradiated (20 to 30 kGy) potato starches were tested. This has been interpreted to indicate the loss of order between crystalline and amorphous regions (Ciesla et al., 1991).

A strong ESR signal was recorded from the sample stored at −40°C for 9 days after irradiation (Fig. 6-1), which weakened after 11 mo storage (Fig. 6-2). Raffi et al. (1981e) reported that haricot bean starch showed a stronger and distinctive "initial" (after 1 day storage) ESR spectra, but a different spectra was obtained after 95 days storage. A final first order rate spectrum appeared to be common to the 8 starches studied, and some observed differences were thought to reflect distinctive initial kinetics.

ESR spectra profiles similar to those we found were reported for French bean and corn starch, maltodextrin, and maltotriose (Raffi et al., 1981e; 1985). Our data suggest that after 11 mo storage, a small portion of radicals with long life were still present in the irradiated bean starch (Fig. 6). However, after hydration and freeze-drying, the bean starch samples showed no ESR spectra, suggesting stabilization of the previous radicals. Raffi et al. (1981e) and Raffi and Agnel (1983) reported similar observations of radical stabilization when eight starch varieties were suspended in water.

CONCLUSIONS

THE CHANGES INDUCED in Great Northern bean starch, irradiated at doses of 2.5–20 kGy, were related to irradiation dose. The swelling power of the bean starches was strongly affected, even at 2.5 kGy. Notable increases in acidity were observed at doses >5 kGy, while increases in water and ethanol-soluble compounds were observed at 20 kGy. The changes induced by irradiation doses did not modify the X-ray diffraction pattern. The spectra of any remaining radicals were lost after adding water and freeze-drying. This suggests no radicals would remain after processing irradiated bean starch before consumption. If irradiation doses >5 kGy would be applied to bean starch, appreciable changes in structure would not be

apparent, but changes in functional properties would be expected. Such properties could be important when starch is the main component of a food ingredient.

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Optimizing the Enzymatic Maceration of Foliole Purée from Hard Pieces of Hearts of Palm (*Euterpe edulis Mart.*) using Response Surface Analysis

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ABSTRACT

Response surface analysis was applied to investigate modifications in viscosity of foliole purée treated with commercial enzyme preparations high in cellulase and endopolygalacturonase (pectinase) with changes in enzyme concentration (0.3–1.2%), incubation time (3.3–6.7 hr) and temperature (40–50°C). Foliole purée was obtained by trituration of hard pieces of hearts of palm (*Euterpe edulis*) and incubated in a rotating agitator. After treatment with 0.81% cellulase for ≈5 hr at 50°C, a fourfold reduction in viscosity was found relative to a control sample. The minimum viscosity for this treatment was within the experimental range investigated. Optimized experimental conditions for treatment with pectinase however were outside the experimental range. The application of the cellulase preparation to the purée resulted in a 10% increase in yield of edible palm.

Key Words: palm hearts, enzyme, softening, texture, core puree, RSM

INTRODUCTION

IN THE INDUSTRIAL USE of the palm tree *Euterpe edulis* Mart., (also known as juçara) the average yield of hearts per tree is 450g (Ferreira et al., 1976, 1981/82b) with a maximum of 1000g (Nogueira, 1979). Thus 1.2–1.5 juçara palm trees are normally necessary to produce a 1 kg of acidified hearts of palm (Ferreira et al., 1981/82b). This yield is very low, considering that the juçara palm tree takes from 6–8 yr to mature, and is eliminated by extraction of hearts of palm. Juçara palm forests are diminishing in the central-south region of Brazil and other tropical regions due to indiscriminate harvesting by industrial processors without corresponding reforestation (Leão and Cardoso, 1974).

To increase yield, some industries include excessively fibrous pieces in the canned product (Hale et al., 1978). This practice is fraudulent, since the texture of such pieces can be perceived by touch or sight, during cutting of the hearts for canning (Ferreira et al., 1976). However, Quast and Bernhardt (1978) attribute the inclusion of fibrous pieces in the canned products to result from lack of qualified personnel. Some producers believe that prolonged cooking can make such pieces edible. However, cooking causes excessive softening of those parts of the raw material considered normal, with accompanying losses in textural characteristics (Quast and Bernhardt, 1978).

Technological information for production of industrial canned hearts of palm is lacking. Several reports (Martin et al., 1969/70; Ferreira et al., 1976; Bernhardt et al., 1978, Paschoalino and Bernhardt, 1978; Paschoalino, 1978) have considered the subject but few research endeavors have dealt with problems involving texture of hearts of palm. No work could be found focusing on the improvement of fibrous parts of the palm tree, considered inedible.

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The texture of the edible portion of the palm *E. edulis* varies considerably along different cross sections of its length (Ferreira et al., 1976; Campos et al., 1978; Ferreira et al., 1981/82a; Paschoalino et al., 1989). This variation is accentuated in the top region near the leaves, where the tissue is differentiated into two principal structures: rachis-precursor of the petiole, and foliole-precursor of the leaf blade. As determined by Kitagawa (1991) the fibrous pieces near the apical region have a texture with a high coefficient of variation (C.V. = standard deviation × 100/average) as compared to the value for cuts from different trees (C.V. = 21.75%) or the longitudinal symmetric sides of the same cut from a given tree (C.V. = 33.42%). This high variation is due to the natural heterogeneity of the palm tree apex.

Hardness of hearts of palm depends on the quantities of cellulose, hemicellulose and lignin (Kitagawa, 1991). Thus possibly their softening may be achieved by employing enzymes which act selectively on those substrates. It may be possible to utilize the semi-rigid parts of hearts of palm and consequently increase the yield of edible palm. However, no conclusive results were found on the effects of enzymic treatment on the softening of whole semi-rigid tissues of hearts of palm (Kitagawa, 1991). An increase in palm yield might nevertheless be achieved by enzymic maceration of comminuted tissues, to produce an edible purée. According to Bernhardt (1993), a potential market exists for sterilized or dried purée, which could be added to instant soup mixtures. Our objective was to explore this possibility, using response surface methodology (RSM) to optimize the lowering of purée viscosity. The effect of enzyme treatment (concentration, time and temperature) on viscosity of foliole purée, was compared to purée obtained from edible hearts of palm.

MATERIAL & METHODS

Raw material

The first semi-rigid portions (cuts), removed from the apical parts of juçara palm (*E. edulis*), and measuring 16 cm in length, were selected from 80 samples obtained from Eldorado (coastal region of São Paulo state). On average, this was about 50 cm from the apical meristem. The criterion for fibrous stalk selection was based on force required by the harvester to effect the cut. When the texture presented resistance to knife penetration, the piece was classified as fibrous stalk. Such stalks were submitted to blanching in tap water, following the method of Paschoalino and Ferreira (1985), with 25 min of additional cooking. Storage was in a cold chamber, with air circulation at 4°C, for a period not exceeding 12 days before processing. After removal of external layers and rachis of the fibrous stalks, the weight of foliole relative to that of edible material was determined. Purée was prepared by trituration of folioles in a domestic multi-processor, at room temperature (≈25°C) until a homogeneous paste was obtained.

Texture and fiber in foliole from fibrous stalks

Texture and fiber quantity determinations on foliole were determined in triplicate on blanched samples. Texture was determined using the Texture Test Systems texturometer (Food Technology Co., Rockville, MD) and method modified by Campos and Pedrassi (1988). Foliole samples, measuring 2 cm in length, were cut with the aid of the cell

Table 1—Enzyme concentrations incubation times and temperatures

	Code[a]	levels		
		−1	0	+
Enzyme concentration, [E] (%)	X_1	0.50	0.75	1.00
Incubation time, t(h)	X_2	4.0	5.0	6.0
Incubation temperature, T(°C)	X_3	40	45	50

[a] $X_1 = ([E] - 0.75)/0.25$; $X_2 = t - 5.0$; $X_3 = (T - 45)/5$

Table 2—Experimental conditions and viscosity values for the central composite design

	Experimental factor values						Viscosity (cP)	
	Real[a]			Codified				
Exp.	[E]	t	T	X_1	X_2	X_3	Y_1[b]	Y_2[b]
1	0.5	4	40	−1	−1	−1	160.0	131.8
2	1.0	4	40	1	−1	−1	91.5	120.0
3	0.5	6	40	−1	1	−1	66.6	110.6
4	1.0	6	40	1	1	−1	55.0	75.0
5	0.5	4	50	−1	−1	1	77.3	130.8
6	1.0	4	50	1	−1	1	85.6	141.6
7	0.5	6	50	−1	1	1	101.3	184.0
8	1.0	6	50	1	1	1	80.0	115.0
9	0.3	5	45	$-\sqrt{3}$	0	0	136.6	103.3
10	1.2	5	45	$\sqrt{3}$	0	0	82.3	55.0
11	0.75	3.26	45	0	$-\sqrt{3}$	0	121.6	105.0
12	0.75	6.73	45	0	$\sqrt{3}$	0	93.3	71.7
13	0.75	5	40	0	0	−1	68.3	128.3
14	0.75	5	50	0	0	1	73.3	117.7
15	0.75	5	45	0	0	0	69.9	90.0
16	0.75	5	45	0	0	0	63.3	95.0
17	0.75	5	45	0	0	0	75.0	89.2
18	0.75	5	45	0	0	0	65.0	80.0
19	0.75	5	45	0	0	0	67.3	90.8

[a] [E] in %, t in hours and T in °C.
[b] y_1 = treatments with cellulase, y_2 = treatments with pectinase.

of a single blade (CA-1). Texture was expressed as the maximum shear force required in kg-force (kgf). It was not necessary to weigh samples. The same methodology was used to cut fibrous stalks, substituting the single blade cell with the standard shear and compression cell (CS-1). For fibrous stalks, texture was determined dividing the maximum shear force by the weight of the samples and expressing results in kg-force per gram (kgf/g). Dry weight was determined by drying in a vacuum oven ($P \leq 25$ mmHg) at 70°C until the sample reached constant weight. The dry material was ground and sieved and contents of acidic detergent fibers (ADF), neutral detergent fibers (NDF) and lignin were determined using the methods of Goldin et al. (1985), Van Soest (1963) and Van Soest and Wine (1968). Hemicellulose was obtained by the difference between percentages of ADF and NDF (Ferreira, 1987) and pectin by the gravimetric method of Carré and Haynes (1922).

Enzymatic treatment

The enzyme preparations Celluclast 1.5 L® and Pectinex Ultra SP®, both derived from *Aspergillus niger*, were donated by Novo Industri (A/S Bagsvaerd, Denmark). The mixture, consisting of 100g of foliole purée (wet weight), 200 mL of 0.05M citrate buffer solution at pH 4.8 and the enzyme preparation, was introduced into a 500 mL conical flask and incubated in a rotating agitator (New Brunswick, NJ) with agitation and temperature control. The values or levels of enzyme concentration, [E], incubation times, t, and temperatures, T were recorded (Table 1) along with their coded or scaled values. The reaction was interrupted by heating in a boiling water bath for 5 min. After cooling to room temperature (25°C) all material was transferred to beakers (600 mL), and 100 mL of distilled water added. The viscosity (in cP) was determined using a model Br RVT Brookfield visocosimeter (Stoughton, MA) at 20°C with #1 and #2 spindles revolving at 10 rpm.

Statistical design

A 2^3 factorial design, (Fig. 1) using the three experimental factors of Table 1, was used initially. To measure possible nonlinearity in viscosity values as a function of these three factors, six axial points were added to the factorial design to form a central composite design (Box et al., 1978). The axial points representing changes in enzyme concentration and incubation time, relative to values of the central point, were a codified distance of $\sqrt{3}$ from the center of the design. Results at temperatures outside the 40–50°C range were not of interest.

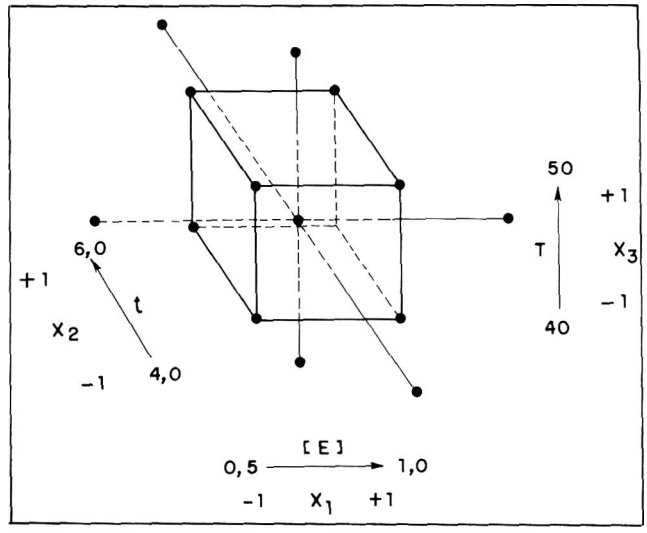

Fig. 1—Central composite design used. X1 X2 and X3 represent codified values of enzyme concentration, [E], incubation time, t, and temperature, T. Investigation at temperatures outside the 40-50°C range was not of interest so design is not completely symmetrical.

Thus, the axial points along the X_3 direction were a codified unitary distance from the central point. To estimate experimental error, 5 replicate experiments were performed at factor values corresponding to the central point. The complete experimental design with real and codified factor values is presented (Table 2). The first 8 lines in the tables are values for the 2^3 factorial experiments, lines 9 through 14 describe the experiments at the axial points and the five replicate experiments at the central point are given in lines 15 to 19.

Response surface method

The enzymatic maceration could be optimized if the viscosity response were known as a function of the experimental factors. A graph of this function forms the response surface for viscosity. This surface, actually a hypersurface since its representation is four-dimensional, is obtained by performing multiple linear regressions of the viscosity values on the values of the experimental factors. The response surface can be represented as

$$y = b_0 - \sum_{i=1}^{3} b_i x_i + \sum_{i=1}^{3} b_{ii} X_i^2 + \sum_{i=1}^{3} \sum_{\substack{j=1 \\ i<j}}^{3} b_{ij}\, x_i x_j + \varepsilon \qquad (1)$$

here y represents the viscosity values in cP units, and X_1, X_2 and X_3 are the codified transformations of [E], t and T. The difference between the values of the viscosity predicted by the model, y and the measured values, y is given by the residuals, i.e.

$$y = \hat{y} + \varepsilon \qquad (2)$$

The data (Table 2) were sufficient to determine all modeling parameters, b_o, b_i, b_{ii}, b_{ij}, for i,j = 1, 2 and 3, $i \neq j$. If a quadratic approximation were sufficient to represent the viscosity data accurately, the residuals should be of the same magnitude as experimental error.

The first eight experiments (Table 2) were sufficient to determine the linear model which included the first two terms in Eq. (1), as well as the cross terms, given by the double summation term in the equation. The experimental design accounted for the possibility that this simplified model, might represent the viscosity data. Statistical F tests were performed to determine significances of the different models. Also, experimental error was propagated to obtain an estimation of errors in each of the b coefficients in Eq. (1). Finally, two responses were of interest in this study, the viscosities of mixtures containing foliole purée of treatments corresponding to the cellulase, and pectinase treatments. Complete statistical analyses were performed separately for each type of treatment.

Table 3—Texture and fiber in the foliole and intermediate portion of stalk[a]

	Fibrous stalk[b]	Foliole[b]
NDC (% d.b.)[c]	37.3 ± 0.7	32.7 ± 1.3
ADF (% d.b.)	28.0 ± 2.0	22.7 ± 1.3
Cellulose (% d.b.)	16*	17.3 ± 1.2
Hemicellulose (% d.b.)	9.0 ± 1.0	10.0 ± 0.0
Lignin (% d.b.)	8.0*	5.0 ± 0.0
Pectin (% d.b.)	3.1*	2.3 ± 0.3
Water (% d.b.)	90.0 ± 0.9	88.4 ± 0.5
Texture (kgf/g)[d]	8.45 ± 1.2	101.0 ± 10.5**

[a] See Kitagawa (1991).
[b] Average of three determinations plus standard deviation, except where marked with (*), which indicates only one determination.
[c] Percent on dry weight basis (% d.b.).
[d] Texture in kg-force per g, except where market with (**), which was determined in kg-force.

Table 4—Least square estimates of model parameters, b, and standard errors for the cellulase and pectinase treatments

Parameters	Estimates ± standard error[a] Cellulase	Pectinase
b_0	69.2 ± 1.9	87.7 ± 2.3
b_1	−13.4 ± 1.2	−12.8 ± 1.5
b_2	−11.5 ± 1.4	−7.7 ± 1.5
b_3	−2.4 ± 1.4	12.7 ± 1.7
b_{11}	12.9 ± 1.2	−2.3 ± 1.4
b_{22}	12.2 ± 1.2	0.8 ± 1.4
b_{33}	−3.4 ± 2.1	37.4 ± 2.5
b_{12}	3.4 ± 1.6	−11.7 ± 1.9
b_{13}	8.4 ± 1.6	−2.6 ± 1.9
b_{23}	18.5 ± 1.6	12.9 ± 1.9

[a] The standard error values should be multiplied by 2.78 if 95% confidence intervals are desired.

RESULTS

Texture and fiber contents in foliole of fibrous stalks

The foliole corresponded to 1/3 of the whole fibrous stalk, weighing about 270g on average. For the 80 samples analyzed, the average value and standard deviation was 94.97 ± 21.03g for the foliole and 909.53 ± 55.07g for the edible part, a ratio of 0.10. Thus, on average, the yield of edible material of the hearts of palm, could possibly be increased by about 10%.

The percentage distribution of the fiber in the foliole, showed that cellulose was the principal component, followed by hemicellulose and lignin (Table 3). Cellulose and hemicellulose in the foliole, were high and similar to those encountered in whole fibrous stalk, indicating concentrations of these components were similar in the rachis. However, lignin levels were slightly higher in the whole stalks, indicating that component was concentrated in rachis. The percentages of ADF and NDF in the foliole, were very close to those reported by Ferreira (1987) for the apical portion of bamboo shoots, used for human consumption.

With respect to texture, it was not possible to determine a reference diameter, due to the irregular geometric form. The shearing force required, depends on the diameter of the cross section of the hearts of palm. Thus, Codex Alimentarius, defines diameter intervals for industrialized canned hearts of palm, small and medium diameters ($15 < D < 25$ mm) giving texture values of about 74.9 kgf, and large diameter samples ($35 < D < 50$ mm), with values of about 113.5 kgf (FAO/OMS, 1989). The foliole gave an average value of about 90.8 kgf, *permitting its classification as an edible product;* however a measure of a reference diameter was not possible.

Response surface

Table 2 includes viscosity values for enzymatic treatments with cellulase and pectinase, for different sets of experimental conditions of the central composite design. The estimates in codified units, of the model parameters, b, of regression Eq. (1) and standard errors were determined (Table 4). At the 95% confidence level, all model parameters for the cellulase treatment, except b_3 and b_{12}, were significant ($P \leq 0.05$). For the pectinase results only b_{11}, b_{22} and b_{13} were not significant. These results, showed that the linear model was not adequate for describing these viscosity variations.

Analysis of variance calculations were performed for the regression results (Table 4). The calculated ratios of the regression mean sum of square to the residual mean sum of squares were 9.00 and 7.26 for the cellulase and pectinase models, respectively. Both values were much larger than the 95% confidence level $F_{9,9}$ value of 3.18, indicating significant regression equations. The statistical F test, established a clear indication of the quadratic model, rather than the linear one.

For the experimental region contained within the limits $0.3 \leq [E] \leq 1.2$, $3.3 \leq t \leq 6.7$ and $40 \leq T \leq 50$ the viscosity, y_1, of foliole purée of the hard ends of hearts of palm *E. edulis* after treatment with cellulase could be estimated using the equations

$$y_1 = 1444 - 734[E] - 310t - 11.8T + 206[E]^2 + 12.2t^2 - 0.136T^2 + 13.6[E]t + 3.70tT + 6.72[E]T \quad (2)$$

where [E], t and T are the cellulase concentration and incubation time and temperature, respectively, in units of %, hours and °C and viscosity has units of cP. For the pectinase treatment and the same experimental region and units, the viscosity, y_2, is given by

$$y_2 = 3411.1 + 330.9[E] - 96.2t - 143.4T - 36.3[E]^2 + 0.795t^2 + 1.496T^2 - 46.8[E]t + 2.57tT + -2.08\,[E]T \quad (3)$$

Cellulase treatment. To facilitate visualization of the response surface, cross sections at constant temperature values were examined (Fig. 2). Contour lines representing constant viscosity values as a function of enzyme concentration [E], and incubation time t, were developed for constant temperature values of 40°, 45° and 50°C. As temperature decreased, minimum viscosity values were found at higher enzyme concentrations and longer incubation times. The concentration and incubation times corresponding to minimum predicted viscosity values at 40°, 45° and 50°C were recorded (Table 5).

To confirm predicted values of viscosity for the cellulase treatment, a verification experiment was performed using a 0.81% cellulase enzyme concentration. 5 hr and 5 min incubation time and 50°C incubation temperature, conditions near to optimum. A 65 cP viscosity, in excellent agreement with the predicted values of 61 cP (Table 5) was found. This was a fourfold reduction in the viscosity value of the sample without enzyme treatment, which was 260 cP. Thus, the viscosity value in the foliole purée after enzyme treatment (65 cP), was in the same range as that obtained in the laboratory with edible hearts of palm (68 cP).

The linear terms in the model Eq. (1) (Table 4), had significant negative b coefficients for cellulase concentration (−13.4) and incubation time (−11.5), indicating that increases in those factors resulted in contributions that lowered viscosity values of the enzyme treated purée. However the quadratic terms with positive coefficients, had an opposite effect and were responsible for the well defined minimum. The significant positive b_{13} (8.4) and b_{23} (18.3) coefficients of the cross terms of incubation temperature with other experimental factors, were consistent with the shift of minimum viscosity values (Table 5) to higher enzyme concentrations and incubation times as incubation temperature was lowered.

The minimization of viscosity of foliole purée treated with cellulase could be attributed to high activities of the cellulase on filter paper (150 IU), determined in the laboratory by the method of Mandels (1976), and xylanase (379 IU), determined as described by Menezes et al. (1976). These were present in the enzymatic preparations, permitting the hydrolysis of substrates present in high concentrations such as cellulose (17%

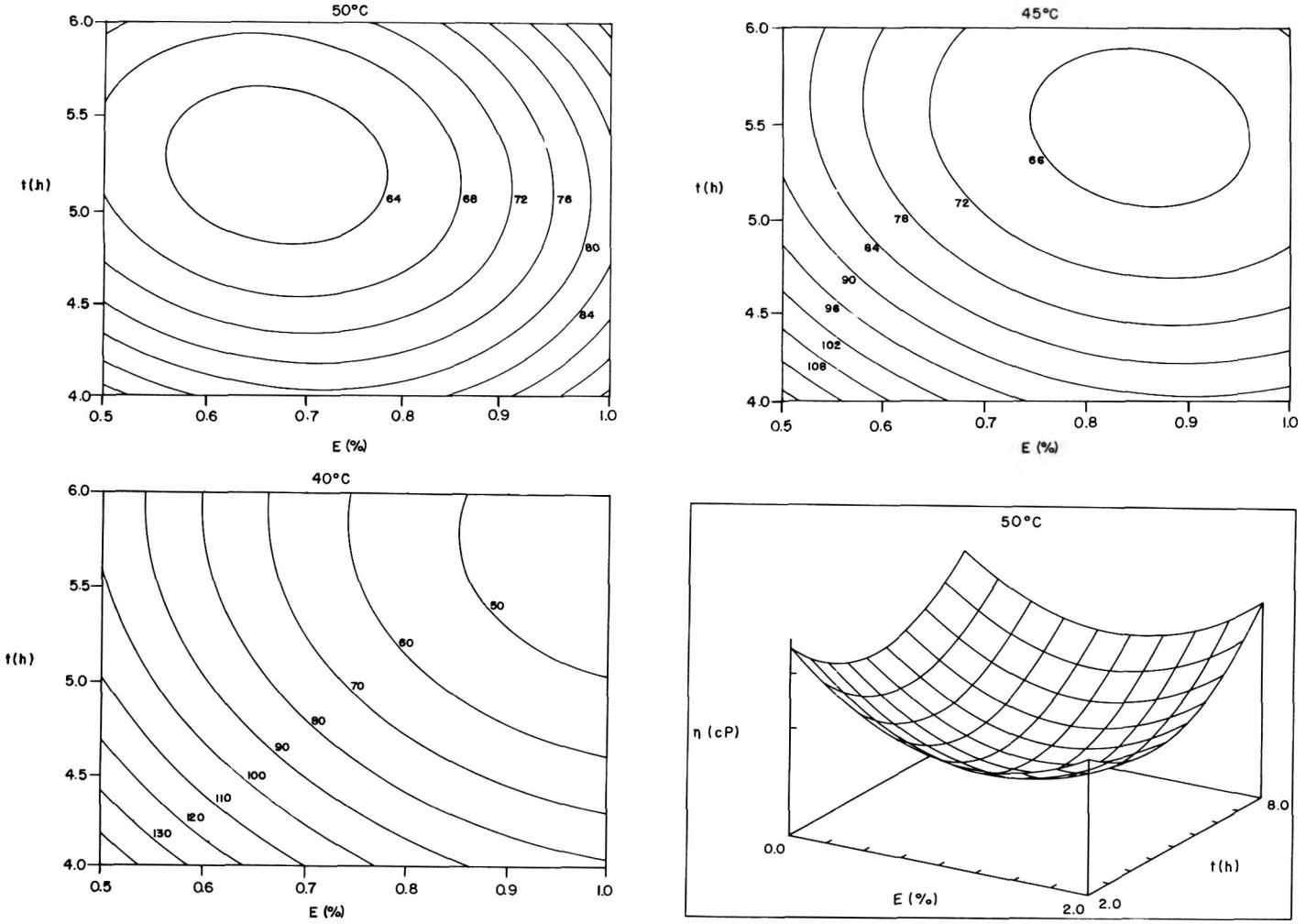

Fig. 2—Constant temperature cross sections of contour lines of response surface for cellulase treatment: (a) 50°C; (b) 45°C; and (c) 40°C. Numbers on contour lines indicate constant viscosity values; (d) constant temperature cross section of response surface for cellulase treatment at 50°C.

d.b.) and hemicellulose (10% d.b.). The relatively low value of lignin (5% d.b.) also provided conditions for minimization of viscosity of foliole purée treated with cellulase.

Pectinase

For treatment with pectinase, the viscosity showed a dependence on experimental factors, which could be represented by a response surface in the form of a saddle point (Box et al., 1978) (Fig. 3). The contour lines of the response surface for the three temperatures showed that increasing enzyme concentration and incubation time resulted in lower viscosity values. This tendency was especially pronounced at lower temperatures. However, the minimum values fall outside the experimental region we investigated.

The linear terms of Eq. (1), had negative concentration (−12.8) and time (−7.7) coefficients (See Table 4), indicating that increases in these values resulted in lower viscosity values. However, the large positive linear temperature coefficient (12.7), contributed to lower viscosity values as temperature decreased. On the other hand, the positive squared temperature coefficient (37.4), provided an opposite effect on viscosity for temperature decreases. Finally, significant negative and positive cross terms for the interaction of incubation time with enzyme concentration (−11.7) and incubation temperature (12.9), corresponded to decreases in viscosity when those factors were simultaneously increased or decreased.

Note that the values of b coefficients are scale dependent. As such, coefficient values for codified factor values (Table 4) could have different signs than these in expressions using variable values with real units (Eq. 2 and 3). This occurred for the coefficient values for the pectinase treatment. Linear and quadratic models in codified units are useful in understanding the forms of contour lines (Fig. 2 and 3). However they are not real effects. Model predictions for viscosity values are sums of all term contributions. Such sums are invariant to the units used for factor values and could be compared with experimental values in Table 2.

Minimum values in viscosity might be encountered for enzyme concentrations >1.2% and incubation times >6.7 hr, about the limits we investigated. This enzymatic preparation had a high activity of endopolygalacturonase (2081 IU), determined in the laboratory by the method described by Thibault and Mercier (1978), but almost no cellulase activity on filter paper (8 IU) or xylanase activity (56 IU). This may explain the need to increase enzyme concentration so that the rate of hydrolysis of the fibers present in the folioles reached the same levels observed for the treatment with cellulase.

CONCLUSIONS

MANIPULATING EXPERIMENTAL FACTORS resulted in a fourfold reduction in viscosity of foliole purée after treatment with cellulase. Conditions for this treatment were 0.81% cellulase and 5 hr and 5 min incubation time, at 50°C. Also, the response surface, indicated the existence of a minimum viscosity of 44 cP under the optimized conditions [E] = 0.92%, t = 6 hr and 5 min and T = 40°C. However, the minimum viscosity for

Table 5—Optimized enzyme concentrations and incubation times for 40, 45 and 50°C and predicted viscosity values for treatment with cellulase

Enzyme conc (%)	Incubation time	Incubation temp (°C)	Predicted viscosity (cP)
0.92	6hr 8min	40	43
0.87	5hr 25min	45	63
0.81	5hr 5min	50	61

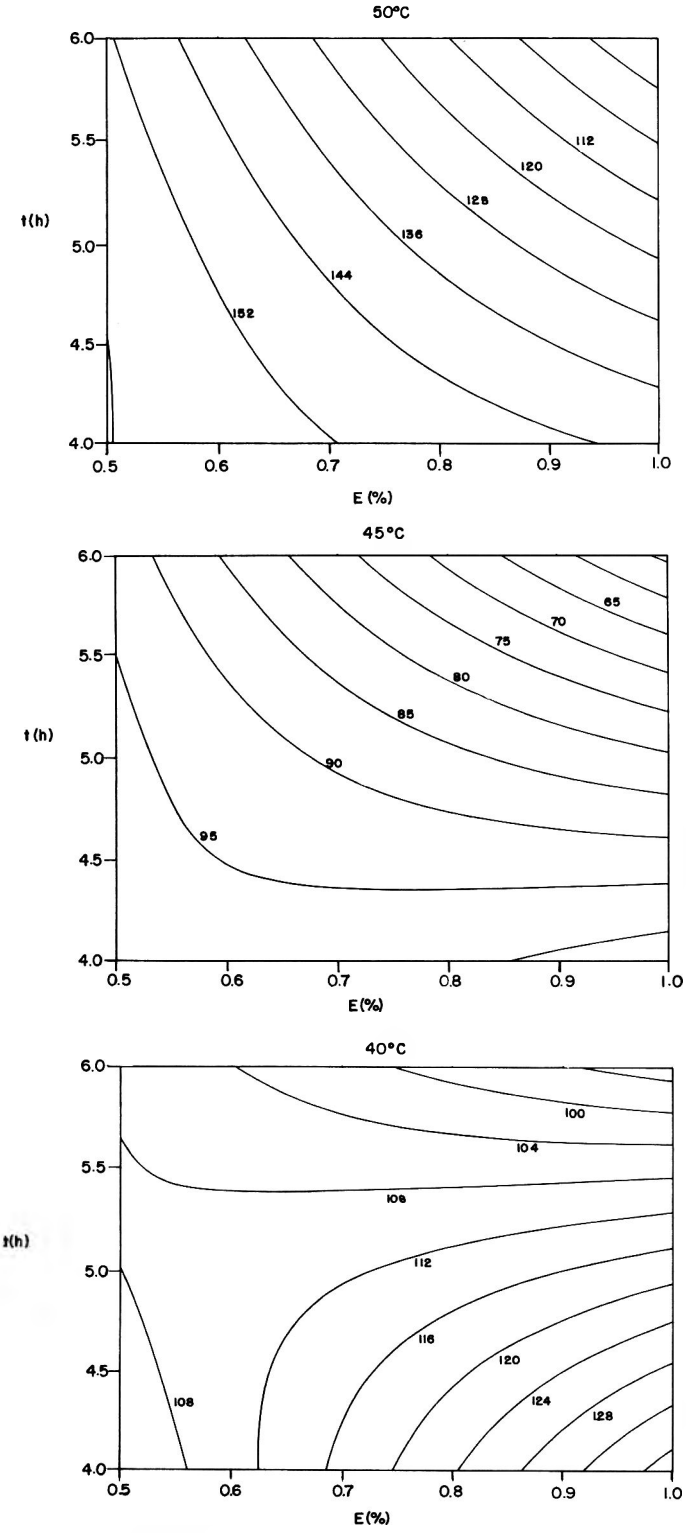

Fig. 3.—Constant temperature cross section of response surface for pectinase treatment: (a) 50°C; (b) 45°C; and (c) 40°C. Numbers accompanying contour lines indicate constant viscosity values.

this purée after treatment with pectinase, was outside the experimental region. Investigations are needed in adjoining experimental regions by simultaneously increasing enzyme concentrations and incubation times. Increases in both incubation time and temperature may also be helpful but should not use temperatures outside the 40–50°C range. Use of foliole purée from fibrous stalks (16 cm in length) resulted in a 10% increase in yield of juçara palm after treatment with cellulase.

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Shelf Life Evaluation of Cling Peaches in Retort Pouches

R.A. KLUTER, D.T. NATTRESS, C.P. DUNNE, and R.D. POPPER

ABSTRACT

Retort pouch sliced peaches in syrup were developed to replace freeze-dried peaches in a military field ration. Two processing variables were investigated: fruit source (fresh and frozen) and syrup pH (3.85 and 3.25). Peaches were stored at 4°, 21° and 38° C and evaluated periodically by sensory panels and biochemical and instrumental analyses. pH had most effect on sensory color, texture and acceptability and instrumental color and sugar composition. High positive correlations existed between a factor (consisting of five sensory quality attributes) and sensory color and texture, Hunter L and a values and sucrose level. Frozen and fresh source peaches at pH 3.85 met shelflife requirements at 21 and 38°.

Key Words: peaches, retort pouches, shelf life, flavor, texture

INTRODUCTION

FOUR PROTOTYPE RETORT POUCH wet pack fruits and freeze-dried counterparts had been evaluated in a prolonged use field ration study with Army troops (Popper et al., 1987). Freeze-dried fruits were a component of the existing Meal, Ready-to-Eat ration menu; wet pack fruits were included as a component of a revised menu. Higher troop acceptance and consumption rates were recorded for prototype wetpack fruit items than for freeze-dried counterparts. Further investigation and refinement of the process for use of the flexible pouch, as well as storage stability evaluations, were needed. Procedures for processing canned cling peaches are well established (Woodroof and Luh, 1986). There are few recent reports on effects of processing variables on sensory and consumer preferences for canned peaches and none on peaches in flexible pouches.

Leonard et al. (1953) investigated the relationship of raw fruit physical and chemical measures of maturity to sensory flavor differences and consumer preferences for canned peaches. Optimal maturity level correlated with optimal sensory flavor, and fresh fruit soluble solids:acid ratio was a potential predictor of canned product flavor. Consumer preferences for sweetness of canned cling peaches were further elucidated by Simone et al. (1956) in a two season/two variety/five brix study. California consumers preferred peaches with a cut-out brix of 24–26° and a brix/acid ratio of 73.7 to 85.4. Joslyn et al. (1957) investigated the effects of sucrose-corn syrup blends on flavor and syrup calcium addition on texture of canned cling peach halves. Up to 25% corn syrup solids replacement in a 40° brix sucrose syrup had minimal effect on flavor. At higher replacement levels, decreases in sensory "fruitiness" were reported. Penetrometer readings maximized at syrup calcium levels of 50 ppm. Pangborn et al. (1958) compared preferences for canned cling peaches based on household consumer panels in California and Midwestern states. Three samples ranged in brix levels from 24.2 to 27.6°. The sample at the higher brix had been acidified with 0.15% additional citric acid to a 0.4 total acidity level, reducing the brix/acid ratio to 67.7 (pH 3.8) compared to 86.9 (pH 4.1) and 97.8 (pH 4.1). Household panels in both geographic areas significantly preferred the acidified samples. Subsequent mass consumer surveys in California, however, indicated equal liking for the three.

Cling peach cultivar and maturity investigations by Kader et al. (1982) confirmed relationships between 8 chemical analyses, 2 physical measures and 6 sensory parameters. For example, in fresh vs canned fruit, titratable acidity and sensory sourness and a firmness tester and sensory firmness significantly correlated. In canned products, a significant correlation occurred between sensory sweetness and peach flavor intensity. The study generally confirmed the positive effects on canned product color and flavor quality when more mature fresh fruit were used.

Critical parameters for processing cling peaches in retort pouches were identified through consultations with other researchers and fruit processors with retort pouch packing capability in California and Oregon. Caution was recommended regarding individual quick frozen (IQF) fruit as a source material because enzyme activity during slow thawing would be maximal prior to further processing and packaging. Otherwise producing an acceptable product using frozen peaches appeared feasible. The same harvest fruit should be used for both frozen and fresh source packs. Control of syrup pH 3.5 ≤ 4.0 or acidification to pH 3.5 were also recommended as possible means of improving color and flavor stability. Nattress et al. (1990) reported that when pH of retort pouch peaches in syrup was adjusted by sodium citrate and/or a citrate/citric acid buffer to 4.0 ± 0.15, quality, acceptability and storage stability were excellent.

Our objectives were to investigate effects of pH and fruit source on quality and acceptability of cling peaches packed in retort pouches and to determine the effects of selected variables on shelflife. The military shelflife requirement was that shelf stable products be acceptable after 24 mo at 21°C and 6 mo at 38°C.

MATERIALS & METHODS

PEACHES WERE PROCESSED in August 1987 by J.R. Wood, Inc. (Sanger, CA) during the cling peach season. They had both an IQF line and a retort pouch line. Fruit source and syrup pH were the two main variables. The two sources of peaches were fresh harvest and frozen using IQF slices. Both sources were processed from the same variety and harvest. Two pH levels were investigated. The higher value was the approximate unadjusted pH of the ripened fruit before processing. Experience has indicated that pH of the processed fruit in syrup is slightly lower. The lower pH value was produced by addition of citric acid without buffer to the syrup. Over all storage temperatures and withdrawals, the measured average pH levels of fruit/syrup homogenates after the equilibration were as follows: Target pH 4.0; frozen product actual pH 3.77 (3.67–3.85) and fresh product actual pH 3.93 (3.77–4.04). For target pH 3.5; frozen product actual pH was 3.27 (3.20–3.30) and fresh product 3.19 (3.10–3.34). Products with target pH 4.0 are designated as "high" and those of target pH 3.5 as "low."

Processing procedures

The processing sequence for both fresh and frozen peaches was identical through slicing. Fruit was conditioned in a ripening room to a penetrometer reading of 1.3 to 2.6 Kg, using a McCormick fruit pressure tester, Model FT011 with a 0.8 cm diameter plunger. Fruit was then washed, pitted, lye peeled, rinsed and sliced. Slices for the

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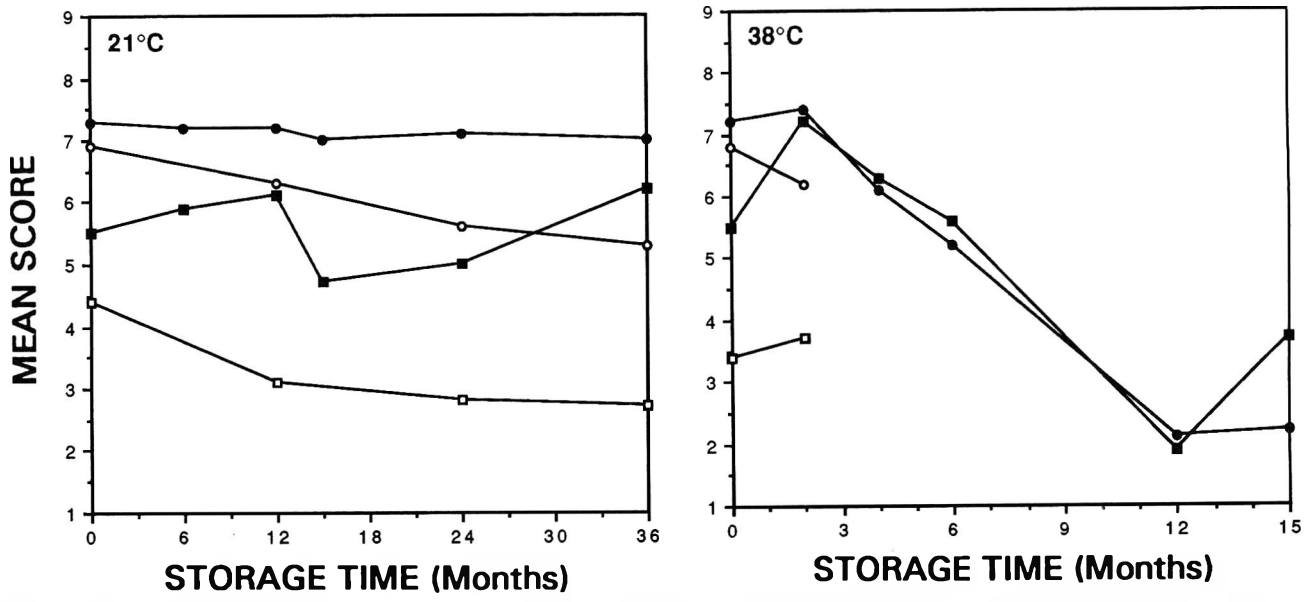

Fig. 1—Relationship of storage time and temperature to sensory color quality of peach slices. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

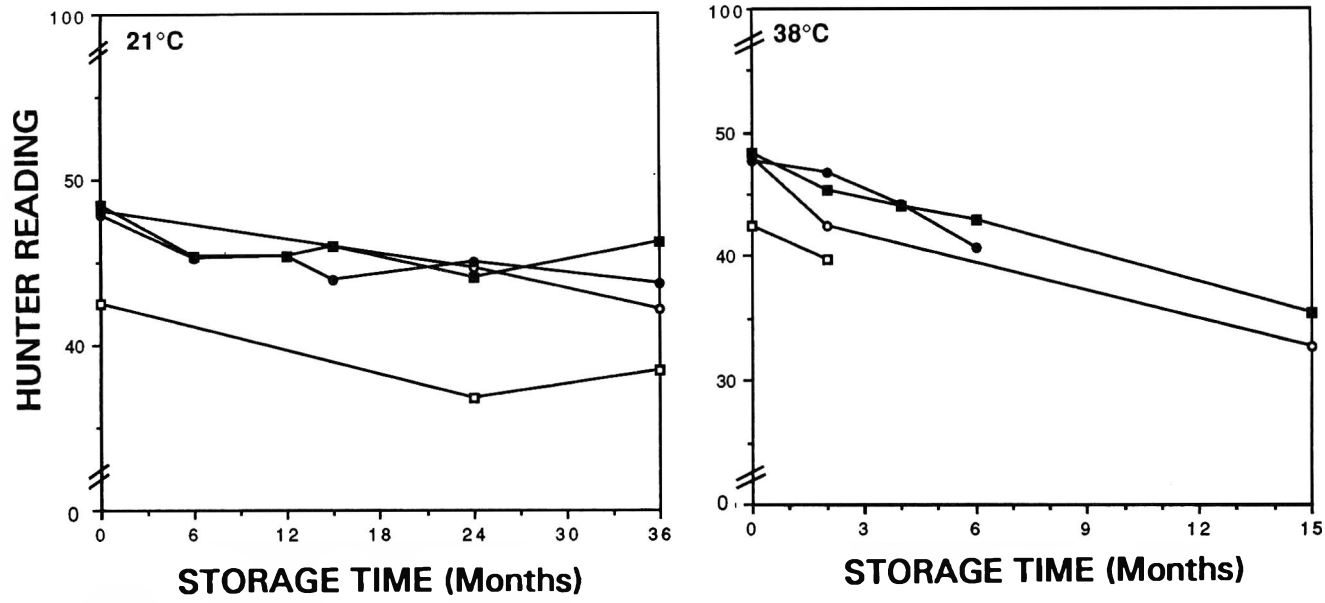

Fig. 2—Relationship of storage time and temperature to Hunter L values of fruit/syrup homogenates. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

IQF line were steam blanched and dipped into an ascorbate solution at ≈10 × the desired end residual, to inhibit browning. Targeted final product level was 200–800 ppm (Anonymous, 1992). The dipped slices were then drained and conveyed to a −29°C blast freezer tunnel, frozen, filled into pails and stored until needed. Prior to further processing, they were partially thawed and rinsed with water to reduce ascorbate residue to the target range. Fresh source slices were not blanched, but were ascorbate dipped and drained before filling and syruping.

Slices from both sources were filled to maximum volume into previously formed retort pouches. Sufficient 60° brix (heavy) sucrose syrup was added to each pouch to achieve a cutout brix after equilibration of 18–22°. This high brix sucrose syrup was used to minimize osmotic shock to peach cellular structures during processing. For low pH packs, the syrup was acidified by adding 0.5% citric acid (anhydrous) without buffer. Pouches were then vacuum sealed.

Pouches were processed for 3–4 min in a still retort to 88°C internal temperature and cooled to <38°C with water sprays. Only peaches packed under these processing conditions were evaluated in the storage study. Two other processing conditions had been evaluated in preliminary trials to represent possible extremes of commercial practice: (1) a "minimal" process to reach internal temperature 76.7°C (1 min hold); and (2) a high temperature process to reach internal temperature 96.1°C (3–4 min hold).

Process adequacy was verified by in-plant enzyme activity tests. Peroxidase and polyphenol oxidase were analyzed by continuous kinetic spectrophotometric assays. Peroxidase was analyzed using a modification of the method of Tate et al. (1964). Polyphenol oxidase enzymes were analyzed using a modification of the method by Gorin and Heidema, (1976). Tests indicated the minimal process inactivated both enzymes.

Target minimum drained weight (Anonymous, 1992) was 100g determined at several storage times on separate pouches other than those used for analytical studies. Post process drained weights averaged higher than targets: 19 pouches from fresh peach source had average drained weights of 123 ± 11g; 11 pouches from frozen sources averaged 126 ± 8g. Variations in drained and net weight were within limits of the check weigher after pouch filling. Pouches with excess (> 10 cc target) headspace were rejected before retorting based on a flotation test. In subsequent laboratory testing, net weights of fresh source pouches averaged 172g (158 to 193g) and frozen source 168g (162 to 185g).

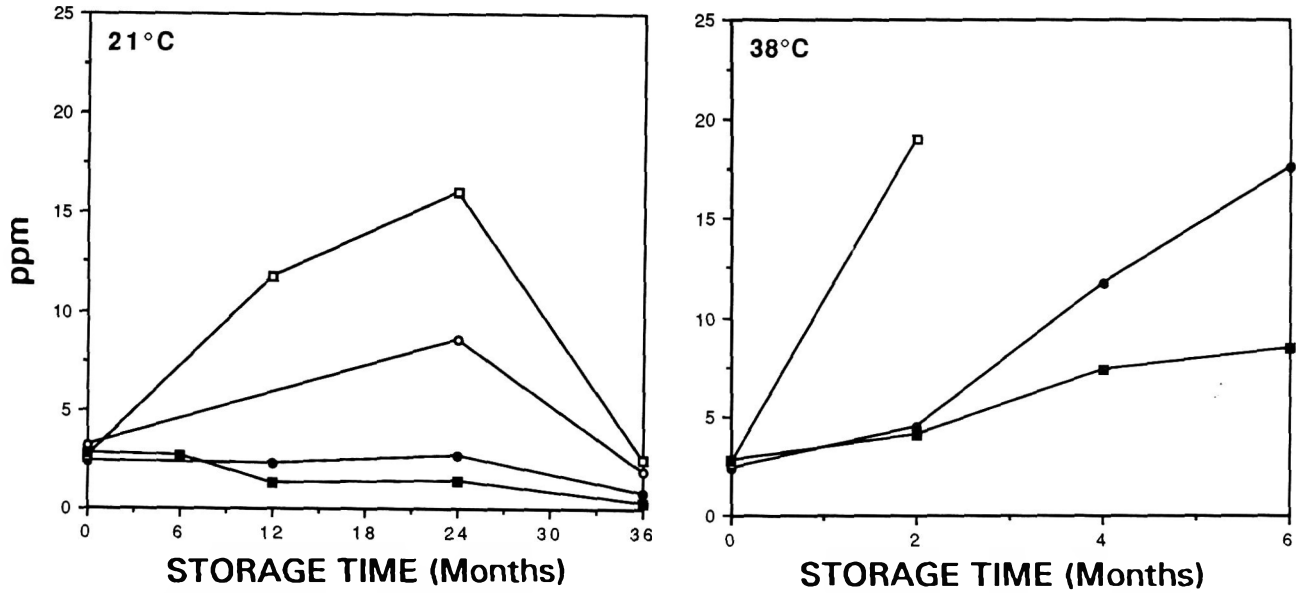

Fig. 3—Relationship of storage time and temperature to hydroxymethylfurfural levels in fruit/syrup homogenates. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

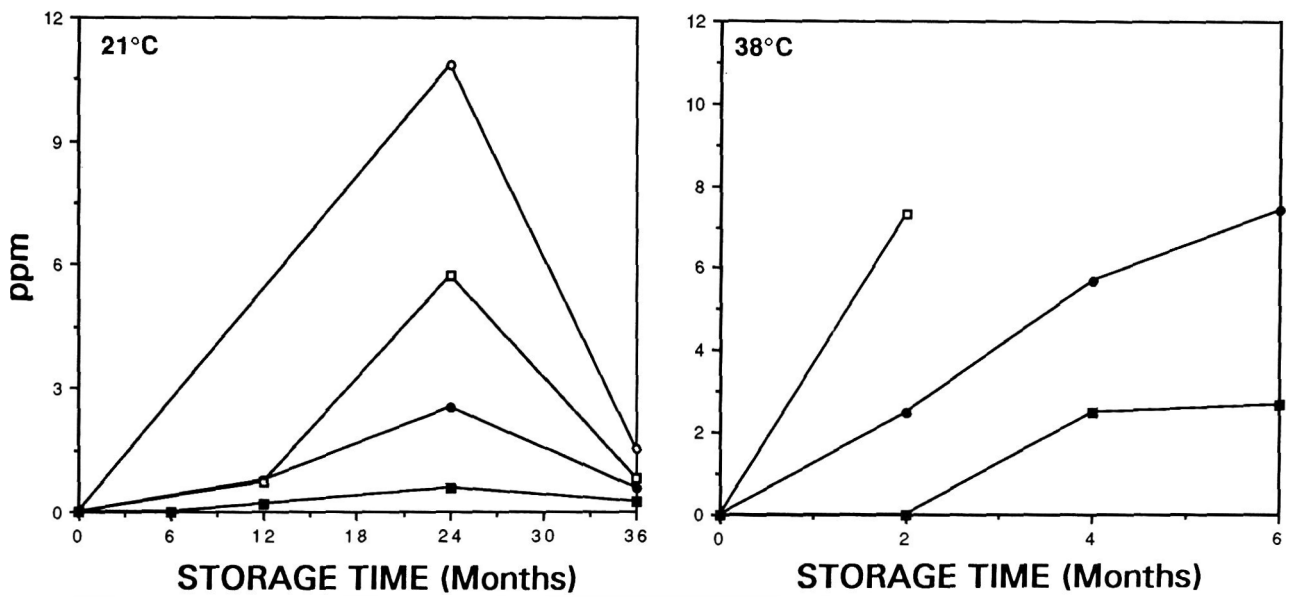

Fig. 4—Relationship of storage time and temperature to furfural levels in whole fruit homogenates. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

Following processing, pouches were held ≈3 mo at room temperature (≈23°C) until brix and pH analyses indicated both syrup and fruit had reached equilibrium. Then, the initial sensory and biochemical analyses were determined, and pouches were placed into storage at 4, 21 and 38°C.

Sensory analyses

Two types of panels were used. Members of the first were food technologists experienced in judging food quality. This panel was used to acquire descriptive sensory attribute data which might be related to biochemical/instrumental values. For this panel, five attributes were evaluated. In order of rating, 9-point attribute scales were used to rate color quality, sweetness, sourness, peach flavor and texture. A sixth attribute, "overall quality" was also included because of its standard use in evaluating military rations. The color and overall quality scales were scored extremely poor = 1 to 5 = fair to excellent = 9. The USDA standard description was used to anchor the "excellent" ratings for color (The Almanac, 1987). The intensity scales for sweetness, sourness and peach flavor were scored extremely low = 1 to extremely high = 9. The texture scale was extremely soft = 1 to extremely firm = 9. For these panels, the same group of 20–25 food technologists, experienced in judging food sensory quality, rated each set of samples. Products were presented simultaneously but evaluated one at a time in counterbalanced order.

The second type of panel consisted of untrained judges selected at random from a roster of U.S. Army RD&E Center employees who had previously volunteered to participate in consumer-type sensory tests. Acceptability was rated using the 9-point version of the hedonic scale. Different groups of 36–38 randomly chosen employee volunteers were selected for each set of samples. They were presented monadically in counterbalanced order. Samples for both types of panels were served at room temperature (≈13°C.).

After initial sensory analyses, planned samplings from storage were: 38°C samples at 2, 4, 6, and 12 mo; 21° samples at 6, 12, 24 and 36 mo; and 4° stored samples at 12, 24 and 36 mo. Due to dissimilar sampling intervals, each temperature series was run and statistically analyzed as a separate experiment.

Biochemical/Instrumental Analyses. Analyses were run at each sampling on whole pouch homogenates which were further extracted/di-

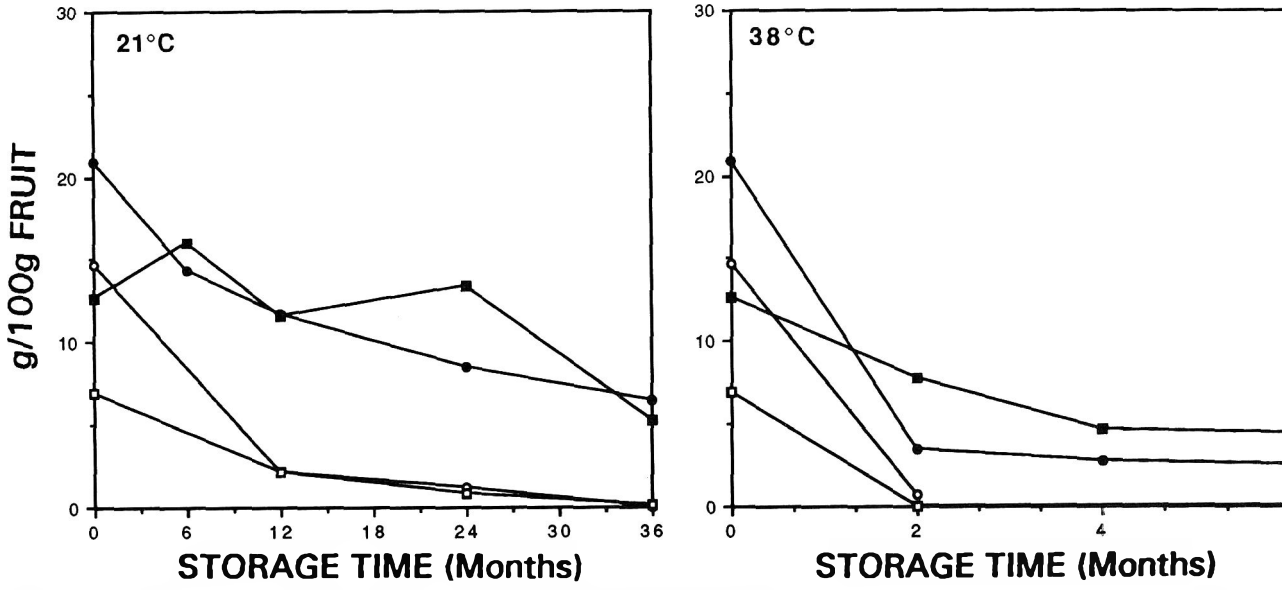

Fig. 5—Relationship of storage time and temperature to sucrose levels in whole fruit homogenates. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

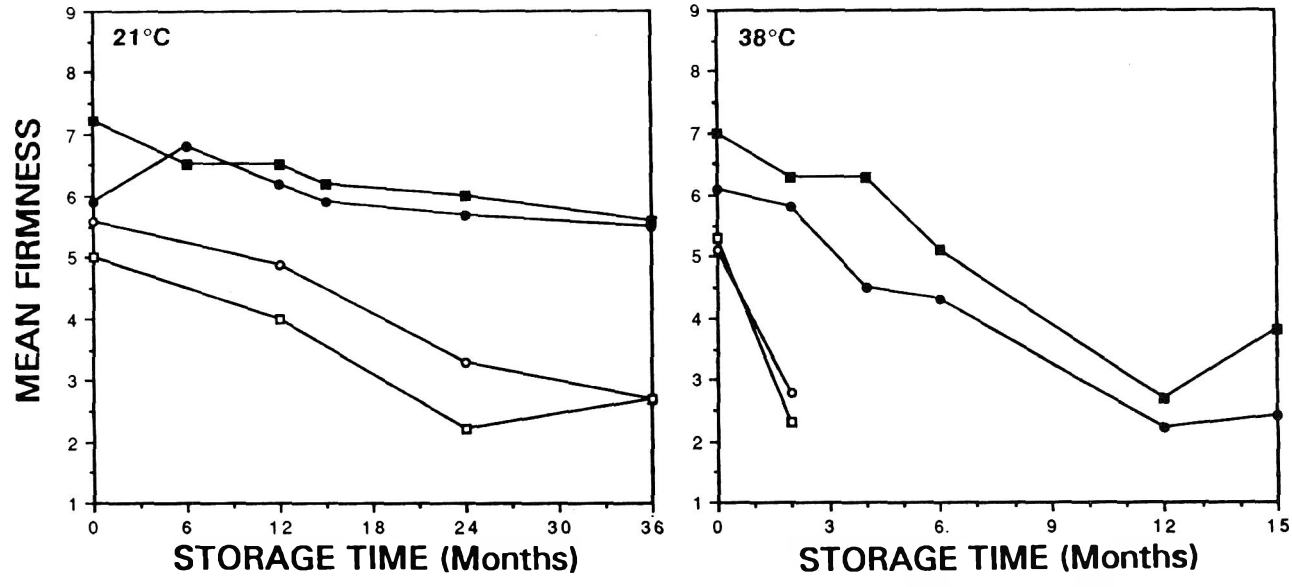

Fig. 6—Relationship of storage time and temperature to sensory firmness of peach slices. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

luted as required. Separate homogenates were prepared from two pouches. For most analyses, three aliquots were removed from each of the duplicate homogenates. The following analyses were run: (1) brix and pH: brix was determined with an Atago Model PR-1 digital refractometer and pH using a Corning Model 150 digital pH meter with a glass electrode standardized with pH 4.0 and 7.0 buffers; (2) reflectance color measurements (L,a,b) were determined with a Hunter Labs Model D25-9 colorimeter; (3) sugar profiles (sucrose, glucose and fructose), were determined by the high performance liquid chromatographic (HPLC) method of Hurst et al. (1979), using the Waters, Inc. aminopropyl carbohydrate analysis column (3.9 × 300 mm); eluent was 80/20 acetonitile/water at 2 mL/min. External sucrose, glucose and fructose standards were used to set response factors for the R.I. detector and Spectra Physics Model 4270 integrator; (4) sugar degradation products, furfural and hydroxymethyl furfural (HMF), using a rapid HPLC procedure modified from the method of Kim and Richardson (1992). An ion retardation (30 × 4.6 mm, Biorad, Inc., catalog No. 125-0114 or 125-0129) was used with 0.001N H_2SO_4 as eluent in a Varian Model 8500 Chromatograph at a 0.5 mL/min flow rate with the UV detector set at 283 nm. External standards were used to set individual response factors for furfural and HMF on a Spectra Physics Model 4270 integrator; and (5) ascorbic acid, estimated for guidance of in-plant quality control testing by EM Quant test strips from EM Science which read colorimetrically in the 50–2000 ppm range. Ascorbic acid was quantified in the post processing storage study by the photometric method of the Association of Vitamin Chemists (1966).

Statistical analyses

At the conclusion of the study, the descriptive attributes and consumer acceptability ratings were analyzed by three-way analyses of variance (ANOVA) to determine significance of pH, fruit source and storage time. $P < 0.05$ was the criterion for significance. In addition, an analysis using the method of partial least squares (Martens and Martens, 1986) was conducted to relate sensory and biochemical/instrumental data. Mean ratings for the six attributes rated by the experienced panel served as dependent variables; 10 biochemical/instrumental measurements, excluding furfural analysis, served as independent variables. The partial least squares method seeks to reveal

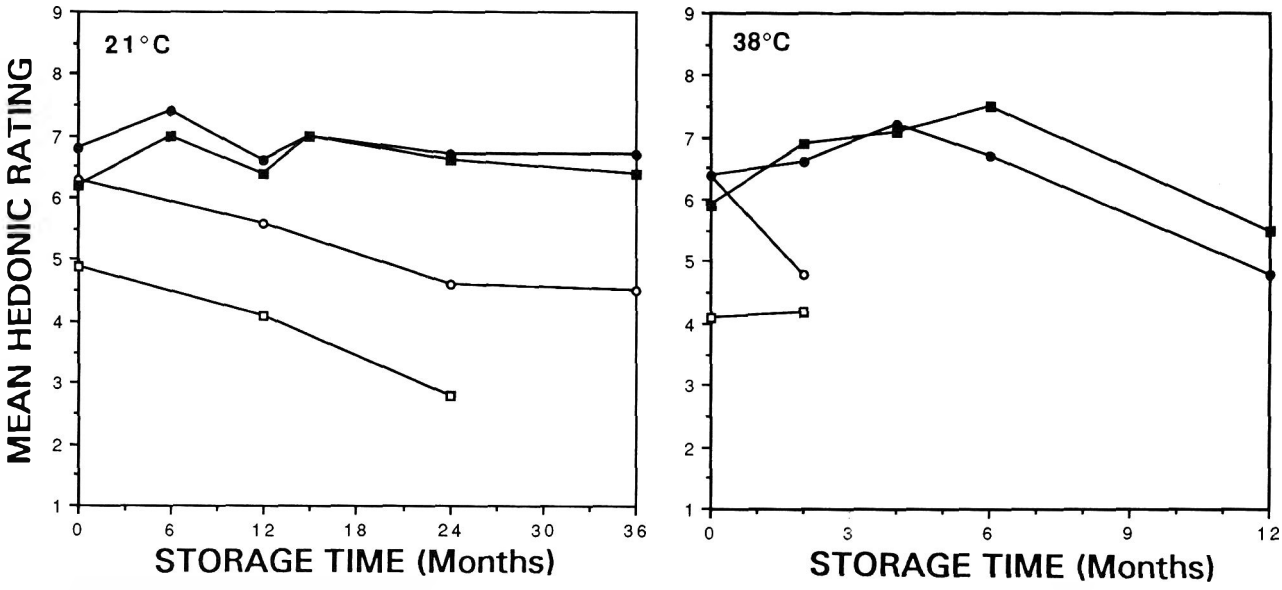

Fig. 7—Relationship of storage time and temperature to acceptability of peach slices. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

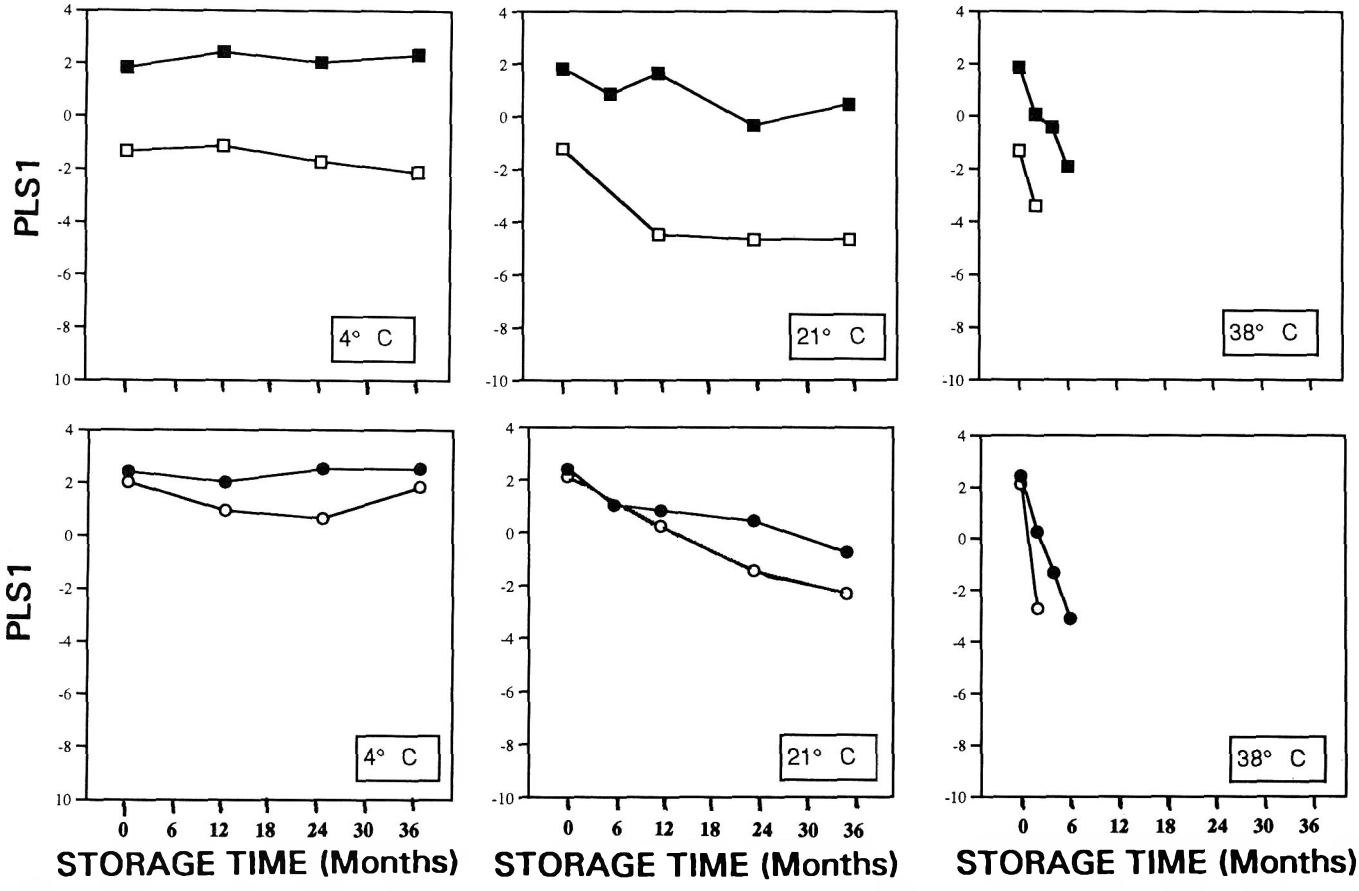

Fig. 8—Relationship of pH to PLS1 values on fresh and frozen source peaches stored at 21 and 38°C. Fresh source: High pH ■, Low pH □; Frozen source: High pH ●, Low pH ○.

latent factors among independent variables that are maximally predictive of dependent variables.

RESULTS & DISCUSSION

Color

In initial evaluations of the 21 and 38°C samples, the panel rated color quality lower for fresh than for frozen source peaches (Fig. 7). The reference 4°C stored fruit (plot not shown) indicated the same. The lower initial color quality of fresh source fruit was likely due to degree of ripeness. Dunne observed that peaches on the fresh source line going directly to the filling operation appeared less ripe than fruit on the frozen line. Higher enzyme levels were found in that fruit prior to retorting than in fruit on the frozen line. Lower initial color quality ratings were also observed for low pH than for high

Table 1—Bivariate correlations (N=46) with PLS Factor 1

Variables	Correlations
Sensory (Dependent)	
Texture	+.82
Color quality	+.75
Overall quality	+.75
Flavor intensity	+.63
Sweetness	+.43
Sourness	−.47
Instrumental/Analytical (Independent)	
Hunter b	+.95
Hunter L	+.95
Sucrose	+.83
Vitamin C	+.51
Total sugars	+.09
Brix	−.39
Hunter a	−.39
HMF	−.39
Fructose	−.83
Glucose	−.83

pH fruit. The low sensory color ratings were supported by panel comments about the dark or deeper color of low pH fruit, particularly the fresh source pack. Darkening likely occurred during the equilibration period.

At all samplings of the 21°C stored products, panelists rated low pH/fresh source peaches lower than the other treatments; the 4°C fruit was rated similarly. Ratings for IQF source peaches at both pH levels were stable over time at 4°C. At 21°C storage, ratings for low pH/frozen source samples gradually decreased through the 36 months. Ratings for the 21°C high pH/fresh source samples varied over time with an apparent "recovery" by 36 mo. The 4°C samples showed a similar trend. ANOVAs of sensory color ratings indicated significant main effects of pH, source and time. Significant interactions between pH and fruit source and pH and time also occurred.

Instrumental/analytical measurements showed trends over time corresponding to sensory ratings. Hunter L (100 = white, 0 = black) measurements over time were grouped similarly (Fig 2) to sensory color ratings. The low pH/fresh source fruit consistently was darker than the other treatments. A somewhat more consistent decrease over time was noted for the high pH/fresh source 21°C samples, in Hunter L readings than in sensory ratings.

Although sensory testing of the low pH/38°C samples was discontinued after 2 mos, HMF (Fig. 3) and furfural (Fig. 4) analyses were continued at planned samplings. The low pH/fresh and frozen samples showed sharp increases in both, peaking at 12 mos. Low levels of HMF and furfural were found in the 4°C samples. At 21°C, furfural and HMF levels reached maximum by 24 mo, then decreased to nearly 0 ppm by 36 mo. The most probable explanation is that long storage time allowed secondary reactions of carbonyl compounds with amino acids (Ledl et al., 1986).

Sweetness and sourness

Data conformed to expectations concerning sensory effects of pH level. Over time at 38°C, sweetness intensity ratings of high pH fresh and frozen samples decreased from the initial "moderate" to "high" rating (mean = 6). Over the 36 mo period, a 0.5 to 3.0 point difference separated sweetness of high from low pH samples stored at 21°C. The degree of difference increased somewhat over time. Smaller sensory sweetness differences between pH levels were observed over time for 4°C than for 21°C fruit. At all temperatures, about a three point difference in sourness means was found over time. High pH means were about 3.0 and the low pH means about 6. The ANOVAs computed for each temperature indicated a significant ($P<0.01$) pH effect for sensory sourness and sweetness attributes at all storage temperatures.

After 2 mos at 38°C, all sucrose in the low pH samples (Fig. 5) had hydrolyzed. Corresponding increases in glucose and fructose occurred. Sharp decreases were also noted in the 38°C high pH peaches by 2 mo, a low level remaining at 6 mo. Sucrose in the 21°C low pH pouches had totally hydrolyzed by 36 mo. In high pH peaches, a more gradual decrease occurred, about a third of the original level remained by 36 mo. Levels measured in the 4°C reference high pH peaches decreased to about half the initial level by 36 mo. Changes in sugar composition did not affect sensory sweetness levels over time.

Peach flavor intensity

Effects of the four processing treatments over time corresponded to sweetness intensity ratings at the three storage temperatures. As noted by Kader et al. (1982), peaches perceived as higher in sweetness were rated higher in flavor intensity. Consistent with sweetness rating patterns for the 4°C stored fruit, flavor ratings for low pH fresh source samples were lower than for the other three treatments. At 21°C, a clear separation of ratings occurred between high and low pH samples. High pH samples were consistently higher at each sampling. At 38°C, ratings for high pH/fresh and frozen source peaches were stable through 6 mo. Between 6 and 12 mo, a sharp drop occurred. The ANOVAs indicated a significant effect of pH at all temperatures.

Texture

Panelists rated peaches at the low pH from both sources lower in texture (i.e., softer) than those at high pH (Fig. 6). As with color, softening of low pH fruit likely occurred during equilibration. In general, the rate of softening was greater with low than for high pH fruit and was also related to storage temperature. Texture and color deterioration of low pH, 38° stored fruit after 2 mo were major reasons for terminating sensory evaluations. ANOVAs at all three storage temperatures indicated significant effects of pH, with significant interactions.

Acceptability

Acceptability of high pH, 38°C stored samples from both sources increased up to 6 mo (Fig. 7) which was likely related to softening of the fruit. In particular, panelists commented that initial samples of fresh source peaches were "hard" and "crunchy." Note that at 12 mo, mean ratings then decreased to below initial levels, near or below the scale mid-point. Panelists rated low pH fresh source samples unacceptable at the outset and frozen source samples unacceptable after 2 mo/storage withdrawal.

Partial least squares analysis

A single factor (PLS 1) was found from instrumental data which accounted for 42% of the variability in sensory data as well as 42% of variability in instrumental data. A second factor also explained variation only slightly. Color quality, texture, overall quality, Hunter b and L values and sucrose highly positively correlated with PLS 1 (Table 1) while fructose and glucose highly negatively correlated.

The PLS 1 factor over time showed clearly the interactive effects (Fig. 8) of temperature, pH and fruit source on sensory and instrumental measurements. Both fresh and frozen source peaches deteriorated in quality more over time at the lower than at the higher pH.

CONCLUSIONS

RETENTION OF QUALITY and acceptability at 21 and 38°C storage for minimum times required for a field ration component could be achieved in peaches for retort pouch packing when processed at their inherent pH (in our study, 3.8–3.9) and not

—Continued on page 865

Nondestructive Methods for Identifying Injury to Vapor Heat-Treated Papaya

KAZUO SUZUKI, TAKASHI TAJIMA, SABURO TAKANO, TUTOMU ASANO, and TADAO HASEGAWA

ABSTRACT

Polygalacturonase, a softening enzyme, influenced the development of physiologically injured fruit treated with vapor heat. Development of a nondestructive method to identify injured fruit was studied. X-ray computed tomography (CT), and ^{1}H-MRI, applied in the medical profession and for food analyses, were investigated for this purpose. Results showed that the X-ray CT, image analyses of the MRI differentiated between normal and injured fruit. The possibility of developing a nondestructive analysis using this technology is suggested.

Key Words: papaya, X-ray-computed tomography, NMR

INTRODUCTION

PAPAYAS HAVE BEEN IMPORTED INTO JAPAN after USDA-APHIS approved a quarantine vapor heat treatment of 47.2°C for 30 min for disinfestation of fruit fly (*Dacus dorsalis*) eggs and larvae as required by the Japanese Ministry of Agriculture, Forestry and Fishery. This treatment sometimes induces physiological injury such as non-softening or non-ripening of flesh between 1 and 1.5 cm thick surrounding the seeds, due to heat shock (Chien and Paul, 1990; Chan and Tam, 1981). We explained this mechanism through studies which measured flesh hardness, softening enzyme activities, and ethylene gas generation by the fruits during ripening. The difference between normal and injured fruits was explained by the activity of enzymes such as polygalacturonase (PG), (Suzuki et al., 1991). In order to measure PG activity fruit tissue must be macerated, if the presence of injured tissue is to be determined with a suitable nondestructive method, ripened fruits could be monitored rapidly without cutting the fruit.

Quality determinations of fruits, vegetables, and other foods (Iwamoto, 1981; Akimoto, 1985; Chen et al., 1989; Horii, 1991), sugar measurements in fruits (Kimura, 1991) and other component analyses of food (Akimoto, 1987) have been carried out by nondestructive methods. We developed a method to differentiate between ripe or normal and injured fruits by image analysis using X-ray absorption (X-ray computed method, X-ray CT) (Fujii, 1989; Fujii et al., 1987) and nuclear magnetic resonance imaging (^{1}H-MRI) (Kawai and Sakata, 1990; Shiow et al., 1988; Chien and Paul, 1989). Such techniques have been formerly applied only in medical fields.

MATERIALS & METHODS

Materials

Papaya fruit (*Carica papaya* cv. Kapoho solo) grown in Hawaii were used. Degree of ripening was classified from yellow to green (1 unripe ~ 7 overripe) based on changes in rind color (Suzuki et al., 1992). Those with green rinds were classified as unripe (1 ~ 2, Green·G), and those that were yellow were classified as ripe (5 ~ 6, Control·C). Dark yellow papayas were classified as overripe (7, Overripe·OR).

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Fruit were physiologically injured artificially by wrapping them with Saran Wrap® during the vapor heat treatment stage. Such fruits were designated as "wrapped-treated fruit" (Wrap·W).

One papaya treated by wrapping was cut through the central part, and the existence of physiologically injured tissue was confirmed. Then both parts of the fruit were held fastened back together with cellophane tape and this was used as a sample of thermally injured fruit (T).

X ray-CT

The CT image of a 10 mm slice from the central part of the papaya was observed with X-ray computed tomography (as used for medical purposes, Toshiba-60A type). A 3 mm radius of CT numbers at three points, such as flesh of the fruit near the seeds (endocarp), near the rind (exocarp) and on the inner portion between the middle and near surface (mesocarp), were measured.

Nuclear magnetic resonance imaging (MRI)

The NMR apparatus was a Philips Gyroscan S-15 (1.5T) (essentially a medical imaging system) and ^{1}H-MRI set at 5 mm slice size at a frequency of 63.78 MHz and ^{1}H-NMR signal intensity. ^{1}H-NMR T_1, and ^{1}H-NMR T_2 were measured at about 5 mm every 2 mm voxel on the points at the middle, midway to the surface and near the surface.

T_1 and T_2 weighted images were developed with a slice of 5 mm thickness. The proton resonance frequency was 63.78 MHz. On these images, T_1 and T_2 relaxation times, and proton density were measured as averages of the 5 voxels located at the center, near surface and between the center and surface, respectively. Each voxel had a dimension of 2×2×5 mm.

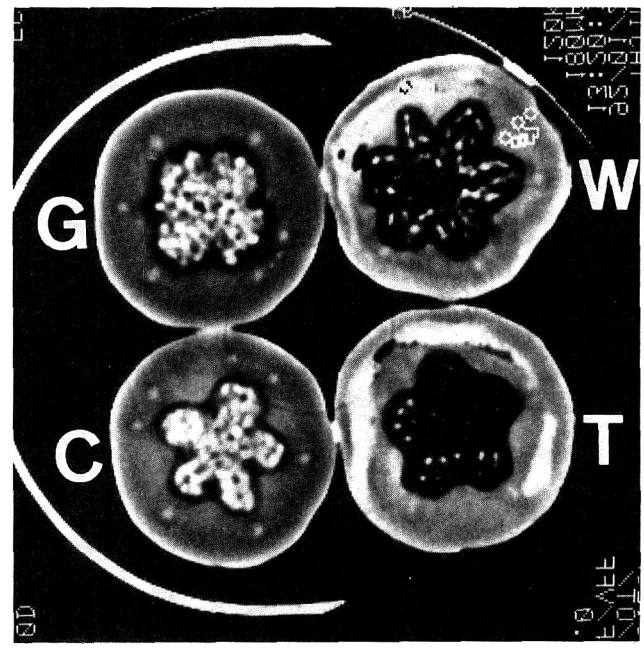
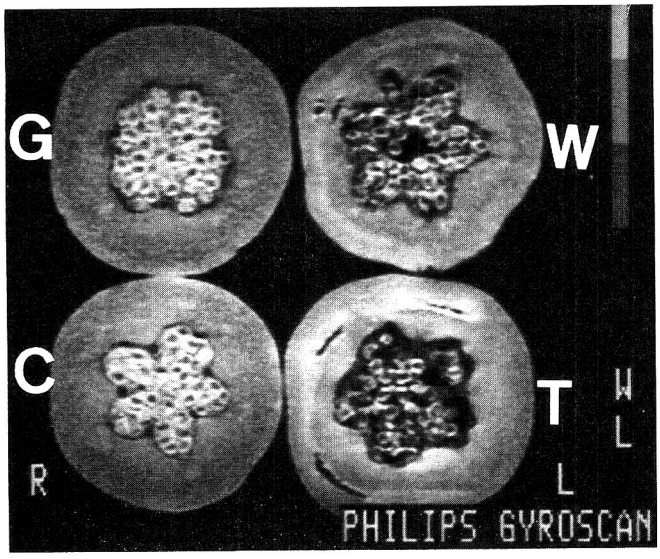
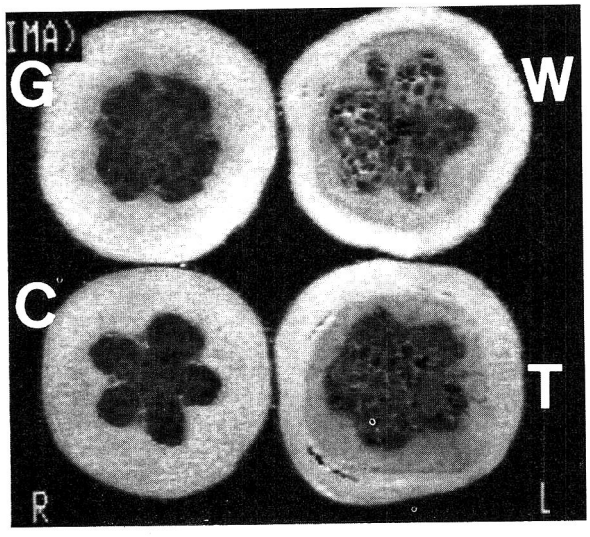


Fig. 1.—X-ray CT images of different samples of papaya fruit: G:unripe fruit; C:control (ripe or normal fruit); W:wrapped treated fruit; and T:thermally injured fruit.





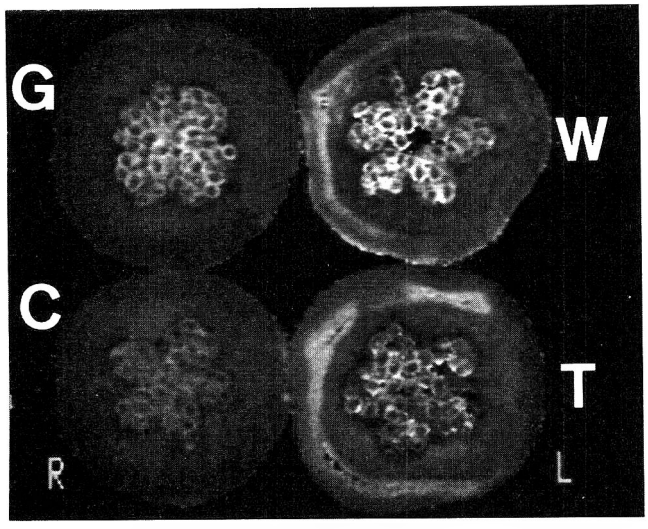
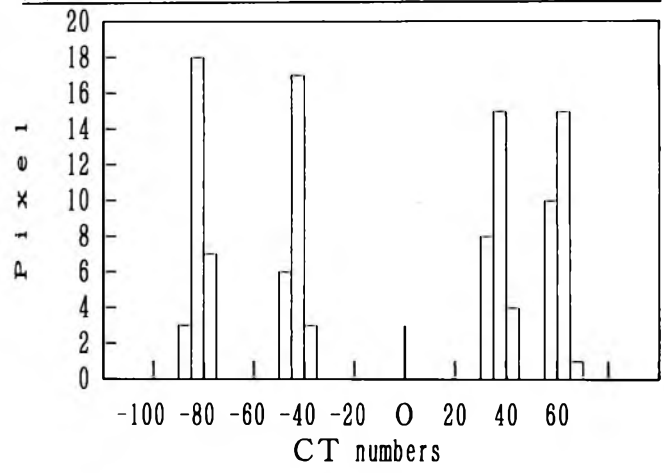


Fig. 2.—^{1}H-NMR images at different relaxation times in different samples of papaya fruit. G:unripe fruit; C:control (ripe or normal fruit); W:wrapped treated fruit; and T:thermally injured fruit.

Table 1—Histogram of X-ray CT numbers-III of different samples of papaya fruit



Mean	−92.5±5.3	−45.0±4.2	+34.0±6.2	+61.1±4.3
highest	−80.8	−37.0	+49.0	+71.0
lowest	−102.0	−54.0	+23.0	+53.0
	G	C	W	T

[180MAS · 120KV]. CT numbers

RESULTS & DISCUSSION

Computed tomographic image (CT imaging) by X-ray computed tomography

CT images were created by X-ray computed tomography (Fig. 1). The flesh tissue of unripe and ripe normal fruits were shown as uniform CT numbers. In contrast, a sample of injured tissue (Fig. 2) consisted of black (high density) and white (low density) parts in the fleshy tissue of the thermally injured sample. This was also seen in different images from the other two samples labeled W and T. Injured fruit wrapped with Saran Wrap was shown by localizing the images of parts of high and low density as with the injured sample. However, the wrapped, injured fruits were surmised to be physiologically injured. Without CT imaging no distinction between normal and damaged fruits was apparent from a casual observation of the whole fruit surface.

CT numbers

The CT numbers were intended to be shown as the numerical value corresponding to parts of black and white on the CT image. However, the fruit flesh was divided into three layers: middle near the seeds, near the rind surface, and in between. Three subsamples of each layer were mechanically measured and CT numbers for a voxel of 3 mm radius was reported. Unripened fruits showed relatively low CT numbers, and normal fruits showed relatively high numbers. In contrast, the injured fruit and the wrapped injured fruit samples showed higher CT numbers for every subsample than did normal fruit. Clearly, the injured areas in the sample fruit, (parts shown in black) were measured as CT numbers at a slice size of 15 mm^2 (equivalent to 27 pixels).

The black part in wrapped fruit, (that thought to have been injured), was measured in CT numbers at a slice size of 15 mm^2. Parts corresponding to the measured areas of the normal, wrapped-injured, and unripened fruits were chosen for measurement with CT numbers under the same conditions as the blank test (Table 1, graph). CT numbers were lowest in unripened fruit, while normal fruit had higher CT numbers.

CT numbers for the middle part of normal fruit were −59.7 at the beginning, −44.4 on the 5th day, and −31.5 on the 9th day. On the 14th day the fruit was rotten. Unripe fruit with no postharvest ripening had the lowest values. CT numbers of normal fruit increased after normal postharvest ripening, but

Table 2—Signal Intensity, T_1 and T_2 with, ^{1}H-NMR in different samples of papaya fruit

	Sample	Endocarp	Mesocarp	Exocarp
		Signal Intensity		
^{1}H-NMR	G	443.2±44.9	477.8±33.5	393.6± 30.0
	C	534.4±31.5	482.8±34.1	462.1± 40.4
	W	753.3±82.5	805.3±59.5	753.5±106.7
	T	852.7±55.7	1279.5±52.6	876.2± 47.4
		T_1 (msec)		
T_1	G	1000.3±70.3	1099.8±58.8	1153.5± 88.2
	C	1009.0±53.2	999.6±57.2	1005.3± 81.2
	W	884.1±31.3	907.3±40.6	1083.9± 45.5
	T	982.4±36.4	959.0±50.1	1145.0± 40.3
		T_2 (msec)		
T_2	G	49.3± 7.0	49.3± 5.8	43.6± 4.8
	C	55.9± 5.2	45.9± 5.2	47.4± 6.6
	W	75.5± 8.0	63.4± 6.9	61.2± 16.3
	T	82.7±11.4	51.4±12.3	78.5± 11.4

SCTIME: 20:30 (4:21) TI: 360
TR SE : 760 (500) FOU: 200 (200)
TE : 20 0/4 (20 1/1) THK: 5. 0 (5. 0)
TR IR : 2240 ANT: 10. 0 (10. 0)
PHILIPS GYROSCAN S15 ( )^{1}H

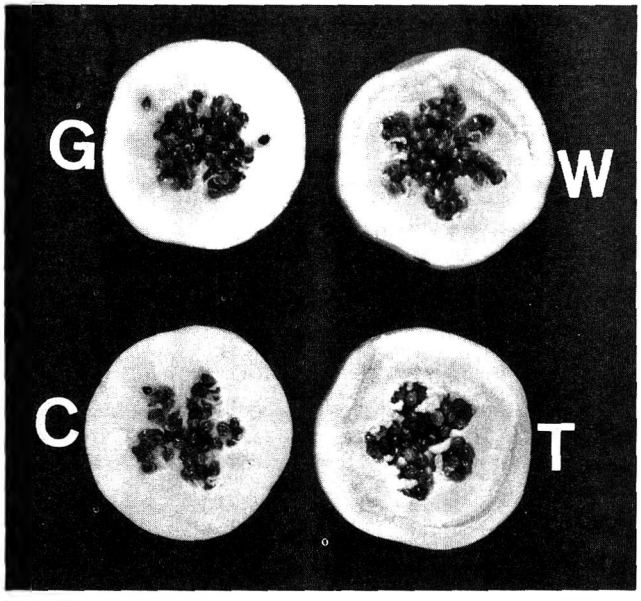


Fig. 3.—Cross sections of different samples papaya fruit. G:unripe fruit; C:control (ripe or normal fruit); W:wrapped treated fruit; and T:thermally injured fruit.

were never larger than 0. However, samples of injured fruit and wrapped-treated fruit showed irregularly high CT numbers, with both being greater than 0. These physiological injuries not only caused postharvest ripening and softening, but might have caused changes in tissue consistency.

^{1}H-MRI by ^{1}H-NMR

Figure 2-1 shows the proton density of MR images. Ripe and unripe fruit had almost uniform proton density images. The wrapped-treated fruit and the injured samples were not uniform and showed high proton density localization. The black areas of those injured fruit samples with irregular low proton density numbers corresponded to that portion of the tissue which was damaged and had a high liquid level, i.e., the overripe part.

The images of T_1 (spin-lattice relaxation time) obtained by the inversion recovery method (Fig. 2-2) showed that normal or ripe fruit gave a uniform T_1 distribution, while the interior of unripe fruit was dark and the T_1 appeared to be shorter. In contrast, the injured fruit clearly showed the different areas with both longer and shorter T_1. Wrapped-treated fruit also showed similar images. The region with a shorter T_1 corresponded to the area of lower polygalacturonase activity as previously reported (Suzuki et al., 1991). This suggested a relationship between T_1 and certain physiological activities.

The spin-spin relaxation time (T_2) images (Fig. 2-3) showed unripe and normal ripe fruit had a nearly uniform distribution of T_2. The injured sample and the wrapped-treated fruit had slightly different distributions of T_2. In them, regions of low density (black), slightly gray areas and high density (white) areas could be well differentiated. The white areas had the longer T_2; which clearly corresponded with crumbled parts of fruit, the overripe tissue.

^{1}H-NMR, T_1, T_2

The proton density, T_1, T_2 values of five voxels, 2 mm^2 each (Table 2) were chosen mechanically for the three (middle in between and near-rind) layers of the fruit (as for X-ray absorption). The wrapped-treated fruit and injured fruit showed high proton signals compared with unripe fruit and ripe or normal fruit. The distribution of T_1, T_2 of ripe and unripe fruit were almost uniform, but wrapped-treated and injured fruit showed nonuniform distributions of T_1 and T_2.

Cross sections of fruit samples

Injured fruit samples were used after confirmation of the injured part by previously cutting the fruit. The injured parts of another three samples were confirmed visually about the cross section. The flesh of unripe, ripe or normal fruit were not seen as abnormal parts, but the wrapped-treated fruit appeared the same as the fruit sample with an injured area. This injured area showed irregularly high CT numbers and also the localization of ^{1}H-MRI. These injured areas corresponded to the partitioned parts which could be differentiated by T_1 and T_2, when compared to normal parts. Nondestructive analyses utilizing CT imaging and ^{1}H-MRI could be effectively employed to identify injured papaya fruits.

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Kiwifruit Juice Clarification using a Fungal Proteolytic Enzyme

H. DAWES, P. STRUEBI, and J. KEENE

ABSTRACT

A commercial fungal proteolytic enzyme from *Aspergillus niger* was used in kiwifruit juice as a replacement for conventional fining agents to produce a stable clarified juice. Reductions in detectable protein levels of 73% and 82% were achieved using 500 mg/kg of enzyme and incubating at 60°C for 20 and 60 min respectively. Concentrates prepared from proteolytic enzyme-treated juice had reduced browning and haze formation compared to a control, without affecting ascorbic acid level. When stored at 20°C, proteolytic enzyme treated concentrates (60 min) remained clear up to 90 days and had minimal haze ($A_{650\ nm}$ = 0.047) and browning ($A_{420\ nm}$ = 0.93) after 6 mo storage. The molecular size of the protein as well as protein concentration are important factors in haze formation.

Key Words: fungal proteolytic enzyme, juice clarification, kiwifruit

INTRODUCTION

CONVENTIONAL PROCESSING OF KIWIFRUIT INTO JUICE is laborious and time consuming. Kiwifruit juice contains a high concentration of soluble protein which, during storage of juice concentrates, results in hazes and sediments which are undesirable in a clarified juice. Fining agents such as bentonite are commonly used for adsorption and removal of protein in clarified juice production. Although bentonite can remove all the heat stable protein in kiwifruit juice, producing a haze-free, stable clarified juice product requires excessive amounts (3,500–4,000 mg/L). Incomplete sedimentation of the floc, which results in large juice losses and prolonged filtration times, can increase costs. Adsorption by bentonite is not specific and may also result in flavor losses (Dawes et al., 1991; Heatherbell et al., 1990).

An alternative to bentonite treatment would be the enzymatic hydrolysis of proteins into more soluble polypeptides. The sulfydryl plant proteolytic enzyme papain has been used for reduction of chill-haze in beer (Nelson and Young, 1987; Schwimmer, 1981). In wine the use of proteolytic enzymes has been less successful due to the lower pH (3.1–3.9) and inhibition resulting from high ethanol and/or SO_2 levels. Fungal acid proteolytic enzymes were more effective under such conditions; however, effective reduction in protein has only been achieved at elevated temperatures (45–50°C) (Lagace and Bisson, 1990; Bakalinsky and Boulton, 1985; Heatherbell et al., 1984; Woiwodov et al., 1982). A high temperature short time treatment (HTST) is applied in production of kiwifruit juice to inactivate enzymes and coagulate heat-unstable protein. The use of proteolytic enzymes, active in acidic medium (pH of 3.4 for kiwifruit juice), may provide an effective means of reducing levels of heat-stable soluble protein (≈2000 mg/L). Protein hydrolysis should, however, be monitored to determine effects on haze formation and to prevent formation of undesirable bitter flavor attributes due to hydrolysis of protein to peptides.

Our objectives were to evaluate the suitability of using a fungal proteolytic enzyme in the acidic medium of kiwifruit juice to reduce soluble protein content; to determine optimum conditions for hydrolysis of protein in kiwifruit juice; to evaluate effects of additional fining (clarification) after proteolytic enzyme treatment; and to design a viable pilot plant (100 L) scale process for production of haze-free, clarified concentrates, without use of fining agents, and to evaluate their storage potential.

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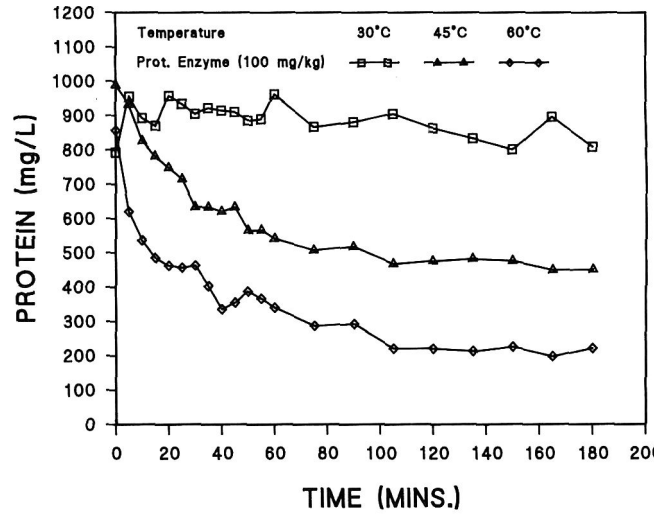

Fig. 1—Changes in soluble protein in kiwifruit juice after addition of proteolytic enzyme (100 mg/kg at 30, 45, 60°C). Each point is average of two determinations.

MATERIALS & METHODS

Preparation of kiwifruit juice

Kiwifruit (*Actinidia deliciosa* (A. Chev.) C.F. Liang et A.R. Ferguson var. *deliciosa* cv. Hayward) were picked at a commercial harvest maturity of 6.5 ± 0.5° Brix and stored at 1°C for 4 mo during which time the fruit softened to average firmness 1.1 kg (Effigi penetrometer, 8 mm head) and reached 13.0 ± 1.0° Brix. Fruit were milled, pectolytic enzymes were added concurrently (50 mg/kg, Rohapect D5L, Röhm, Darmstadt, Germany), the mash was then held at room temperature and pressed 1 hr after addition of press aid (2% w/w cellulose fibers) in a rack-and-cloth press. The pressed juice (average soluble solids 12.4° Brix, titratable acidity of 12.5 g/L as citric acid) was heat treated at 90–92°C for 15 sec in a tubular heat exchanger to inactivate enzymes and microorganisms and to coagulate heat-labile, soluble protein. An additional 150 mg/kg pectolytic enzymes were added to produce a pectin-free juice (ethanol test). Instead of treating the pectin-free juice with bentonite (Heatherbell et al., 1990), a commercial fungal (*Aspergillus niger*) proteolytic enzyme (Rohapect VR Super, Röhm, Darmstadt, Germany) was then added under different conditions to clarify the juice. Additional trials with differing levels of bentonite (sodium bentonite, Volclay, 5% w/w aqueous solution) and silica sol (Baykisol, 30% v/v), added individually and in combination after proteolytic enzyme treatment, were also evaluated. Fining agents were mixed with the juice by stirring for 15 min.

Proteolytic enzyme treatment of juice

Initial time, temperature, and concentration trials with proteolytic enzyme were undertaken on a laboratory scale. Juice samples (5 mL,

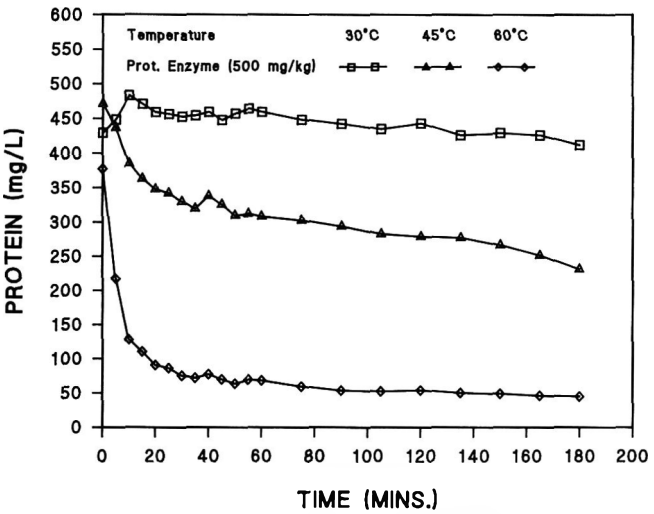

Fig. 2—Changes in soluble protein in kiwifruit juice after addition of proteolytic enzyme (500 mg/kg at 30, 45, 60°C). Each point is average of two determinations.

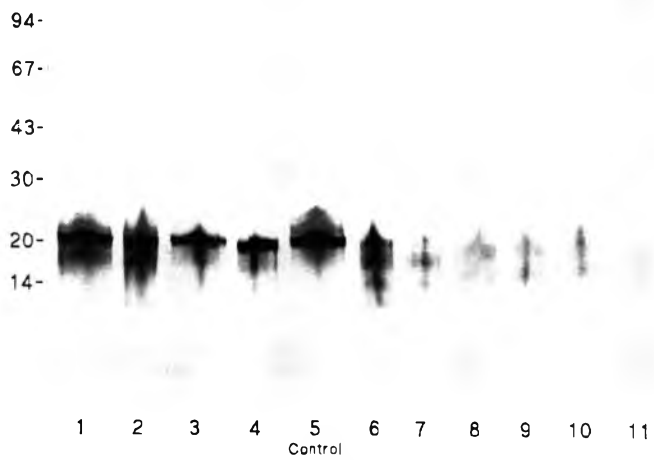

Fig. 3—SDS-PAGE gel of protein fractions in proteolytic enzyme treated kiwifruit juice (500 mg/kg at 30, 45, 60°C). Lane 1-2 60, 180 min 30°C; Lane 3-4 60, 180 min 45°C; Lane 5 control, pectin-free pasteurised juice; Lane 6-11 5, 10, 15, 20, 40, 60 min 60°C. Molecular weight standards (Daltons × 10^3) are indicated on the left hand side of the gel.

in duplicate) in test tubes were heated in 30, 45 or 60°C waterbaths. Different concentrations of proteolytic enzyme (100, 300, 500 mg/kg) were added to a series of test tubes. Individual samples were removed from the waterbath at 5 min intervals for the first hour followed by 15 min intervals for the second and third hours. Samples were immediately heated at 100°C in a boiling waterbath for 2.5 min to inactivate the enzyme and then cooled in cold running tap-water.

Preparation of kiwifruit juice concentrate for storage trials

Based on the laboratory experiments, optimized conditions were chosen for proteolytic enzyme treatment of juice for preparation of juice concentrates. Kiwifruit juice was prepared as described above. After siphoning off the clear supernatant the juice was separated into three 25L samples. Samples 1 and 2 were treated with proteolytic enzyme (500 mg/kg) for 20 min and 60 min respectively. The samples were then heated at 90°C for 10–15 sec and after cooling mixed with diatomaceous earth (0.2% w/w, Celite, grade 535) and filtered through Carlson-Ford filters (grade 5 and 7) in a plate and frame filter press. A control sample (sample 3) without proteolytic enzyme treatment was identically heat treated and filtered. Each sample was then concentrated (Alfa Laval Centritherm, CT-1B) to 65 to 70° Brix.

Concentrates of the three samples were stored in glass vials (20 mL) with minimal headspace at −20°C and 20°C. The analytical composition of the juices was determined at specific intervals up to 6 mo storage. Individual vials were sampled in duplicate and diluted with distilled water to ≈ 13.0° Brix.

Table 1—Fining of proteolytic enzyme treated kiwifruit juice and stability of the samples to the heat/cold test

Fining agents (g/hL)				Total protein (mg/L)	Haze after heat/cold test: Visual clarity[a]	Haze after heat/cold test: A (650 nm)
Proteolytic enzyme treated juice (500 mg/kg; 20 min; 60°C)				129	−	0.180
Bentonite	500			91	++	0.036
	1000			81	+	0.022
	1500			60	++	0.030
	2000			18	+	0.022
	2500			5[b]	+−	0.007
	3000			3[b]	+−	0.006
Silica Sol	500			96	++	0.046
	1000			95	++	0.049
	2000			85	++	0.052
Silica Sol	500	Bentonite	500	84	+	0.034
	1000		500	85	+	0.038
	2000		500	72	++	0.080
Silica Sol	1000	Bentonite	1000	72	+++	0.053
	1000		1500	35	+	0.026
	1000		2000	8[b]	−	0.008

[a] Visual clarity: − = clear; +− = trace haze, + = slight haze, ++ = moderate haze, +++ = strong haze.
[b] Stable juice.

Table 2—Composition of kiwifruit juice concentrates before storage[a]

Parameter	Control	Concentrate 1[b]	Concentrate 2[c]
°Brix	13.0	13.0	13.0
pH	3.3	3.3	3.3
Titratable acidity (% w/v as citric acid)	1.20	1.19	1.20
Total ascorbic acid (mg/100 mL)	67.0	67.0	67.0
Formol index (meq/100 mL)	1.03	1.05	1.04
Total soluble protein (mg/L)	478	130	86
Haze ($A_{650\ nm}$)	0.008	0.007	0.006
Browning ($A_{420\ nm}$)	0.147	0.157	0.151

[a] Analyzed as reconstituted juice, 13°Brix.
[b] Proteolytic enzyme; 500 mg/kg; 20 min; 60°C.
[c] Proteolytic enzyme; 500 mg/kg; 60 min; 60°C.

Analytical methods

Conditions for determination of total soluble protein, using a Coomassie assay with bovine serum albumin as standard, and molecular weight of protein fractions, using SDS-PAGE electrophoresis were described previously (Dawes et al., 1991). Juice samples were analyzed for titratable acidity (TA), ascorbic acid, pH and Brix (IFFJP, 1985). Formol levels were determined by the AOAC method (1990).

Juice concentrates from storage trials were also analyzed for haze ($A_{650\ nm}$), browning ($A_{420\ nm}$ corrected for $A_{650\ nm}$), and heat stability (Dawes et al., 1991). Compositional data and absorbance readings for all samples were normalized to 13.0° Brix to facilitate comparisons between samples.

RESULTS & DISCUSSION

Proteolytic enzyme treatment of kiwifruit juice

The effect of proteolytic enzyme treatment under different conditions on reduction of heat-stable protein in kiwifruit juice was investigated. The change in total soluble protein with time was compared for original levels of 100 mg enzyme/kg juice (Fig. 1) and 500 mg/kg (Fig. 2). The initial values of protein in the heat-treated and pectolytic enzyme-treated juices for the two experiments were 798 mg/L and 480 mg/L respectively. Control samples which were heat treated identically without

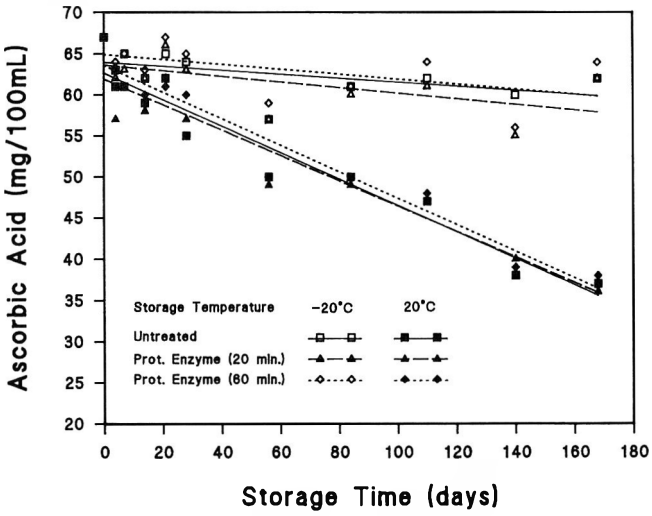

Fig. 4—Changes in ascorbic acid concentration of proteolytic enzyme treated kiwifruit juice concentrates as related to time and temperature (analyzed as reconstituted juice, 13° Brix).

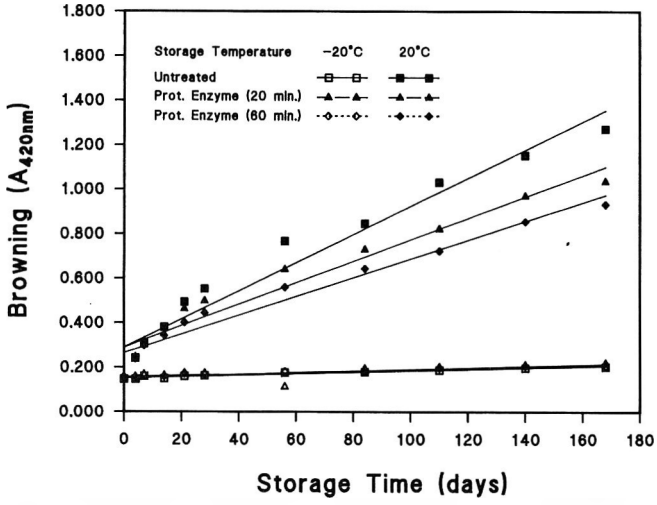

Fig. 5—Changes in browning of proteolytic enzyme treated kiwifruit juice concentrates as related to time and temperature (analyzed as reconstituted juice, 13° Brix).

addition of proteolytic enzyme showed little change in total protein. At 30°C, minimal change in protein concentration was observed over 3 hr with both proteolytic enzyme concentrations. At elevated temperatures significant reductions in soluble protein occurred. Addition of 100 mg/kg proteolytic enzyme reduced total soluble protein after 3 hr at 45°C to 451 mg/L and at 60°C to 221 mg/L (Fig. 1). With 500 mg/kg proteolytic enzyme at 45°C protein was reduced after 20 min by 27%, and by 52% after 3 hr. At 60°C 81% reduction of the original protein occurred after 20 min with a further reduction of 91% after 3 hr (Fig. 2). When 300 mg/kg of proteolytic enzyme was used the reduction in protein during the 3 hr treatment period at 60°C was intermediate (results not shown).

Fractionation of kiwifruit juice protein

The hydrolysis of kiwifruit protein treated with the proteolytic enzyme is shown in the SDS-PAGE gel (Fig. 3). In HTST pectolytic enzyme treated kiwifruit juice 3 major protein fractions were present with molecular weights (MW) of ≈ 21,000, 23,000 and 25,000 (Fig. 3, lane 5). At 30° and 45°C incomplete hydrolysis of the 21,000 MW band was observed when the juice was treated with 500 mg/kg proteolytic enzyme for 1 and 3 hr (lanes 1,2 and 3,4). The same enzyme concentration at

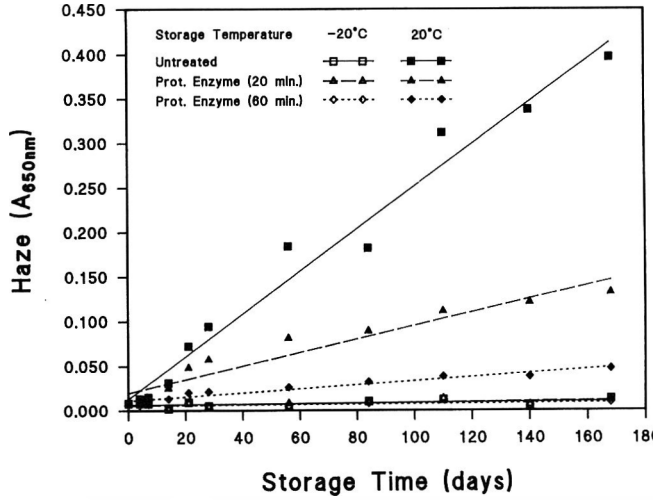

Fig. 6—Changes in haze formation of proteolytic enzyme treated kiwifruit juice concentrates as related to time and temperature (analyzed as reconstituted juice, 13° Brix).

Table 3—Nonenzymatic browning rates and rates of haze formation for kiwifruit juice concentrates at 20°C

Treatment	Nonenzymatic browning rate ($A_{420\ nm}.wk^{-1}$)	Correlation coefficient	Haze formation rate ($A_{650\ nm}.wk^{-1}$)	Correlation coefficient
Control	0.044	0.96	0.017	0.98
Proteolytic enzyme (500 mg/kg; 20 min; 60°C)	0.034	0.95	0.005	0.93
Proteolytic enzyme (500 mg/kg; 60 min; 60°C)	0.029	0.96	0.002	0.94

60°C, and a reaction time up to 20 min, resulted in degradation of major protein fractions into the ranges of ≈ 14,000 to 20,000 MW (lane 9). Additional hydrolysis to fractions of < 14,000 MW was observed after 60 min (lane 11).

Stability of proteolytic enzyme treated juice

The stability of juices containing hydrolyzed protein fractions was assessed. The standard heat/cold test used in the wine industry was a good indicator of kiwifruit juice stability in storage (Dawes et al., 1991). After heat/cold testing, no visible haze formation was observed in juice samples treated with 500 mg/kg proteolytic enzyme at 60°C for 20 or 60 min, although they contained 91 mg/L and 45 mg/L protein respectively. Spectrophotometric readings ($A_{650\ nm}$) of < 0.03 (limit of visible haze) were measured. A control juice had considerable haze ($A_{650\ nm}$ = 0.1, 480 mg/L protein). Heat/cold testing of wine has shown low molecular weight protein fractions (<14,000 MW) were more heat stable (Dawes et al., 1990; Hsu and Heatherbell, 1987) than those of higher molecular weight.

The removal of hydrolyzed protein fractions by fining agents was investigated. Experiments on bentonite fining of pasteurized kiwifruit juice have shown that stability in the heat/cold test and in long term storage only occurred after essentially protein-free juice was produced (< 10 mg/L protein) (Dawes et al., 1991). Increasing concentrations of bentonite, silica sol and their combinations were added to enzyme treated juice samples (500 mg/kg, 60°C, 20 min; 129 mg/L protein). The total protein concentration and stability in the heat/cold test were compared (Table 1). Increasing amounts of bentonite decreased total soluble protein. A level of 2500 mg/L bentonite was required to essentially remove the protein. The bentonite required was not much different from that required for clari-

fication of control juice (3500 to 4500 mg/L) (Dawes et al., 1991). The amount required for both was high. This may be because not only are high molecular weight proteins adsorbed by the negatively charged silicate platelets, but also low molecular weight proteins (<3,000 MW) which were not measured by our assay. Fining with silica sol resulted in a slight reduction in total soluble protein when used alone or in combination with low levels of bentonite. Increased haze formation after heat/cold testing was observed on addition of low concentrations of fining agents. Absorbance values (at 650 nm) were lowered to <0.01 after protein levels were <10 mg/L confirming earlier observations.

Stability of proteolytic enzyme treated kiwifruit juice concentrates

Long term stability of the unfined proteolytic enzyme treated juice concentrates was assessed over 6 mo storage. The total soluble protein level in the juices was reduced from 482 mg/L before proteolytic enzyme hydrolysis to 130 and 87 mg/L when treated for 20 and 60 min respectively at 60°C. Protein levels on pilot plant (100 L) scale were comparable to laboratory trials (≈30 mg/L higher). The composition of the three juice concentrates was compared (Table 2).

Formol levels remained virtually unchanged from controls throughout storage, indicating that protein degradation was stopped before complete hydrolysis to peptides and amino acids resulted. The total amount of ascorbic acid remained constant on storage at −20°C while at 20°C all concentrates had similar losses of 30 mg/100 mL after 6 mo (Fig. 4). These values are similar to reports of earlier studies where the loss during storage of concentrates coincided with increased browning and haze (Wong et al., 1991; Heatherbell et al., 1990).

The development of browning in proteolytic enzyme treated kiwifruit juice concentrates was followed (Fig. 5). Minimal browning occurred in concentrates stored at −20°C. The overall increase in browning at 20°C was nearly linear with time although a higher increase occurred in the initial phase up to 20 days. Average browning rates (0 to 24 weeks) with correlation coefficients were compared (Table 3). The browning in kiwifruit juice concentrate was highly temperature related (Wong and Stanton, 1989; Wong et al., 1991). Markedly different rates of browning but a similar loss of ascorbic acid were found for the three concentrates at 20°C.

Haze development during storage of concentrates (Fig. 6) was compared with rates of haze formation with their correlation coefficients (Table 3). No haze formed in any samples stored at −20°C. At 20°C haze increased rapidly in the control sample with time ($A_{650\ nm}$ = 0.40 after 6 mo). Less haze formation occurred in enzyme treated samples: the sample treated for 20 min had gradual increase in haze ($A_{650\ nm}$ = 0.13 after 6 mo); while in the sample treated for 60 min, no visible haze was present after 75 days and $A_{650\ nm}$ = 0.047 after 6 mo. In comparison, kiwifruit juice concentrate which had been bentonite fined (near zero protein) had $A_{650\ nm}$ = 0.06 after 6 mo at 20°C (Dawes, 1992). Haze development has been attributed primarily to precipitation of unstable protein (Dawes et al., 1991). Non-enzymatic browning products and polymerized phenolics may contribute to haze formation after extended storage.

CONCLUSIONS

SOLUBLE PROTEIN CONTENT OF KIWIFRUIT JUICE was reduced by treatment with acid fungal proteolytic enzyme. Protein hydrolysis increased with reaction temperature and enzyme concentration. Filtered proteolytic enzyme-treated juices had visual clarity and were stable to heat/cold testing. Concentrates from enzyme treated juice were stable at −20°C. At 20°C they developed much less haze and less browning than controls. Haze formation was virtually absent in concentrates from enzyme-treated juice stored up to 90 days at 20°C. Control concentrate showed considerable haze. Kiwifruit juice concentrates containing only low molecular weight hydrolyzed protein fractions were stable to long term storage. Ascorbic acid loss was identical in all treatments. Browning rates decreased with increased enzyme treatment time and were considerably lower than in controls. Additional clarification of enzyme-treated juice by bentonite removed hydrolyzed protein fractions. However, an essentially protein-free juice required a large amount (2500 mg/L) of bentonite. Removal of all hydrolyzed protein fractions was not necessary to produce stable juice.

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Pigments of *Monascus*

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ABSTRACT

A chemically defined medium with glutamic acid as nitrogen source was devised for the culture of two species of *Monascus* (*M. ruber* and *M. purpureus*), resulting in optimum production of their pigments and their chemical structures (both free and complexed) were compared. Structural data on two major complex pigments in aqueous solution were obtained by IR, UV, NMR and MS. They were free pigments linked to glutamic acid by amino groups, where nitrogen replaced the pyronoid oxygen.

Key Words: *Monascus*, fungi, pigment, colorant, mass spectra

INTRODUCTION

SPECIES OF THE FUNGUS *MONASCUS* have been utilized for making red rice wine, red soya bean cheese and anka (red rice). They were identified in Europe (van Tieghem, 1884) and in Indonesia (Went, 1895). Anka was traditionally produced by inoculating moistened rice with *Monascus*, and the product contained various pigments linked to proteins, peptides and amino acids. Many studies (Yoshimura et al., 1975; Wong, 1982; Wong and Koehler, 1983; Shepherd and Carels, 1983; Lin and Demain, 1991) were done on submerged fermentations using complex media, increasing production of pigments.

Some synthetic colorants have been implicated as carcinogenic and teratogenic. This has increased interest in the natural pigments of *Monascus*. Some toxicity studies of *Monascus* pigments have been done; but their toxicities have not been clearly defined. Acute oral and intraperitoneal toxicity on mice showed LD_{50} values of 33.3g and 8.7g/kg body weight respectively (Huang, 1981; Kaio et al., 1978). Commercial preparations of *Monascus* pigments had increased effects on fetal rat hepatocyte enzymes important in detoxification processes (Sako et al., 1983). These results must be confirmed (according to EU legal procedures) for authorization of their use as food additives. Thus, the molecules must be identified. The fungi *Monascus* produces six major free pigments (Fig. 1): rubropunctatine (1), monascorubrine (2), rubropunctamine (3), monascorubramine (4), monascine (5) and ankaflavine (6) (Kumasaki et al., 1962), as well as complexed pigments. Although the free pigments were water-soluble, their complexation with amino groups of the culture medium made them soluble in aqueous media (Lin et al., 1992). Our attention was focused on the production of extracellular pigments on a chemically defined medium in order to obtain complexed pigments where chemical structures could be readily identified.

Only pigments of *M. purpureus* are authorized for food use in Japan. In our study the chemical structures of *M. purpureus* pigments were compared to those of a high pigment-producing *M. ruber* strain (Zhang, 1989). Our objective was to obtain and compare structural information on two major water-soluble complexed pigments (labeled 7 and 8) by means of ^1H one- and two-dimensional Nuclear Magnetic Resonance (NMR) spectroscopy and also by Mass Spectrometry (MS).

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MATERIALS & METHODS

Microorganism and media

Strains were either a high pigment-producing isolate from anka (Zhang, 1989), identified as *Monascus ruber* van Tieghem by CBS in Baarn (Netherlands), or *Monascus purpureus* CBS 10907.

The inoculum medium composition was: malt extract 5g, yeast extract 3g, glucose 5g and agar 1 g/L ultrapure water. The initial pH of the medium was 8. The synthetic culture medium contained: monosodium glutamate (MSG), 5g; K_2HPO_4, 5g; KH_2PO_4, 5g; $MgSO_4 \cdot 7H_2O$, 0.5g; $CaCl_2$, 0.5g; $FeSO_4 \cdot 7H_2O$, 0.5g; $ZnSO_4 \cdot 7H_2O$, 0.01g; $MnSO_4 \cdot H_2O$, 0.03g; ethanol or glucose, 20 g/L deionized tap water. Initial pH of the medium was adjusted to 6.5 with ammonia and was not regulated but the medium was buffered with K_2HPO_4 and KH_2PO_4.

Cultivation methods

The slant culture was kept on potato dextrose agar (PDA) Difco. Spores of strains were prepared by growth on PDA slants for 10 days at 28°C. Spores were washed with sterile water. A suspension of 10^8 spores was used to inoculate a 5L baffled Erlenmeyer flask containing 2L inoculum medium which was incubated at 28°C on a rotary shaker (150 rpm) for 3 days. This inoculum was then transferred to a Incelltech/SGI(Toulouse, France) 20L fermentor containing 15L of synthetic medium. The culture was incubated at 28°C, with an aeration rate of 0.04 vvm and using agitation speed increased from 300 to 600 rpm.

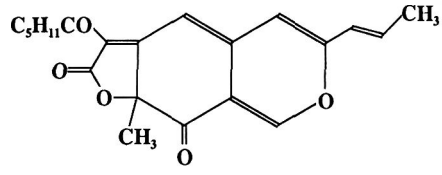
Analytic methods

Ammonia and Isolation and purification of pigments. Ammonia in the culture medium supernatant was evaluated using a Boehringer kit.

The mycelium was separated from the culture broth by an ultrafiltration M14 membrane from Tech-Sep (Bollene France), then the filtrate was lyophilized. Pigments were extracted from the lyophilized powder (crude extract) with chloroform/methanol (50/50, v:v) until exhaustion (total colorant extract). This extract was applied to chromatographic columns packed with silica gel (70-230 mesh) suspended in chloroform. The adsorption column was first eluted with chloroform/methanol (90/10, v:v) to elute less polar products containing free pigments; then, the complexed pigments were eluted with chloroform/methanol (50/50, v:v). The pigments were isolated from fractions thus obtained by preparative thin layer chromatography (TLC) on silica gel using chloroform/methanol/water (65/25/4, v:v) as solvent system. This system was also used with TLC (HPTLC silica gel) to separate components of the total colorant extract.

The free and complexed pigments thus isolated were then repurified by HPLC on a C18 column by using the following separation gradient: water/methanol (80/20, v:v)→ water/methanol (0/100, v:v) in 30 min. The flow rate was 0.8 mL/min. The detector used was a Waters Lambda Max spectrophotometer at 480 nm. Retention times of the two complexed pigments (8 and 7) were respectively 15 and 18 min while the retention time of the free pigment was 35 min. The complexed pigments were afterwards identified by NMR spectroscopy and MS. This HPLC method was also used to evaluate concentrations of the 2 complexed pigments.

NMR Spectroscopy. One- and two-dimensional NMR spectra were recorded at 298 K using a Bruker AC 200P spectrometer. For assignment purposes Hohaha experiments (Davis and Bax, 1985) were performed with mixing times of 70 ms. The water resonance was suppressed by gated irradiation during the relaxation delay times (1.5 sec). Spectra were acquired with 32 scans per t1 value and 256 t1



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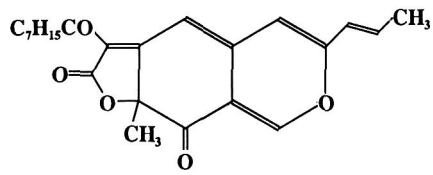

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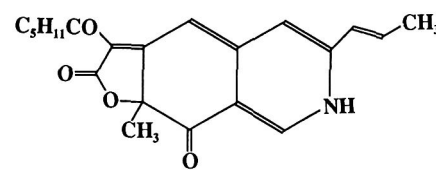

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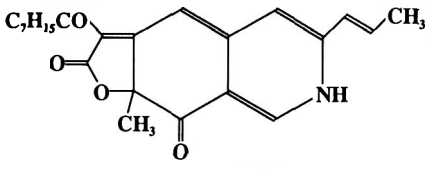

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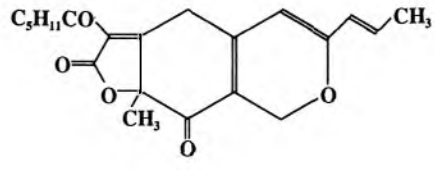

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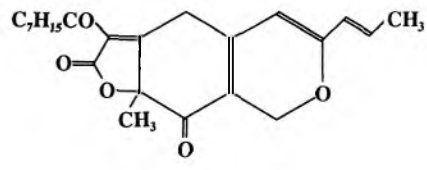

No. 6 - Ankaflavine M = 386

Fig. 1—Structure of the free pigments of *Monascus*.

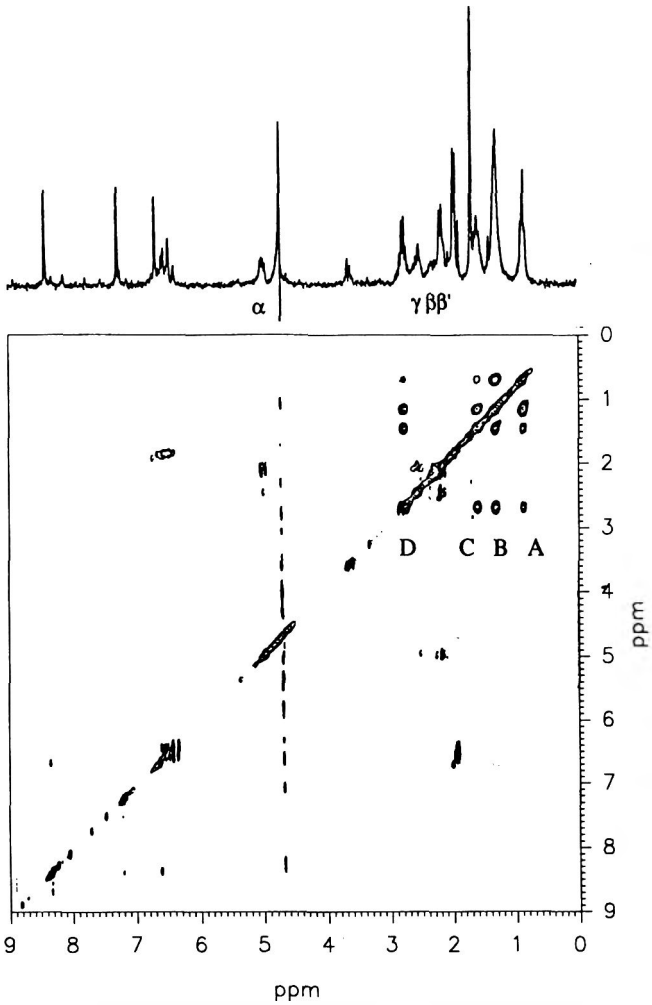

Fig. 2—200 MHz Hohaha spectrum for component 8 in D_2O recorded with a mixing time of 70 ms. The α,β,β' and γ point to the chemical shift position of the glutamic residue; while the A, B, C and D resonances at 0.76, 1.20, 1.46 and 2.69 ppm correspond to the -$(CH_2)_2$-CH_3,-CH_2-$(CH_2)_2$-CH_3,-CH_2-CH_2-$(CH_2)_2$-and-CO-CH_2-CH_2-protons of the hydrophilic side chain.

values. Data points in t1 were zero filled to give a 1024 × 1024 data matrix and sine bell apodization was performed in both dimensions. The pigment was dissolved in D_2O (99.97% D) from Spectrométrie Spin et Techniques (France).

Table 1—Retention factors (R_f) of components separated on HPTLC plates [solvent system chloroform/methanol/ethanol (65/25/5 v:v)]

Color of components	Component	R_f
Yellow	Free component 5	0.98
Red	Free component 4	0.92
Red	Complexed component 7	0.44
Red	Complexed component 8	0.39

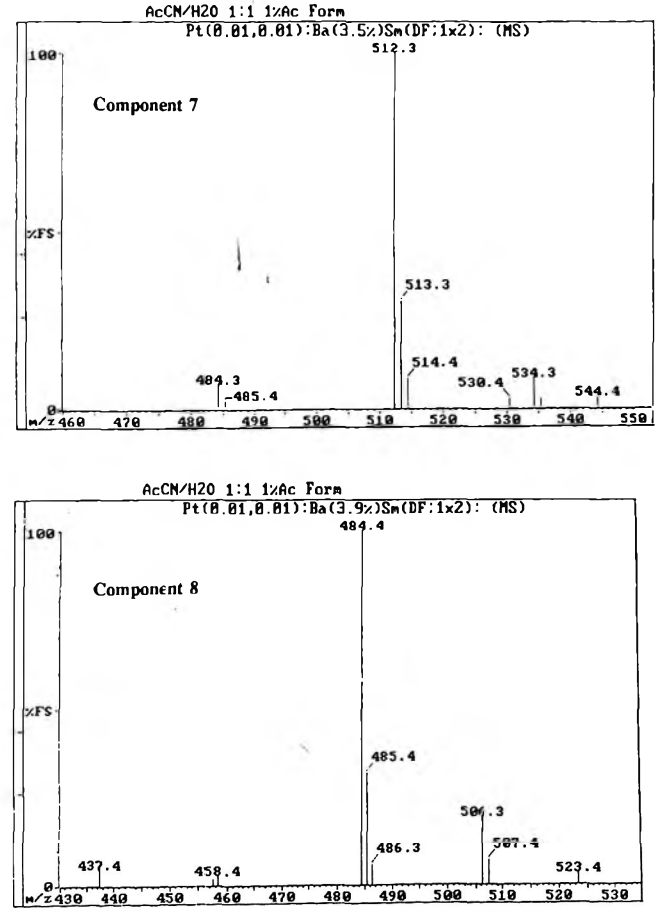

Fig. 3—Mass spectrum for component 7 and 8.

Mass IR and UV Spectrometry. Molecular weight of pigments was determined by FAB (fast atom bombardment) on a VG-ZAB 2F instrument. The matrix was glycerol acidified with trichloroacetic acid. Complexed pigments were analyzed by electrospray on a Fion-Biotech

Table 2—Retention factors (R_f) and m/z of the colored components from *Monascus ruber* and *M. purpureus* on two organic substrates (glucose or ethanol)

	M. ruber		*M. purpureus*	
Substrate	R_f	m/z	R_f	m/z
Glucose	0.39	484.4	0.39	484.4
Glucose	0.44	512.3	0.44	512.3
Ethanol	0.39	484.4	0.39	484.4
Ethanol	0.44	512.3	0.44	512.3

Trio 2000 instrument. Solvent was a 1:1 acetonitrile-water mixture containing 2% formic acid. Both methods gave $(M+H)^+$ ions from the samples. The IR spectra were recorded on a Perkin-Elmer 1310 Infrared spectrophotometer while the UV spectra were recorded on a Kontron Instruments Uvikon 860.

RESULTS & DISCUSSION

Chemically defined medium

Glucose or ethanol was used as carbon source. The use of one amino acid as sole nitrogen source provided a homogenous family of complexed pigments. For this purpose, we needed an amino acid involved in reactions of transamination leading to synthesis of amino acids useful for the microorganism. MSG was chosen, since it has been described as optimal for both growth and pigment production (Yoshimura et al. 1975). The production of complexed pigments for identification by structural methods was conducted on such media.

Complexed pigments production

After treatment of 1L of culture supernatant 12.5g of lyophilized powder resulted. With chloroform/methanol (50/50, v:v), pigments were extracted from this powder yielding 5.8 g/L of total colorant extract. Adsorption chromatography was used to separate and isolate complexed pigments representing a major part of this colorant extract. Retention factors observed on TLC of these various components (both free and complexed) were recorded (Table 1). At the end of purification by preparative TLC, a gravimetric estimation of concentration of complexed pigments gave a value of 2 g/L. The evolvement of percentages of each of the total complexed pigments during a production batch was evaluated by HPLC. The percentage of complexed pigment 7 varied from 25 to 35 while the percentage of 8 varied from 65 to 75.

Chemical structure of complexed and free pigments

Two water-soluble complexed pigments (7 and 8) represented the major part of the colorant extract. They consisted of one molecule of glutamic acid substituted respectively to monascorubrine or to rubropunctatine and were differentiated by the side chains: capryl (C7) or caproyl (C5). The IR and UV spectra of the complexed pigments (data not shown) were similar to those reported previously (Lin et al. 1992).

Resonance assignments

Assignments of the ^1H NMR resonances of complexed pigment (8) were obtained by using the Hohaha spectra (Fig. 2), recorded with a mixing time of 70 ms in order to detect direct and relayed through-bond connections. All spin systems were unambiguously identified. As an example, correlation signals were obtained for the four different methylene protons corresponding to the hydrophobic side chain ($-CO-\underline{CH_2}-\underline{CH_2}-(\underline{CH_2})_2-\underline{CH_3}$), (Fig. 2). These assignments differed slightly from those reported elsewhere (Sweeny et al., 1981) in $CDCl_3$ solution. Note the characterization of all spin systems due to the glutamic residue. The most downfield resonances corresponded to the $C\alpha H$ protons at 4.93 and 4.89 ppm. A cross-peak to these resonances led to the direct assignment of the $C\beta H_2$ protons at 2.18 and 2.07 ppm while the relayed assignment of the $C\gamma H_2$ protons was observed at 2.41 ppm. These results showed clearly that the glutamic residue was anchored to the rubropunctatine ligand. In order to know how glutamic acid was connected, the mass spectrum was evaluated.

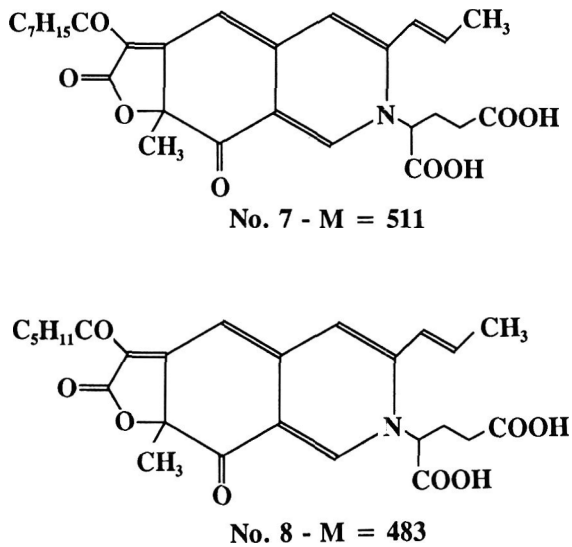

Fig. 4—Structure of colored components from *Monascus ruber* and *M. purpureus* on chemically defined media (organic substrates: glucose or ethanol).

Mass spectrometry

The glutamic acid addition could occur in two ways. Ammonia, which is always present in the culture medium, may lead to conversion of the pyronoid oxygen of monascorubrine into a NH- group (Kumasaki et al., Haws et al., 1959). This reaction proceeds through a nucleophilic reaction of ammonia on the pyrone ring, inducing ring opening, followed by ring closure on the newly introduced nitrogen group with water elimination. This conversion product was called monascorubramine. A first alternative to producing a glutamic acid conjugate would be through an amide bond between this amino acid and the intracyclic NH-group.

Alternatively, the amino group of glutamic acid may also react with the pyrone ring of monascorubrine as NH_3 did. By a similar set of consecutive reactions, this would give another type compound in which the amino group of glutamic acid was inserted into the ring in place of the pyronoid oxygen. The mass difference between these two possible conjugates would be one dalton.

Electrospray mass spectrometry of pigment 7 showed a large peak at m/z 512.3 (Fig. 3) that was consistent with the $(M + H)^+$ ion of the compound produced by the other alternative. However, a peak at m/z 513.3 was also present. It may correspond to the superimposition of the ^{13}C or ^{15}N isotopic contribution of the preceding ion and to the $(M + H)^+$ ion of the amide linked glutamic acid conjugate, if any. Calculation of the isotopic ratio points to a m/z 513 / m/z 512 ratio = 0.310. Measurement gave 0.311. Thus the amide linked conjugate does not exist. A similar result was obtained with pigment 8, showing the presence only of the glutamic acid conjugate with insertion of the α-amino group into the ring (Fig. 3).

A compound resulting from ammonia nitrogen insertion was also detected by its mass spectra (pigment 4). The red pigment 4 had a molecular weight of 381 daltons which corresponded to monascorubramine. The yellow pigment 5 had a molecular weight of 358 daltons which corresponded to monascine. Their UV spectra were similar to those published for the described pigments. Note that the pyronoid precursors (rubropunctatine and monascorubrine) were not detected in the supernatant.

CONCLUSION

BOTH COMPLEX (GLUTAMIC ACID CONJUGATES) and free pigments isolated from the broth of *M. ruber* and *M. purpureus* were the same, after growing on MSG as nitrogen source and using either glucose or ethanol as carbon source. The formation of these compounds seemed to arise from the same process:insertion of the amino group in place of the pyronoid oxygen through Schiff base intermediates (Fig. 4). These structural determinations of *Monascus* pigments confirmed previous results.

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*SHELF LIFE OF RETORT POUCH PEACHES. . .*From page 854

acidified. Though targeted pH of 3.5 was missed, any benefits of acidification to lower pH were questionable. Overall, use of IQF slices resulted in higher quality, more acceptable and less variable retort pouch products than did fresh unfrozen peaches.

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Strength of Protein Gels Prepared with Microbial Transglutaminase as Related to Reaction Conditions

HIROKO SAKAMOTO, YOSHIYUKI KUMAZAWA, and MASAO MOTOKI

ABSTRACT

Influence of gelling reaction conditions on the strength of several protein gels prepared with microbial transglutaminase (TGase) was investigated. A method was developed to gel proteins and measure gel breaking strength in a micro well plate. Enzyme concentration range for maximum gel breaking strength varied from 10 to 40 units/g protein. Maxima gel breaking strengths were achieved at 50°C for SPI, caseinate and gelatin and 65°C for egg yolk and egg white proteins. Optimum pH resulting in strong gels was pH 9 for SPI, caseinate, and egg yolk, and pH 6 for gelatin and egg white. Adjusting pH was promoted in egg white the formation of ε-(γ-glutamyl)lysine crosslinks and increased its gel breaking strength.

Key Words: protein gels, breaking strength, transglutaminase, casein, albumin

INTRODUCTION

FOOD PROTEINS ARE IMPORTANT COMPONENTS for human nutrition and also for texture or viscoelastic properties of foods. Modification of food proteins has been investigated to improve their physical functionality, i.e., gelation, viscosity, emulsification, foaming (Feeney and Whitaker, 1977). In practice, enzymic modifications of the functional properties of foods have been investigated (Hamada, 1992).

TGase is a Ca^{2+}-dependent enzyme which catalyzes an acyl transfer reaction between γ-carboxyamide groups of glutamine residues of proteins and primary amines (Folk and Chung, 1973; Folk and Finlayson, 1977). When the primary amines are ε-amino groups of lysine residues of protein, ε-(γ-glutamyl)lysine (GL) crosslinks are formed. Ikura et al. (1980 a, b), Motoki and Nio (1983) and Kurth and Rogers (1984) reported polymerization and cross-linking of some food proteins which were catalyzed by mammalian TGases. However, the use of such mammalian TGase for food production has been impractical because of its high cost.

The situation was changed by discovery of a microbial TGase extracellularly produced by *Streptoverticillium* species (Ando et al., 1989). The remarkable characteristic of this enzyme is its Ca^{2+}-independent catalytic property. The enzyme catalyzed formation of intermolecular GL crosslink in several food proteins and polymerization and gelation (Nonaka et al., 1989, 1992; Tanaka et al., 1990), and was similar to guinea pig liver TGase.

The gelation with TGase may affect qualities of many foods, since gel properties are important in many foods. They can contribute firmness and texture to products, such as jelly desserts, hams, sausages, fish paste products, etc. Gel properties can be determined by a variety of methods, of which the breaking strength of gels by puncture test is one of the most widely used.

Effective gels, required study of the influence of enzymic reaction conditions using food proteins. In previous experiments, the influence of conditions for enzymic reaction were investigated using synthetic substrates (Ando et al., 1989). Determination of the influence of conditions for enzymatic reactions on gelation of food proteins has not been reported using food proteins. Also comparisons have not been reported among different food proteins.

The objective of our research was to investigate the influence of protein gelling reaction conditions (using microbial TGase) on breaking strength of gels prepared from different food proteins. To prepare gels under the same conditions from several proteins and measure breaking strengths of many samples, we developed a method using a micro well plate. Gels of commercially available food proteins, including soy protein isolate (SPI), sodium caseinate, gelatin, egg yolk and egg white were compared. Heat treatment followed incubation with TGase, considering practical applications and required inactivation of the enzyme.

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MATERIALS & METHODS

Enzyme

Microbial transglutaminase (1.0 unit/mg) was prepared from the culture of *Streptoverticillium* sp. by the method previously described (Ando et al., 1989). Enzyme activity was measured by the hydroxamate procedure with CBZ-L-glutaminyl glycine (Folk and Cole, 1966).

Substrate proteins

Sodium caseinate, Alanate 180®, was purchased from New Zealand Dairy Board, Inc. (Wellington, New Zealand), referred as caseinate. SPI, Ajipron SU® was purchased from Ajinomoto Co., Inc. (Tokyo, Japan). Acid-treated gelatin, S-1093® was purchased from Nippi Gelatin Co., Inc. (Tokyo). Fresh egg was purchased from Maruto Chicken Egg Co., Inc. (Kanagawa, Japan). Egg white, whose chalaza was removed, was stirred gently with a magnetic stirrer so as not to produce foams at room temperature, providing homogeneous egg white. Egg yolk, with surface membrane removed, was stirred similarly to egg white.

Protein concentrations, measured by Kjeldahl of SPI, caseinate, gelatin, egg yolk and egg white were 87, 78, 86, 15 and 11 (w/w%), respectively. The solution pH of SPI, caseinate, gelatin, egg yolk were 7.0, 6.5, 7.0, 6.3, respectively. The pH of egg white was about 9, and was adjusted to pH 6.0.

Preparation of gels by microbial TGase treatment

Protein substrates were blended with distilled water using a magnetic stirrer, and adjusted to desired concentrations. Substrate concentrations for SPI, caseinate, gelatin, egg yolk and egg white were 10, 12, 10, 80 and 100 (w/w%), respectively. Protein concentrations were adjusted to about 10 (w/w%). The pH of these suspensions was adjusted with 1N HCl or 1N NaOH to the required pH. Proteins were mixed with required amounts of microbial TGase. The mixtures (0.4 mL) were applied into each well of the micro well plate with care to avoid entrapment of air bubbles. Each well was 7 mm diameter and 11 mm height, 96 wells/plate (Nunclon Micro Well Plates®, Japan Inter Med Co., Inc., Tokyo). The micro plate was then sealed and placed into a water bath (5, 20, 37, 50, 65, 75°C) for 0–300 min. Just after incubation, the plates were placed into a hot water bath (85°C) for 20 min to inactivate the enzyme. After that the plates were cooled to room temperature (≈23°C) and stored for 16 hr at 5°C.

Instrumental assessment of gel breaking strength

The strength of gel was evaluated by measuring breaking strength (g). A puncturing test was performed at 25°C as follows: The micro well plate was set on the detachable table of a Tensipressor® (Taketotmo Denki K. K., Tokyo) equipped with a cylindrical plunger (2.5 mm diameter) (Fig. 1). The Tensipressor is a sensitive instrument well suited for texture profile analysis under a wide range of test conditions (Bourne, 1982). The plunger was placed over the center of each well, then the sample was compressed with a table speed of 120 mm/min. The breaking strength was measured and read on the force vs. deformation curve by the value of the first force peak. When the sample solution was not gelled, the force peak was not observed and breaking strength was referred to as zero. This was repeated 10 times on each sample gel, and averages and standard errors were calculated.

SDS-polyacrylamide gel electrophoresis analysis

A portion of each gelled SPI and egg white was treated for sodium dodecyl sulfate - polyacrylamide gel electrophoresis (SDS-PAGE) by the procedure described by Nonaka et al. (1992). The content of ovalbumin and conalbumin were analyzed by a quantitative densitometer (Ultroscan XL Enhanced laser densitometer, Pharmacia LKB Biotechnology, Sweden). The analysis was repeated four times on each sample and the average and standard error were calculated.

Analysis of the ε-(γ-glutamyl)lysine (GL) content

SPI and egg white gels were lyophilized. An aliquot of 0.1M boric acid buffer (pH 8.0) and a crystal of thymol were added to the protein sample (20 mg protein). Proteolytic digestion of this mixture and the quantitative measurement of GL content were done by the same procedure we reported earlier (Kumazawa et al., 1993).

RESULTS & DISCUSSION

Enzyme concentration

The changes in breaking strength varied with microbial TGase concentrations (Fig. 2). The breaking strength of SPI, caseinate, gelatin, egg yolk and egg white increased with addition of the TGase. The breaking strength of caseinate and gelatin increased sharply with increase in TGase concentration. The maximum breaking strengths at a given enzyme concentration largely differed among protein substrates. Those were obtained with an enzyme concentration of 40–50 units/g protein in SPI, 15 units/g protein in caseinate, 30 units/g protein in gelatin and egg yolk, 10–30 units/g protein in egg white gels. In the caseinate, gelatin and egg yolk gels, the decrease in breaking strength was observed above 15, 30 and 30 units/g protein, respectively. Those gels at high enzyme concentration were not only soft but also fragile. We hypothesized that excessive formation of GL crosslinks would inhibit uniform development of the thermally induced protein network.

Incubation time

Incubation time also affected the enhancement in breaking strength (Fig. 3). When not treated by TGase, the gels were not formed from SPI and caseinate solutions. Thirty minutes of incubation with the TGase provided gels with the maximum breaking strength for SPI gel. The breaking strength of caseinate and gelatin gels treated by the TGase increased sharply with longer incubation time. For caseinate gel and gelatin gel, 120–250 min were required to obtain maximum breaking strength. As for egg yolk and egg white, the breaking strength slightly changed with incubation time, even in control gels (without TGase treatment). In the case of egg yolk, the difference in breaking strength between the TGase-treated sample and the control increased with incubation time, although the rate degree of increase was slower than with SPI, caseinate and gelatin. For egg white, the breaking strength, when treated by TGase, increased rapidly for 30 min. After that, the increments in breaking strength were smaller. For egg yolk and egg white, the differences in breaking strengths between treated and non-treated samples by the TGase, were observed even at 0 min. Apparently, TGase could catalyze the enzymic reaction just after starting heat treatment, but it did not increase the temperature of the samples enough to inactivate TGase.

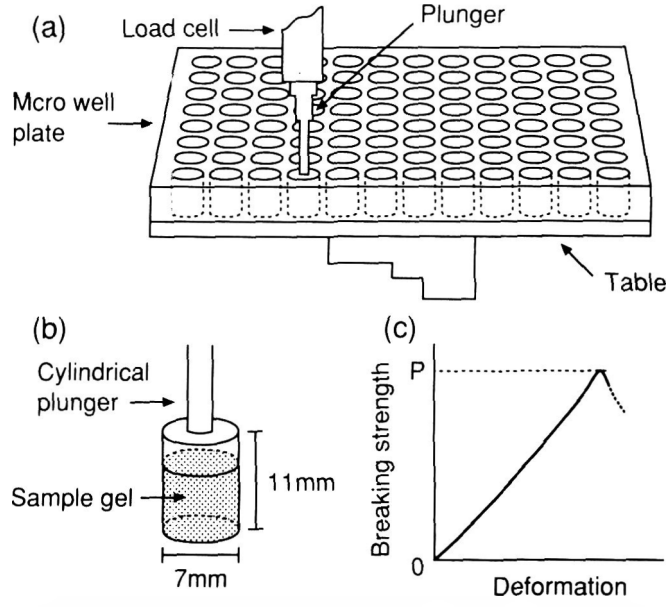

Fig. 1—Instrumental assessment of gel breaking strength. (a) Cylindrical plunger attached to Tensipressor and micro well plate; (b) Gel in a well and plunger; (c) Typical force vs deformation curve.

Incubation temperature

Breaking strength was also influenced by incubation temperature (Fig. 4). For gelatin, the test was performed above 37°C, because at lower temperatures, the gelatin solution was too viscous to mix with TGase. The maximum breaking strengths occurred with a temperature ≈50°C in SPI, caseinate and gelatin gels, and 65°C in egg yolk and egg white gels. When hydroxylamine and CBZ-glutaminylglycine were substrates, the optimum temperature, at pH 6.0 for 10 min, was ≈50°C. Regarding thermal stability, 100% activity remained at 40°C on treatment at pH 7.0 for 10 min, 74% activity at 50°C, and 10% activity at 60°C (Ando et al., 1989). Increases in breaking strengths were observed at higher temperatures in comparison with results of the hydroxamate procedure. In our experiments, food proteins were the substrates. The stability of the TGase was probably affected by coexisting with substrate proteins. The results were also affected by conformational changes of substrate proteins which had been incubated and heated. Except for egg white, little or no increase occurred at 75°C. The increase in the breaking strength was probably reduced at high temperature due to thermal inactivation of the TGase.

Relatively high activity was observed for egg white incubated at 5°C. The increased from the TGase at low-temperature incubation could have been due to heat treatment (80°C, 20 min) used for inactivation of the TGase. The TGase could catalyze the enzymatic reaction just after starting the heat treatment, which did not increase the temperature of samples enough for the inactivation.

pH dependence

The enhancement in breaking strength, by the TGase treatment, was related to the pH value (Fig. 5). For SPI, caseinate and egg yolk gels, enhancements in breaking strength at pH 9 were most significant, as compared with other pHs. The pH

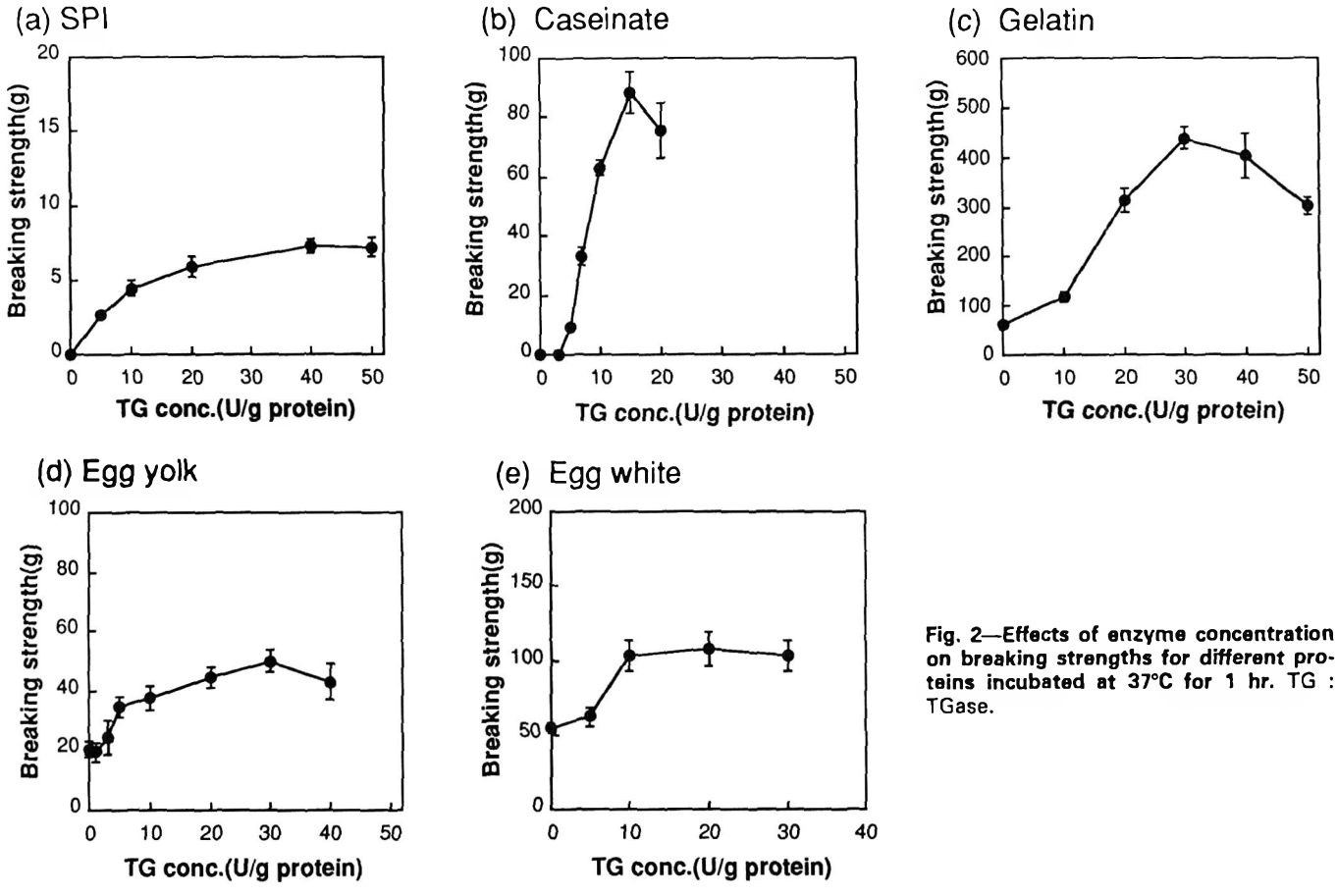

Fig. 2—Effects of enzyme concentration on breaking strengths for different proteins incubated at 37°C for 1 hr. TG : TGase.

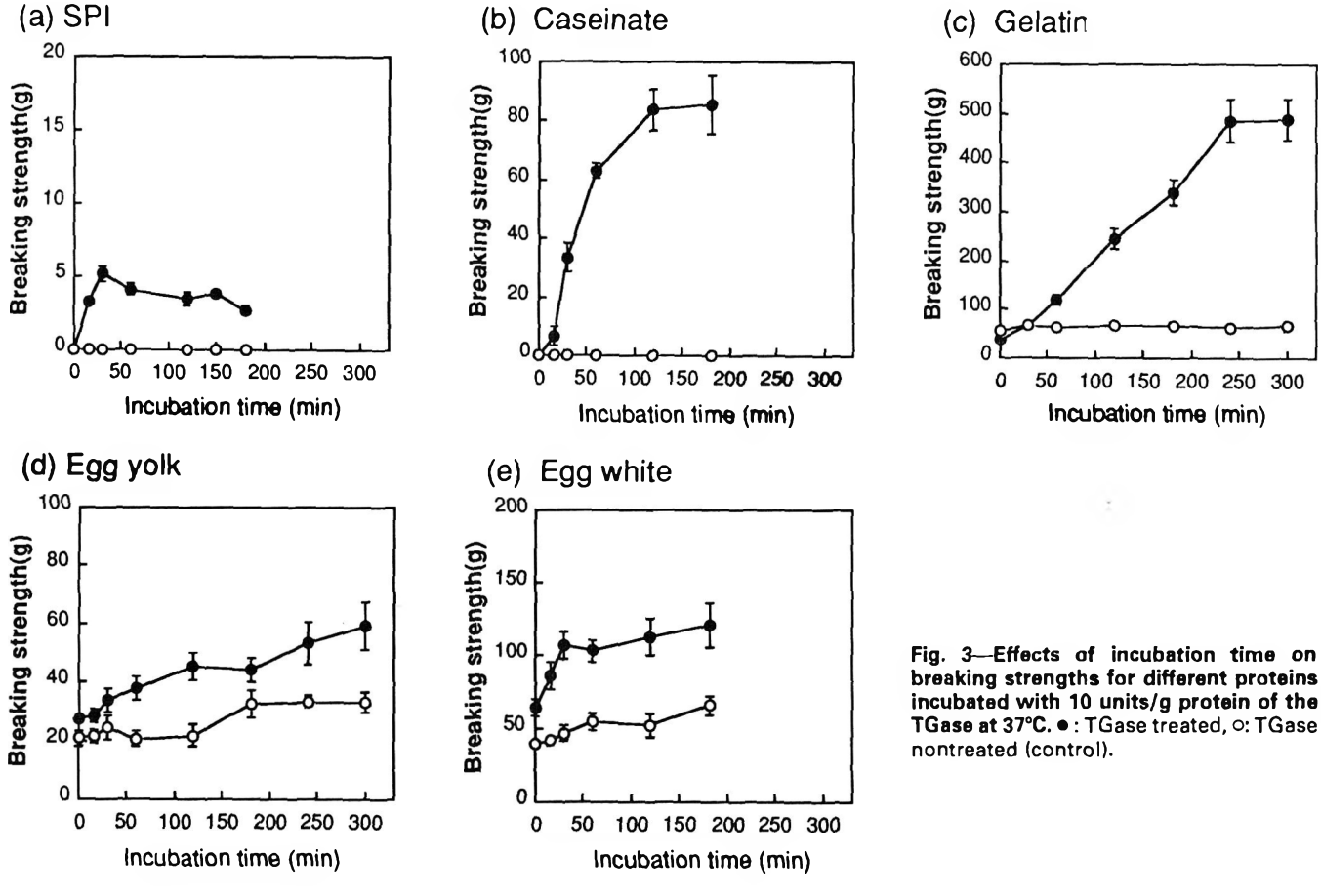

Fig. 3—Effects of incubation time on breaking strengths for different proteins incubated with 10 units/g protein of the TGase at 37°C. ●: TGase treated, ○: TGase nontreated (control).

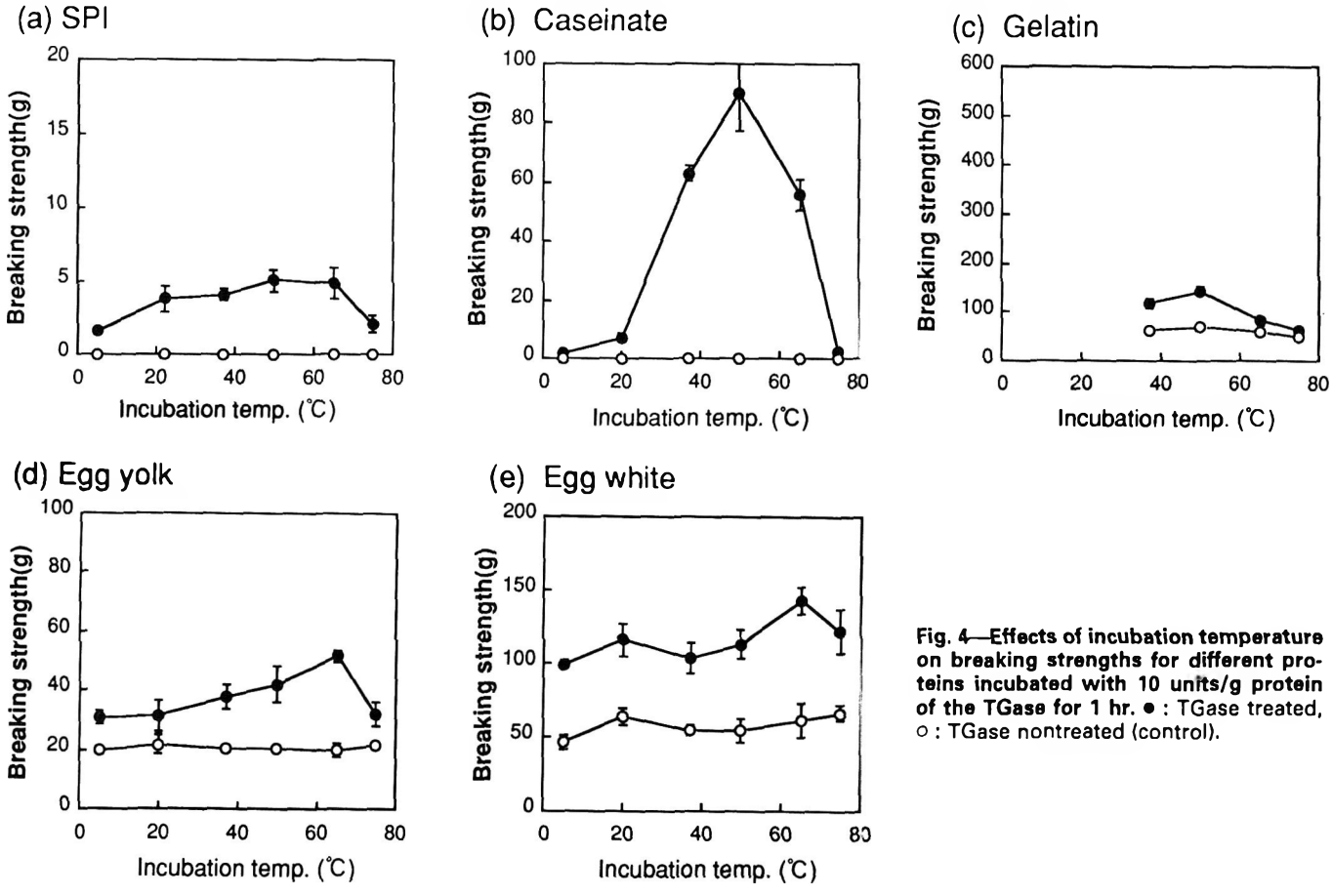

Fig. 4—Effects of incubation temperature on breaking strengths for different proteins incubated with 10 units/g protein of the TGase for 1 hr. ● : TGase treated, ○ : TGase nontreated (control).

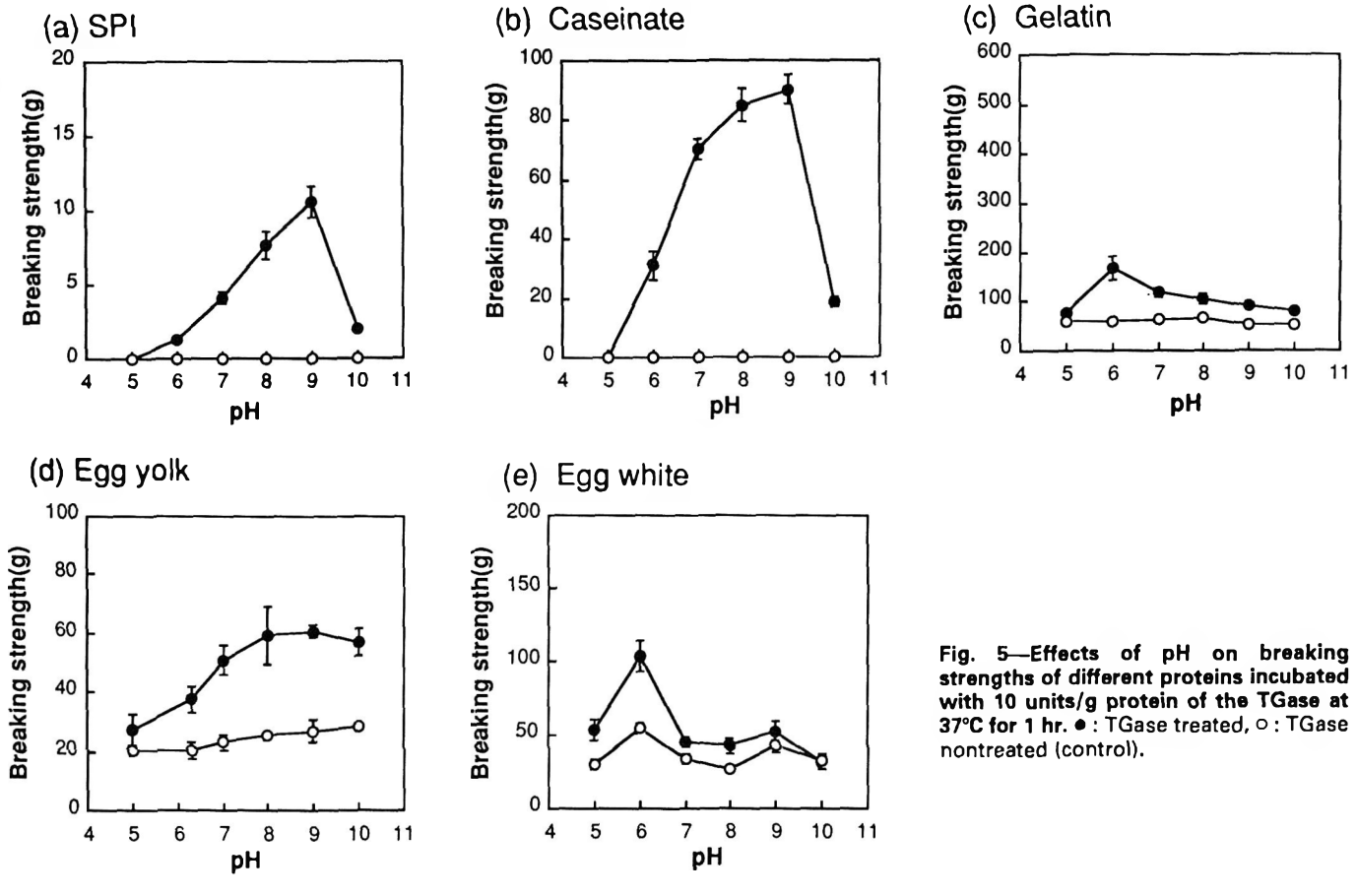

Fig. 5—Effects of pH on breaking strengths of different proteins incubated with 10 units/g protein of the TGase at 37°C for 1 hr. ● : TGase treated, ○ : TGase nontreated (control).

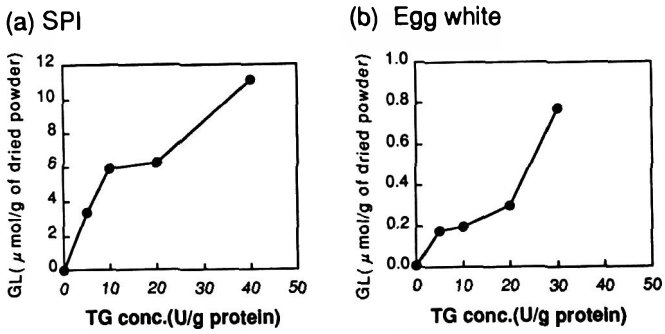

Fig. 6—Relationship between TGase concentration and amount of ε-(γ-glutamyl)lysine for two proteins incubated at 37°C for 1 hr, and subjected to ε-(γ-glutamyl)lysine analysis. TG : TGase.

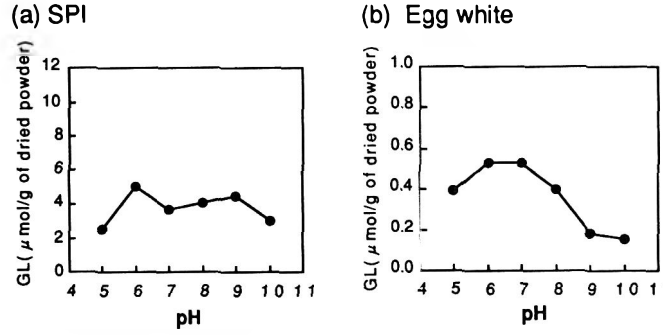

Fig. 8—Relationship between pH of gels and amounts of ε-(γ-glutamyl)lysine from two proteins incubated with 10 units/g protein of TGase at 37°C for 1 hr.

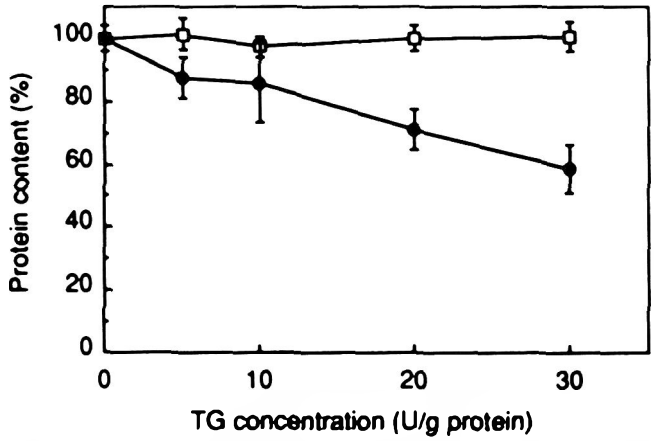

Fig. 7—Changes in the content of ovalbumin (□) and conalbumin (●) of egg white gel as related to TGase concentration. Gels were incubated at 37°C for 1 hr. TG : TGase.

dependence of caseinate gel was in accord with that reported in α_{s1}-casein solution treated by guinea pig liver TGase (Nio et al., 1986). Our result on SPI was different from the results on 7S gel treated by human placental Factor XIII (Backer-Royer et al., 1992). Our microbial TGase retained enough activity and stability at pH 9 and the SPI contained other proteins besides 7S. Also the protein gels were heat-set in our study. Thus, the difference in results was probably affected by those factors, besides substrate specificity of both enzymes. For gelatin and egg white gels, the enhancement of breaking strength at pH 6 was most significant compared with other pH's. Probably the isoelectric point of gelatin, which is in the alkaline region, affected the pH dependence of the gel.

No reports on cross-linking, polymerization or enhancement of physical properties of egg white proteins by TGase have been found. Conformational changes due to reduction of disulfide bonds were probably necessary for bovine and human serum albumins to be the substrate for the TGase (Nonaka et al., 1989). As with those serum albumins, some reducing agent was needed to make ovalbunmin a substrate of the TGase. At pH 9, the increase in breaking strength was not obtained with various TGase concentrations, incubation times, and incubation temperatures. Note that the enhancements in physical properties for egg white gel formed by TGase at pH 6 were without any reducing agents.

Increase of gel strength related to formation of GL or polymerization

With SPI and egg white gels, the amount of GL was analyzed to confirm the generation of cross-linking by TGase. TGase concentration was related to GL (Fig. 6). The amount of GL increased with TGase concentration. The enhancement in breaking strength by the TGase was due to formation of GL. As a result of SDS-PAGE for SPI, the monomeric fraction of the intact proteins diminished, and a novel polymer fraction, which could not enter the gradient gel (5–20%), was formed (data not shown).

As a result of analyses of GL and SDS-PAGE, we confirmed that egg white proteins could be the substrate for TGase and enhance gel properties. To confirm that egg white was the substrate for TGase, changes in protein content of the gel incubated at pH 6 were analyzed by SDS-PAGE. Although content of ovalbumin (Fig. 7) did not change significantly, the content of conalbumin decreased directly with the TGase concentration and reached about 60%. A novel polymer fraction, which could not enter the gel, was formed in the sample treated with the TGase. Large molecular-size components might have been formed by cross-linking of conalbumin. A decrease in conalbumin content in SDS-PAGE was observed. Analyses of the changes in contents of other components besides ovalbumin and conalbumin were not reported, because the density was not high enough even in gels which were not treated with TGase.

To investigate the reason why the increases in breaking strength of SPI gel formed at pH 9.0 were significant as compared with those formed at other pH values, the amount of GL was analyzed (Fig. 8a). No correlation was found between pH of gels produced by TGase and the amount of GL. From SDS-PAGE, the ratio of polymerization at pH 9 was not higher than those at other pH ranges (data not shown). The isoelectric points of most amino acid residues in SPI are pH 5–7. Many negatively charged amino acid residues exist at pH 9. For instance, pK values of aspartyl and glytamyl side chains are about 4. Therefore, the interaction between those negatively charged residues and water molecules could increase the solubility and water-holding capacity of SPI. Therefore, the thermally induced global network was well produced in the gel. The increase in breaking strength at pH 10 was slight. It might be influenced by the activity and stability of the TGase at that pH. With egg white gel, correlation was found between pH of the TGase-treated gel and the amount of GL (Fig. 8b). Amounts of GL at pH 6 and pH 7 were higher than those at other pH's. At those pHs, glutaminyl residues and lysyl residues of conalbumin were probably more accessible than at other pH ranges, and therefore the strong gels were formed.

Correlation of two variables for increases in breaking strength

As mentioned, reaction conditions largely influenced the gel breaking strength. In SPI, the correlation of two conditions for increase in breaking strength were investigated. Breaking strength of SPI gels was a function of both enzyme concen-

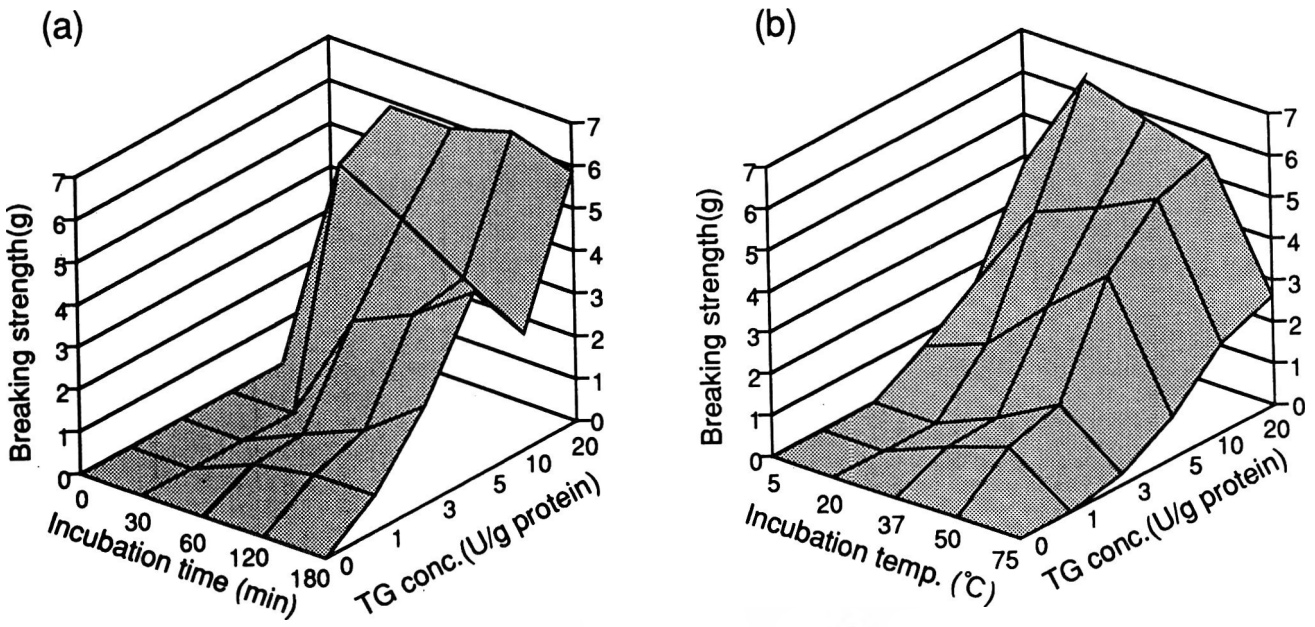

Fig. 9—Correlations of two conditions for the increase of gel breaking strength. TG: TGase.

tration and incubation time (Fig. 9a), and of enzyme concentration and incubation temperature (Fig. 9b). The gels were produced in duplicate at the same conditions, and good reproducibility in data was obtained.

To obtain higher breaking strengths, it was more advantageous to increase the enzyme concentration than to extend incubation time (Fig. 9a). Similarly, it was more advantageous to increase the enzyme concentration than to raise incubation temperature (Fig. 9b).

CONCLUSIONS

GELLING REACTION CONDITIONS using the microbial TGase very much influenced the strength of gels from several food proteins. Degree and nature of the relation to reaction conditions varied among proteins. In egg white, control of pH was advantageous for formation of ε-(γ-glutamyl)lysine crosslinks and enhanced gel breaking strength. Correlation between formation of GL and enhancement of strength of egg white gel using TGase has not been previously reported.

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Gelation Enhancement of Soy Protein Isolate using the Maillard Reaction and High Temperatures

OSCAR CABODEVILA, SANDRA E. HILL, HELEN J. ARMSTRONG, ISOBEL DE SOUSA, and JOHN R. MITCHELL

ABSTRACT

Soy protein isolate gels prepared by autoclaving solutions in the presence of xylose of glucono-δ-lactone (GDL), were compared. In both cases, the pH decreased from neutral to pH 5.5 during gel formation. In the xylose systems, this pH decrease was a consequence of the Maillard reaction. The Maillard gels showed less syneresis, had a higher breaking force and were more elastic, as determined by stress relaxation, then the GDL gels. The differences were attributed to formation of additional covalent crosslinks due to the Maillard reaction, as evidenced by greatly reduced solubility in sodium dodecyl sulfate + β-mercaptoethanol.

Key Words: soya, protein isolates, gelation, Maillard, browning

INTRODUCTION

THE PROTEIN CONCENTRATION required for the heat-induced gelation of globular proteins can be substantially reduced by incorporation of reducing sugars (Hill et al., 1992). Temperatures >100°C are required and the resulting gels have low syneresis and are brown. Gel formation appeared to be promoted by the Maillard reaction. In our previous work, we used this technique to gel bovine serum albumin (BSA) although we reported some preliminary data on commercial soya isolates, which suggested that the approach could improve their gelation behavior. The soya system is of particular interest because high concentrations of protein are usually required to form homogeneous gels (Grinberg et al., 1992).

The Maillard reaction has two or more major effects on protein gelation. A reduction in pH occurs at high temperatures due to formation of acidic by-products of the reaction. This will occur subsequent to protein denaturation. The critical concentration for protein gelation is usually highly pH dependent, with a minimum at the protein isoelectric point (Bikbov et al., 1985; Stading and Hermansson, 1990). Therefore, the pH changes induced by the Maillard reaction would be expected to alter characteristics of the gel. Also increased covalent protein crosslinking probably would occur since the Maillard reaction is known to reduce protein solubility (Kato et al., 1986). The presence of such additional crosslinks might be expected to alter both the rupture strength and viscoelastic properties of the gel. Our objective was to distinguish between these two effects by comparing rheological properties of soya isolate gels that were prepared by heating the protein either with the reducing sugar xylose or the acidulant glucono-δ-lactone. In both cases a pH decrease would occur during heating. Solubilities in sodium dodecyl sulfate (SDS) and β-mercaptoethanol (β-ME) were measured to identify differences in bonding between the two types of gels.

Soya protein gels have been extensively investigated (Utsumi and Kinsella, 1985; Hermansson, 1986; Van Kleef, 1986; Shimada and Cheftel, 1988; Grinberg et al., 1992). Also, studies were reported on the gelation of the 11S component using glucono-δ-lactone (Kohyama and Nishinari, 1992; Kohyama et al., 1992). However in those studies gelation was observed at 60°C, as compared to the higher temperatures we studied.

We found no reports of previous work on use of the Maillard reaction to enhance soya protein gelation.

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MATERIALS & METHODS

Materials

Soya protein isolate PP™ 500E (SPI) was obtained from Protein Technologies International. The reported protein content was 91.5% (solid weight basis) and the moisture content was 5.5%. D(+)xylose and glucono-δ-lactone were obtained from Sigma Chemical Co. Ltd. Other reagents were purchased from Fisons PLC and from Sigma Chemical Co. Ltd.

Gel preparation

Soy protein gels were prepared following the method described by Hill et al. (1992), with slight modifications. Solutions of protein in the presence or absence of D(+)xylose and glucono-δ-lactone (GDL) were prepared in distilled water. Maillard gels were prepared by addition of 3% (w/v) D(+)xylose, a level established in a previous study using BSA (Armstrong et al., 1994), for different concentrations of SPI in the range 10% to 20%. GDL gels were prepared by adding GDL to SPI solutions to maintain the final pH within the range for the Maillard gels. This had to be adjusted depending on concentration of SPI used, due to the buffering effects of the protein. Ordinary gels were prepared from SPI solutions without adding GDL or xylose. Gelation was initiated by heating 20 mL solutions in dialysis tubing (14 mm diameter) in a steam atmosphere in a laboratory autoclave at 121°C for 60 min. For the GDL gels autoclaving was carried out as rapidly as possible after addition of GDL to ensure that the pH decrease, which resulted from hydrolysis to gluconic acid, occurred during, rather than prior, to gel formation. In order to reduce losses of water during the retorting process, the dialysis tubing was covered with a double layer of polyethylene film. Following formation, the gels were aged inside the dialysis sacs at 4 ± 1°C for about 18 hr.

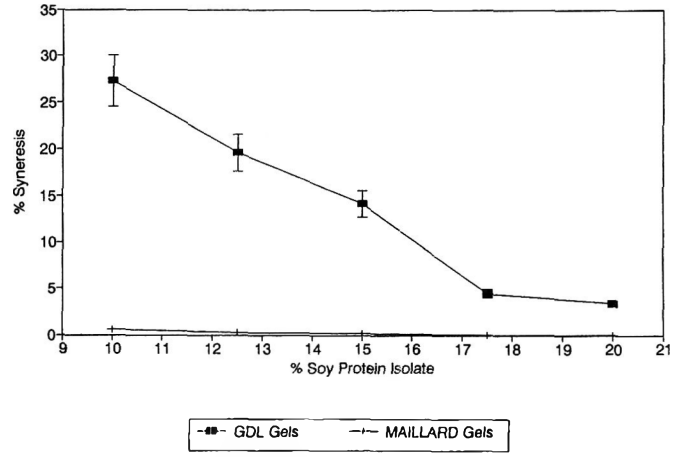

Fig. 1—Percentage syneresis for Maillard (+) and GDL (□) gels prepared from SPI and 3% xylose (retorted at 121°C for 1 hr).

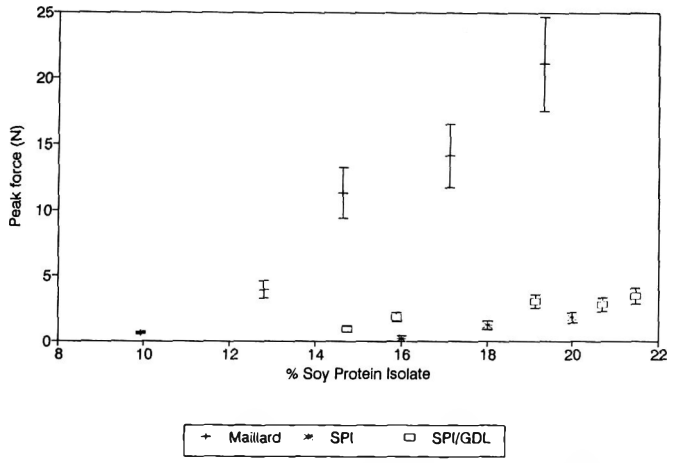

Fig. 2—The force to break gels prepared as in Fig. 1 without (*) and with acidulants GDL (□) and the Maillard reaction by-products (+). The force was related to the protein estimated to be in the gel network.

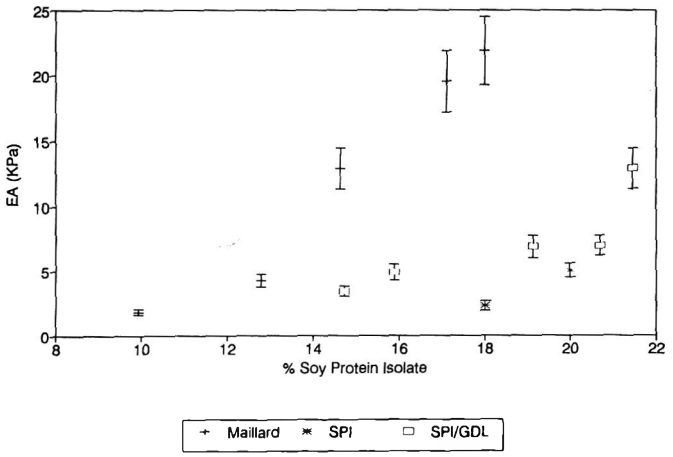

Fig. 3—The asymptotic residual modulus (E_A) measured at 25% deformation as related to protein concentration. Details as in Fig. 2.

Evaluation of gels

The original solution and gel pH were determined using a laboratory pH meter (WPA CD 620 Digital pH-meter). The percentage of material difference after autoclaving was determined by weighing the protein solution inside the dialysis tubing before and after autoclaving. Syneresis liquid was recovered from the dialysis tubing and weighed.

Losses and syneresis data were expressed as a percentage of total weight of the gels, i.e.,

$$\%\text{Losses} = \frac{(W_0 - W_f)}{W_0} \cdot 100 \quad (1)$$

$$\%\text{Syneresis} = \frac{W_s}{W_0} \cdot 100 \quad (2)$$

where W_0 = initial weight of solution (before autoclaving); W_f = final weight of gel (after autoclaving); and W_s = weight of syneresis liquid.

Gel protein solubility

Gels were homogenized by pressing through a copper sieve (linear size of aperture, 211 μm). Samples of 0.5 g of the homogenized gel were extracted in 10 mL of three different solvents: 1% sodium dodecyl sulfate (SDS), 1% β-mercaptoethanol (β-ME) and a mixture of both. The extraction time was about 14 h (overnight). Any undissolved material was removed by centrifugation (1500 × *g* for 10 min). The concentration of protein in the supernatant was determined by the Lowry method (Lowry et al., 1951).

Rheology

Rheological properties of gels were evaluated by compressing gel cylinders (2 cm height and 1.4 cm diameter) using a TAXT-2 Texture Analyser (Stable Microsystems Ltd.). The plunger speed was 2 mm/sec and measurements were performed in an air-conditioned room at 15 ± 2°C. Stress relaxation was followed for 4 min after compression to (usually) a deformation of 25%. In addition, gels were deformed until rupture as evidenced by a peak in the force-time curve. This peak determined the gel breaking force. The data from the stress relaxation determinations were linearized using the method proposed by Peleg (1979). Parameters k_1, k_2 and the asymptotic residual modulus E_A were obtained as described by Nussinovitch et al. (1990), employing the equations,

$$\frac{F_0 \cdot t}{F_0 - F(t)} = k_1 + k_2 t \quad (3)$$

$$E_A = \frac{F_0}{A(\varepsilon)\,\varepsilon}\left[1 - \frac{1}{k_2}\right] \quad (4)$$

where F_0 and $F(t)$ are maximum and momentary force and k_1 and k_2 are constants; and, where E_A is the asymptotic residual modulus—that serves as a measure of the gel solidity—, ε is the imposed strain and $A(\varepsilon)$ is the calculated cross-sectional area of the relaxing specimen, i.e.

$$A(\varepsilon) = \frac{A_o L_o}{L_o - \Delta L} \quad (5)$$

RESULTS & DISCUSSION

MAILLARD REACTIONS involving the reducing sugar xylose and the protein were evidenced by several physicochemical changes occurring during heating. These included a color change from cream to dark brown and a pH decrease. In the absence of xylose or GDL the initial pH of 7.15 ± 0.1 did not alter by more than 0.2 units on heating. However, the pH of the Maillard system decreased to 5.5 ± 0.1. Achieving similar gel pH's using GDL gels over a range of protein concentrations required incorporating different levels of GDL to allow for the buffering effect of SPI. Thus, pH's for the Maillard and GDL gels were comparable.

Although the dialysis tubing was covered with polyethylene film, some weight loss was observed. This was around 10% for Maillard gels and 17% for GDL gels. Within the dialysis tubing a big difference occurred between the two systems with the Maillard gels showing much less syneresis than GDL gels (Fig. 1). Syneresis decreased as protein concentration increased. The higher water holding ability of the Maillard gels probably reflected the higher net charge of the Maillard network at pH 5.5. A reduction would occur in number of positively charged amino groups present, resulting in a reduction in the isoelectric point of the protein. The water holding ability of SPI gels decreases as the isoelectric pH is approached (Van Kleef, 1986). In plots of rheological data, the protein concentrations were calculated assuming the syneresis liquid and the material lost from the dialysis tubing contained no protein.

The dependence of gel strength on protein concentration for the different kinds of gel tested was apparent (Fig. 2). The breaking force of the Maillard gels increased as related to protein concentration at a higher rate than for GDL gels. In confirmation of our preliminary work, the minimum gelling concentration was much higher for gels not containing a reducing sugar (Hill et al., 1992). Both the breaking force and E_A were substantially lower for the SPI gels compared with BSA Maillard and GDL gels prepared under comparable conditions (Armstrong et al., 1994). The asymptotic residual modulus E_A showed a similar protein concentration relationship to that of breaking force (Fig. 3). The relation of k_1 and k_2 on protein concentration was also shown (Fig. 4). These parameters can be considered as a measure of gel elasticity (Peleg, 1979). They may have values between 1 and ∞. For an ideal

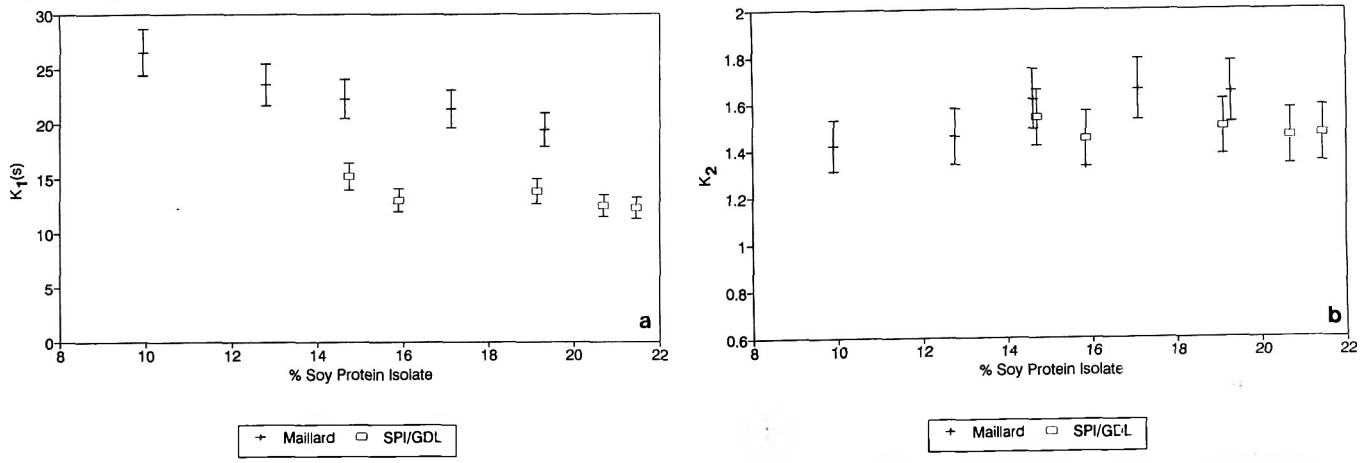

Fig. 4—The gel elasticity parameters (K_1 and K_2) as calculated by Peleg (1979) as related to protein concentration. Details as in Fig.

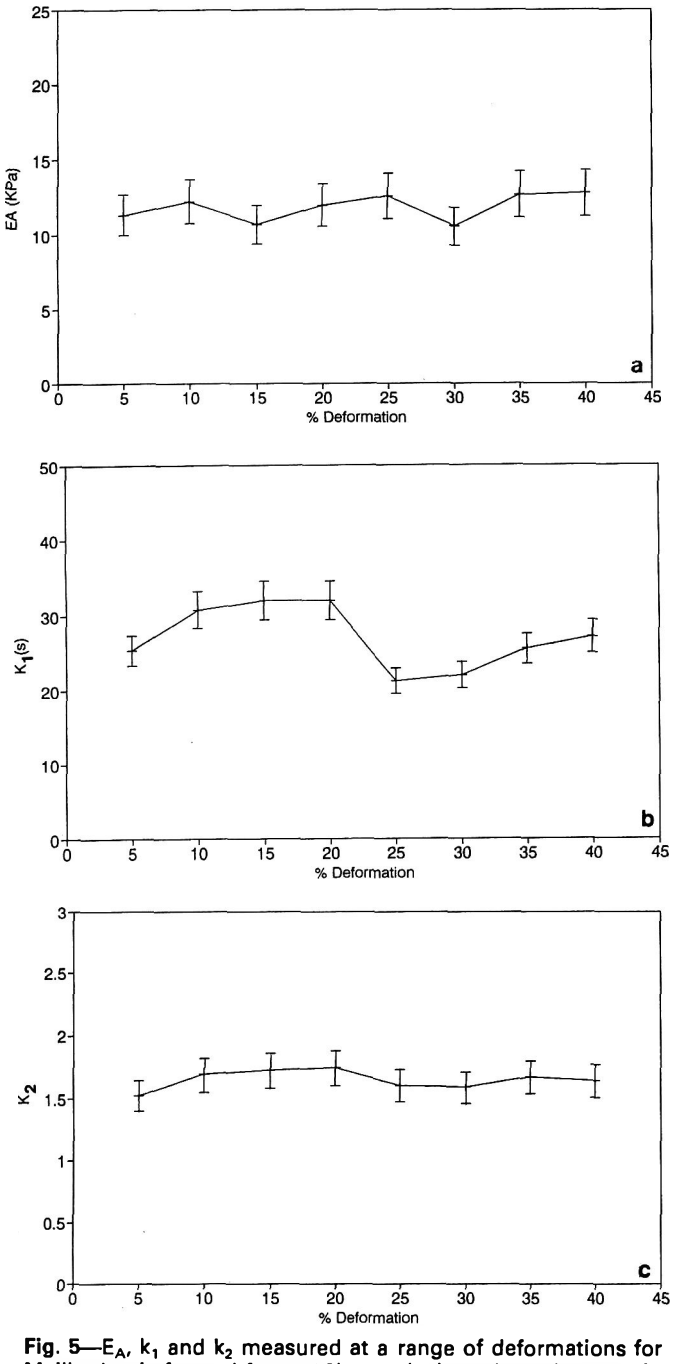

Fig. 5—E_A, k_1 and k_2 measured at a range of deformations for Maillard gels formed from 15% soya isolate plus 3% xylose in water and retorted at 121°C for 1 hr.

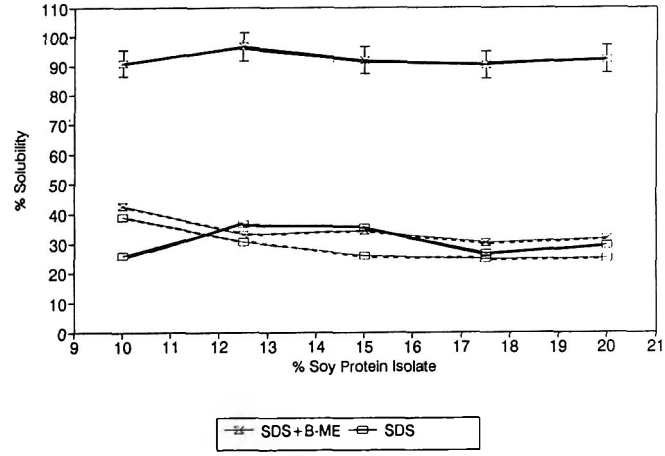

Fig. 6—The percentage of protein solubilized from gels in a mixed solvent containing 1% β-ME + 1% SDS or 1% SDS alone as related to the protein estimated to be in the gel network. GDL gels (−), Maillard gels (−−).

elastic solid, $k_1 = k_2 \rightarrow \infty$, whereas for a nonelastic liquid, $k_1 = k_2 \rightarrow 1$.

Note that our compression test was done at large deformations which must be outside the linear viscoelastic region. The decay in stress with time reflects the rearrangement and/or rupture of crosslinks within the gel structure. Increasing the degree of covalent bonding within the network, as a result of formation of additional Maillard induced crosslinks, reduced this tendency and hence resulted in a more elastic gel. In this respect, note that for the Maillard gels, the related parameters K_2 and E_A show very little relation to the degree of deformation (Fig. 5). This contrasted to the results for polysaccharide gels which did not contain covalent crosslinks (Nussinovitch et al., 1990). K_1 showed a significant decrease at 20% compression which contrasted with our results for Maillard BSA gels where this parameter was independent of compression (Armstrong et al., 1994). Possibly the decrease in K_1 found for SPI gels reflected changes occurring in the region of the gel which was not covalently crosslinked.

Supporting evidence for the increased level of covalent crosslinks in Maillard gel was provided by the studies of solubility in SDS and β-ME. When the solubility in SDS + β-ME were compared (Fig. 6), almost all of the GDL gel was solubilized, while only about 40% of the protein in the Maillard gel became soluble. The insoluble fraction must represent a matrix held by nondisulphide covalent bonds, presumably formed as a consequence of the Maillard reaction.

The solubility in 1% SDS alone was also determined for both types of gel (Fig. 6). Note that, despite the different sol-

ubility in the mixture of solvents shown by each kind of gel, their solubility in SDS was very similar (about 30% of total protein). This may suggest that Maillard crosslinking occurs in a region of the network containing disulphide bonds. We could postulate that the region solubilized by SDS consisted of the 7S soya globulin that contains no disulphide crosslinks (Van Kleef, 1986). This is somewhat supported by the observation that Maillard gels formed from BSA, (a protein high in disulphide crosslinks) were almost completely insoluble in SDS + β-ME (Armstrong et al., 1994).

CONCLUSION

WHEN SUBJECTED TO FOOD STERILIZATION temperatures the gelation behavior of SPI may be substantially improved by incorporation of reducing sugars. The improvement is a consequence of both the pH decrease and the formation of covalent crosslinks. The SPI gels have a lower rigidity and breaking force than the BSA gels prepared under comparable conditions. Probably Maillard crosslinks occurred in the regions of the network which contained disulphide bonds and, thus, the Maillard reaction affected the 11S rather than the 7S globulin.

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*INTERACTION OF FLAVORS WITH FAT REPLACERS. . .*From page 815

ther would affect foods somewhat differently in flavor profiles so flavor reformulation would likely be required.

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*NONDESTRUCTIVE ANALYSES OF VAPOR HEAT-TREATED PAPAYA. . .*From page 857

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Food Protein Nutrient Improvement by Protease at Reduced Water Activity

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ABSTRACT

Protein resynthesis catalyzed by α-chymotrypsin was studied to modify the nutritive quality and physical properties of albumin. The increase in substrate concentration produced an increase in enzyme synthetic action. At low substrate concentration, several water activity depressing additives (salts or polyols) in the reaction media enhanced plastein synthesis. Polyols enhanced the synthetic reaction proportionally to an increase in their molecular size. The positive effect of alkali halides was related to an increase in cation size and decrease in anion size. All plastein products showed a clear increase in nutritional value with respect to the substrate.

Key Words: egg albumin, protein, plastein, chymotrypsin, essential amino acids

INTRODUCTION

UTILIZED PROTEINS are usually characterized by a lack of important physical, nutritional or sensory properties, necessary for their use as human nutrients (Kilara, 1985). High-quality proteins, such as albumin, may be modified into products of added value which could be used as additives for foods with better acceptance (flavor, texture and eye appeal). Supplementing certain food proteins with essential amino acids is important to improve their nutritional quality. Covalent attachment of amino acids by peptide bonding is effective because the amino acids are more stable during food processing, storage and cooking, and have better nutritional efficiency in digestibility and lumen transport. Chemical methods to incorporate amino acids into food proteins have not been practical (Whitaker, 1986).

Enzymatic protein degradation and resynthesis (plastein reaction) have been described as methods to improve both the functional quality and the nutritive values of food proteins (Fujimaki *et al.*, 1977; Edwards and Shipe, 1978). By this technology, several food proteins (i.e., soybean protein, gluten, zein) have been nutritionally improved by incorporation of essential amino acids (i.e., Leu, Lys, Met Thr, Trp) into the polypeptide chain, using L-amino acid esters as substrates. Several physical and functional properties (solubility, emulsifying capacity, antifreeze properties) of food proteins have been changed by the plastein reaction. Thus, the covalent attachment of a highly hydrophilic (glutamic acid-ethyl ester) or hydrophobic (leucine-n-dodecyl ester) amino acid ester to a food protein has increased the solubility of denatured soybean protein or produced effective food surfactants (Arai *et al.*, 1986).

Generally the plastein reaction involves formation of polypeptides by a reversal of the usual peptide bond hydrolysis to produce synthesized proteins (plasteins) from a protein hydrolysate. Three general conditions are required for the plastein reaction to occur: a low molecular weight substrate, a substrate concentration > 10% (w/v) and optimum pH value for synthetic activity of the enzyme (Tsai *et al.*, 1972; Fujimaki *et al.*, 1977; Lozano and Combes, 1991). Moreover, previous results (Lozano and Combes, 1992; Combes and Lozano, 1992) showed water activity (a_w) of the reaction medium was a fourth important factor in control of plastein synthesis.

The effects of proteolytic enzymes and their kinetic mechanisms in the plastein reaction are not clearly understood. In the hydrolytic reactions, catalyzed by serine-proteases, a transitory acyl-enzyme intermediate transfers the acyl group to water. However, in enzymatic peptide synthesis, the acyl group is transferred to other nucleophiles, such as the amino groups amino acids, resulting in synthesis by a transpeptidation mechanism, as postulated for the plastein reaction (Tsai *et al.*, 1972; Hofsten and Lalasidis, 1976; Fujimaki et al., 1977; Edwards and Shipe, 1978). Other researcher (Determann *et al.*, 1965; Yamashita *et al.*, 1973), suggested that plastein synthesis occurs by a condensation mechanism, as a reversal of the usual hydrolytic reaction. Lozano and Combes (1992) proposed an overall integration of all proteolytic reactions (hydrolysis, transpeptidation and condensation) as a general mechanism of plastein synthesis. They discriminated between the effects of substrate and water. Thus, at low substrate concentrations (<10% w/v), which induce a high a_w, both transpeptidation and hydrolytic reactions would be involved. However, at high substrate concentration (>30% w/v), the condensation catalytic pathway would occur. At low substrate concentration and low a_w, produced by manipulation with additives (polyols or salts), the plastein reaction was enhanced via the condensation pathway (Iborra *et al.*, 1992; Lozano and Combes, 1993). Our objective was to establish a relationship between microenvironmental conditions of the plastein reaction (defined by type additive and substrate concentration) and amino acid composition of the synthesized plasteins, for possible use of proteins in novel food applications.

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MATERIALS & METHODS

Materials

Bovine serum albumin (Sigma Chem. Co., St. Louis, MO) was used for preparation of substrate. Pepsin (EC 3.4.23.1.) from porcine stomach mucosa, was the hydrolyzing catalyst to prepare the substrate. α-chymotrypsin (EC 3.4.21.1.) from porcine pancrease was used to catalyze the plastein reaction. Both enzymes were obtained from Sigma Chem. Co. All other reagents were analytical grade.

Substrate

A 10% (w/v) albumin solution (pH = 1.6) was treated by pepsin (1% w/w) with magnetic stirring for 48 hr at 40°C. The reaction was stopped by increasing the pH to 7.0 with NaOH, and the resulting hydrolysate solution was ultrafiltered through polysulphone membranes (10,000-dalton cut-off) and then concentrated to 55% (w/v) in rotoevaporator (Lozano and Combes, 1991).

Plastein reaction and activity

Into 1 mL Eppendorf tube 100 mg concentrated hydrolysate of albumin was placed, and 3.5 mg α-chymotrypsin was added. The reaction volume was adjusted to 1 mL with water or 3M aqueous additive

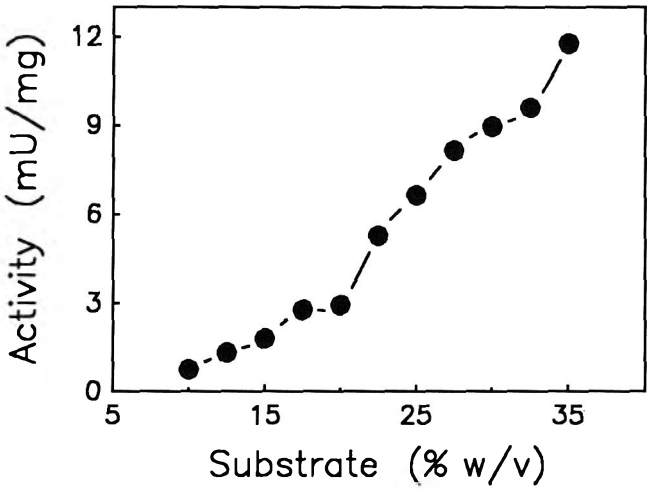

Fig. 1—Effect of substrate concentration on the plastein activity of α-chymotrypsin. A peptic hydrolysate of albumin as substrate under optimal conditions of assay (40°C, pH 7.0) was used.

(polyol or salt), and the reaction mixture incubated without stirring at 40°C (Lozano and Combes, 1991). Aliquots of 50 μL were extracted from the reaction mixture, previously homogenized by shaking, at different times for plastein product quantification.

Total plastein products were determined as the fraction that precipitated in a 10% (w/v) trichloroacetic acid solution (TCA) and quantified spectrophotometrically at 280 nm, by redissolving in 50% (v/v) acetic acid solution (Sukan and Andrews, 1982). One unit of plastein activity (U) was defined as the amount of enzyme that produced 1 mg plastein/min under optimum conditions (Lozano and Combes, 1991).

Water activity

Water activity was determined using a humidity and temperature digital indicator, HUMIDAT-IC II (Novasina, Zürich, Switzerland), with a humidity sensor model BS - 3(4)/PP (Novasina) 20°C. The humidity sensor was checked and periodically recalibrated at three points, with control saturated salt solutions (LiCl, a_w = 0.113; $Mg(NO_3)_2$, a_w = 0.544; and $BaCl_2$, a_w = 0.905) for the overall measuring range.

Amino acid analysis

A lyophilized sample (1 mg) was hydrolyzed in a Pico-Tag system (Waters Chromatography, Milford, MA) as follows: first, air was removed exhaustively from the system by a repeated freeze-thaw technique and then, the hydrolysis was carried out in 6N HCl at 110°C for 24 hr. Later, free amino acids were determined with an AminoQuant (Model 1090 Series II, Hewlett-Packard France, Toulouse, F) automatic amino acid analyzer, using both o-phthalaldehyde and 9-fluorenylmethyl chloroformate pre-column derivatization methods for spectrophotometric quantification. All the amino acid analyses were performed in duplicate (Kamp, 1991; Lozano and Combes, 1993).

RESULTS & DISCUSSION

Effects of substrate concentration on plastein synthesis

The effect of substrate concentration in the range 10–35% (w/v) on plastein activity of α-chymotrypsin, under optimal assay conditions (Fig. 1) showed plastein activity increased proportionally with substrate concentration. This was explained by the increase in concentration of free amino groups, acting as nucleophile acceptors of acyl groups transferred by the enzyme, and by the reduction in water activity, favoring the synthetic over hydrolytic pathway (Tsai *et al.*, 1972; Hähn-Hagerdal, 1986; Combes and Lozano, 1992).

When the influence of substrate concentration was determined on characteristics of plastein products, the amino acid composition (Table I) of plastein products generally increased in the hydrophobic amino acids. Thus, Asp, Glu, Ser and Thr

Table 1—Amino acid (AA) composition, based on hydrophobicity, of a paptic hydrolysate of albumin (substrate), and resynthesized plastein products[a]

AA	HΦ (kcal/mol)	Substrate (%)	Plastein products[b] Substrate concentration 10%	15%	20%	25%	30%
Ser	−0.30	4.5	2.3	3.8	3.8	3.9	3.9
Glu	0.00	15.5	5.9	7.6	8.5	8.4	8.5
Asp	0.00	9.4	4.6	5.6	6.5	6.5	6.3
Gly	0.00	3.1	3.5	5.1	3.0	2.5	2.6
Thr	0.40	6.1	2.2	4.4	5.1	5.7	5.6
Ala	0.50	9.0	6.0	7.0	6.4	6.4	6.4
His	0.50	2.5	0.7	1.4	1.9	2.5	2.5
Arg	0.75	3.7	1.8	3.0	3.5	4.1	4.2
Cys	1.00	2.1	0.0	3.3	3.9	4.6	4.4
Met	1.30	0.8	0.7	0.9	1.0	1.1	1.2
Lys	1.50	9.1	2.8	5.6	6.0	7.1	7.3
Val	1.59	6.7	2.6	6.3	7.8	9.0	9.1
Leu	1.80	12.1	3.3	13.8	18.0	20.8	21.2
Tyr	2.30	3.9	54.7	20.8	11.2	4.7	4.3
Phe	2.50	6.1	5.3	5.1	5.5	5.1	4.9
Pro	2.60	2.9	1.6	2.0	2.9	2.2	2.4
Ile	2.95	2.4	2.0	4.3	5.0	5.4	5.2

[a] Obtained by α-chymotrypsin at different substrate concentrations (10–30% w/v), under optimal conditions of assay (pH 7.0 and 40°C).
[b] amino acid.

decreased, while Ile, Leu, Val and Tyr contents increased. The increase in substrate concentration resulted in plastein products of very different amino acid compositions. Thus, at the lowest substrate concentration, with highest water activity (0.98), the plastein product showed high Tyr (54.7%). This could be explained by the specificity of the α-chymotrypsin for aromatic amino acids and the low solubility of the peptides containing Tyr. However, Phe did not show similar results. The increase in substrate concentration produced a decrease in Tyr content of the products, and the concomitant increase in other hydrophobic amino acids, such as Lys, Val, Pro, Ile and Leu (from 3.3 to 21.2%).

The important effects of hydrophobic amino acids on physical and functional properties of food proteins emphasized the need for quantification of an overall index of protein hydrophobicity. Different scales have been proposed to measure the average protein hydrophobicity, based on several criteria. Molar free energy transfer (ΔG^o) for each amino acid from water to vapor phase or from water to organic solvent was proposed. The empirical distribution of amino acids in protein structure may be a criterion. Some consensus scales may be derived by considering both experimental partition data and empirical distribution of amino acids (Thorton and Taylor, 1989). In our case, a hydrolysate and denatured polypeptide were used as substrate. The amino acids were arranged in order of hydrophobicity of their side chain ($H\Phi_I$, the molar free energy transfer for the amino acid with respect to Gly, from aqueous solution to solution in organic solvent at the same mole fraction at infinite dilution), as described by Bigelow and Channon (1976). Thus, an average hydrophobicity ($H\Phi_{avg}$) of the substrate and the plastein products could be determined as follows:

$$H\Phi_{avg} = \sum_{i=q}^{n} X_I \cdot H\Phi_I$$

where X_I is the molar ratio of the amino acid and $H\Phi_I$ is the hydrophobicity of the amino acid. On the other hand, Ikai (1980) proposed the use of aliphatic amino acid content as a measure of protein hydrophobicity, establishing an Aliphatic Index (AI) of protein, which could be determined as follows:

$$AI = X_A + a \cdot X_V + b \cdot (X_I + X_L)$$

where X_A, X_V, X_I and X_L are molar ratios of Ala, Val, Ile and Leu in the protein, respectively, and **a** and **b** are numerical coefficients of molecular size relative to the molecular size of Ala (a = 1.39; b = 1.59).

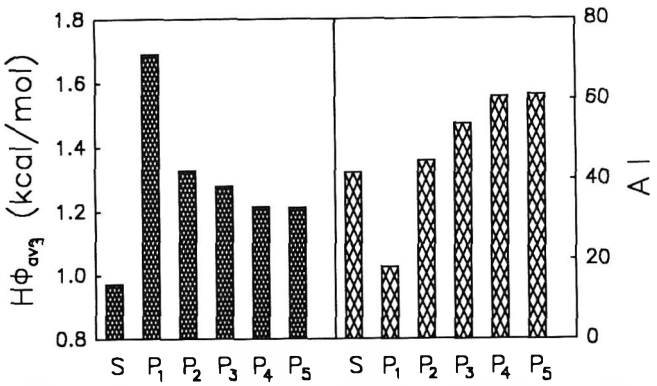

Fig. 2—Average hydrophobicity ($H\Phi_{avg}$) and aliphatic index (AI) of the substrate (S) and plastein products (P_1, P_2, P_3, P_4 and P_5). A peptic hydrolysate of albumin as substrate at concentrations of 10, 15, 20, 25, and 30% w/v was treated with α-chymotrypsin.

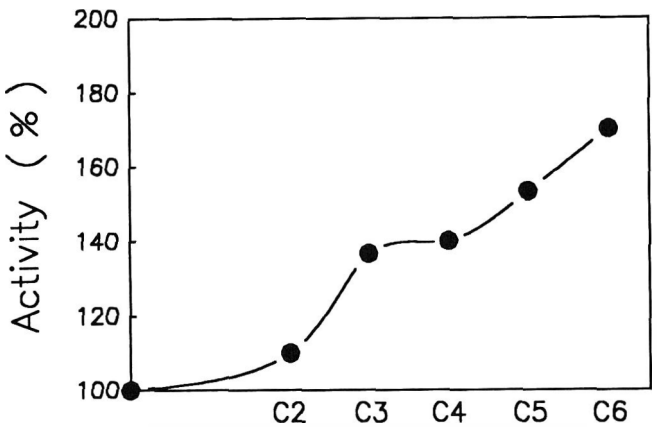

Fig. 3—Effect of size of polyol molecule (C2, ethylene glycol; C3, glycerol; C4, erythritol; C5, xylitol and C6 sorbitol) at 3M concentration on the plastein reaction catalysed by α-chymotrypsin. A 10% (w/v) peptic hydrolysate of albumin as substrate under optimal conditions of assay (40°C, pH 7.0 was used.

In all cases, the $H\Phi_{avg}$ of the plastein products was higher than those of the substrate (Fig. 2) probably favored by their lower water solubility, shifting the reaction equilibrium to the synthetic path. Furthermore, when the substrate concentration was increased, the $H\Phi_{avg}$ of the plastein products decreased. On the other hand, at the lowest substrate concentration (P_1), the **AI** of the plastein products was lower than that of the substrate. However, the increase in substrate concentration from 15 to 30% (w/v) increased the **AI** of the products more than that shown by the substrate.

These results were in agreement with the proposed mechanism of plastein synthesis (Lozano and Combes, 1992). Thus, at low substrate concentration (a_w>0.97), where hydrolytic and transpeptidation activities are involved, only plastein products with high content of low-water-soluble amino acids (i.e., Tyr) would be in the protein precipitate. When substrate concentration was increased (a_w<0.97), the high content in free amion groups influenced the synthesis towards hydrolytic reactions. Transpeptidation and condensation plastein products could be formed with reduced aromatic amino acids and increases in other hydrophobic amino acids in the product (Ala, Ile, Leu and Val). As postulated, if the increase in substrate concentration enhanced the condensation pathway, the amino acid composition of the plastein product would become like that of the substrate, decreasing the average hydrophobicity of the product (Iborra *et al.*, 1992; Lozano and Combes, 1993).

Effects of polyols in plastein synthesis

Enzymic hydrolyses are reactions in which the equilibrium is shifted in the direction of hydrolysis, which consumes one or more water molecules (Hähn-Hagerdal, 1986). It is then reasonable to assume that a medium with decreased water activity, produced by high substrate concentration or water activity reducing agents, should shift the equilibrium toward synthetic reactions (Iborra *et al.*, 1992). Therefore, the effects of different 3M solutions of polyhydroxylated additives containing 1 to 6 carbon atoms (ethylene glycol, glycerol, erythritol, xylitol and sorbitol) on the plastein reaction catalyzed by α-chymotrypsin at the lowest substrate concentration (10% w/v) was studied. The increase in number of hydroxy groups per molecule of additive increased plastein activity (Fig. 3). This enhancement of enzyme activity could be explained by the substantial reduction of a_w in the reaction media (from 0.98 to 0.89) caused by the presence of the additives (Combes and Lozano, 1992; Lozano and Combes, 1992).

Table 2—Effect of different 3M polyol solutions[a] on the amino acid (AA) composition of the resynthesized plastein products[b]

	Plastein products (%)				
AA	C2	C3	C4	C5	C6
Ser	3.9	4.0	3.9	3.8	3.7
Glu	9.0	9.3	9.4	9.6	10.0
Asp	6.5	6.6	6.7	6.8	7.4
Gly	1.8	1.9	1.8	1.7	1.6
Thr	6.0	6.1	6.0	6.0	6.0
Ala	5.8	6.0	6.1	6.1	6.4
His	1.8	1.9	2.0	2.1	2.2
Arg	4.1	4.0	4.2	4.3	4.4
Cys	5.3	4.8	4.7	5.2	5.5
Met	0.9	1.1	0.9	0.9	0.9
Lys	6.2	6.5	7.3	7.4	7.9
Val	9.7	9.6	9.5	9.0	8.2
Leu	23.9	23.4	23.2	22.0	21.1
Tyr	3.5	3.0	3.0	3.0	3.1
Phe	4.7	4.6	4.5	4.3	4.2
Pro	2.0	2.4	2.0	3.0	3.1
Ile	4.9	4.9	4.8	4.8	4.2

[a] C2, ethylene glycol; C3, glycerol; C4, erythritol; C5, xylitol and C6, sorbitol.
[b] Obtained by α-chymotrypsin, using a 10% (w/v) peptic hydrolysate of albumin as substrate, under optimal conditions of assay (pH 7.0 and 40°C).

The amino acid composition of the plastein products (Table 2) showed those products obtained in the presence of polyols were very similar. However, the amino acid contents were very different from those at the same substrate concentratons (10% w/v) without additives and very similar to those at high substrate concentrations (Table 1). However, a slight decrease in both the $H\Phi_{avg}$ and the **AI** of the plastein products occurred (Fig. 4) when the molecular size of polyol was increased. In all cases, both parameters were higher than that shown by the substrate, which proved that the changes were produced in the physical properties of the protein. These results indicated that the effect of reduction in water activity induced by polyols should be similar to that from increase in substrate concentration (Combes and Lozano, 1992). However, the enhancement of plastein synthesis by increased substrate concentration was greater than that from decreased water activity, produced by polyols. Thus, a_w should be regarded as a main influence on the reaction, but substrate concentration is the most important factor in enzyme activity (Lozano and Combes, 1992).

Effects of salts in plastein synthesis

The influence of six different alkali halides on plastein synthesis catalyzed by α-chymotrypsin at the lowest substrate concentration (10% w/v) was also studied with both anions (Fig. 5) the increase in cation size enhanced the catalytic capability of the enzyme in plastein synthesis, but the effect was lessened by the cation of highest size (Li^+<Na^+=K^+). The degree of activation decreased with increasing anion size (Cl^->Br^-). Moreover, this effect was additive: when the largest anion (Br^-) and the smallest cation (Li^+) were assayed together, no effect on activity was observed. With the smallest anion (Cl^-) and the larger cations (Na^+ or K^+) activity was higher than

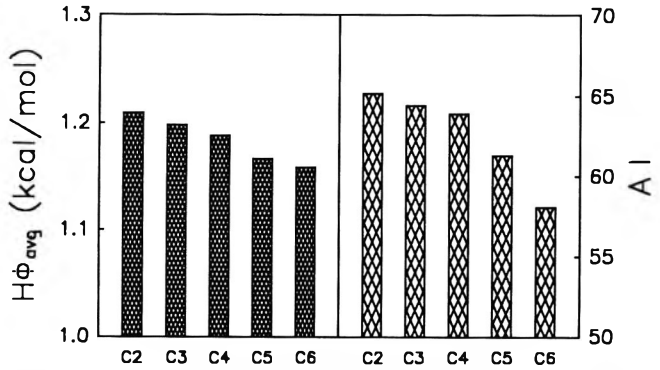

Fig. 4—Average hydrophobicity ($H\Phi_{avg}$) and aliphatic index (AI) of the plastein products obtained by α-chymotrypsin in the presence of different 3M polyol solutions. C2, ethylene glycol; C3, glycerol; C4, erythritol; C5, xylitol and C6 sorbitol. A 10% (w/v) substrate concentration was used.

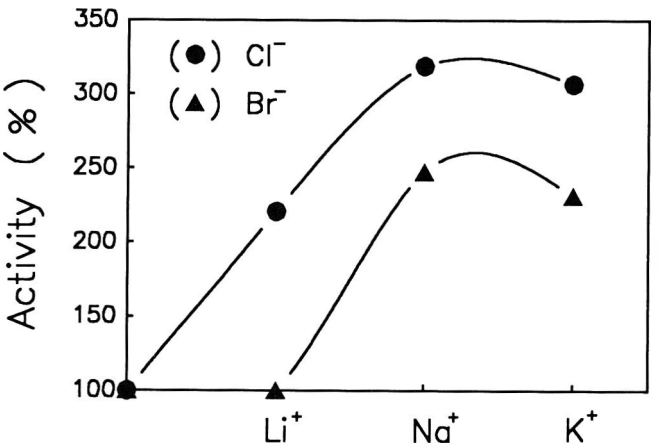

Fig. 5—Effect of alkali halides at 3M concentration on the plastein reaction catalyzed by α-chymotrypsin as a function of the assayed ions. A 10% (w/v) peptic hydrolysate of albumin as substrate under optimal conditions of assay (40°C, pH 7.0) was used.

Table 3—Effect of different 3M alkali halide solutions on the amino acid (AA) composition of the resynthesized plastein products[a]

	Plastein products (%)					
AA	LiCl	NaCl	KCl	LiBr	NaBr	KBr
Ser	4.3	4.0	3.8	6.0	4.2	3.9
Glu	8.9	9.1	9.3	5.8	7.6	7.8
Asp	6.3	6.2	6.1	5.9	5.6	5.2
Gly	2.9	2.0	1.8	5.0	2.3	1.9
Thr	5.7	5.7	5.7	6.7	5.9	5.8
Ala	7.3	7.1	7.0	7.1	6.6	6.5
His	2.2	2.5	2.7	2.1	2.3	2.4
Arg	4.6	4.7	4.9	4.1	5.0	5.3
Cys	3.9	3.7	1.6	3.5	3.1	2.5
Met	0.8	1.0	1.0	1.0	0.7	1.0
Lys	7.3	7.5	8.0	5.5	7.2	7.5
Val	9.5	8.2	8.0	12.3	10.2	8.8
Leu	19.7	21.0	21.9	20.5	22.4	23.5
Tyr	4.0	4.2	4.6	3.5	4.2	4.4
Phe	5.6	6.0	6.2	4.0	5.6	5.8
Pro	3.3	3.2	3.1	3.1	3.0	2.9
Ile	3.7	3.9	4.3	3.9	4.1	4.8

[a] Obtained by α-chymotrypsin, using a 10% (w/v) peptic hydrolysate of albumin as substrate, under optimal conditions of assay (pH 7.0 and 40°C).

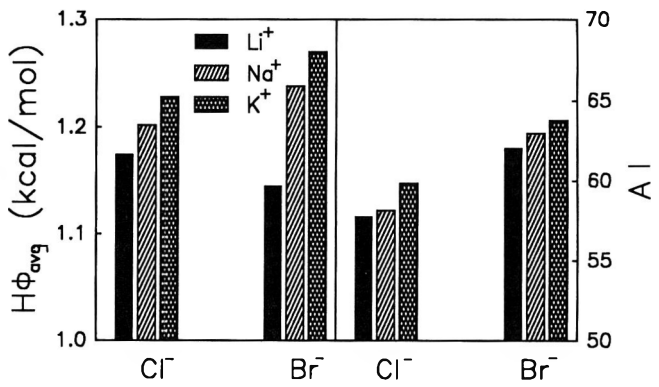

Fig. 6—Average hydrophobicity ($H\Phi_{avg}$) and aliphatic index (AI) of the plastein products obtained by α-chymotrypsin with different 3M salt solutions, LiCl, NaCl, KCl, LiBr, NaBr and KBr. A 10% (w/v) substrate concentration was used.

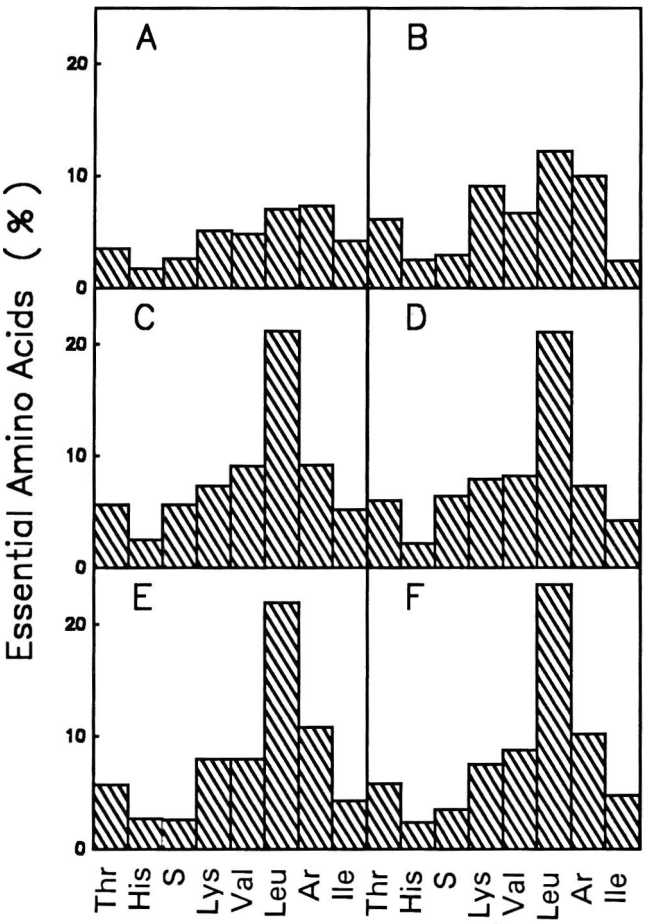

Fig. 7—Essential amino acid patterns for human nutrition of different products. S represent the S-amino acids content and AR represent the aromatic amino acids content; A: Minimum requirements for human adult, according to FAO/WHO standards; B: Plastein product obtained by α-chymotrypsin using a 30% (w/v) substrate concentration; D: Plastein product obtained by α-chymotrypsin with 3M sorbitol, using a 10% (w/v) substrate concentration; E: Plastein product obtained by α-chymotrypsin with 3M KCl, using a 10% (w/v) substrate concentration; F: Plastein product obtained by α-chymotrypsin with 3M KBr, using a 10% (w/v) substrate concentration.

with Li^+. These results of the water activity reducing power of salts (between 0.88 and 0.83, as a function of type of salt) could be related with the Hofsmeister lyotropic series. The property of a salt that affects hydrophobic interactions was quantified by the molal surface tension increment of the medium, which was classified in the order of the Hofmeister series (Melander and Horvath, 1977). These effects enhanced plastein product precipitation by salting-out in Na^+ and K^+ cases, and reduced the synthetic reaction by salting-in Li^+ (Lozano and Combes, 1993).

The amino acids of the plastein product synthesized by α-chymotrypsin in the presence of alkali halides (Table 3) were also different from those at the same substrate concentration (10% w/v) without additives. They were also very similar to those at high substrate concentration (Table 1). On the other hand, the increase in cation size resulted in an increase in the Ile, Leu, Lys Phe and Tyr contents, and a decrease in the Ala, Asp, Cys, Gly and Val contents. This effect was clearly evident (Fig. 6) where both the $H\Phi_{avg}$ and the **AI** of the plastein products were related to anion and cation types. The increase in cation size increased both the $H\Phi_{avg}$ and the **AI** hydrophobic parameters. Furthermore, this effect was enhanced by the increase in anion size ($Br^- > Cl^-$). These results were in agreement with the proposed mechanism of plastein synthesis and the salting-out hypothesis. Thus, the effect of the decrease in a_w induced by salts should be similar to those caused by the presence of polyols or by the increase in substrate concentration (Lozano and Combes, 1992, 1993). As a function of salting-out, the presence of salt produced a high exposure of hydrophobic amino acid residues of the polypeptides. This increased plastein synthesis by the specificity of the enzyme towards aromatic residues and the shift of equilibrium to synthesis by a decrease in solubility of reaction products.

Nutritional properties of plastein products

Nutritional quality of plastein products synthesized at different conditions under depressed a_w (i.e., high substrate and low substrate with additives) was calculated and compared to standards of the Joint FAO/WHO *Ad Hoc* Expert Committee (Fujimaki *et al.*, 1977), for minimum nutritional requirements of essential amino acids for human adults (Fig. 7). The peptic hydrolysate of albumin used as substrate (Fig. 7B) showed an effective essential amino acid content, except for Ile. In the case of plastein products, (Fig. 7 C-F), the essential amino acid patterns were very similar and independent of assayed reaction conditions. In all cases, essential amino acids patterns were more effective than that shown by the substrate. Thus, the Leu, Val and S-amino acids were 2× higher than that of the substrate and the FAO/WHO standards. These results clearly showed improved nutritional properties in albumin from the plastein reaction, yielding a product with increased value as a food supplement.

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Free D- and L-Amino Acid Evolution During Sourdough Fermentation and Baking

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ABSTRACT

The evolution of free D- and L-amino acids in sourdoughs started with various lactic acid bacteria (LAB) and yeasts was studied. *Lactobacillus brevis* subsp. *lindneri* CB1 and *Lactobacillus plantarum* DC400 had high proteolytic activity. During sourdough fermentation, *Saccharomyces cerevisiae* 141 and *Saccharomyces exiguus* M14 sequentially utilized free amino acids produced by bacterial activity. Due to increased cell yeast autolysis, more *S. exiguus* M14 inocula caused more free amino acids which were partially utilized by LAB without causing hydrolysis of wheat flour protein. D-alanine, D-glutamic acid and traces of other D-isomers were observed in sourdoughs fermented with *L. brevis* subsp. *lindneri* CB1 and *S. cerevisiae* 141. Free total D- and L-amino acid content decreased by more than 44% after baking the sourdoughs. No abiotic generation of new D-amino acid isomers was detected in the baked sourdoughs.

Key Words: sourdough, lactic acid bacteria, amino acids, bread, fermentation

INTRODUCTION

THE NUTRITIONAL AND SENSORY CHARACTERISTICS of bread are influenced by the ingredients, sourdough fermentation and baking process (Hansen et al., 1989). Sourdough fermentation is necessary to render the flour suitable for baking, to control development of characteristic flavor components, to achieve dough leavening with yeast and to inhibit undesiderable fermentations by other bacteria and yeasts (Spicher, 1986). The main consideration concerning effects on flavor is carbohydrate metabolism by lactic acid bacteria (LAB) (Spicher and Stephan, 1984). Nevertheless, amino acids, originating from degradation of flour proteins during sourdough fermentation, can be important for the microbial dynamic and for bread crust flavor (Rothe, 1975). Spicher and Nierle (1988) investigated the proteolytic activity of rye sourdough bacteria, while Barber et al. (1987a,b) reported the evolution of water- and salt-soluble proteins during bread-dough fermentation. The main difficulties in the study of a complex biological system such as sourdough, result from the numerous interactions among the environment, microorganisms and technological treatments (Kennes et al., 1991). Little research has been reported on the proteolytic activity of wheat sourdough starters or on amino acid evolution due to biological interactions and the baking process. Studies have been reported on the biotic or abiotic generation of D-amino acid forms in other foods (Bruckner and Hausch, 1990a,b) but none has been reported for the wheat bread-making process.

Our objective was to determine the proteolytic activity of the most important wheat sourdough starters (*Lactobacillus brevis* sups. *lindneri* CB1, *Lactobacillus plantarum* DC400, *Saccharomyces cerevisiae* 141 and *Saccharomyces exiguus* M14) and analyze the D- and L-amino acid evolution as the consequence of LAB-yeast interactions during leavening or heat treatment during sourdough baking.

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MATERIALS & METHODS

Microorganisms and media

Lactobacillus brevis subsp. *lindneri* CB1 (heterofermentative), *Lactobacillus plantarum* DC400 (homofermentative), *Saccharomyces cerevisiae* 141 (maltose-positive), and *Saccharomyces exiguus* M14 (maltose-negative), all isolated and identified from Italian wheat sourdoughs (Gobbetti and Rossi, 1991), were selected because they were typical sourdough microorganisms for Italian bread-making. Lactic acid bacteria and yeast were cultured on sour dough bacteria (SDB) (Kline and Sugihara, 1971) and Saboureaud (Difco Laboratoires, Detroit) media, at 28°C for 24 hr. Cells were harvested at 10,000 × *g* for 10 min, washed twice with sterile, distilled water and then resuspended in sterile water at optical density (620 nm) of 12.0, yielding about 10^9 and 10^7 CFU/mL for LAB and yeasts respectively.

Dough kneading

Type 0 wheat flour contained: moisture 12.8%, protein (N × 5.83) 10.6% of dry matter (d.m.), fat 1.79% of d.m., and ash 0.56% of d.m. Wheat flour (187 g), 60 mL tap water and 30 mL of the cellular suspension, containing one or more microorganisms, at the concentration of 10^9 or 10^7 CFU/mL for LAB or yeasts, respectively, were used to produce 280g of dough (dough yield = 148) with a continuous high speed mixer (60 × *g*; dough mixing time = 5 min) (Boulogne, Seine, France). The initial cellular concentration in the dough corresponded to about 10^7 and 10^5 CFU/g for LAB and yeasts, respectively. The assay conducted with more *S. exiguus* M14 inocula had 10^7 CFU/g.

Sourdough fermentation and baking

Sourdoughs were fermented with individual microorganisms or with various associations of selected LAB and yeasts. For a better understanding of the proteolytic activity, the fermentation process at 28°C was first checked at 4 hr and also extended to 24 hr. After 4 hr fermentation, sourdoughs prepared with the most interesting microbial combinations were baked in a peel baking oven at 250°C for 30 min. After baking, amino acid analysis was conducted either on whole bread or on the crumb and crust, separately. Also for these assays results were expressed as mg free amino acids/kg of initial sourdough, considering the water loss (ranged from 7 to 13% of dough weight) due to baking.

Bacteria and yeasts viable cell counts

To determine the influence of amino acid availability on starter cell yield, 10g of each fermented sourdough plus 90 mL sterile distilled water were homogenized with a Classic Blender (PBI International, Milan). LAB and yeasts were determined on the homogenized sample by plating on SDB and Wallerstein Laboratory (WL) Nutrient (Difco) media at 28°C for 72 hr, respectively.

Isolation of amino acids and high resolution gas chromatography (HRGC)

Fifteen mL of a D-L-nor-leucine reference solution (300 ppm) were added as double internal standard (I.S.) to 10g of fermented, or fermented-baked sourdough. The samples were homogenized with a Classic Blender, 100 mL of hexane added and then shaken at 100 rpm for 30 min at 30°C to completely remove the fatty fraction. After centrifugation at 12,000 × *g* for 30 min, the organic supernatant was removed and the sample dialyzed (tubing size ≈ 11.9 cm, Medicell International LTD, London) against bi-distilled water at 4°C for 24 hr. The liquid fraction from dialysis was concentrated to ca. 5 mL (under reduced

pressure, at 40°C) and a 2 mL aliquot of concentrated sample was passed through an AG 50W-X8 ion-exchange column (Bio-Rad, Hercules, CA), with 1M HCl eluent. After washing with 45 mL of distilled water, a 60 mL fraction was collected and concentrated to 5 mL under reduced pressure at 40°C. A 0.5 mL aliquot of concentrated sample was treated to obtain amino acid isopropyl-trifluoroacetyl derivatives which were used for HRGC analysis (Gobbetti et al., 1993). Amino acid profiles were obtained using a gas chromatograph VARIAN 3400 (Varian Instrument Group, Sunnyvale, CA) with a split-splitless injector (in split mode ratio 1 :30) and flame ionization detector on a 25m × 0.25 mm chirasyl-L-val column (Chrompack, Midelburg, The Netherlands). The column oven was temperature programmed: 60°C (15 min), to 88°C at 1.5°C/min, 88°C (6 min), to 195°C at 2°C/min and held at 195°C for 15 min. Other GC conditions were: injector and detector temperatures, 230°C; H_2 flow rate, 20 mL/min; N_2 flow rate (make up), 20 mL/min; air flow rate, 220 mL/min; carrier gas He, head pressure, 780 mmHg, and injection size, ca 0.1μL. The amino acids were identified by comparing retention times with amino acids from a reference solution.

Statistical analyses of data

Results are means of three replicates obtained by three different sourdough and bread productions. Standard deviation (SD) ranged between 0.1 and 14.5 for values below 100 mg/kg and from 11.3 to 125.8 for values between 100 and 1900 mg/kg. ANOVA of the data were carried out (Stanton, 1988).

RESULTS & DISCUSSION

Total D- and L-amino acid evolution during sourdough fermentation

Individual amino acids were grouped (Table 1) as aliphatic (leu, ala, val, gly, ile), dicarboxylic (asp, glu), hydroxy (ser, thr), basic (arg, lys, his), sulphur-containing (met, cys), aromatic (phe, tyr) and cyclic (pro) as reported (Morimoto, 1966; Spicher and Nierle, 1984) to make discussion and statistical analyses clearer. The wheat flour dough was characterized by a total amino acid concentration of 736 mg/kg with high levels of aspartic and glutamic acids (285 and 70 mg/kg, respectively). These amino acids are the major components of the glutenin subunits (Khan and Bushuk, 1979). The addition of the cellular suspension during dough preparation did not modify ($P < 0.05$) the total amino acid composition of wheat flour (data not shown).

The sourdoughs started with LAB or with associations of LAB and yeasts showed at 24 hr pH values of 3.7 ≤ 4.0, while those produced with yeasts or the reference test (unstarted) did not drop below pH 6 0. LAB had high proteolytic activity. At 24 h *L. brevis* subsp. *lindneri* CB1 caused an increase of 1037 and 818 mg/kg of free D- and L-amino acids with respect either to spontaneously fermented sourdough (unstarted) or to initial concentrations in the dough, respectively (Table 1). The proteolytic activity of *L. brevis* subsp. *lindneri* CB1 was in particular shown by changing amounts of specific amino acids. Compared to unstarted sourdough, its action increased ($P < 0.05$) the concentration of aliphatic (alanine 101, valine 131, isoleucine 80, and leucine 270 mg/kg as partial increases, respectively), dicarboxylic (glutamic acid increase of 184 mg/kg) and hydroxy (seryne increase of 82 mg/kg) amino acid groups. All aliphatic and dicarboxy amino acids, with exception of glycine and aspartic acid, are essential for optimal sourdough LAB growth (Spicher and Shroder, 1979; Gobbetti et al., 1994a). By contrast, no differences ($P < 0.05$) between unstarted and *L. brevis* subsp. *lindneri* CB1 sourdough were detected for the other amino acid groups. *L. plantarum* DC400, with a final D- and L-amino acid concentration of 1510 mg/kg, showed no significant ($P < 0.05$) difference in proteolytic activity with respect to *L. brevis* subsp. *lindneri* CB1, while reflecting a common amino acid metabolism of the main sourdough LAB.

In sourdoughs fermented with yeasts the total D- and L-amino acid concentration was lower than the initial levels in the dough. *S. cerevisiae* 141 and *S. exiguus* M14 did not give differences ($P < 0.05$) in the total concentration. Their amino acid profiles were about the same with a difference only in the partial amount of the aliphatic amino acid group. Compared to the initial concentration in the wheat dough, the yeast profile was characterized by a marked reduction of dicarboxylic amino acid groups with particular relevance for aspartic acid concentration (from 285 to 37 mg/kg in the sourdough started with *S. exiguus* M14) and by a small increase of lysine (53 to 96 mg/kg), only for *S. cerevisiae* 141. These findings strongly confirmed the weak proteolytic activity of sourdough yeasts (Spicher and Nierle, 1988) and suggested the partial integration of wheat flour lysine by those derived from autolysis of the baker-yeasts (Becker, 1966).

The sourdoughs fermented by associations of each LAB and yeasts showed a total D- and L-amino acid concentration intermediate between the high value reached in presence of the bacteria and the low value in presence of the yeasts. The sourdough produced by the association of *L. brevis* subsp. *lindneri* CB1 and *S. cerevisiae* 141 had fewer total D- and L-amino acids (1037 mg/kg) than the yeast free sourdough (1549 mg/kg) with a noticeable decrease of aliphatic, dicarboxylic and hydroxy amino acid groups (169, 208 and 128 mg/kg, respectively). This specific reduction and in particular the greater aspartic acid decrease (192 mg/kg) was in agreement with the amino acid profile observed in the sourdough fermented with *S. cerevisiae* 141. This indicated that after the proteolytic activity of *L. brevis* subsp. *lindneri* CB1, the yeast selectively utilized the suitable amino acids. With the greater availability of amino acids, *S. cerevisiae* 141 enhanced its own growth passing from 10^7 CFU/g (sourdough without LAB), to 10^8 CFU/g (sourdough fermented in association with *L. brevis* subsp. *lindneri* CB1) after 24 hr at 28°C. The association involving *L. brevis* subsp. *lindneri* CB1 and *S. exiguus* M14 was characterized by lower partial and total amino acid concentrations and it further strengthened the hypothesis of a sequential utilization of amino acids by yeasts. The same conclusions were reached with both associations involving *L. plantarum* DC400. In an attempt to simulate the most frequent microbial composition of Italian sourdoughs (Gobbetti et al., 1994b), all of the selected microorganisms were used together (Table 1). The amino acid concentration (1006 mg/kg) and the partial amounts of individual amino acids, could probably be explained, after the previous experiments, as the result of interactions between bacterial proteolysis and use of amino acids by the yeasts.

Collateral biological actions could take place. In agreement with studies conducted on San Francisco sourdough (Sugihara,

Table 1—Total D- and L-amino acids in sourdoughs produced with individual and associated starters (initial cellular concentration of 10^7 and 10^5 CFU/g for lactic acid bacteria and yeasts, respectively) at 28°C for 24 hr

	Amino acids mg/kg of dough					
Starters*	Aliphatic	Dicar-boxylic	Hydrohy	Basic-Sul.cont.-Aromatic-Cyclic	Total	SD
Wheat flour	90[a]	355[a]	41[ad]	245[a]	731[a]	46.5
Unstarted	146[a]	75[b]	59[ac]	232[a]	512[bd]	44.3
141	112[a]	108[b]	30[a]	190[a]	440[b]	42.9
M14	208[b]	69[b]	35[ad]	223[a]	535[bd]	43.1
DC400	672[c]	455[c]	155[b]	228[a]	1510[c]	113.2
CB1	674[c]	476[c]	164[b]	235[a]	1549[c]	71.5
141-DC400	276[db]	187[d]	51[ad]	142[b]	656[da]	65.4
141-CB1	505[f]	268[e]	36[ad]	228[a]	1037[e]	90.8
M14-DC400	456[f]	271[e]	37[ad]	192[a]	956[e]	77.7
M14-CB1	378[g]	224[e]	25[c]	171[a]	798[ed]	74.5
141-M14-DC400-CB1	501[f]	248[e]	58[ac]	199[a]	1006[e]	85.4
M14**	941[h]	315[a]	92[c]	552[c]	1900[f]	125.8
M14-DC400**	604[c]	130[b]	70[cd]	500[c]	1304[g]	66.2

* The starters used were named with the Collection number reference.

** Initial cellular concentration of 10^7 CFU/g for both M14 and DC400.

[a-h] Values in the same column with different superscript letters differ ($P < 0.05$). The SD was calculated on the total values.

Table 2—D-amino acids in the sourdoughs produced with individual and associated starters (initial cellular concentration of 10^7 and 10^5 CFU/g for lactic acid bacteria and yeasts, respectively) at 28°C for 24 hr

	Amino acids mg/kg of dough			
Starters*	D-Alanine	SD	D-Glutamic acid	SD
unstarted	1^{a}	0.1	1^{a}	0.2
CB1	18^{bcf}	2.3	19^{b}	2.5
DC400	25^{b}	3.2	6^{c}	0.9
141	19^{bcf}	2.5	1^{a}	0.1
M14	13^{ce}	1.3	1^{a}	0.1
CB1-141	38^{d}	3.4	34^{d}	3.4
CB1-M14	10^{e}	1.2	25^{d}	3.3
DC400-141	16^{ef}	1.6	14^{b}	1.1
DC400-M14	12^{ef}	1.7	7^{c}	1.5

* The starters used were named with the Collection number reference.
[a-d] Values in the same column with different superscript letters differ ($P < 0.05$).

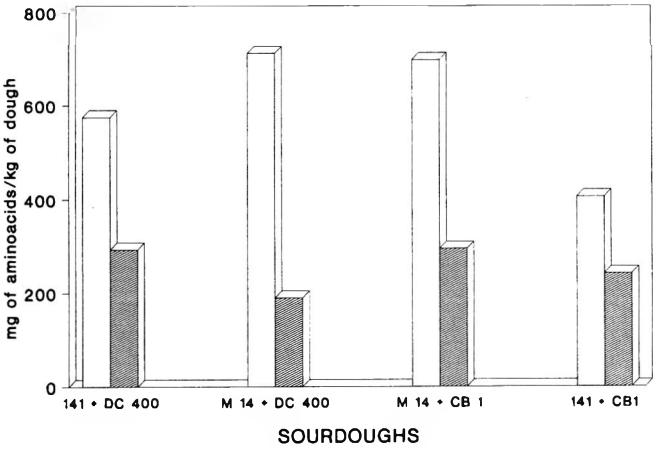

Fig. 1—Total D- and L-amino acid concentrations (mg/kg of dough) in the different sourdoughs before (▨) and after (▨) baking. The starters used were named with the Collection number reference.

1983), an initial higher cellular concentration of *S. exiguus* M14 (10^7 CFU/g) (Table 1) did not modify the initial amino acid composition of wheat flour (788 vs 731 mg/kg) but resulted, after 24 hr fermentation, in a higher amino acid level than with standard conditions of inoculum (1900 vs 535 mg/kg). In agreement with Spicher and Nierle (1984), *S. exiguus* M14 was apparently demonstrated not proteolytic. The increase of amino acid concentration was thus probably due to the greater inoculum which enhanced amino acid synthesis (Challinor and Rose, 1954; Lyons and Rose, 1977) and especially amino acid excretion by cell yeast autolysis during the 24 h period (Selby Smith et al., 1975). In presence of increased total amino acids, *L. plantarum* DC400 did not cause further degradation of wheat flour proteins, as previously demonstrated, but used available amino acids (decrease of 596 mg/kg compared with the sourdough started only with *S. exiguus* M14). This suggested that amino acid synthesis and excretion by a higher inoculum of yeast could represent a collateral source of free amino acids which satisfied LAB requirements (Gobbetti et al., 1994a) and which may have enhanced bacterial growth rate.

D-amino acid evolution during sourdough fermentations

Severe treatment of food proteins, in particular by alkali and heat, leads to conversion of L-amino acids into their optical isomers, the D-amino acids (Master and Friedman, 1979; Schwass and Finley, 1984). Bruckner and Hausch (1990a) demonstrated that in dairy products, the synthesis of D-amino acids could be attributed to the typical microflora of each product. All sourdoughs started with individual microorganisms had, at 24 hr a higher ($P < 0.05$) total concentration of D-amino acids than the reference test incubated without starters (Table 2). Though traces of D-leucine, D-aspartic acid and D-lysine were also detected (data not shown), D-alanine and D-glutamic acid were determined at higher and comparable values. The fermentation with *L. brevis* subsp. *lindneri* CB1 was associated with 18 mg/kg of D-alanine and 19 mg/kg of D-glutamic acid and the total synthesis by both LAB was greater ($P < 0.05$) than those by the yeasts. As with the metabolism of amino acids by yeasts, this result suggested that during sourdough fermentation there was probably a selective utilization of L-isomers, whereas the cellular excretion of amino acids could provide L- and D-forms. The possible biotic generation of D-forms was further confirmed by analysis of all sourdoughs produced with associations between LAB and yeasts (Table 2). In particular the association of *L. brevis* subsp. *lindneri* CB1 and *S. cerevisiae* 141 had almost the cumulative effect of the individual syntheses of D-alanine and D-glutamic acid (72 mg/kg of total D-amino acids vs 2 mg/kg in the unstarted sourdough).

Total D- and L-amino acid evolution during sourdough baking

The total D- and L-amino acid concentrations after 4 h fermentation reached values between 75 and 85% of total values observed at 24 h. According to the Italian wheat bread-making process (Quaglia, 1984), the sourdoughs started with associations LAB-yeasts for 4 hr at 28°C, assumed a suitable structure for baking. As a consequence results from baked products were referred to sourdoughs fermented 4 hr at 28°C. Gas production and expansion during baking increased the sourdough volume by about 30%, the surface area by about 10% and the whole breads contained 35–43 wt % of moisture (depending on crust thickness). Each whole (crust plus crumb) baked sourdough decreased between 40 and 75% the initial total D- and L-amino acid concentration (Fig. 1). No particular differences, as reported by Spicher et al. (1980), were noted with sourdoughs fermented with different starters. In general, sourdoughs with initial higher concentrations had greater decreases of free amino acids. The reduction was most pronounced (>64%) for proline, leucine, aspartic acid, glutamic acid and lysine. The marked reductions of free amino acids were positively related to the aromatic characteristics of the bread (Rothe, 1975) and have been attributed to various causes. The nonenzymatic Maillard browning reaction in the crust can cause condensation between reducing sugars and free amino acids (mainly lysine) to form N-glycosides (Thomas and Rothe, 1956). Also the amino acids can be converted to aromatic carbonyl compounds by Strecker degradation (eg. leucine to 2-methylbutanol) (Rothe, 1975). In the same way, the direct heat degradation of amino acids was also possible. No differences were detected in reduction of amino acid content between the whole baked sourdough and the crust and crumb fractions (data not shown).

D-amino acid evolution during sourdough baking

The D-amino acid isomers were also separately analyzed for the baked sourdoughs (Fig. 2). Appreciable increases of total D-forms were not observed after the baking process at 250°C for 30 min. In contrast with the sharp drop observed for L-isomers, the not significant ($P < 0.05$) increases of D-forms after baking, could be probably attributed to the balance between partial degradation and generation of new D-isomers by heating (Master and Friedman, 1979; Schwass and Finley, 1984). Consequently we concluded that under the experimental conditions microbial metabolism was responsible for D-amino acid synthesis during sourdough fermentation and the level reached before baking could influence the final concentrations in baked products.

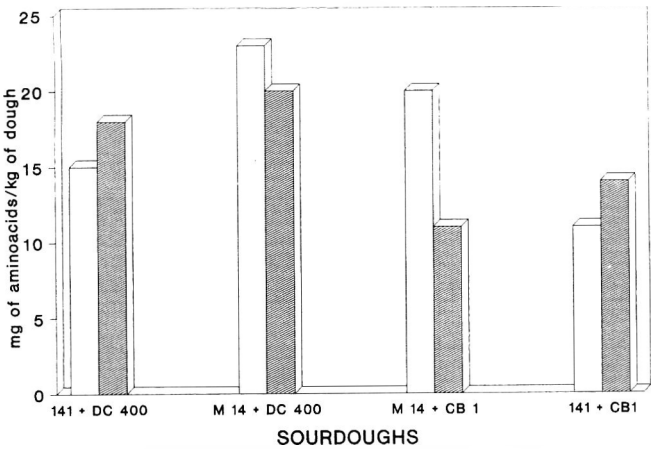

Fig. 2—Total D-amino acid concentrations (mg/kg of dough) in the different sourdoughs before (▨) and after (▨) the baking. The starters used were named with the Collection number reference.

CONCLUSIONS

THE DYNAMICS OF AMINO ACIDS during sourdough fermentation are complex. Homo- (*L. plantarum* DC400) and heterofermentative (*L. brevis* subsp. *lindneri* CB1) LAB were the higher proteolytic sourdough microorganisms. Their activity was necessary to obtain greater concentrations of free amino acids before baking and to enhance yeast growth during fermentation. The yeasts (*S. cerevisiae* 141 and *S. exiguus* M14) did not show appreciable proteolytic activity, but with greater inoculum (10^7 CFU/g), due to more yeast cells undergoing autolysis, excreted significant amounts of free amino acids. Consumption and liberation of amino acids during fermentation correlated with the metabolic requirements of the microorganisms. All starters used synthesized D-amino acid isomers during sourdough fermentation. After baking a loss of amino acids ranged from 40 to 75%. Amino acids represent precursors of aromatic compounds produced during heating. Thus, selection and utilization of proteolytic LAB have important effects on overall quality of bread.

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Two Lipoxygenases from Germinated Barley—Heat and Kilning Stability

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ABSTRACT

Using ammonium sulfate precipitation followed by hydrophobic and ion exchange chromatography, two lipoxygenase isoenzymes, LOX 1 and LOX 2, were 18.3- and 44.5-fold purified from germinated barley, with 18 and 24% recovery of activity respectively. LOX 1 and LOX 2 were characterized by isoelectric points 4.9 and 6.4, and molecular weights of 90 kd and 110 kd, respectively. Apparent Km values for linoleic acid were 0.06 mM for LOX 1 and 0.18 mM for LOX 2. LOX 1 converted linoleic acid to 9 and 13 hydroperoxides at about 4:1, whereas the 13 hydroperoxide was the major product formed by LOX 2 (ratio 3:7). For both isoforms, thermal inactivation data indicated first order kinetics with activation energies influenced by ionic strength and pH. Isoenzymes composition was analyzed for three kilning schemes: the 1:3 ratio between LOX 1 and LOX 2 observed in germinated barley increased during the course of kilning.

Key words: barley, lipoxygenase, thermal inactivation, malting, kilning, germination

INTRODUCTION

REFERRED TO AS EC 1.13.11.12 IN ENZYMATIC NOMENCLATURE, lipoxygenases (LOX) or lineoleate oxygen oxydoreductases are found throughout plants and animals (Whitaker, 1991; Sanz et al., 1992a,b). In plants, they catalyze the hydroperoxidation of cis-cis 1-4 pentadiene structures in unsaturated fatty acids, mainly linoleic and linolenic acids. Further breakdown of thus formed hydroperoxides (and those from lipid autoxidation) generates volatile carbonyl compounds, which are a major cause of rancidity and off-flavors in inadequately processed and stored foodstuffs. Brewers encountered this problem in stored beer in which Palamand and Hardwick (1969), Drost et al. (1990), and Angelino (1991) identified trans -2-nonenal and 2,4 nonedial as main products responsible for the "cardboard off flavor" generated during storage. However, it has not been determined whether enzymatic or auto-oxidation contributes more to the oxidation of fatty acids during the beer process. Yabuuchi (1976) reported a concomitant increase in carbonyl compound development and in lipoxygenase activity during malting. He hypothesized lipoxygenase would be the main factor in off-flavor production. Moreover, Kobayashi et al. (1993b) reported a similar behavior between lipoxygenase activity and hydroperoxides formation during wort production.

Due to its probable involvement in off flavor production, lipoxygenase from barley has been well studied. Several works reported the presence of one isoform in sound barley, which has been purified to homogeneity and characterized (Yabuuchi and Ahama, 1975; Van Aarle et al., 1991). However, few studies have been devoted to germinated barley. Doderer et al. (1992) and Yang et al. (1993) identified two isoforms, one present in both quiescent and germinated barley. The two isoenzymes were separated by Doderer et al. (1992) using a S-Sepharose fast flow column and Yang et al. (1993) after hydroxylapatite chromatography. However, their results had some discrepancies and in those two studies, lipoxygenase activity was spectrophotometrically assayed. That method has limitations due to absorbance of secondary products, which may not be taken into account.

Our objective was to prepare stable and purified extracts of LOX 1 and LOX 2 in sufficient amount, to investigate some properties such as kinetics, thermostability and stability during kilning. The two latter characteristics have not been previously reported and will aid malters and brewers in developing means to limit lipoxygenase activity and thus reduce staling in beer.

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MATERIALS & METHODS

Materials

Triumph barley (*Hordeum vulgare*, 1992 Harvest), selected for its high lipoxygenase activity, was malted in the IFBM (Institut Français des Boissons de la Brasserie Malterie) micromalting. The procedure used included 46 hr steeping at 15°C with varying wet and dry periods followed by 5 days germination at 17°C and 100% humidity. The malt was then lyophilised and stored at −20°C until use. Kilning was performed by the IFBM institute following three procedures (A, B and C) described in Fig. 1. Soybean LOX 1 was purchased from Sigma (St Louis, MO) and used without further purification (nominal activity, 150,000–350,000 units per mg protein). Fractogel butyl TSK 650 M was from Merck (Darmstad, Germany), (CM) Sepharose CL6B and Phastgels® were from Pharmacia (Uppsala, Sweden), linoleic acid and

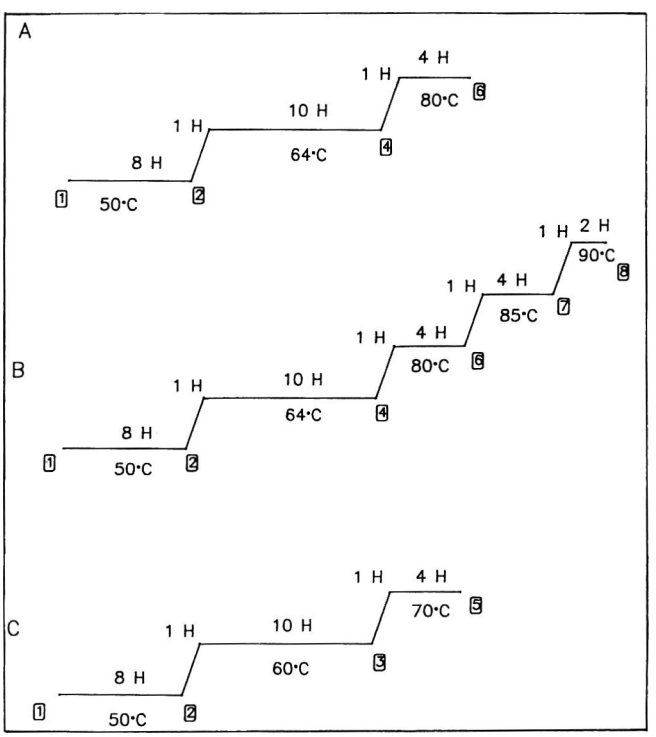

Fig. 1—Kilning schemes studied. Numerals one to eight indicate samples analyzed for isoenzyme composition.

Table 1—Summary of purification of germinated barley lipoxygenases

Purification step	Volume (ml)	Activity µkat	Proteins g	Yield %	Specific activity µkat.g^{-1}	Purification factor
Crude extract	435	25.9	1.8	100	14.4	1
$(NH4)_2SO_4$ fractionnation						
Supernatant 25%	450	24.5	1.6	94.6	15.3	1.1
Precipitate 60%	56	18	0.8	69.5	22.5	1.6
Precipitate 60%, dialyzed and centrifuged	60	15.7	0.6	60.5	26.2	1.8
Hydrophobicity						
Active fractions	48	14.8	0.12	57	123.3	4.7
Ion exchange						
LOX 1	25	5	0.02	19.4	275	19
LOX 2	29	6.8	0.01	26.2	724	50.3

all other chemicals were reagent grade supplied by Sigma (St Louis, MO).

Extraction procedure

Lyophilised material (48g) was ground during 3 × 10 sec in a beat mill IKA, suspended in 480 mL of a 0.1M phosphate buffer (pH 7.5) and stirred gently during 40 min at 4°C. The homogenate was centrifuged (30000 × *g*, 30 min) at 4°C and the supernatant was used as crude extract.

Purification procedure

All steps were carried out at 4°C. A 25–60% saturation precipitate in ammonium sulfate was prepared from the crude extract. The resulting pellet was resuspended in 30 mL of 0.1M phosphate buffer at pH 7.5 containing 0.6M ammonium sulfate and 0.6M KCl and dialyzed overnight against the same buffer. After centrifugation (30000 × *g*, 30 min), the resulting supernatant was loaded onto the top of a Fractogel TSK butyl 650 M (Merck) column (8 × 2.5cm) previously equilibrated with buffer at 120 mL·h^{-1}. After elution of unbound proteins by the equilibration buffer, the LOX was eluted with a 0.1M phosphate buffer at pH 7.5, 0.3M in ammonium sulfate and KCl. Proteins that were still bound to the gel were eluted with 150 mL H_2O. Absorbance at 280 nm and LOX activity were determined on each 6 mL fraction.

Active fractions from the TSK butyl 650M column were pooled and the enzyme was precipitated by ammonium sulfate at 60% saturation. After centrifugation (30000 *g*, 30 min), the pellet was redissolved in 20 mL of 0.1M acetate buffer (pH 4.65), dialyzed overnight against the same buffer and applied onto a CM Sepharose CL6B column (8 × 2.5 cm) preequilibrated with the former acetate buffer at 100 mL·h^{-1}. The column was washed with equilibration buffer and the eluted protein was monitored at 280 nm. After absorbance returned to the base level, further elution was carried out with a linear salt gradient of 0–0.3M NaCl in 0.1M acetate buffer, pH 4.65. Proteins still bound to the gel were eluted by 150 mL acetate buffer (pH 4.65) containing NaCl 2 M. Absorbance at 280 nm and LOX activity were assayed on each 4 mL fraction.

LOX activity and protein assays

The substrate was 2.5 mM in linoleic acid dispersed in phosphate buffer (0.1 M, pH 6.5) containing Tween 20 (0.1%), according to Nicolas and Drapron (1977). LOX activity was routinely assayed by polarography at 30°C using air saturated substrate solution. Activity was expressed as nmol of O_2 consumed per second (nkat). Protein contents were determined by the Bradford method (1976) using bovine serum albumin as standard.

Reaction product specificity

20 mL of air saturated substrate were oxidized at 30°C by 40 nkat of enzymatic extract (LOX 1, LOX 2 and soybean LOX 1). With soybean LOX 1, enzymatic reactions were carried out at pH 9. After 10 min incubation, reactions were stopped by adding 20 mL of ethanol and adjusting to pH 2 with 6N HCl. Hydroperoxides were reduced with sodium borohydride (250 mg) and three times extracted with hexane:ether (1:1) mixture. The three organic phases were combined, washed several times with distilled water, dried with anhydrous sodium sulfate, evaporated to dryness and resuspended in 5 mL hexane. Adsorption chromatography was carried out using a Varian HPLC (Workstation 9020, pump 9010, UV-visible detector 9050) with a Lichrosorb Si60 (Merck) column (15 × 3 cm, 5 µm). To improve separation of the hydroperoxide, we used a hexane:isopropanol:acetic acid (984:15:1,v/v/v) eluting solvent according to Nikolaev et al. (1990), delivered at 1 mL·min^{-1}. The eluent was monitored at 234 nm.

Electrophoresis experiments and Mr determination

Electrophoresis experiments were performed with the Phastsystem® (Pharmacia) using Phastgels pH 4.65 (isoelectrofocusing, IEF) or Phastgels 10-15% (native electrophoresis). The migration and $AgNO_3$ staining conditions were those described in the Phastsystem instruction. IEF in liquid medium was carried out at 4°C with a 110 mL column (LKB type 81011) in the pH range 3.5–10 as described by Fils et al. (1985). A 25-60% ammonium sulfate saturation precipitate was loaded onto the column.

A column packed with Ultrogel AcA 54 (IBF) (90 × 0.8cm, 180 mL bed volume) was calibrated with the gel filtration calibration kit of high Mr proteins from Pharmacia. 1.5 mL of purified LOX (60% ammonium sulfate fraction) or standard proteins dialysed overnight against a 0.1 M phosphate buffer at pH 7.5 were applied onto the column previously equilibrated with buffer and eluted with the same buffer at 8.8 mL.h^{-1}. Absorbance at 280 nm and LOX activity were determined on each 4 mL fraction.

RESULTS AND DISCUSSION

Preliminary studies reported major differences in LOX activity according to cultivars (Hughes M., unpublished results). A systematic study carried out on 12 cultivars has shown that the malt from "Triumph" barley had the highest activity. Therefore, our study was performed with "Triumph" malt as enzyme source.

Purification of germinated barley lipoxygenase isoenzymes

Results from the purification procedure were summarized (Table 1). After extraction, the ammonium sulfate treatments yielded a 1.6-fold purification with a yield near 70%. All LOX activity was eluted from the butyl TSK 650 M column in a single peak representing 90% of the loaded activity, with less than 20% of the applied proteins, which indicated an overall purification factor of 4.7. The purification efficiency of the chromatographic step was largely exploited in other LOX purification procedures (Flurkey et al., 1978; Cabibel and Nicolas, 1991). As soon as they were eluted from butyl TSK 650M, active fractions were set to 60% ammonium sulfate saturation to avoid the 30% loss in LOX activity observed when no sulfate ammonium precipitation was done. This loss could have been the result of a partial denaturation occurring in dilute enzyme solutions as reported by Nicolas et al. (1982). An alternative to prevent such denaturation could lie in the enrich-

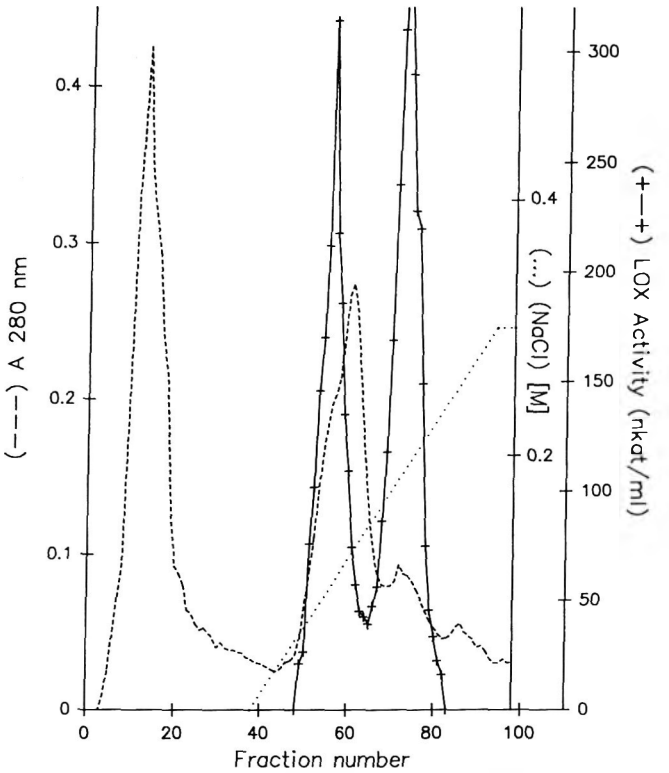

Fig. 2—Separation of two barley lipoxygenases (LOX 1 and LOX 2) using ion exchange chromatography on CM-Sepharose CL6B.

ment of our buffer solutions with a detergent, as recommended by Yang et al. (1993).

Further purificaiton was accomplished by ion exchange chromatography on CM Sepharose CL6B. LOX activity was eluted in two peaks (Fig. 2) LOX 1 and LOX 2. Results indicated the presence of two isoenzymes characterized by different isoelectric points >4.65 in confirmation of the works of Yabuuchi (1976), Doderer et al. (1992) and Yang et al. (1993). The yield was always >80%, leading to an overall purification factor near 20 for LOX 1 and 50 for LOX 2. The purification protocol described by Yang et al. (1993) for preparative purposes included three chromatographic steps (DEAE CL6B, Sephacryl S300 and hydroxylapatite) and resulted in high purification factors, around 120 for LOX 1 and 250 for LOX 2. For analytical purposes, using ammonium sulfate precipitation followed by a hydroxylapatite column, LOX 1 and LOX 2 had been 25- and 30-fold purified by the previous researchers.

Some physicochemical properties of LOX 1 and LOX 2

The isoelectric points of LOX 1 and LOX 2 were determined by isoelectric focusing in liquid medium with a 3.5-10 pH gradient. The elution profile confirmed the two peaks of LOX activity (LOX 1 and LOX 2) with maxima at pH 4.9 and pH 6.5, respectively. Moreover, a 1:3 ratio between LOX 1 and LOX 2 was determined. These results were in good agreement with values reported by Yabuuchi (1976). However, Doderer et al. (1991) and Yang et al. (1993) reported higher values, around 5.2 and 6.8. In each case a 1.6 pH unit difference occurred between the two isoelectric point values.

Despite the heterogeneity of the germinated barley LOX system, when active fractions eluted from hydrophobic chromatography were applied onto a AcA 54 gel filtration column, activity was eluted in a single peak, corresponding to a 100000 Mr value. The electrophophoretic patterns of purified LOX 1 and LOX 2 on Phastgel 10-15 allowed us to designate a Mr of 100 kd to LOX 1 and of 110 kd to LOX 2.

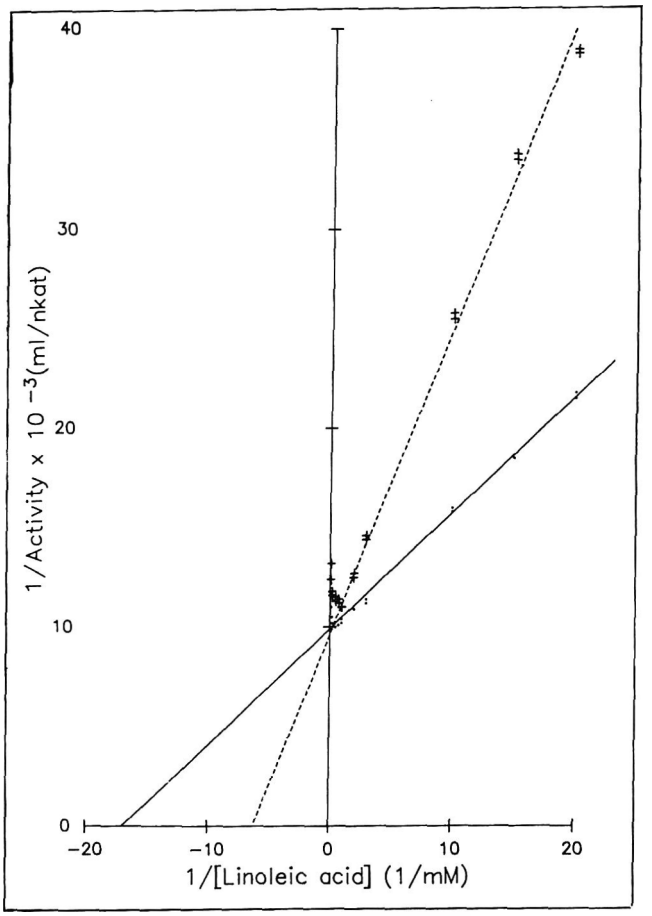

Fig. 3—*Effect of concentration of linoleic acid dispersed in Tween 20 on LOX 1 (——) and LOX 2 (---) activity.* For all concentrations, the ratio linoleic acid/Tween 20 was the same. Apparent Km values were determined (from the linear part of the curves) using a nonlinear regression data analysis program developed for IBM PC by Leatherbarrow (1987).

Some kinetic properties of LOX 1 and LOX 2

For each isoform, initial velocity was determined as a function of concentration for linoleic acid dispersed in Tween 20. Results (Fig. 3) using a double reciprocal plot showed LOX 1 with an apparent Km close to 0.06 mM and a three fold higher affinity for linoleic acid dispersed in Tween 20 compared to LOX 2 (Km = 0.18 mM). Using spectrophotometric assays, Doderer et al. (1992) and Yang et al. (1993) reported also a higher linoleic acid affinity for LOX 1, compared to LOX 2. However, apparent Km values they reported were 1.3 and 1.9 $\times 10^{-5}$ M, i.e. mostly lower than the polarographically calculated constants. These differences could also be the result of different substrate preparation procedures characterized by different detergent concentrations (Whitaker, 1991).

In addition, inhibition by excess linoleic acid was noted (Fig. 3) for >5 mM for LOX 2 and 10 mM for LOX 1. This inhibition has not been reported by Yang et al. (1993) who routinely assayed LOX activity with 80μM in linoleic acid. This substrate inhibition seemed to result from a competition between fatty acids and hydroperoxide for a regulatory site on the enzyme (Egmond et al., 1976; Schilstra et al., 1992; Wang et al., 1993).

Chromatograms were compared (Fig. 4a,b,c) on the mixtures of hydroperoxides from incubating linoleic acid with soybean LOX 1 and pH 9 (a), LOX 1 and LOX 2 at pH 6.5 (b,c). At pH 9, soybean LOX 1 oxidized linoleic acid mainly to the 13 hydroperoxide (Nicolas and Drapron, 1981). Thus we could identify the fraction eluted at 7 min as the 13 hydroperoxide and that eluted at 12 min as the 9 hydroperoxide. Consequently, LOX 1 and LOX 2 have been characterized by ratios of 9 hydroperoxide to 13 hydroperoxide equal to 80:20

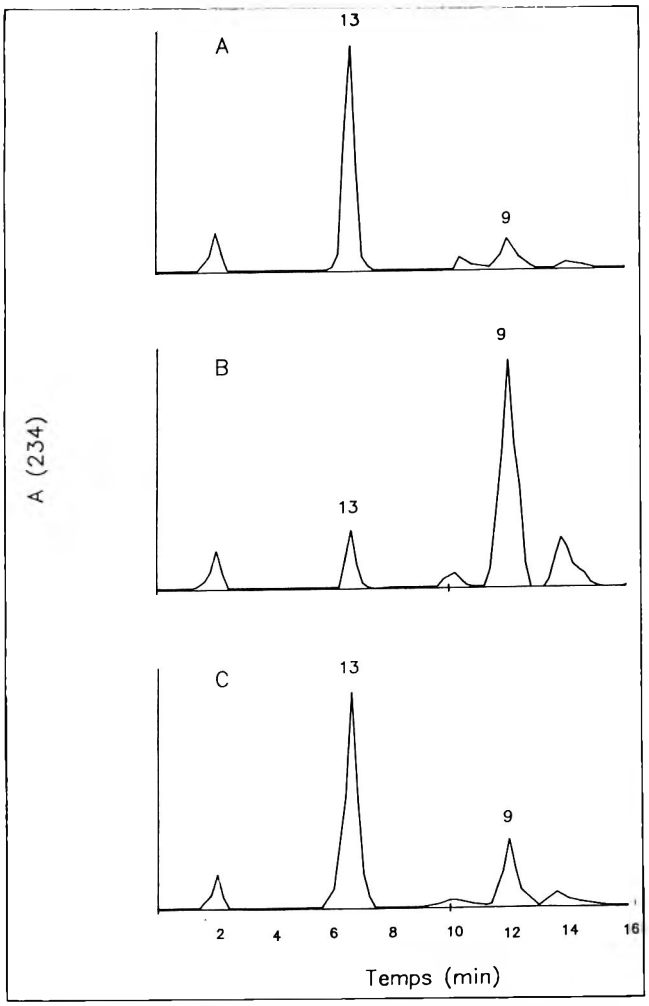

Fig. 4—Chromatograms of hydroperoxide mixtures after incubating linoleic acid with soybean: LOX 1 at pH 9 (a); germinated barley LOX 1 (b); and LOX 2 (c) at pH 6.5.

and 30:70 respectively, in confirmation of the results of Yang et al. (1993). However, the low percentages of 13 and 9 hydroperoxide found for LOX 1 and LOX 2 respectively, could result from autoxidation of linoleic acid which was difficult to avoid, and must be considered. Since the 9 hydroperoxide is involved in trans 2 nonenal formation (Galliard and Chan, 1980), the results suggested that LOX 1 is mainly responsible for off flavor development.

Heat and kilning stability of LOX 1 and LOX 2

The effect of temperature on isoform stability was investigated by measuring residual activity after heat treatment of LOX 1 and LOX 2 for various times at 50–65°C. A preliminary study had shown that ionic strength had a negligible effect on heat stability (data not shown). In a second experiment, the two fractions eluted from CM Sepharose CL6B were dialyzed overnight against phosphate buffers at various pH values (4.65; 5.0; 5.5 and 7) and analyzed for heat stability. For each temperature/pH combination, the two parameters used as "standard" designations by the industry were defined, i.e.;

D : time (min) required to reduce initial activity by 90%

Z : temperature increase (°C) required to reduce D by 90%

These values were compared (Table 2). When residual activities of LOX 1 and LOX 2 were plotted against heating time (Fig. 5) both isoenzymes showed first order inactivation kinetics. At pH 4.65, LOX 2 was clearly more thermostable than LOX 1. Thus the D value of LOX 2 was ≈ 1.6 times higher than that associated with LOX 1. However, an opposite result was noticed at pH 7, where LOX 1 was more thermostable

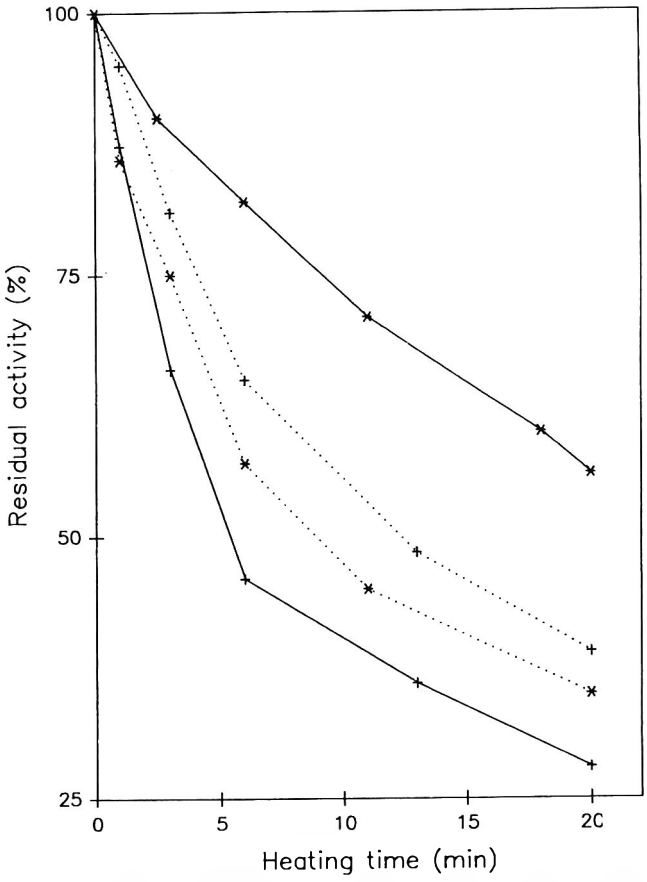

Fig. 5—Residual activity (% of initial activity) relationship to heating time for LOX 1 (——) and LOX 2 (----) at 50°C, pH 4.65 (+) and 7 (*).

Table 2—D and Z values for LOX 1 and LOX 2

	D (min)				
pH	50°C	55°C	60°C	65°C	Z (°C)
4.65					
LOX 1	57	13	4	0.5	8.6
LOX 2	87	44	9	3	9.8
5.5					
LOX 1	65	22	6	1.3	8.8
LOX 2	75	28	7	2	9.3
6					
LOX 1	78	30	11.5	2	9.6
LOX 2	60	20	4	1.4	8.8
7					
LOX 1	84	38	13	4	11.3
LOX 2	45	10	1.5	1	8.7

than LOX 2. Increasing pH from 4.65 to 7 led to an increase in heat stability of LOX 1 (Table 2) and a decrease in heat stability of LOX 2 (Table 2). Thus minimal heat stability was noted for both isoenzymes at pH near the isoelectric point. This was not in agreement with results reported by Bohran and Snyder (1979), concerning soybean LOX 1. No study on germinated barley isoenzyme heat stability as a function of pH could be found.

Ungerminated and germinated Triumph barleys and a series of samples corresponding to various stages of three kilning procedures (Fig. 1) were analyzed for total LOX activity and isoenzyme composition (Table 3). As indicated, barley germination led to the appearance of a new isoform LOX 2. Moreover, a 3.5-fold increase in LOX amount was noted between ungerminated and germinated barley. In green malt, LOX 2 represented 75% of the total LOX. The percentage gradually decreased during the course of kilning procedures,

Table 3—Residual LOX activity and isoenzyme composition during the course of three kilning procedures (A,B,C)

		Percentage of LOX 1 and LOX 2 (%)					
		A		B		C	
Samples	Activity (nkat/g)	LOX 1	LOX 2	LOX 1	LOX 2	LOX 1	LOX 2
0 (Barley)	200	100					
1 (green malt)	700	25	75	25	75	25	75
2	280	30	70	30	70	30	70
3	255					42	58
4	200	45	55	45	55		
5	120					66	34
6	80	70	30	70	30		
7	40			80	20		
8	25			92	8		

to attain 8% for the high kilning, Special process (B), 30 and 34% for the A and C kilning processes, respectively. Results were in good agreement with our previous study of isoform heat stability where LOX 1 was the most thermostable for pH >5.5 (malt conditions). Moreover, at the end of each kilning, a low but significant residual activity was observed, (25, 80 and 110 $nkat.g^{-1}$ for the B, A and C kilning processes, respectively). This residual activity as well as the ratio LOX 1 to LOX 2 were directly related to severity of heat treatment. LOX activity was present after kilning and its probable involvement in off flavor development after that stage of brewing is possible. Most residual activity was represented by the LOX 1 isoenzyme which mainly produced the 9-hydroperoxide, precursor of trans 2 nonenal in beer. This supported the hypothesis of Kobayashi et al. (1993a) who presented LOX as the main factor involved in off flavor production.

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Barrel-Valve Assembly Affects Twin-Screw Extrusion Cooking of Corn Meal

M. LIANG, F. HSIEH, H.E HUFF, and L. HU

ABSTRACT

The effects of a barrel-valve assembly on extrusion system dynamics and product properties with various screw speeds and specific feeding loads (SFL) were studied using an APV Baker twin-screw extruder. Turning the barrel-valve from the open to closed position resulted in a convex response of die pressure due to the axial offset of paired orifice plugs, with concomitant changes in system responses and extrudate properties. Variations in SFL by changing feed rate or screw speed caused more pronounced effects on product properties compared with barrel-valve control.

Key Words: twin-screw extrusion, corn meal, barrel-valve

INTRODUCTION

HIGH QUALITY OF EXTRUDATE in a process that occurs at relatively short residence times requires that flow of the dough mass in the extruder and factors affecting it are carefully controlled (Kumar et al., 1989). The product quality of extruded materials, on an industrial scale, are mostly empirically optimized (Meuser and van Lengerich, 1984). In an extrusion-cooker, operational settings in the screw profile can be manipulated for a variety of mixes, to give a fluid compound with desired characteristics. This is particularly true for twin-screw extruders, typically operated in the "starve-fed" mode. This mode allows material to be pumped at a lower torque, using less energy to turn the screws (Bigio and Wigginton, 1990). Twin-screw extruders also provide effective material conveying, mixing, kneading, heat transfer, and are self-cleaning (Noguchi, 1990).

Flexibility is provided by varying the screw profile with different screw elements. However, it is usually not convenient to change these elements to accommodate frequent changes in feeds (Todd, 1980). A feature on some twin-screw extruders is the barrel-valve assembly which allows flow control inside the extruder without the need to change screw configurations. The barrel-valve position may be varied while the extruder is running. However, this device has found few applications in the food industry because of a lack of development research.

Todd (1980) introduced the concept of the barrel-valve and its functions, which influence the extruder's hold-up, compounding, and energy input. Using a co-rotating twin-screw extruder operated at different conditions, the general effects of barrel-valve on residence time distribution (RTD), degree of fill, and flow pattern were reported by several researchers (van Zuilichem et al., 1989; Jager et al., 1991; Liang et al., 1993). The effect of changing the orifice plug size in the assembly was also found to be similar to effects when the barrel-valve opening was changed (Liang et al., 1993). Results demonstrated that the barrel-valve assembly provides a means for moderate adjustment of degree of fill and more precise RTD control of extrusion. As the degree of fill and RTD are influenced by inclusion and variation of the barrel-valve assembly, it is reasonable to expect that extrusion dynamic parameters, (motor torque, die pressure, and dough temperature) would be to some extent, simultaneously affected. These effects would affect the change of the amount of work done on the feed material and thereby product quality. However, no studies concerning the influence of the barrel-valve device on extrusion system parameters and product characteristics have been published.

Our objective was to determine the effects of the barrel-valve control on the extrusion system's dynamic responses and extrudate properties under different processing conditions using an intermeshing, co-rotating twin-screw extruder.

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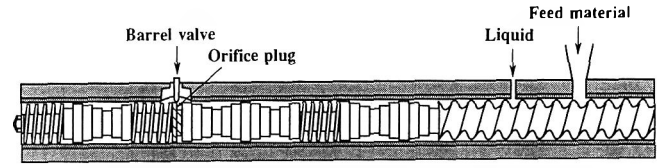

Fig. 1—The screw profile comprised (from right to left): 200 mm, twin lead feed screw; 125 mm, 30° forwarding paddles; 50 mm, single lead screw; 150 mm, 30° forwarding paddles; 12.5 mm, orifice plug; 50 mm, single lead screw; 75 mm, 30° forwarding paddles; 12.5 mm, 30° reversing paddle; and 50 mm, single lead end screw.

MATERIALS & METHODS

Material

Commercial degermed yellow corn meal (CCM 250, Lauhoff Grain Company, Danville, IL) was the feed material. The proximate composition of the corn meal was: moisture 13.2%, protein 7.0%, fat 0.7%, fiber 0.5%, ash 0.4%, and carbohydrate 78.2% (by difference). Final moisture of the feed was adjusted to 18% (wb).

Extrusion

Each extrusion experiment was carried out on a co-rotating, intermeshing twin-screw extruder (MPF 50/25, APV Baker Inc., Grand Rapids, MI). This pilot scale model was driven by a 28 kW DC motor with a rated speed of 500 rpm. A barrel length-to-diameter ratio (L/D) of 15:1 was used. The barrel contained five heating zones and six cooling zones with independently controlled electrical cartridge heaters and water cooling channels.

The screw configuration is shown in Fig. 1. It consisted of 8 sections. The barrel-valve assembly (Fig. 2) was at the end of the fourth section. Corn meal was fed into the extruder using a K-tron Type T-35 twin-screw volumetric feeder with a Series 6300 controller (K-tron Corp., Pitman, NJ). A die head with two circular die holes of 3.18 mm was used. The adjustable die face cutter with four blades was operated at 325 rpm. Barrel temperatures were set at 23.9°C (feed zone), 23.9°C, 51.7°C, 93.3°C, 121.1°C and 121.1°C throughout all experiments.

All barrel and product temperatures, die pressure, die temperature, and percent motor torque, feed rate, screw speed, and cutter speed were monitored through the fully-equipped sensor system of the extruder and control panel, and logged via an RS-232C port to a PC computer (Northgate, Plymouth, MN). MACS II software coupled with a PL-1000 programmable controller (Elexor Associates, Morris Plains, NJ) was used for data acquisition. The sampling data were recorded every 15 sec during steady-state operation. Steady-state conditions were assumed when there were no visible drifts in product temperature at the die or in percent torque for at least 5 min. Samples were then collected

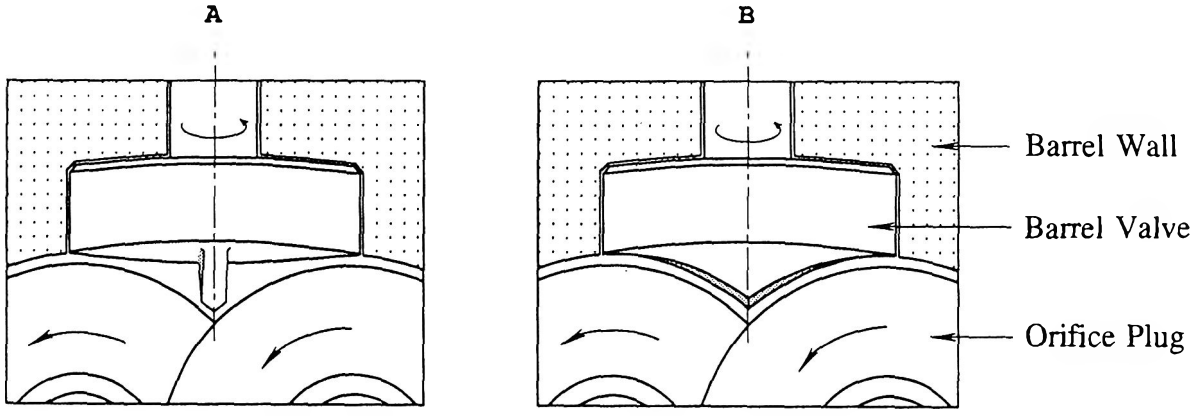

Fig. 2—Barrel- valve assembly with (A) barrel-valve open and (B) barrel-valve closed.

Table 1—Regression models for extrusion system parameters and extrudate physical properties

	System parameter				Extrudate property			
Variable[a]	Torque (%)	Die pressure (kPa)	Product temp (°C)	Specific energy (kJ/kg)	Length (mm)	Diameter (mm)	Specific volume (cm^3/g)	Breaking strength (g/mm^2)
Intercept	51.426	12073.625	209.658	1701.904	-19.036	19.750	6.980	-3.459
N (rpm)	-9.2×10^{-2}		-4.38×10^{-1}	-1.20	-1.28×10^{-1}			-3.95×10^{-2}
SFL (kg/rev)	1.045×10^{4}	-4.558×10^{6}	6.30×10^{3}	-3.632×10^{5}	2.929×10^{4}	-3.378×10^{3}	6.829×10^{3}	
Angle (deg)	-5.8×10^{-2}	1.383×10^{1}	-7.7×10^{-2}	-5.74×10^{-1}	-3.1×10^{-2}	1.7×10^{-2}	-2.5×10^{-2}	
N^2	1.2×10^{-4}	-1.2×10^{-2}	7.0×10^{-4}	1.3×10^{-3}	3.0×10^{-4}	9.0×10^{-6}	3.0×10^{-5}	-6.0×10^{-5}
SFL^2		6.697×10^{8}		3.460×10^{7}	-3.967×10^{6}	6.434×10^{5}	-9.185×10^{5}	-7.581×10^{4}
$Angle^2$	2.5×10^{-4}	-1.3×10^{-1}	1.2×10^{-3}	2.60×10^{-3}	5.0×10^{-4}	-2.0×10^{-4}	2.0×10^{-4}	
N × SFL		2.257×10^{3}		7.063×10^{1}	1.282×10^{1}			
N × Angle								
SFL × Angle	1.217×10^{1}	-1.730×10^{3}		1.142×10^{2}			5.648	
R^2 for regression	0.995	0.930	0.995	0.995	0.994	0.920	0.985	0.862

[a] N=screw speed; SFL=specific feeding load; Angle=barrel valve angle. All variables shown in each model are s gnificant at 0.05 level.

and dried in a fluidized bed drier at 65°C for 5 min. The final product moisture was 7.0 ± 0.5% (db). The specific mechanical energy (SME) input was calculated using the following formula (Hsieh et al., 1990):

$$\text{SME (kJ/kg)} = \frac{\text{rpm (run)}}{\text{rpm (rated)}} \times \frac{\text{\% Torque (run)}}{100} \times \frac{\text{Rated power (kW)}}{\text{Feed rate (kg/s)}} \quad (1)$$

Experimental design

A 3×3×3 factorial design was used and each treatment was run in duplicate. Independent variables were: (a) barrel-valve angle: 0 (fully open), 45°, and 90° (fully closed), in conjunction with a pair of orifice plugs of 47.5 mm in diameter and 6.25 mm in land length; (b) screw speed: 300, 375, and 450 rpm; and (c) specific feeding load: 0.0026, 0.0032, and 0.0038 kg/rev.

The term of specific feeding load (SFL) is defined as (Della Valle et al., 1987):

$$\text{SFL (kg/rev)} = \frac{\text{Feed rate}}{\text{Screw speed}} \quad (2)$$

Thus SFL is an extruder screw load index which is either feed rate- or screw speed-dependent. Since this extruder had a rated power of 28 kW and a rated screw speed of 500 rpm, once the % torque of motor and SFL were known, the specific mechanical energy in Eq. (1) could be calculated as:

$$\text{SME (kJ/kg)} = 3.63\times10^{-2}\,\frac{\text{\% Torque}}{\text{SFL (kg/rev)}} \quad (3)$$

Product properties

The lengths and diameters of 50 pieces of extrudates taken at random were measured and averages calculated (Fletcher et al., 1985). The specific volume measurement was done by the rapeseed displacement method which was modified from the sand displacement method (Park, 1976). The average of 5 samples was reported. Ten pieces of extrudates were chosen at random and sheared at the central cross section using a Warner-Bratzler shear on a Model 1132 Instron (Instron Corp., Canton, MA) to determine breaking strength. The maximum breaking force was divided by sample cross-sectional area for each piece and the average was calculated and reported as breaking or shear strength.

Statistical analysis

Statistical analysis was conducted using the General Linear Model procedure of SAS Institute, Inc. (1991). Procedure STEPWISE (with options) was used to develop quadratic regression models based on experimental data since a second order polynomial, is commonly used in food industry for modelling (Olkku et al., 1983). Multiple regression analysis was performed by plotting each model as a 3-dimensional graph with the response surface analysis procedure of G3D in SAS.

RESULTS & DISCUSSION

In regression models and R^2 values for extrusion parameters and product properties (Table 1) some processing variables might seem unimportant regarded separately in a model, but their joint effects could be important. All regression equations accounted for 86% or more of the total variation ($P<0.05$).

Motor torque and die pressure

Response surfaces for motor percent torque and die pressure were recorded (e.g. Fig. 3 and 4). As mentioned, the specific feeding load (SFL) is dependent on either feed rate or screw speed, according to Eq. (2). The effect of varying SFL generally represents the response caused by a change in either feed rate or screw speed. An increase in SFL by increasing feed rate or decreasing screw speed caused both torque and die pressure to be higher at all barrel-valve positions. Changing the barrel-valve position from open to closed, for all SFL lev-

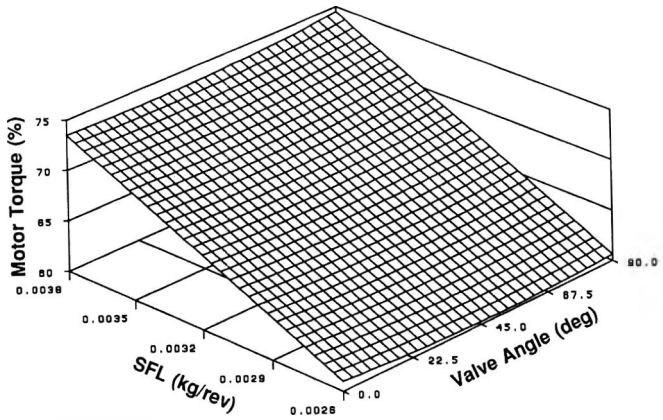

Fig. 3—Effects of specific feeding load and barrel-valve angle on motor torque at 375 rpm.

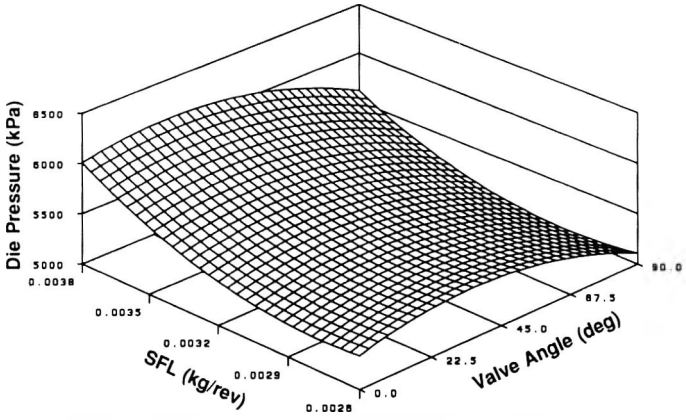

Fig. 4—Effects of specific feeding load and barrel-valve angle on die pressure at 375 rpm.

els, increased the die pressure moderately at first, and then decreased, while an opposite trend for torque was observed.

The simultaneous increase in torque and die pressure with increasing feed rate or decreasing screw speed has been universally observed in cereal extrusion processing. This can be readily explained by the following expressions developed by Martelli (1983) for twin-screw extruders:

$$Z_t = C\bar{\mu}N_f\omega^2 + \frac{Q_v^2\,\mu}{K_f} \quad (4)$$

$$T = \frac{Z_t}{\omega} \quad (5)$$

$$P = \frac{Q_v\,\mu}{K_f} \quad (6)$$

where Z_t is the total motor power transmitted to screws, T the motor torque, P the flow pressure, C a constant based on screw geometry, N_f the number of filled flights, ω the screw angular velocity, Q_v the volumetric output rate, K_f the conductance of die, μ the melt viscosity, and $\bar{\mu}$ the average viscosity over the filled channels. Using the concept of specific feeding load (SFL) (Eq. 2), the equations can be modified:

$$Z_t = C\bar{\mu}N_f\omega^2 + \frac{(SFL)^2\omega^2\mu}{K_f} \quad (7)$$

$$T = C\bar{\mu}N_f\omega + \frac{(SFL)^2\omega\mu}{K_f} \quad (8)$$

$$P = \frac{(SFL)\omega\mu}{K_f} \quad (9)$$

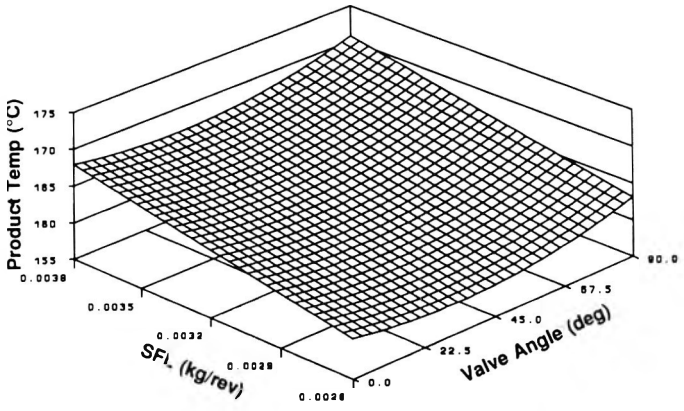

Fig. 5—Effects of specific feeding load and barrel-valve angle on product temperature at 375 rpm.

An increase in SFL by increasing feed or decreasing speed would cause the extruder fill level to increase (Liang et al., 1993). The dough viscosity variation could be considered to be less pronounced due to a relatively small change of product temperature, as observed. Thus, the pressure built-up from material behind the die and the power consumption increased, leading to an increase in motor torque as well.

The inconsistent trends for die pressure and motor torque and the negative correlation between them, as influenced by varying the barrel-valve angle for a given SFL, were unexpected (Fig. 3 and 4). The reason the maximum pressure and minimum torque occurred not when the barrel-valve was fully, but when it was partially closed is not clear. A possible explanation is that the least resistance to dough flow caused by the barrel-valve assembly was not when the barrel-valve was at 0°, but when it was turned to a certain angle because of the axial offset of the paired full bore orifice plugs. This inference could be justified by the observance reported by Todd (1980). In his experiment, the product temperature was found to be a function of barrel-valve position. The minimum and maximum restrictions to the flow that led to lowest and highest product temperatures were found when the valve was angularly offset from the 0° and 90° position, respectively. This phenomenon was also observed in our study. Based on this inference, the sections between the die and barrel-valve assembly would be filled more. The overall extruder fill level would decrease relatively when the valve angle was set at the position which provided the least flow restriction. This caused the die pressure to increase whereas the motor torque decreased. On the contrary, when the valve was turned towards being closed, to give a greater flow resistance, the filled channels between die and barrel-valve would be reduced, but the overall degree of fill would increase. This resulted in decreased die pressure but an increased torque.

Product temperature and specific energy

Response surfaces for product temperature in the melting zone and specific mechanical energy (SME) (Fig. 5 and 6) showed an increase in SFL was associated with an increase in product temperature but a decrease in specific energy at all barrel-valve positions. When the barrel-valve was turned from open to closed, the product temperature decreased slightly at first, and then increased. However, SME had a similar trend as that of torque when the barrel-valve position was changed.

An increase of the product temperature in the melting zone with increasing barrel-valve angle resulted from the increased degree of fill, and hence increased residence time inside the extruder (van Zuilichem et al., 1989; Liang et al., 1993). The slight decrease of temperature at the beginning of the valve angle change was in agreement with the observation by Todd

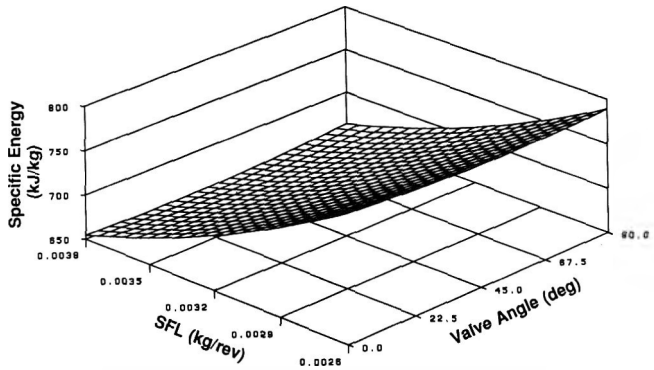

Fig. 6—Effects of specific feeding load and barrel-valve angle on specific mechanical energy at 375 rpm.

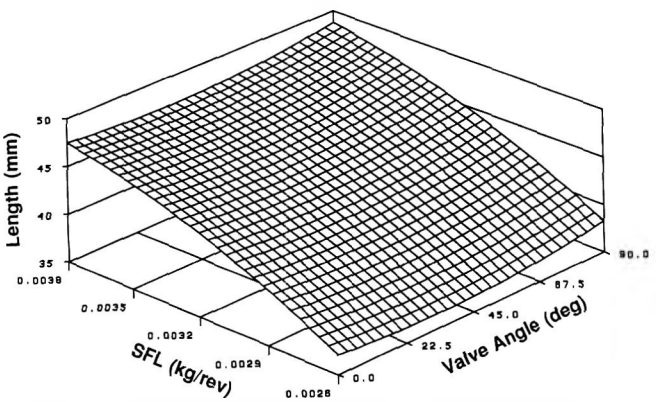

Fig. 7—Effects of specific feeding load and barrel-valve angle on extrudate length at 375 rpm.

(1980). A 90° rotation of the barrel-valve from fully open to fully closed elevated the product temperature by 3 to 4°C for all SFL levels (Fig. 5). Since the specific energy input was dependent on torque only for a fixed screw speed and feed rate (Eq. (1)), very little change in SME was observed when the valve position was varied (Fig. 6). This corresponded to the insignificant change in motor torque (Fig. 3).

It has not been well recognized that an increase in screw speed would introduce more mechanical energy in the extrusion system, generally resulting in a higher product temperature in the melting zone. Increasing product temperature but decreasing specific energy with increasing feed rate were reported by Fletcher et al. (1985), Senouci and Smith (1988) and Peng (1991). However, a decrease in product temperature with increasing feed rate was also reported (Della Valle et al., 1987). If the rise of feed temperature is considered to depend only on barrel temperature and screw speed, the product temperature would be expected to drop when feed rate was increased because less energy would be provided per unit mass of product. Nevertheless, increasing feed rate, hence the number of filled flights, would result in a net increase in motor power consumption according to Eq. (4). A larger extruder fill level would also allow more efficient utilization of available hot barrel surface (Jager et al., 1991). It would be thus possible that increasing feed rate would not generate less heat to each particle of dough, though the mechanical energy consumption per unit mass of product might be lower.

Expansion

Typical expansion characteristics for the axial and radial dimensions of the extrudate (Fig. 7 and 8) showed an increase in SFL at all barrel-valve positions favored both axial and radial expansions. Reducing the opening of the barrel-valve at

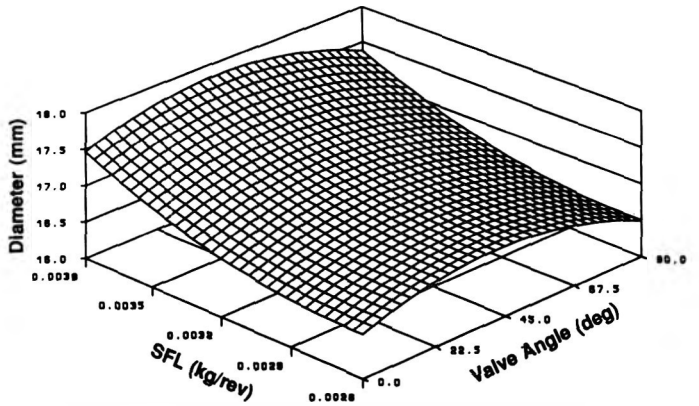

Fig. 8—Effects of specific feeding load and barrel-valve angle on extrudate diameter at 375 rpm.

all SFL levels increased the diameter of extrudate at first and then decreased. The trend reversed for the axial dimension.

Extrudate expansion affected by feed rate and screw speed has been attributed to several factors such as die pressure, residence time, viscosity, moisture, and elastic effects of the dough (Fletcher et al., 1985; Harmann and Harper, 1973; Launay and Lisch, 1983; Padmanabhan and Bhattacharya, 1989). An increase in extrudate length with a higher feed rate mainly resulted from a higher throughput from the die. Although increasing the feed rate would build up a higher die pressure (Fig. 4), which would give more resistance to flow. The increased mass flow rate could dominate the pressure rise until both balanced at a higher throughput. The net effect was an increase in extrudate length since the cutter speed was fixed. An increase in radial expansion (Fig. 8) was also expected with increasing feed rate due to higher die pressure (Fig. 4) and product temperature (Fig. 5). These enhanced the effects of moisture force and favored puffing (Padmanabhan and Bhattacharya, 1989).

Increasing screw speed resulted in a higher axial expansion but lower radial expansion (data not shown). This has been commonly reported for starch-based material extrusion (Grenus et al., 1992; Hsieh et al., 1991; Richburg and Garcia, 1988; Srivastava et al., 1988). An increase in screw speed decreased die pressure (Fig. 4), but increased product temperature (Fig. 5). The dough viscosity could thus be reduced. Launay and Lisch (1983) reported that the extrudate's axial expansion and dough viscosity were inversely related. Thus, extrudates with a greater axial expansion were expected. A lower radial expansion could be explained by the decrease in die pressure according to the elastic effect (Padmanabhan and Bhattacharya, 1989), and the inverse relationship between extrudate axial and radial expansions (Alvarez-Martinez et al., 1988).

The effect of changing the barrel-valve opening on extrudate dimensions (Fig. 7 and 8) could also be explained by the corresponding changes in die pressure and dough temperature at the metering section. However, the die pressure variation showed a more pronounced influence than that of dough temperature on expansion in both axial and radial directions, as the valve angle changed. The results suggested that the extrudate expansion, particularly in the radial direction, was closely related to the die pressure (Fig. 4 and 8). This was probably because the extrudate expansion depended on the pressure differential between die and atmosphere (Bhattacharya et al., 1986).

Density and breaking strength

The extrudate density and strength properties represented as specific volume (Fig 9) and breaking strength (Fig 10) showed

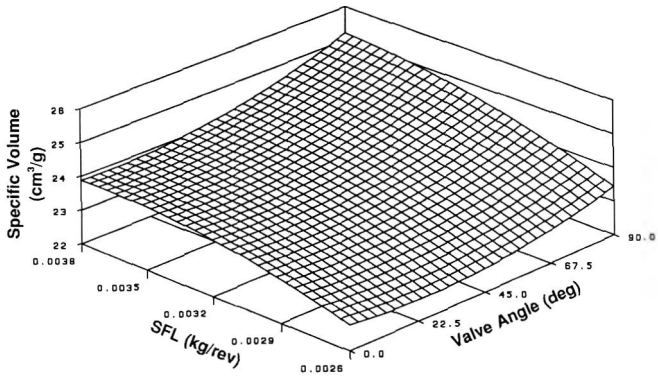

Fig. 9—Effects of specific feeding load and barrel-valve angle on extrudate specific volume at 375 rpm.

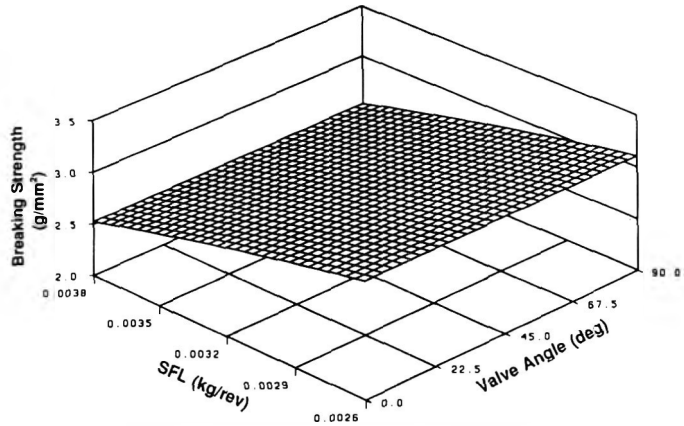

Fig. 10—Effects of specific feeding load and barrel-valve angle on extrudate breaking strength at 375 rpm.

increasing SFL at any barrel-valve angle resulted in higher extrudate specific volume but lower breaking strength. Reducing the barrel-valve opening decreased the specific volume slightly at first and then increased. The extrudate breaking strength, however, was not significantly affected by the valve position.

Volume expansion phenomena are basically dependent on viscous and elastic properties of melted dough (Launay and Lisch, 1983). The increased specific volume with higher SFL (Fig. 9) could be explained by the greater expansion in both axial and radial directions as discussed. This was mainly attributed to the increased product temperature (Fig. 5), which increased the dough visco-elasticity and caused the dough to expand more readily as moisture flashed off at the die (Harmann and Harper, 1973). Changes in the barrel-valve opening, influenced specific volume in a similar manner to the effects they had on extrudate length change (Fig. 7), which was inverse to the effects on die pressure (Fig. 4).

Since the extrudate breaking strength was determined by shearing at the central cross section, it was related to the extrudate's diametral expansion. Thus, the increased density or decreased expansion in the radial direction would result in a higher shear strength. This can be seen (Fig. 10) where a higher SFL generated an extrudate with a lower shear strength because of its higher radial expansion (Fig. 8).

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Screw Configuration Effects on Corn Starch Expansion During Extrusion

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ABSTRACT

Normal corn starch was extrusion cooked in a Brabender single-screw extruder. Three screws with no mixing, one mixing or two mixing elements were used to extrude the samples through a 3 mm cylindrical die nozzle at 140°C barrel temperature and 140 rpm screw speed. Dependent variables included overall and radial expansion ratios, bulk density and specific mechanical energy (SME). Extrudates were ground and re-extruded using the same three screws, and the same extrusion conditions. Significant differences ($P > 0.05$) in bulk density, SME and radial expansion ratio were found on re-extrusion. No changes occurred in overall expansion ratio ($P > 0.05$).

Key Words: corn-starch, extrusion, radial expansion, bulk density

INTRODUCTION

EFFECTS OF MOISTURE CONTENT and extruder barrel temperature on expansion properties of starch extrudates from single or twin-screw extruders have been extensively reported (Faubion and Hoseney, 1982; Owusu-Ansah et al., 1984; Bhattacharya and Hanna, 1987; Chinnaswamy and Hanna, 1988b). Decreasing moisture content or increasing extruder barrel temperature increased expansion ratios of corn starch extrudates (Owusu-Ansah et al., 1984; Bhattacharya and Hanna, 1987; Chinnaswamy and Hanna, 1988b). Moisture content of starch affected expansion ratio more than temperature of the extruder barrel (Bhattacharya and Hanna, 1987; Chinnaswamy and Hanna, 1988b). Expansion ratio of starch increased with increasing amylose content up to 50% (Chinnaswamy and Hanna, 1988a). Screw speed had insignificant or slight effect on expansion ratio (Owusu-Ansah et al., 1984; Bhattacharya and Hanna, 1987). Expansion is a function of the amount of shear force during extrusion. Low moisture content caused high shear resulting in higher expansion (Davidson et al., 1984; Diosady et al., 1985; Chinnaswamy et al., 1989).

Some reports exist on effects of screw design on extrudate properties such as solubility and viscosity (Anderson et al., 1969a,b; Barrès et al., 1990). However, none is available on the effect of screw configuration on expansion properties of corn starch extrudates. Our objective was to investigate the effect of mixing elements on expansion properties of extrudates when moisture content, barrel temperature and screw speed were kept constant.

MATERIALS & METHODS

Materials

Normal corn starch (25% amylose, 0.3% protein, 0.1% fat, 0.2% ash, 9.5% moisture, 0.014% phosphorous) was donated by American Maize Products Co. (Hammond, IN). Powdered starch was agglomerated by steadily spraying distilled water onto starch, while gently tumbling in an inclined rotating pan and subsequently drying to 9–12% (dry basis) moisture content at room temperature (≈23°C). The dry granules (average diameter 2.4 ± 0.3 mm) were stored in air tight containers.

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Extrusion

Extrusion of starch samples was carried out in a single-screw extruder (Model #2003, C.W. Brabender, South Hackensack, NJ) with a 1.9 cm barrel diameter, a 20:1 barrel length to diameter ratio, and a cylindrical die nozzle with 3 mm diameter. Three different extruder screws (Fig. 1), one with no mixing element (part number 05-00-035, referred to as NM), one with a single stage mixing element (part number 05-00-52, referred to as 1M), and one with a single stage mixing element (part number 05-00-52, referred to as 1M), and one with a dual stage mixing element (part number 05-00-054, referred to as 2M) were purchased from C.W. Brabender. The temperatures of the three metering sections and die, were set at 140°C. The temperature at the feed section was kept as low as possible by circulating ambient temperature air through the heating/cooling jacket for smooth and uninterrupted feed flow. Bulk densities of the feed and screw speeds were not changed, in order to maintain uniform feed rate for a given screw configuration. Screw speed was maintained at 140 rpm. The moisture content of starch was 18% (dry basis), unless otherwise noted. Screw speed and torque, for the purpose of calculating specific mechanical energy were recorded by a computer interface and PL2000 controller unit (C.W. Brabender, NJ) using a software Programloader version 1.9.5 (Brabender (R) OHG).

Starch samples were first extrusion cooked using NM, 1M or 2M (Extrusion 1). Extrudates were coarsely ground to pass through a screen with 2 mm openings and moisture contents were adjusted to 18% (dry basis) before being re-extruded with each of the three screws (Extrusion 2). All extrusions were performed in triplicate. Data are averages of the three replications.

Specific mechanical energy (SME), bulk density, and expansion ratios

SME was determined by dividing net energy input to the screw by extrudate flow rate using the method outlined by Sokhey and Chinnaswamy (1992) except that the torque and the screw speed were measured by the PL2000 controller unit. Bulk density of extrudates was determined by the glass beads displacement method outlined by Sokhey and Chinnaswamy (1992). Overall expansion ratios were determined by dividing solid density of extrudates by bulk density. Solid density of ground samples was determined using an air comparison pycnometer (Multivolume pycnometer model 130-50000-00, Micrometrics, Norcross, GA) by the method outlined by Sokhey and Chinnaswamy (1992). Radial expansion ratios were calculated by dividing the extrudate cross sectional area by the cross sectional area of die nozzle.

Statistical analysis

Data were analyzed using a statistical computer package (SAS Institute, Inc., 1989). The experimental design was a randomized complete block design with three replicates for each of 12 treatments. The treatments were blocked over 3 replicates as each experiment (total 36 runs) was performed on a separate day.

RESULTS & DISCUSSION

EXTRUDER SCREW TYPE AND CONFIGURATION can affect the SME input to the material being extruded and consequently can affect expansion ratios. The bulk density, overall and radial ex-

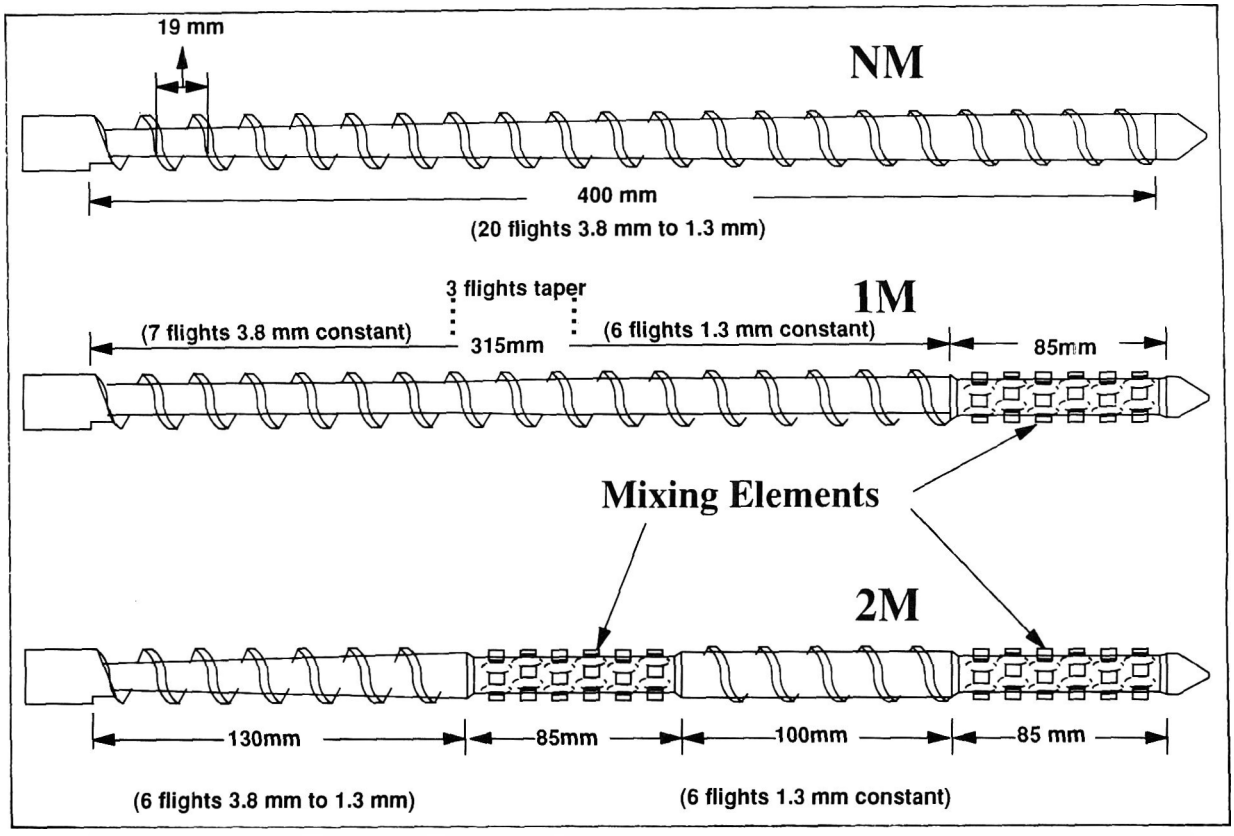

Fig. 1—Single-screws, showing mixing elements, used in extrusion. NM: screw with regular flights; 1M: screw with flights of mixing elements towards the tip of the screw; 2M: screw with two sets of six flights of mixing elements, one in the middle and one at the tip of the screw.

pansion ratios, and SME values for normal corn starch extruded with NM, 1M and 2M screws (Extrusion 1) were compared (Table 1). Overall expansion differences were not significant (P>F=0.6814). Radial expansion ratio differences were also insignificant (P>F=0.6568). All expansion values as well as bulk densities of extrudates with the three screw configurations were also not significantly different (P>F=0.6814).

Changes in overall and radial expansions and SME values of samples after extrusion 2 were compared (Fig. 2, 3, and 4). Symbols in Table 2 refer to screw type used during either Extrusion 1 or Extrusion 2; e.g., NM1 indicates no mixing element screw in Extrusion 1. Reference to a particular screw type used in Extrusion 2 is given by using a symbol such as NM1-Extrusion 2, which means samples extruded with any of the three screws where Extrusion 1 was carried out with NM. Similarly, NM1-NM2 means that Extrusion 1 was carried out with NM and Extrusion 2 was carried out with NM.

On re-extrusion, we observed that bulk densities (P>F=0.0140), SME's (P>F=0.0011) and radial expansion ratios (P>F=0.0005) were different when different screw configurations were used for re-extrusion. Overall expansion ratios had a P>F value of 0.0873. Overall expansion ratios of all samples in 2M1-extrusion 2 were not significantly different (P>F=0.1123) than those in 2M1 with 2M1-1M2 showing the highest overall expansion ratio of 5.68 amongst all samples (Fig. 2C). Overall expansion ratio of the 1M1-1M2 sample increased from 3.99 to 4.9, and those of 1M1-NM2 and 1M1-2M2 decreased to 3.36 and 3.66, respectively (Fig. 2B). NM1-extrusion 2 samples had no (P>F=0.1755) change in overall expansion ratios over those for NM1. The 2M1-Extrusion 2 samples exhibited a much higher (P>F=0.0058) increase in overall expansion ratios than either NM1-Extrusion 2 or 1M1-Extrusion 2.

Radial expansion ratios of extrudates increased (P>F=0.0001) from 8.44 for NM1 to 12.37, 12.05 and 10.29 for NM1-NM2, NM1-1M2 and NM1-2M2, respectively (Fig. 3A). Extrudates from 2M1-1M2 and 2M1-2M2 had small increases in radial expansion ratios to 10.64 and 9.85, respectively, over their corresponding 2M1 value of 9.23 (Fig. 3C). However, the 2M1-NM2 extrudate exhibited a decrease in radial expansion ratio to 8.27 (Fig. 3C). Changes in 2M1-Extrusion 2 were insignificant (P>F=0.1991). Extrudates from 1M1-Extrusion 2 showed increases (P>F=0.0013) in radial expansion ratios similar to their corresponding overall expansion ratios (Figs. 2B and 3B). NM1-Extrusion 2 increased radial expansion (P>F=0.0001) more than overall expansion ratios (Fig. 2A and 3A) which disagreed with the findings of Chinnaswamy et al. (1989). They extruded normal corn starch with NM at 14% starch moisture content and re-extruded with NM at 14% ground extrudate moisture content and found that the radial expansion decreased after re-extrusion. They attributed the decreased radial expansion to re-associations of starch molecules after the first extrusion. The higher radial expansion we found in this study could have been due to the fact that the higher moisture content (18%) of starch in Extrusion 1 may have decreased the number of re-associations of starch molecules after extrusion. The starch molecules, when reextruded, probably had a better mobility along the radius resulting in a higher radial expansion. Interestingly, 2M1-Extrusion 2 seemed to increase overall expansion ratios more than radial expansion ra-

Table 1—Effect of type of screw during first extrusion on bulk density, overall and radial expansion, and SME[a]

Screw type	Bulk density (g/cm^3)	Overall expansion	Radial expansion	SME (Wh/kg)
NM	0.316±0.02	4.76±0.35	8.44±1.07	323±45
1M	0.384±0.06	3.99±0.68	9.31±0.95	239±27
2M	0.364±0.12	3.85±0.62	9.23±1.27	311±47

[a]Average of three replications.

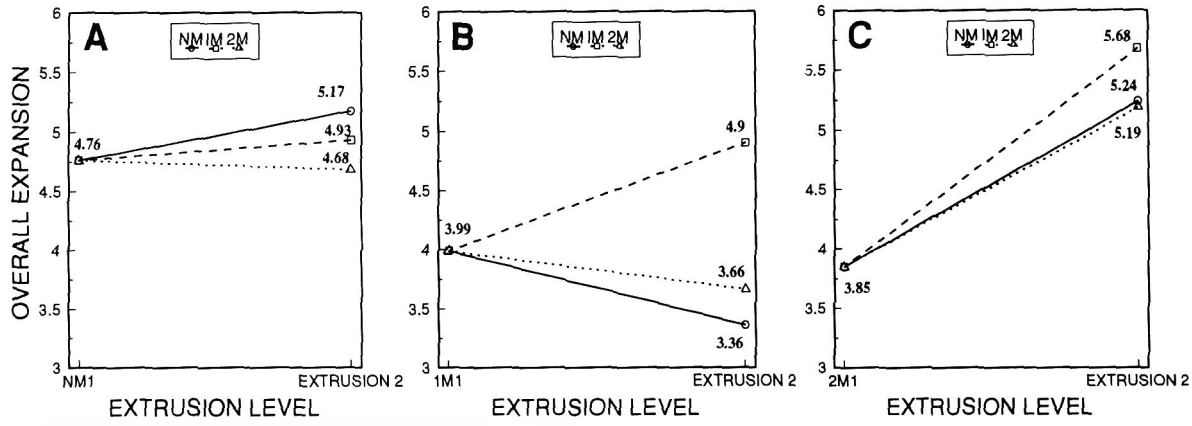

Fig. 2—Effects of screw configuration on overall expansion ratios. Extrusion 2 was carried out with all three screws, i.e. NM, 1M and 2M. A—Extrusion 1 with NM; B—Extrusion 1 with 1M; and C—Extrusion 1 with 2M.

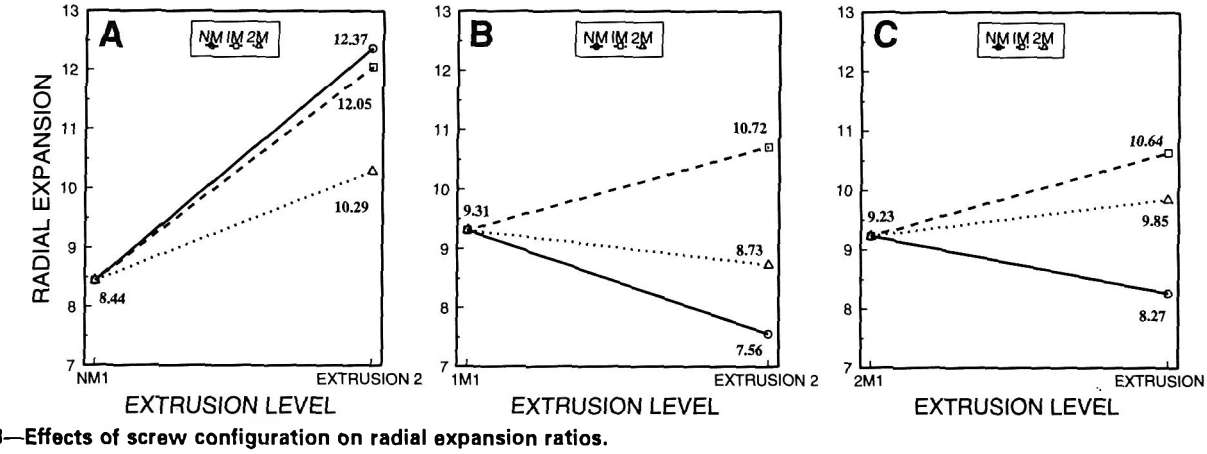

Fig. 3—Effects of screw configuration on radial expansion ratios.

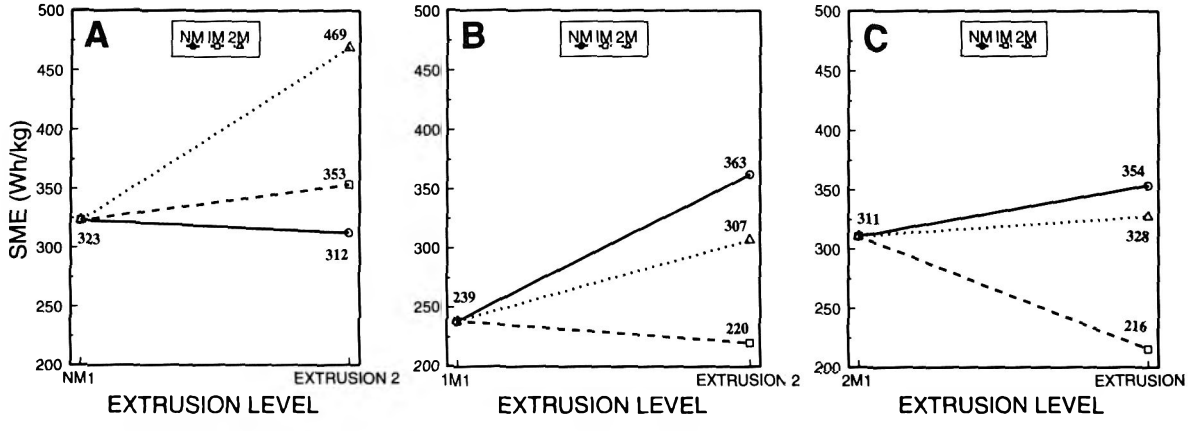

Fig. 4—Effects of screw configuration on specific mechanical energy (SME).

tios (Figs. 2C and 3C). Clearly the NM1-Extrusion 2 exhibited increased radial expansion ratios more than their corresponding overall expansion ratios, 1M1-Extrusion 2 showed similar changes in radial and overall expansion ratios, and 2M1-Extrusion 2 increased overall expansion ratios more than their corresponding radial expansion ratios.

SME inputs into the samples in Extrusion 2 are shown in Fig. 4. SME values for NM1-Extrusion 2 were different ($P>F=0.001$) from 1M1-Extrusion 2 and 2M1-Extrusion 2. SME inputs for NM1-2M2 and NM1-1M2 increased and those for NM1-NM2 decreased compared to the SME input for NM1 (Fig. 4A). Extrudates from 1M1-NM2, 1M1-2M2, 2M1-NM2 and 2M1-2M2 showed increased SME values over their corresponding 1M1 or 2M1 values (Fig. 4B and 4C). The 1M1-1M2 and 2M1-2M2 extrusions required less SME input than their corresponding 1M1 or 2M1 values. The 1M1-Extrusion 2 and 2M1-Extrusion 2 also had significant SME changes ($P>F=0.0260$ and 0.0367 respectively). In Extrusion 2 decreased SME values generally corresponded with increased radial expansion (Fig. 3 and 4). However, overall expansion did not entirely follow the pattern of increases or decreases in SME, which was probably affected by expansion along the axial direction

Results of overall and radial expansion ratios were compared for extrudates for which Extrusion 1 and Extrusion 2 were carried out with the same screw type (Table 3). 2M1-2M2 brought about the highest change in overall expansion (34.8%) and the least change in radial expansion (6.7%).

Table 2—Symbols used in the text to refer to the extrusion using a particular screw in either extrusion 1 or extrusion 2

Screw type in extrusion 1[a]	Screw type in extrusion 2	Symbol used in text
NM	—	NM1
NM	NM, 1M & 2M	NM1-Extrusion 2
NM	NM	NM1-NM2
NM	1M	NM1-1M2
NM	2M	NM1-2M2
1M	—	1M1
1M	NM, 1M & 2M	1M1-Extrusion 2
1M	NM	1M1-NM2
1M	1M	1M1-1M2
1M	2M	1M1-2M2
2M	—	2M1
2M	NM, 1M & 2M	2M1-Extrusion 2
2M	NM	2M1-NM2
2M	1M	2M1-1M2
2M	2M	2M1-2M2

[a] NM—no mixing element; 1M1—one mixing element, first extrusion; 2M2—two mixing elements, second extrusion; NM1-NM2—no mixing first extrusion, re-extruded using no mixing element; 1M1-Extrusion 2—first extrusion using one mixing; all re-extrusion (NM, 1M & 2M).

NM1-NM2 had the highest change in radial expansion (46.6%) and the least change in overall expansion (8.6%) (Table 3, set A). Radial expansion was the highest for NM1-NM2 (12.37) which decreased to 10.72 and 9.85 for 1M1-1M2 and 2M1-2M2, respectively, whereas, overall expansion did not follow this pattern. The changes in expansion of samples were also compared when the screw type in Extrusion 1 was varied and those in Extrusion 2 were kept constant (Table 3, sets B, C and D). Changes in expansion ratios for NM1-1M2, 1M1-1M2 and 2M1-1M2 were similar to those extrudates where extrusion 1 and extrusion 2 were carried out with the same screw type (Table 3, set B). Changes in overall and radial expansion for NM1-1M2, 1M1-1M2 and 2M1-1M2 over their corresponding Extrusion 1 values were 3.6, 22.8 and 47.5%, and 42.8, 15.1 and to 15.3%, respectively. In a set where Extrusion 2 was carried out with NM or 2M and the screws in Extrusion 1 were varied, the overall and radial expansion followed no apparent patterns. Clearly 2M1-Extrusion 2 showed a higher increase in overall expansion than those by either NM1-Extrusion 2 or 1M1-Extrusion 2, irrespective of screw type in Extrusion 2 (Table 3).

Bulk density and SME values of samples in Extrusion 2 were compared (Table 4). Bulk densities of samples, first extruded with 1M and then re-extruded with 1M1-NM2, 1M1-1M2 and 1M1-2M2 were 0.461, 0.307 and 0.378 g/cm^3, respectively, which were higher ($P>F=0.0008$) than samples which were first extruded with either NM or 2M. As expected total SME input (total of SME of Extrusion 1 and Extrusion 2) for 1M1-NM2, 1M1-1M2, and 1M1-2M2 were the lowest at 602, 459, and 546 Wh/kg, respectively (Table 4).

Expandability of starches can change depending on molecular structure, amylose:amylopectin ratio, moisture content and processing conditions. We demonstrated that screw configuration as well as the order of screw type used in a re-extrusion process had notable effect on the expansion properties of corn starch. Changes brought about in extrudate properties by such process variations can affect the product quality. Although physical properties were measured, it was not clear whether variations in expansion ratios or bulk densities of extrudates were due to changes at the molecular level. Further research is needed to determine the effects of screw configurations on molecular and functional properties of extruded starches. Some applications of no mixing and/or mixing elements could be applied for extruded marine feed with high bulk densities to effect controlled sinking, manufacture of water soluble starches without mono or disaccharides, or for applications where process conditions must be mild and only pre-gelatinized starches could be used.

CONCLUSIONS

SCREWS WITH NM, 1M OR 2M ELEMENTS affected the expansion properties of corn starch differently. Extruding with different screws did not influence the variables studied but re-extrusion of the samples had significant ($P>0.05$) effects on all variables studied except overall expansion ratio. NM used in the first extrusion gave the highest overall expansion and the lowest radial expansion. Samples extruded with NM, and then re-extruded, had the greatest increases in radial expansion

Table 3—Effect of type of screw during extrusion 2 on overall and radial expansion ratios[a]

Set	Extrusion 1 screw type	Extrusion 2 screw type	Overall expansion	Radial expansion	Change over extrusion 1 (%) Overall expansion	Change over extrusion 1 (%) Radial expansion
A	NM	NM	5.17±0.39	12.37±1.22	8.6	46.6
	1M	1M	4.90±0.38	10.72±1.48	22.8	15.1
	2M	2M	5.19±0.20	9.85±0.41	34.8	6.7
B	NM	1M	4.93±0.20	12.05±0.60	3.6	42.8
	1M	1M	4.90±0.38	10.72±1.48	22.8	15.1
	2M	1M	5.68±0.28	10.64±0.51	47.5	15.3
C	NM	2M	4.68±0.40	10.29±0.90	−1.7	21.9
	1M	2M	3.66±0.48	8.73±0.64	−8.3	−6.2
	2M	2M	5.19±0.20	9.85±0.41	34.8	6.7
D	NM	NM	5.17±0.39	12.37±1.22	8.6	46.6
	1M	NM	3.36±0.69	7.56±0.58	−15.6	−18.8
	2M	NM	5.24±0.74	8.27±0.82	36.1	−10.4

[a]Average of three replicates.

Table 4—Effect of type of screw during extrusion 2 or extrusion 1 + 2 on bulk density (g/cm^3) and SME (Wh/kg)

Extrusion 1 screw type	NMS Bulk density[a]	NMS SME[a] 2[b]	NMS SME 1+2[c]	1MS Bulk density[a]	1MS SME[a] 2[b]	1MS SME 1+2[c]	2MS Bulk density[a]	2MS SME[a] 2[b]	2MS SME 1+2[c]
NM	0.292±0.03	312±11	635	0.305±0.01	353±28	676	0.322±0.03	469±76	792
1M	0.461±0.01	363±26	602	0.307±0.02	220±29	459	0.378±0.07	307±37	546
2M	0.290±0.04	354±22	665	0.265±0.02	216±04	527	0.296±0.01	328±06	639

[a] Average of three replicates.
[b] Stands for Extrusion 2 values.
[c] Stands for sum of Extrusion 1 and 2 values.

Microwave Bumping: Quantifying Explosions in Foods During Microwave Heating

Y.C. FU, C.H. TONG, and D.B. LUND

ABSTRACT

An apparatus was developed to quantify the degree of bumping due to the explosion of food particulates during microwave heating. The apparatus consisted of a sound recording device, a digital storage oscilloscope, and an electric filter. Degree of bumping for a product was defined by integration of the magnitude of an explosion over time of bumping. The sound spectrum of explosion was analyzed by Fast Fourier Transform (FFT) analysis. Frequency of bumping was usually in the range 1.6 kHz to 8 kHz. The character of microwave bumping varied according to localized microwave superheating effects, product formulations, and prior heat treatment of the food.

Key Words: bumping, explosions, microwave heating

INTRODUCTION

SHELF-STABLE MICROWAVE MEALS (established by the introduction of Lunch Bucket® in 1985) is one of the fastest growing product types in the food industry. Shelf-stable foods are available in many different retortable plastic containers (Labell and Rice, 1985). An important and attractive feature of such containers is their microwaveability. Consumers can store, heat, and eat food in them (Farrell and Organ, 1988). The plastic is usually a multilayered construction, consisting of 5 or 7 layers, with an oxygen barrier material of either ethylene vinyl alcohol (EVOH) or polyvinylidene chloride (PVDC) at the center. Foods packaged in such containers have shelf life up to 2 yr (Wachtel et al., 1985). Shelf-stable microwavable prepared foods generally are marketed in tubs, trays, or pouches.

Further requirements for microwave packages are that they should not be messy to use, and potential physical hazards from heated products such as eruptions of viscous materials upon reaching their boiling points should be controlled (Perry, 1987). The term, "microwave bumping," also called popping or splattering, is descriptive of an explosion phenomenon and is characterized by a jostling or shaking of the container, usually accompanied by an audible, abrupt, explosive sound. When microwave bumping occurs, such explosive sounds, which may be heard several meters away are annoying and may be an unexpected surprise to consumers. Splattering of soup and food particles around the inside of a microwave oven is a common consequence of microwave bumping. When very severe, the container may invert or spill during microwave heating because of the momentum of such explosions.

Although microwave bumping has been observed and reported, few published studies of the phenomenon are available. Our objectives were to develop a method to quantify microwave bumping and to demonstrate the applicability of such method to study factors influencing microwave bumping.

MATERIALS & METHODS

Apparatus

The system to generate, measure and quantify bumping explosions during microwave heating consisted of components to record this sound spectrum and its amplitude variations with time (Fig. 1). A Toshiba Model ERS 6831B microwave oven with 720 watts of power was used. The power supply circuit was modified so that the field intensity in the cavity could be changed continuously (Tong et al., 1993). The food container was inserted and fixed in the cavity by being placed in a hole at the center of a polyfoam plate (2 cm thick) which covered the horizontal area of the cavity. The vertical position of the container could be adjusted by moving the polyfoam. Temperatures and pressures at several locations inside the container were monitored by fiber optic temperature and pressure probes and sensing units (Model 1400, MetriCor, Inc., Woodinville, WA).

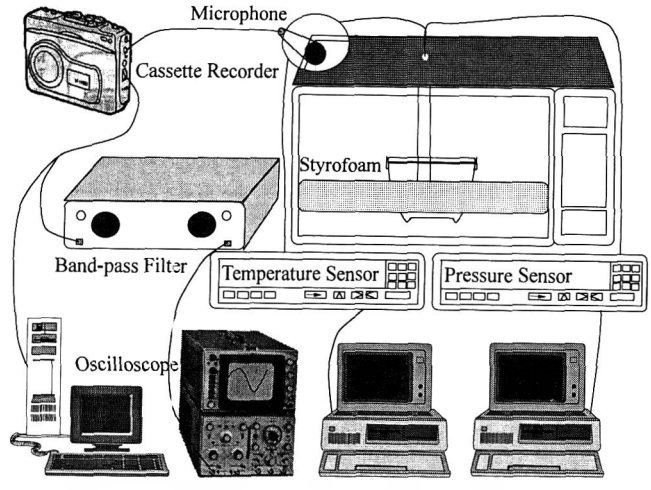

Fig. 1—Apparatus for measuring bumping intensity.

The explosion sounds produced in the microwave oven were recorded and measured during the process to gain a broad understanding of auditory information in microwave heating. The sound pick-up device was a dynamic miniature microphone of a low-impedance omnidirectional type (Realistic Brand, Fort Worth, TX) which was placed behind the manufacture-installed ventilation holes, just between the microwave cavity and the outer metal cover. The microphone was positioned ≈20 cm horizontally and 10 cm vertically from the experimental subject. The frequency response of the microphone was 30–18000 Hz and its sensitivity was 72 db ± 4 db. Sounds were recorded on Maxell brand XLII-S 90 tape using a Panasonic RQ-330 mini cassette recorder equipped with a stable 3 volt AC Adapter. To obtain a stable recording and playback level, a constant volume level of sound was used.

Two methods for analyzing the sound spectrum from the tape recorder were used. In one we used a digital storage oscilloscope (Model 2221, Tektronix, Inc., Woodbridge, WA) and an electronic filter (Model 3100R, Krohn-Hite Corp., Cambridge, MA), so that the playback of a short recording was first fed into the filter to remove background noises. Both the high and low cut-off frequencies of the filter were independently adjustable over the frequency range 10 Hz to 1 MHz. Output from the filter was fed directly into the digital oscilloscope which enabled automatic storage of waveforms introduced by the trigger mechanism.

In the second case we used the playback of a short recording which included the bumping spectrum which was fed into a computer equipped with a data acquisition system and a commercial software package, EASYEST LX (Asyst Software Technologies, Inc., Rochester, NY). Data were sampled and stored directly to the file. The data acquisition rate was 30,000 Hz and EASYEST LX collected and stored a 2 min segment of the sound spectrum into four separate files. Each data file was up to 18.9 Mbytes. Then, data from each file were analyzed. The frequency spectrum was further analyzed by Fast Fourier

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Transform (FFT) which is a widely used signal-processing and analysis concept. The essence of the Fourier Transform of a waveform is to decompose and separate the waveform into a sum of series of oscillations with various frequencies and amplitudes. Hanning weighting or window function was used throughout to generate the spectrum (Brigham, 1988). The forward FFT decomposed the signal into its periodic component waves of various frequencies and amplitudes.

Food products

During initial stages, commercially available Lunch Bucket® and Top Shelf® products, purchased from a local supermarket, were used as model systems. Lunch Bucket® was a bowl-shaped container with a bottom diameter of 5.5 cm, a larger top diameter of 9 cm, and a depth of 7 cm. Top Shelf® was a shallow tray (13 cm L × 10 cm W × 3 cm H). Later work was performed mostly with fresh, blanched-frozen, and sterilized diced potatoes, green beans, diced carrots, and lima beans. An emptied bowl-shaped Lunch Bucket® was used as a container. To study effects of salt content and heat treatment of foods on microwave bumping, all the above food particulates were sterilized in 0%, 1% and 2% salt solutions for 60 min at 121° C. Then, all food particulates were stored for 10 to 20 days to achieve equilibrium with the brine prior to evaluation.

RESULTS & DISCUSSION

ABILITY TO DEFINE THE DEGREE OF BUMPING quantitatively is essential to investigate its causes. Based on our experience and the magnitude of the explosion sound, we arbitrarily established four degrees of bumping: small, medium, large and extremely large. Small and medium bumping are not much different. They are audible but not loud enough to give people an uncomfortable feeling. Large and extremely large bumping are loud explosion sounds, are annoying, and would probably surprise consumers. For those two levels, consumers might think something was wrong inside the microwave oven. Of these bumpings, ≈90% will be accompanied by jostling or shaking and the container may spill or invert. Microwave bumping was qualitatively assessed for several commercial supermarket products using such judgment (Table 1). Most bumping occurred with potatoes, carrots, and green beans.

Frequency-time studies

In one method to quantify sounds from bumping we used a digital storage oscilloscope and an electronic filter to remove background noises by setting low and high cutoff frequencies of an electronic filter. In principle, if the explosion frequency of these four degrees of bumping was quite different from that of background noise, a rather clean and pure explosion spectrum should be obtained. The background noise was complicated, and it was difficult to isolate small and medium bumping using the trigger mechanism of the digital oscilloscope. This probably indicated that the frequency of small and medium bumping and background noises were too close to separate by cutoff frequency. In the microwave oven, the background sound spectrum is usually complicated by other noises in the room, the magnetron cooling fan, the mode stirrer, and/or boiling of food product. Also, using the trigger mode and storage function of our oscilloscope we could not isolate and save the entire waveform of a single bump.

In another method, the entire spectrum from the original tape including the bumping and background noises were reproduced and stored by a computer. The data acquisition rate was 30,000 Hz, fast enough to isolate and record the bumping spectra. A typical background noise spectrum (Fig. 2) by Fast Fourier Transform (FFT) analysis indicated high levels of low frequencies with most of the noise being < 1000 Hz. Some frequencies of background noise occurred in the range 1000–1600 Hz but at very low levels. Typical spectra of small, medium, large and extremely large bumping were compared (Fig. 3).

Using FFT analysis, determining the frequencies of small bumping was difficult because they were similar to background noises or not of sufficient amplitude. For medium, large and extremely large bumping, however, FFT analysis enabled separation of bumping from background noise. Using the low (1600 Hz) and high (8000 Hz) cutoff frequency to remove background noise (Fig. 4) we could identify characteristic frequencies of the three kinds of explosion. Selected peaks were identified by frequency and intensity. Basically not much difference occurred in frequencies of the classified bumping although amplitude of selected frequencies was related to degree of bumping. Furthermore, extremely large bumping was characterized by a second peak at about 6000 Hz. For a given characterized bumping, dominant peaks could be reproduced ± 300 Hz, and associated intensities ± 10%. The only difference between medium and large bumps was amplitude. That of the highest peaks for a medium bump was about 30% of that for a large or extremely large bump.

Degree of bumping (DOB)

We could identify the bumping waveform by finding a group of peaks which were obviously different from the background noises (Fig. 3). Spectra for medium, large and extremely large bumps were compared (Fig. 5) after the band pass filter removed background noise outside the range 1600-8000 Hz. From this, degree of bumping was defined by the area of intensity over time of the bump:

$$DOB = \int_{t_i}^{t_f} ABS(V_i - V_{baseline}) \cdot dt \qquad (1)$$

where V_i is the magnitude of an explosion i measured in volts, $V_{baseline}$ is the magnitude of the baseline of an explosion (always equal to 0.1 volt), ABS ($V_i - V_{baseline}$) is the absolute value of ($V_i - V_{baseline}$), and t_i and t_f are the initial and final time of a section of the spectrum, respectively.

The degree of bumping as produced by the integral bumping area method (Eq. 1) was superimposed with the average produce temperature for one Lunch Bucket® sample (scalloped potatoes with ham chunks) during 2 min of microwave heating (Fig. 6). Using auditory perception to characterize degree of bumping (identified by the bands of dashed lines), the value of degree of small bumps was less than 0.04, for medium bumps 0.04–0.05, for large bumps, 0.05–0.065, and for extremely large bumps >0.065. The degree of bumping was related to temperature. The explosion spectra would vary with the oven due to sound resonant properties of the oven cavity. Thus, such DOB values would be oven dependent.

Explanation for microwave bumping

There are at least two apparent explanations for microwave bumping. Food being cooked in a microwave oven is coupled with microwave energy at a rate similar to that when lightning strikes a tree. The mode of action is similar except for magnitude. As water (sap) in a tree flashes to steam, the tree may explode due to internal pressure. This also occurs when microwave energy is applied to foods that are nonporous to steam and represents one mechanism for producing the sound associated with microwave bumps.

When food is heated in a microwave oven, steam bubbles nucleate, grow, and explode. This produces the bumping sound. Figure 7 shows temperature and pressure profiles at three different locations of a Lunch Bucket® containing Country Vegetable Soup with the plastic lid on. Most bumping occurred between 90 sec and 150 sec heating time. Temperature and pressure increased slowly near top and bottom. Increase in pressure was probably due to the temperature coefficient of the pressure probe. Temperature and pressure sharply changed

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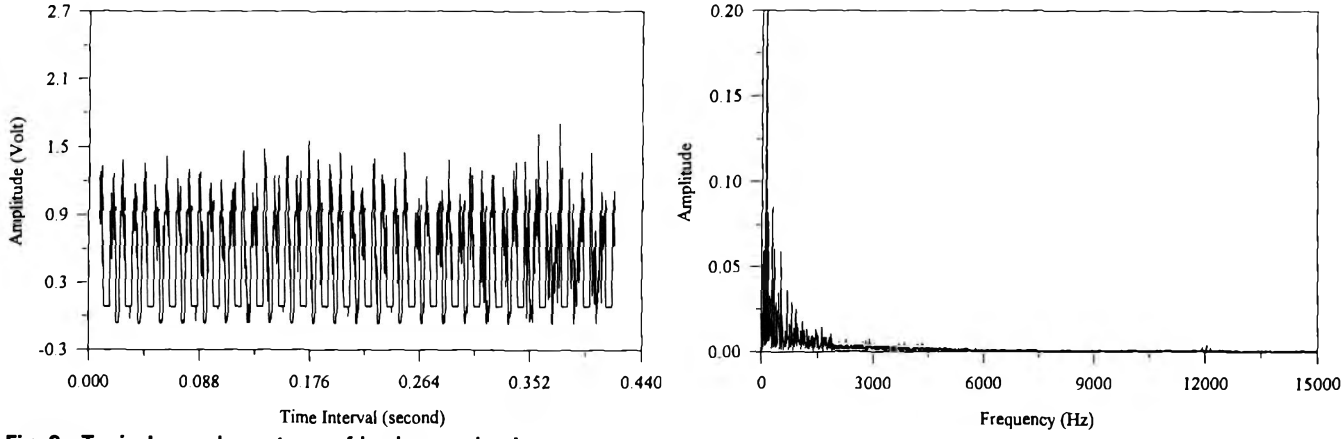

Fig. 2—Typical sound spectrum of background noises.

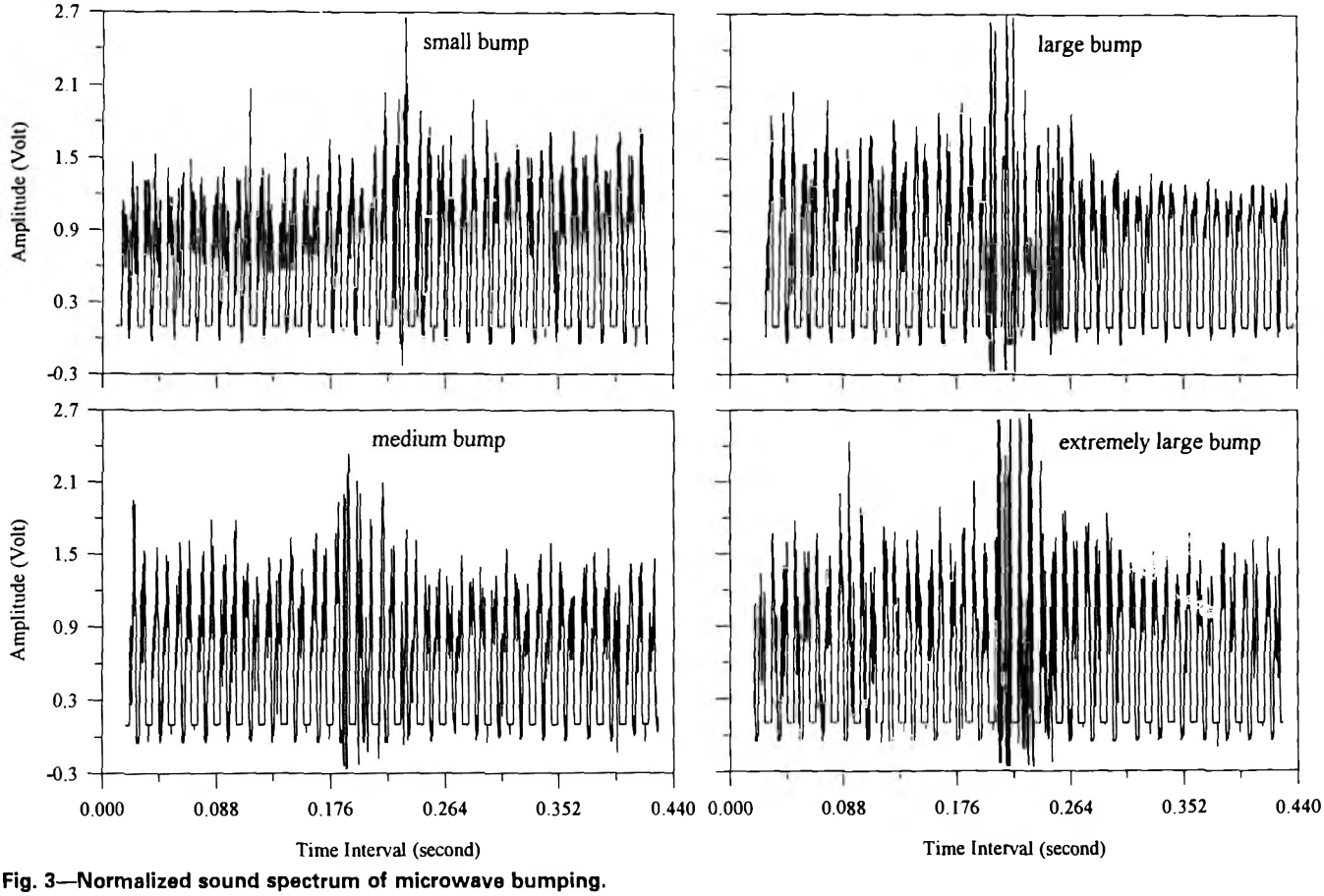

Fig. 3—Normalized sound spectrum of microwave bumping.

Table 1—*Qualitative degree of microwave bumping for commercial products*

Experimental Products	Major Particulates	Salted	Plastic container	Number of bumps[a] Small bump	Medium bump	Large bump	Extremely large bump
Scalloped Potatoes with Ham Chunk	Potatoes, Ham	Yes	Bowl	5	7	5	4
Potatoes with Cheese	Potatoes	Yes	Tray	3	4	4	5
Beef Stew (company A)	Potatoes, Carrots, Beef	Yes	Bowl	2	2	5	2
Hearty Beef Stew (company B)	Potatoes, Carrots, Beef	Yes	Bowl	2	3	2	1
Country Vegetable Soup	Potatoes, Carrots, Green Beans	Yes	Bowl	6	2	3	2
Chicken Noodle Soup (company A)	Noodles	Yes	Bowl	4	3	1	0
Chicken Noodle Soup (company B)	Noodles	Yes	Bowl	5	3	0	0
Beef Noodle Soup	Noodles	Yes	Bowl	5	2	0	0
Bean with Bacon'n Ham	White Beans	Yes	Bowl	3	2	3	1
Macaroni & Cheese	Macaroni	Yes	Bow	1	0	0	0
Spaghetti's Meatsauce	Spaghetti	Yes	Bow	1	0	0	0
Pasta'n Chicken with Vegetables	Noodles	Yes	Bowl	1	0	0	0
Pasta Primavera	Macaroni	Yes	Tray	0	0	0	0
Lasagna (company B)	Macaroni	Yes	Bowl	0	0	0	0
Hearty Lasagna (company B)	Macaroni	Yes	Bowl	0	0	0	0
Italian Style Lasagna	Macaroni	Yes	Tray	0	0	0	0

[a] *Mean value of three samples, heating time = 105 sec, 720 watts microwave oven.*

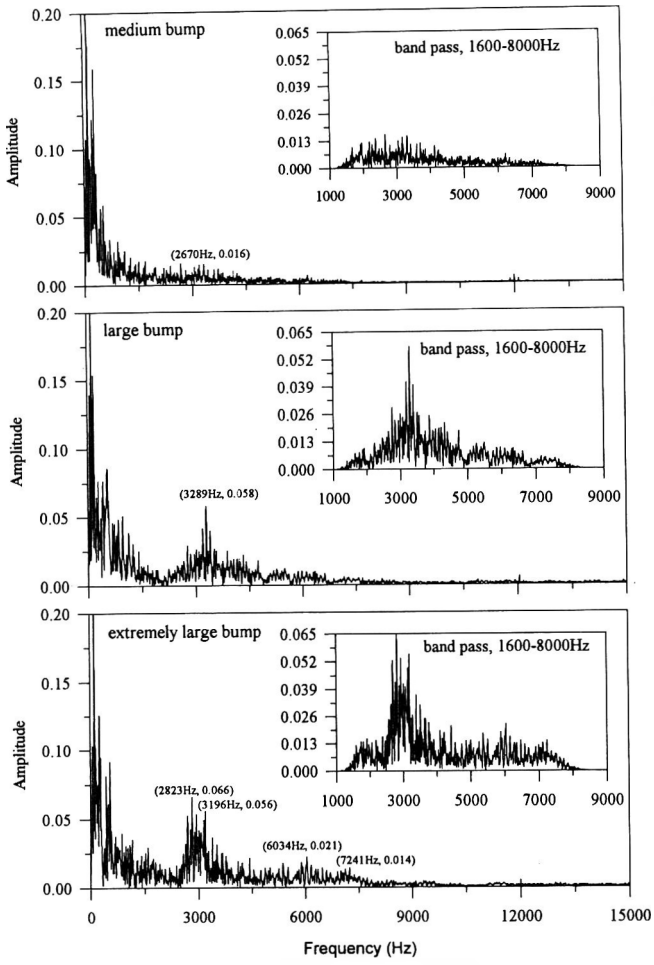

Fig. 4—FFT analysis for medium, large and extremely large bumping. Insets: All frequencies except 1600—8000 Hz are removed (background noises).

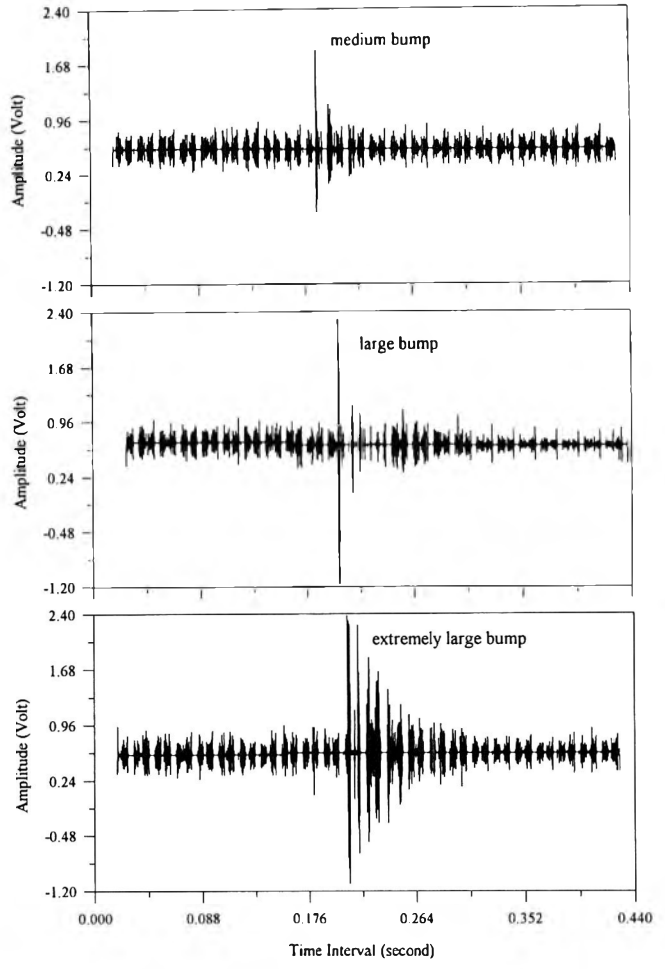

Fig. 5—Normalized sound spectra of medium, large and extremely large bumping in the frequency range (1600–8000 Hz).

at the geometric center. This increase can be explained by the focus heating effect near the geometric center of the container, a common phenomenon for cylinders and spheres (Ohlsson and Risman, 1978). The temperature and pressure probes at the geometric center sensed high heat and severe pressure changes, respectively, which coincided with bumping either at that specific location or in the immediate vicinity of the probes. A sharp increase in pressure occurred at 95 sec which exactly coincided with one extremely large bump. However, it does not follow that all bumps could be correlated with pressure measurements since all bumps did not occur at the probe location. It would probably not be feasible to map bumping throughout the container even if more temperature and pressure probes were used.

Microwave bumping can occur within the food particulate or the viscous liquid phase. When bumping occurs in the food particulate, the pressure would be sufficiently high to break the cell wall, probably producing large or extremely large bumps. If bumping occurs in the viscous liquid phase, or near the surface of a particulate, only small and medium bumps would be produced. Stirring or shaking the food material in its container did not reduce the incidence of bumping.

Microwave bumps did not occur in products such as pasta (spaghetti and noodles) (Table 1) but very notable bumps occurred in products containing food particulates such as potatoes, carrots, green beans, and lima beans. Notable bumping was also observed with vegetable soup, containing both liquid and food particulates. No bumps were observed when particulates were removed and only the liquid was heated. However, microwave bumps were again observed when the particulates were reconstituted with distilled water and microwave heated.

These observations led us to study the effects of treatment of particulates on microwave bumping. In studies using 35 size-graded cut green beans (3 cm long, green bean/liquid = 4/1, w/w) which were thermally processed for commercial sterility in 1% salt solution, six extremely large bumps occurred when the product was microwave heated in the plastic container for 1.75 min. Examining the green beans after the bumps, we observed many small broken pieces and only 29 of 35 green beans were still in their original shape. Three additional experiments also resulted in the same degree of bumps and many broken pieces of green beans. This was evidence that explosion of food particulates can produce large microwave bumps.

A second explanation for the sound associated with microwave bumps could be localized boiling of the liquid. If this were true, we might expect the magnitude of bumps to be directly related to viscosity of the liquid since more viscous liquids should produce broader local thermal gradients and provide more resistance to bubble rise. However, when we increased the viscosity of the liquid by adding corn starch, no difference in intensity or frequency of bumping was observed. In addition, no bumping occurred if we only heated the liquid without food particulates. Thus, though bumping may occur in the liquid phase, the magnitude of such bumps was not comparable to the bumps which occurred within food particulates.

Localized heating rate

Bumping is hypothesized to be primarily caused by local superheating effects in particulates, where convection and conduction heat transfer cannot remove heat from superheated

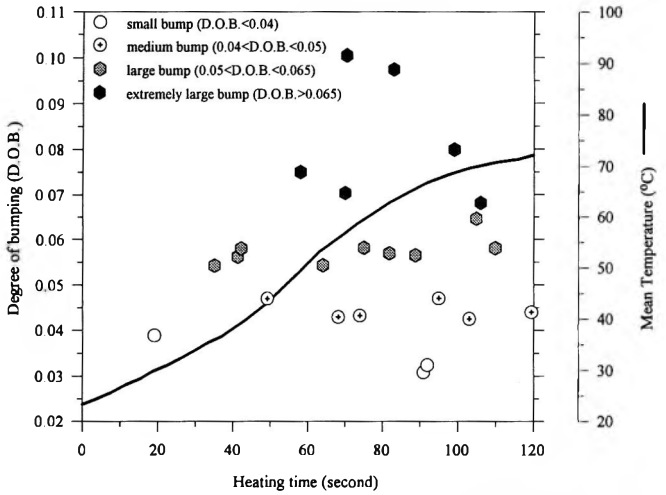

Fig. 6—Degree of bumping and average product temperature profile for microwaving Lunch Bucket® (Scalloped Potatoes with Ham Chunks) in a 720 watts full power microwave oven for 2 min.

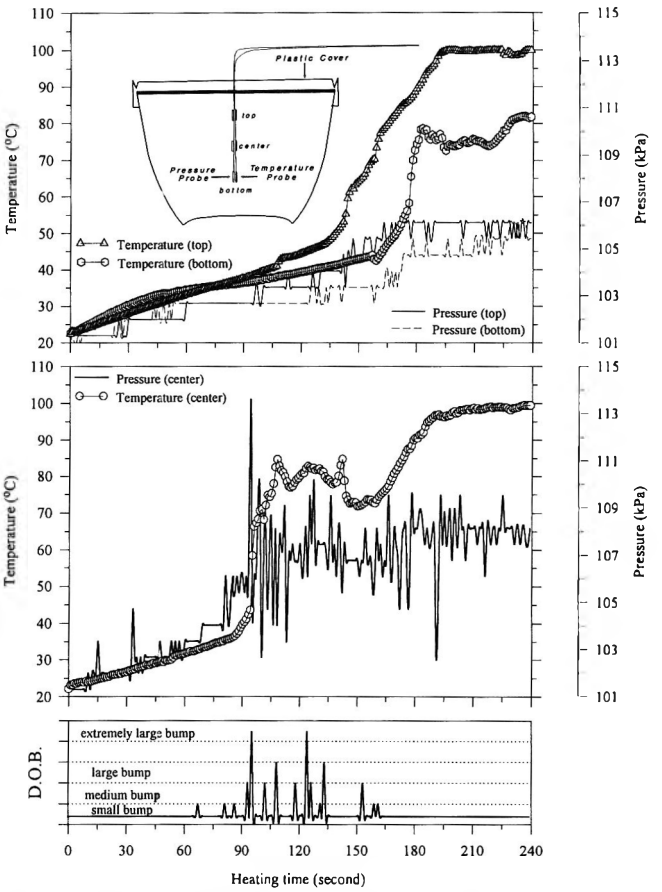

Fig. 7—Typical temperature and pressure profiles for microwave heating in the liquid phase at the geometric center of a Lunch Bucket® containing Country Vegetable Soup.

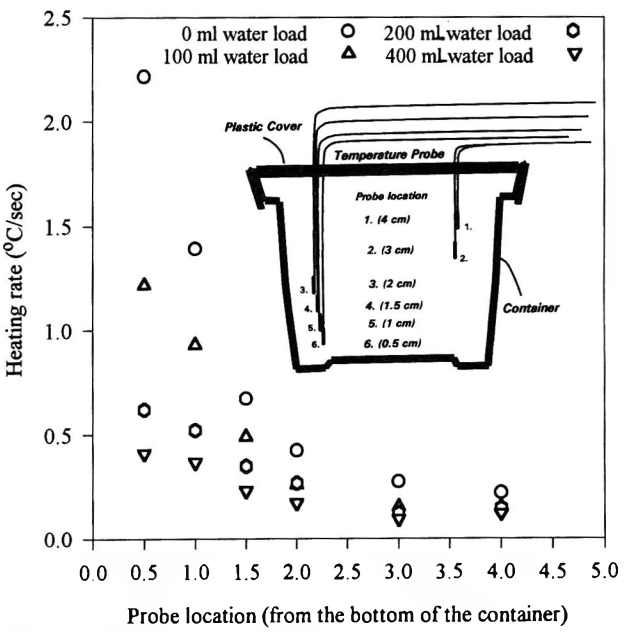

Fig. 8—Effect of water load on localized heating rate in a microwave oven.

regions before a violent bump occurs. Thus, higher power, electric field intensity, and heating rate, would produce greater incidences of bumping. Microwave oven power output can be adjusted in two ways. Conventional microwave ovens operate with duty cycle control to achieve proportional power (i.e. 50% power means on at 100% power for 50% of the time). The other method is to have true proportional power from the magnetron tube. We modified our oven for this control operation (Tong et al., 1993). For the same final average product temperature, data in Table 2 showed a higher incidence of bumps when duty cycle control was used. That was because the oven on duty cycle control promoted local hot spots of higher temperature than occurred in the oven with proportional power control.

The same effect as proportional power control could be achieved by increasing the load in the oven. The localized heating rate was reduced when more water load was present in the microwave oven (Fig. 8). We also found that moving the food container from the bottom plate to the center of the oven tended to reduce bumping. Since the microwave energy inlet was at the bottom the electric field intensity would probably be higher at the bottom.

Container shape

Bumping is a food product related phenomenon that must be addressed for microwavable containers. We observed bumping in Top Shelf® as well as OMNI® and Lunch Bucket® containers. Bumping occurred in trays, bowls, cylinders, and glass beakers. Generally, bumping was independent of container shape and material. However, container shape may influence heating performance of a food product and location of bumping in the container. This could be due to edge, corner, and focus heating effects.

In focus heating, maximum heating occurs in the center for certain spherical and cylindrical geometries (Ohlsson and Risman, 1978). The explosion of eggs during microwave heating is one of the best known demonstrations of core heating effect. This is due to center heating releasing a steam energy impulse with such high power it moves the surrounding mass parts away from each other (Ohlsson and Risman, 1978). The maximum heating region also moves from the center toward the surface when the diameter increases.

Another reason for uneven heating is the electromagnetic boundary conditions at edges and corners (Pearce et al., 1988). This uneven heating is the "edge and corner" effect. Square containers can result in overheating at the corners of the product due to a greater surface area/volume ratio in these areas, resulting in more microwave energy absorption. Circular or oval containers help reduce such edge and corner effects as energy absorption occurs evenly around the edge. From our results, container geometry changed the microwave heating pattern but did not reduce the degree of microwave bumping. Due to edge

Table 2—Effect of magnitude of microwave power on degree of bumping

	Number of bumps[c]						
	Continuous power 100%[b]	Continuous power 70%	On/Off power 70%	Continuous power 50%	On/Off power 50%	Continuous power 30%	On/Off power 30%
Small bump[a]	3	2	2	1	2	5	4
Medium bump[a]	5	4	3	2	4	1	2
Large bump[a]	6	6	7	1	4	0	4
Extremely large bump[a]	4	3	2	0	2	0	0
Heating time (sec)	90	120	120	165	165	240	240
Mean temp (°C)	65	64	68	63	65	63	62

[a] As defined by degree of bumping (DOB)(Eq. 1).
[b] 100% full power = 720 watts.
[c] Mean value of three samples.

and corner effects and core heating effects, explosion of food particulates usually occurred either at corners or edges for trays or at the center for bowls and cylinder-like containers.

Heat treatment and salt concentration

Heat treatment is an essential step in preserving most foods by sterilization, freezing or drying. Texture and cell structure changes are influenced by the cellular nature of vegetables and the degree of heat treatment. Such treatments weaken and eventually destroy cell walls, allowing air and cell sap to escape usually resulting in softening. We reasoned that raw vegetables may be more susceptible to microwave bumping since rupture strength would be greater for raw than for previously heated vegetables. Pressure could build up inside the vegetable tissue ultimately reaching sufficient magnitude to cause an internal explosion. However, we found that only sterilized vegetable products produced microwave bumping.

No bumping was found when cut fresh green beans and water mixtures were microwave heated. The same results of no bumping were also observed when blanched frozen-thawed green beans, carrots, and lima beans, purchased from the local supermarket were microwave heated. We applied additional heat treatments to those thawed food particulates by either cooking them in 0, 1 or 2% salt water at 100° C for 30 and 60 min or steaming them at 100° C for the same time and then mixing them with 0, 1 or 2% salt solution. All food particulates were stored for 10 to 20 days to achieve equilibrium with salt water before exposing them in brine to microwave heating. No bumping was observed when these food particulates and their brine were microwave heated.

Using the same sources of vegetables, canned foods were prepared with 0, 1 or 2% salt water solution and exposure for 60 min at 121° C in a retort. Notable bumping occurred when salted canned foods were microwave heated. In another experiment, green beans were separated from the liquid after they were sterilized in pure water or 1% salt solution. Both the unsalted and salted green bean particulates were then mixed with pure water or 1% salt solution (green bean/liquid = 4/1, w/w) and tested for microwave bumping. Only the "salted" green beans produced bumping in both pure water and 1% salt solution. The sterilized "unsalted" green beans did not produce bumping in either pure water or 1% salt water solution. Similar results were found for lima beans and diced carrots.

In order to explain these observations, it is necessary to understand what happens to salt in canned vegetables and the influence of salt on microwave absorption. During retort processing, the degree of heat treatment is extremely high which causes excessive softening (Huang and Bourne, 1983). Salt diffuses into the vegetable, while osmotic effects tend to remove water and cause surface cell collapse. With the diffusion of salt into the particulate, microwave power adsorption is significantly affected. Salt has the effect of increasing heating rate of a particulate due to the contribution of d.c. conductivity which increases the dielectric loss factor and reduces penetration depth. Consequently, salted particulates are more likely to have local hot spots which can result in explosions. Thus, high heat treatment of food particulates which causes excessive softening and salting of food particulates which causes increased microwave heating rate are two conditions which apparently cause microwave bumping. The maximum heat treatment which can be applied to vegetables without bumping during subsequent microwave heat treatment has not been determined.

CONCLUSIONS

SOUND GENERATED BY EXPLOSION OF FOOD during microwave heating was studied and a technique to quantify the degree of microwave bumping was developed. The degree of bumping could be determined using the area of intensity over time of the acoustic signal. Microwave bumping is hypothesized to be caused by several factors including product composition. Not all food particulates caused bumping when heated in a microwave oven. Meat (i.e. beef stew, ham chunk, meat balls), fish, chicken, and pasta did not bump. Vegetables such as potato, green bean, carrot, and lima bean bumped. Some vegetables like tomato, celery, and green pepper did not bump. Microwave bumping is due to explosion of food particulates, not localized boiling of liquid. Increasing viscosity of the liquid did not affect intensity or frequency of bumping. Salted vegetable particulates are more likely to have local hot spots which can result in bumping. Degree of microwave bumping is apparently directly related to local superheating effects. The higher the electric field intensity, the greater the incidence of bumping. Container shape influenced heating pattern of a food product and location of bumping in the container. Sterilizing vegetable particulates which causes excessive softening and salting food particulates which increases microwave heating rates are conditions which caused microwave bumping.

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Caffeic Acid Activity Against *Clostridium botulinum* Spores

BOBBY L. BOWLES and ARTHUR J. MILLER

ABSTRACT

Caffeic acid (CA) is widely distributed among higher fruits and vegetables. While CA has antimicrobial activity, little information exists on its utility as a food additive. As such, CA was tested for activity against *Clostridium botulinum* spores. At 0.78 and 3.25 mM, CA inhibited germination for 6 and 24 hr, respectively, with >100 mM required to render spores nonviable. CA concentrations ≥ 50mM reduced 80°C spore thermal resistance. Sporostatic activity was retained when tested in commercial meat broths, and 5.0 mM CA delayed toxigenesis. Caffeic acid has potential as a food additive to inhibit growth of *C. botulinum*, and reduce thermal processing requirements of heat sensitive foods.

Key Words: caffeic acid, antibacterial, microbes, *Clostridium botulinum*, food additives

INTRODUCTION

CLOSTRIDIUM BOTULINUM is an anaerobic, Gram-positive, endosporulating bacillus that synthesizes a potent neurotoxin. The disease induced by the neurotoxin is of international concern with distinct seasonal and strain regional differences (Hauschild, 1989). Foodborne botulism outbreaks are a frequent form of the disease, and are usually associated with home prepared products. Recent U.S. and Canadian (1977-1985) outbreaks were attributed to foods at restaurants (Sugiyama, 1990). Vegetable and fruits are the most often implicated food vehicles, followed by fish and fish products, condiments, and beef or pork products. *C. botulinum* spores are the important initial contaminants, and inhibition is primarily directed against spore transition phases (e.g., activation, germination and/or outgrowth) to ensure botulism-safe foods.

The incidence of botulism is low in the U.S. but toxin potency, health concerns about some antimicrobial agents such as $NaNO_2$, and growth potential in low oxygen-tension packaging, necessitate further investigations to identify alternative antibotulinal agents (Bean and Griffin, 1990; Conner et al., 1989). Organic acids inhibit growth of several bacterial pathogens, and their study as antimicrobial food additives has been reported (Debevere, 1988; Miller et al., 1993; Mountney and O'Malley, 1965; Palumbo and Williams, 1992).

Hydroxycinnamic acids, their methylated and sugar derivatives, are ubiquitous throughout the plant kingdom (Harborne and Conner, 1961). Although hydroxycinnamic acids, such as caffeic acid (CA) are not approved as food additives, they are common naturally occurring components of most daily dietary intakes. The total human dietary intake of plant phenols may approach 1.0 g/day (Brown, 1980). CA reduces the incidence of cancer by preventing *in situ* formation of carcinogens from precursors (Yamaguchi and Iki, 1986). CA and its sugar esters are antibacterial (Ravn et al., 1989; Toda et al., 1989), antifungal (Valle, 1957), antiviral (John and Mukundan, 1979), and inhibit mycotoxin production (Paster et al., 1988). Furthermore, CA interacts with metal ions such as iron (Kontoghioghes et al., 1986), that have been demonstrated to be essential *C. botulinum* growth factors.

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The antibotulinal activity of hydroxycinnamic acids is not well defined, and few have been tested for spore inhibition. The inhibitory activity of CA, coumaric, ferulic and sinapic acids against Gram-positive bacteria (Herald and Davidson, 1983; Nowak et al., 1992) suggests, however, that they may have potential as antibotulinal agents. CA is a natural component of raw plant materials for which antibotulinal activity has been reported (Ismaiel and Pierson, 1990). Our objective was to test CA to determine activity against *C. botulinum* spores. The antibotulinal efficacy was defined by assessing its effect on germination rates, dipicolinic acid release, spore thermal resistance, and vegetative cell toxigenesis.

MATERIALS & METHODS

Cultures

A spore mixture containing three type A (33, 62A, 69) and 3 type B (999, 169, ATCC 7949) proteolytic *C. botulinum* strains was used. Individual strain spore suspensions were prepared by culturing in botulinal assay medium (Huhtanen, 1975) without thioglycollate (BAM) for 21 days at 32°C in a flexible anaerobic chamber (Coy Laboratory Products, Ann Arbor, MI). Anaerobiosis was maintained using a gas mixture consisting of 10% CO_2, 5% H_2, and 85% N_2 by periodic gas-exchange flushes and palladium catalyzed O_2 removal. Spore crops were harvested by 3 successive centrifugations at 17,310 × *g* for 10 min at 5°C with sterile distilled/deionized H_2O washes between centrifugations. Spore pellets were suspended in sterile distilled/deionized H_2O, heat-shocked (10 min at 80°C) and stored at 5°C prior to use. *C. botulinum* confirmation was based on Gram-reaction cellular morphology, neurotoxin production confirmed by mouse bioassay, lipase, catalase, and oxidase activities (Centers for Disease Control, 1974). Each spore crop was quantified and the six-strain spore mixture prepared by combining equal numbers of individual strains to provide a final concentration of 4.7×10^5 spores/mL. Viability and germination rates of individual strains were tested initially, and monthly on spore mixtures (Centers for Disease Control, 1974). All analyses, unless otherwise indicated, were conducted using heat (10 min at 80°C) activated 4.7×10^5 CFU/mL *C. botulinum* spores.

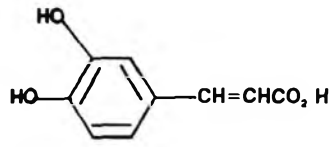
Caffeic acid

CA (cis-butanedioic acid) was purchased as the 99.9% free acid from Sigma Chemical Company (St. Louis, MO) and used as supplied. A 0.2 M stock solution was prepared in 95% ethanol and stored at 5°C. Final ethanol concentrations of treatments for antimicrobial testing were below those reported to be sporostatic or sporicidal (Koransky et al., 1978). The structure and some properties of CA are listed (Table 1).

Determination of spore minimal inhibitory concentrations (MIC)

A quantitative broth dilution method was used to determine the effect of CA on C. *botulinum* spores (Bowles and Miller, 1993a,b). CA was serially diluted (200, 100, 50, 25, 12.5, 6.25, 3.13...-0.05 mM) in 1.0 mL BAM broth (pH 7.0 ± 0.2), and inoculated. The CA concentrations represented a final pH range 7.0–4.0. The tubes were incubated anaerobically for 6 or 24 hr at 32°C, heat-shocked for 10 min at 80°C to destroy germinated and outgrown spores, and 0.1 mL subcultured into 9.9 mL thioglycollate (TG) broth. A separate set of TG tubes inoculated with non-heat-treated culture was included to determine spore viability without thermal treatment. Five replicate TG tubes inoculated with heat- or nonheat-treated culture, were used for each concentration. TG tubes were incubated aerobically for 48 hr at 32°C, then examined for turbidity. A sporicidal effect was defined as no growth in at least 4 TG tubes containing non-heat-treated cultures. A sporo-

Table 1—Description and properties of caffeic acid

Type	Description[a]
Structure	
Synonyms	3-(3,4-Dihydroxyphenyl)-2-propenoic acid 3,4-dihydroxycinnamic acid
Molecular weight	180.15
Melting point	152–153°C
Decomposition	223–225°C (softens at 194°C)
Normal state (25°C)	Yellow-brown crystals
Food regulatory status	Nonapproved
Carcinogenicity[b]	Gastrointestinal tumors in 6 wk old male rats by 2% oral administration
Natural occurrence	Plants Conjugated form (e.g. chlorogenic acid)
Solubilities	Sparingly in cold H_2O; Freely in hot H_2O or cold alcohol

[a] Budavair et al., (1989).
[b] Hirose et al. (1990).

static effect was defined as growth in at least 4 TG tubes inoculated with heat-treated culture.

Dipicolinic acid (DPA) release

DPA release was estimated using the colorimetric assay of Janssen et al. (1958). CA was added to 9.9 mL BAM broth tubes to provide a concentration series of 0, 1, 2, 3, 4, and 5 mM, with pH range 7.0-5.0. Test media were inoculated and incubated 9 hr anaerobically at 32°C. Thereafter, cultures were centrifuged at 1,500 × *g* for 10 min and the supernatant fluid was sampled for colorimetric analysis. One mL of a freshly prepared 0.5M acetate buffered chromogenic reagent was added to 4.0 mL of the culture supernatant. Optical density was measured at 440 nm using a Shimadzu UV-VIS Model 160 spectrophotometer (Kyoto, Japan) and DPA content calculated from a DPA standard curve (0-160 µg/mL).

Effect of CA on spore thermal resistance

C. botulinum spores (8.2×10^6 CFU/mL) were aerobically exposed to 50 or 100 mM (both pH 4.6) of CA in 5.0 mL glass vials containing BAM broth for 30 min at 25°C, and the exposure medium transferred to an 80°C Exacal high temperature water bath (NesLab Instruments Inc., Newington, N.H.) for 5–20 min. A Keithley Metrabyte datalogger model DDL 4100 (Taunton, MA) was used to monitor temperature and equilibration time. After heat treatment, samples were removed, cooled in an ice bath, and 0.1 mL diluted into 9.9 mL 1% peptone-H_2O (pH 7.2). Diluted samples were plated in duplicate onto BAM agar plates using a Spiral Systems Model D plating instrument (Cincinnati, OH) and incubated anaerobically at 32°C for 48 hr. Plates were enumerated using a Spiral Systems Model 500A, then converted into bacterial counts with Spiral Biotech CASBATM II BEN software (Bethesda, MD). Spore thermal resistance was evaluated by comparing the population densities of CA-treated and -untreated BAM samples. A 50 min exposure control (25°C) of non-heat-treated spores with 100 mM CA was included to confirm spore viability in the absence of thermal treatment.

Inhibitory activity of CA in chicken and beef broth

Commercially prepared canned chicken and beef broths were dispensed in 9.9 mL portions to sterile test tubes and caffeic acid added to provide concentrations of 2.0, 3.0, 4.0 and 5.0 mM, with pH range 7.0–5.0. The broths were inoculated and incubated anaerobically at 25°C and examined visually at 24 hr intervals for turbidity. The commercial chicken and beef broths each contained, (manufacture's description) 1% each (w/v) of protein carbohydrate, and fat. Inhibition was defined by comparing the turbidity of inoculated CA treatments to those of uninoculated and unsupplemented controls.

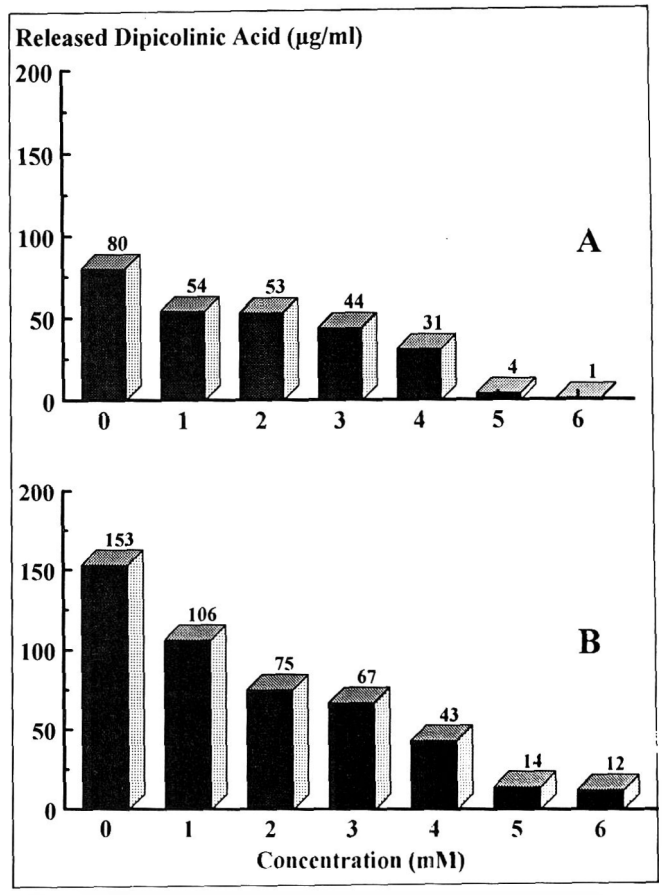


Fig. 1.—Effect of caffeic acid on dipicolinic acid (DPA) release from *C. botulinum* spores in BAM broth after 9 (A) and 24 (B) hr anaerobic incubation at 32°C.

Effect of CA on toxigenesis

Five mL BAM broth tubes containing 0, 5, 10, 25, 50 or 100 mM of CA were inoculated. All tubes, including an uninoculated set of CA test concentration controls, were incubated anaerobically for 48 hr at 32°C, then centrifuged (1500 × *g* for 10 min) to remove cellular debris. A 72 hr bioassay was conducted on duplicate Swiss-Webber 15–20g mice of either sex by intraperitoneal injection (0.5 mL) of undiluted culture supernatant fluid. Polyvalent antiserum controls were included on some samples to confirm clinical symptoms as botulism (Centers for Disease Control, 1974).

RESULTS & DISCUSSION

CA INHIBITED GERMINATION for 6 hr at 0.78 mM and 24 hr at 3.25 mM (data not shown). CA concentrations > 100 mM were sporicidal at both incubation periods (data not shown). Controls with no CA addition germinated, as confirmed by lack of growth in TG broth after heat shocking. Although bacterial spores may remain viable under extreme environmental conditions, germination may be inhibited by minor physical or chemical changes (Gould et al., 1970; Halvorson et al., 1966). Relatively small amounts of a chemical agent can be sporostatic, yet considerably higher concentrations are required to render spores non-viable or to inhibit vegetative cell growth (Bowles, 1991; Bowles and Miller 1993a,b; Smith and Dawes, 1989).

In the presence of CA DPA release was markedly less than unsupplemented controls for up to 24 hr at 32°C. Inhibition was time- and concentration-related, with an average 90% reduction observed at concentrations ≥ 4.0 mM (Fig. 1). Unsupplemented cultures contained 80 and 152.7 µgDPA/mL after 9 and 24 hr at 32°C, respectively. During initial germination phases, degradative processes of cortex embedded lytic en-

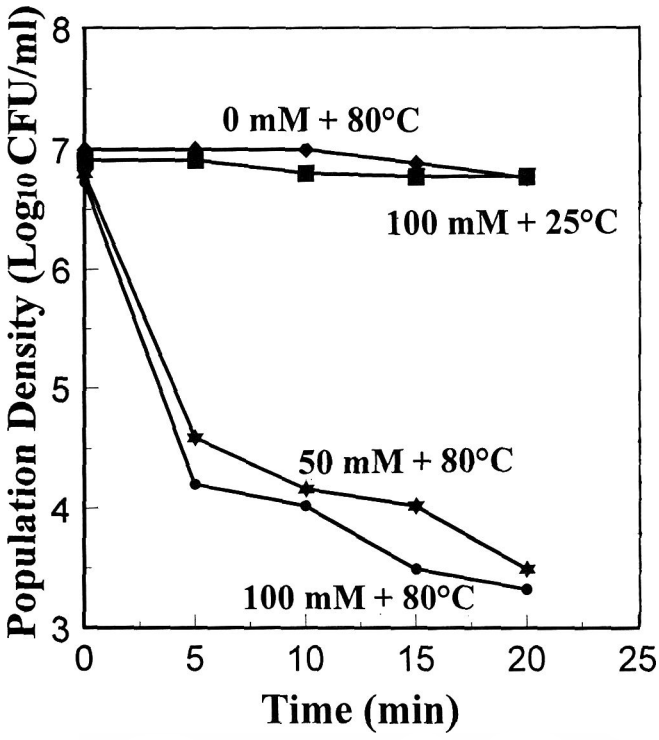


Fig 2.—Effect of caffeic acid on thermal resistance of *C. botulinum* spores in BAM broth. Spores were incubated with various levels of CA at 25°C for 30 min, then transferred to 80°C for 0–20 min. A control was maintained for 50 min at 25°C.

Table 2—Activity of caffeic acid against *C. botulinum* spores in commercial meat broths[a]

Incubation (d)	Minimal inhibitory concentration (mM)	
	Chicken broth	Beef broth
2	2	2
4	2	2
6	3	>5
8	>5	>5

[a] Caffeic acid was tested at 2.0, 3.0, 4.0, and 5.0 mM. Growth was assessed by comparing cultures with unsupplemented (inoculated and uninoculated) commercial broths.

Table 3—Bioassay of the effect of caffeic acid on 48 hr *C. botulinum* challenge in BAM broth at 32°C

Caffeic acid (mM)	Culture toxicity assessment (dead mice/total mice)
0[a]	2/2
C[b]	0/2
5	0/2
10	0/2
25	2/2[c]

[a] Unsupplemented BAM control.
[b] Polyvalent antiserum control, containing spores and no compound.
[c] Mice died of unconfirmed causes.

zymes alter spore permeability with concomitant DPA release (Gould et al., 1970; Halvorson et al., 1966). Reduced DPA release suggested that CA precluded changes in spore coat permeability (Halvorson et al., 1966).

Proteolytic *C. botulinum* spores were reduced by ≤ 100 mM CA for 30 min, followed by 80°C heating for ≤ 20 min (Fig. 2). Temperature equilibrium was reached in 2.7 min and as expected, no decrease in population density occurred in 0 mM CA controls. There was an indirect relationship between the two CA doses and population densities. After 5 min at 80°C a 2.4 or 2.8 $\log_{10}$ CFU/mL reduction occurred at CA levels of 50 and 100 mM, respectively. Inhibition as time-dependent, and after 20 min at 80°C viable spore population densities at 50 and 100 mM CA fell 3.4 and 3.6 $\log_{10}$ CFU/mL with respect to the control. Viability was retained when 100 mM CA was added to challenge spores at 25°C for 50 min, the full chemical exposure time. This indicated that CA had lowered the thermal resistance of proteolytic *C. botulinum* spores to a normally innocuous thermal challenge. Furthermore, although 100 mM samples had a pH of 4.6, there was no evidence that this acid level had any inhibitory effect.

Thermal resistance of sporeforming spoilage organisms has been reduced by edible green plant extracts (LaBaw and Desrosier, 1953), and certain synthetic plant auxins modulated *Bacillus coagulans* spore resistance (LaBaw and Desrosier, 1954). Thermal resistance of *C. botulinum* spores, moreover, may be altered by the Fe^{++} and Ca^{++} levels or fatty acid content in the sporulation substrate (Sugiyama, 1951). CA inhibitions may be attributed to its reactivity at 80°C and changes in the tertiary structure or spore coat proteins. Mild heating activates bacterial spores by inducing spore coat structural changes (Gould et al., 1970; Halvorson, et al., 1966). Thus, spore core incorporation of CA may be enhanced with subsequent structural damage that render spores non-viable. The observed activity suggests CA may be useful to lower thermal processing requirements for some foods.

Two mM CA prevented germination for up to 4 days in both chicken and beef broths, and >5.0 mM was required for inhibition for 8 days at 32°C (Table 2). CA MICs in these foods were ≈5 times higher than those in BAM broth. Antibacterial agents are often less active in foods (Raccach, 1984), because many food components (e.g. protein, fats) can lower activity.

In the mouse bioassay, toxigenesis was delayed or inhibited by CA concentrations ≥ 5.0 mM (Table 3). Unsupplemented controls were toxigenic at 48 hr at 32°C, and the observed clinical symptoms were confirmed as botulism by neutralization with polyvalent antiserum. CA concentrations ≥ 25 mM were lethal. CA has a mouse LD_{50} of > 721 mg/kg (Sigma Chemical Co, St. Louis, MO).

CONCLUSIONS

CAFFEIC ACID, A HYDROXYCINNAMIC ACID DERIVATIVE, is a naturally occurring food component that was demonstrated to be sporostatic and sporicidal, to reduce spore thermal resistance, and to inhibit *C. botulinum* toxigenesis. The antibotulinal properties suggest that CA may have potential to control *C. botulinum* in foods or to lower required thermal processing conditions.

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From page 898

whereas those with 2M, after re-extrusion, showed the greatest increases in overall expansion. Samples first extruded with 1M caused similar changes in both overall and radial expansion.

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Properties of Edible Films from Total Milk Protein

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ABSTRACT

The mechanical properties and water vapor permeability of edible films made from various total milk proteins (TMPs) were investigated. Two TMPs obtained from nonfat dry milk (NDM) by removing lactose and three TMPs obtained from a commercial source were studied. Lactose was extracted from NDM by ultrafiltration or suspension in ethanol followed by filtration. TMP concentrate obtained by ultrafiltration (UF) produced films with the lowest water vapor permeability (WVP) and the highest tensile strength at break. Commercial TMP concentrates produced films more ductile than those from the UF-TMP or retentate from ethanol extraction. Further research is needed to improve mechanical properties of UF-TMP films without increasing the WVP.

Key Words: milk protein, edible films, food preservation, water vapor permeability

INTRODUCTION

EDIBLE, BIODEGRADABLE PACKAGING MATERIALS have been the focus of much research. Properties and applications of edible films have been thoroughly reviewed (Kester and Fennema, 1986; Guilbert, 1986; Krochta, 1992). Proteins and polysaccharides can form films which are good barriers to oxygen with good mechanical properties, but such films tend to be quite permeable to water vapor (Gennadios et al., 1991; Krochta, 1992; McHugh et al., 1993).

Research on milk proteins has focused on film-forming properties of whey proteins and caseinates. Because of associations and properties of different proteins, total milk protein (TMP) films might be stronger and have lower water vapor permeabilities (WVP) than films from individual proteins. Of the proteins in milk ≈80% are caseins, while whey proteins comprise about 20% (Glover, 1986). Caseins occur in complexes of protein and salts commonly called micelles (Farrell et al., 1990). Caseinates can form films without plasticizers and tend to have lower permeabilities than whey protein films (Avena-Bustillos, 1993; Avena-Bustillos and Krochta, 1993; McHugh et al., 1993). Caseinates do not require denaturation to form films (Avena-Bustillo, 1993). Native whey proteins have globular structures and will not form films unless denatured (McHugh et al., 1993). Once denatured, whey proteins form strong films but need plasticizers. These are smaller molecule compounds which inhibit chain interactions between larger protein polymer chains and give films flexibility, but which tend to increase WVP. The most commonly used plasticizers are polyols, mono-, di-, and oligosaccharides, lipids and derivatives (Gontard et al., 1993).

Individual caseins have random structures and are not notably denatured by heat. The size of casein micelles is not greatly changed by heat, but heat can induce interactions between whey proteins and caseins and cause dissociation of micellar protein (Singh and Fox, 1989). Such resulting associations/dissociations are very pH- and temperature-related. Whey proteins are substantially denatured around 100°C (Creamer and Matheson, 1980). At that temperature they form disulfide bonds with other whey proteins and κ-casein, which remains attached to the micelle (Noh et al., 1989). At elevated temperatures, β-casein is closely associated to the micelle, but upon cooling below 40°C as much as 50% can dissociate (Singh and Fox, 1989).

Total milk protein (TMP) can be isolated by several techniques, most of which involve acid and/or base precipitation. Each separation technique affects the structure and functionality of TMP differently. Most separations isolate the caseins from whey proteins and the fractions must be recombined to obtain TMP (Pearce 1991; Lonergran, 1983; Premaratne and Cousin, 1991). NDM as a source of TMP is low cost and readily available, but NDM contains over 50% lactose (Bassette and Acosta, 1988).

Lactose is more soluble in alcohols than are the proteins of milk (Hoff et al., 1987). Extraction of lactose with ethanol yielded a TMP concentrate where the caseins and whey proteins aggregated together. The TMP isolated had low lactose (<3%) with minimal protein loss (<5%). However, ethanol denatures some proteins, and alterations to functionality undoubtedly occurred using their technique.

Ultrafiltration (UF) is widely used to concentrate milk, and is also well suited for separating milk proteins from lactose, salts and other smaller molecules (Bastian et al., 1991). In the UF process, the molecular size of material retained, and that which permeates the membrane, is dependent upon membrane pore size. Concentration is limited by osmotic pressure of constituents on both sides of the membrane. Separation can be enhanced by diluting the retentate with solvent, lowering osmotic pressure on the incoming side of the membrane. This process, diafiltration, can be repeated several times, but a point is reached where further filtration is ineffective due to reduced concentrations of permeable solutes.

Most milk proteins have molecular weights > 14,500, whereas lactose has a molecular weight 342 (Glover, 1986). A membrane with a nominal molecular weight limit (NMWL) of 10,000 could separate lactose from protein. Additionally, free amino acids, polypeptides, unassociated vitamins and minerals would pass with the permeate (Premaratne and Cousin, 1991). To provide a better assessment of film characteristics of TMP, such smaller compounds should be removed.

Our objective was to evaluate five TMPs, two of which we prepared and three we obtained commercially. Films were formed with each of these and WVP, elastic modulus, yield strength, tensile strength, and elongation at break were compared.

MATERIALS & METHODS

UF-TMP powder

Milk (4 L) was made by reconstituting low-heat NDM in distilled water to a 10% solids solution. The solution was warmed to 30°C, then concentrated 2× in a UF unit by recirculating filtrate and removing the permeate. When the volume of filtrate was reduced to 2L, distilled water (2L) was added to begin diafiltration. This step was repeated three more times. When the volume reached 2L for the fourth time, filtration was stopped. The solution was then frozen and freeze-dried to provide a UF-TMP powder. Ultra- and diafiltration were performed with a Millipore lab scale UF unit comprised of a Millipore peristaltic pump and a 10,000 NMWL, 0.5M² cassette, Pellicon tangential cross flow membrane (Millipore Intertech, Bedford, MA), with a transmembrane pressure of 8.8 MPa.

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Table 1—Tensile properties of total milk protein films

Film Type	Thickness (μm)	Yield Strength (MN/m^2)	Elastic modulus (MN/m^2)	Tensile strength[z] (MN/m^2)	Elongation at break (%)
UF-TMP[y]	70	—	70.7[c]	10.0[a]	5.2[g]
EER[x]	81	—	29.6[g]	5.1[b]	12.2[f]
TMP 1100	75	9.5[d]	59.3[e]	8.6[a]	22.1[e]
TMP 1230	72	13.3[c]	67.9[d]	9.1[a]	33.3[d]
TMP 1350	84	5.9[e]	41.6[f]	6.3[b]	38.5[c]
LDPE[w]	25	42.6	—	8.6-17.3	500
PVDC[w]	—	—	—	48.4-138	20-40
Cellophane[w]	25	27.4	—	48.4-110	15-25

[z] Tensile strength at break.
[y] Total milk protein obtained by ultrafiltration.
[x] Total milk protein concentrate obtained by lactose extraction with alcohol.
[w] Briston, 1988.
[a,b] Films different at confidence level p=0.001.
[c-g] Films different at confidence level p=0.01.

Ethanol extraction retentate (EER) powder

Lactose was removed from NDM by selective solubilization in alcohol using the optimum conditions outlined by Hoff et al. (1987). These were: solvent-to-solids ratio 20:1, 25°C, and 72.9% ethanol concentration with no citrate. NDM (20g) was suspended in 316g of 95% ethanol with stirring for 10 min. Water (84g) was added to the suspension and stirred for 2 min. The resulting suspension was then rapidly filtered through Whatman #1 filter paper. After most of the filtrate had passed through, a vacuum was connected to finish removing solvent. The retentate was immediately washed from the filter paper with a minimum amount of water, resuspended by stirring (magnetic stir bar) and freeze-dried to provide an EER powder which could be rehydrated. When vacuum drying immediately after filtration was attempted, the resulting powder could not be readily rehydrated.

Protein determination

Protein contents of UF-TMP powder and EER powder were determined based on nitrogen determination (N × 6.38) with a Kejldahl lab apparatus. Protein contents of commercial samples (also determined by Kejldahl method), were provided by manufacturer specifications for TMP 1100, 1230 and 1350 (New Zealand Milk Products, Santa Rosa, CA).

Film solutions

Glycerin was added at 25% of total solids weight to plasticize all films. Solutions were made of 7.5% milk protein solids, 2.5% glycerin, and 90% water, then heated to 100°C for 30 min and allowed to cool to room temp. After they cooled, they were vacuum deaerated.

Water vapor permeability

Each film for WVP determination was made by pipetting 10g of solution onto a 90 mm diameter leveled casting plate of polytetrafluouroethylene (PTFE) or high density polyethylene (HDPE) and allowing to dry at ambient room conditions (RH~40%, T~25°C). After the water evaporated (≈24 hr), resulting films were removed from casting plates and thickness measured with a gauge (Mitutoyo Precision USA, Inc.; Elk Grove Village, IL). Five films were cast and tested from each sample solution. WVP of films was determined by ASTM E96-80 (1980), as modified by McHugh, et al. (1993). Water (6mL) was placed in a polymethylmethacrylate permeability cup. A film was then sealed onto the cup with silicone vacuum grease and a bolted retaining ring to hold the film in place. The cup with water and film was placed in a desiccator cabinet at 0% RH, 30°C and allowed to equilibrate for ≈90 min. After a steady rate of weight loss was achieved, the cups were weighed 5 times at intervals > 2 hr over a 24 hr period. This entire procedure was replicated three times, for a total of 15 tests on each type of film for WVP.

Mechanical properties

Mechanical properties were determined using three films cast from each solution. Due to the low elasticity, a minimum test length of 100 mm was needed (ASTM D882-91, 1991). Films were prepared by casting 20g of solution on 152 mm diameter HDPE plates. This yielded films of about the same thickness as those used for WVP testing. Three strips (25.4 mm × 127 mm) were cut from each film, for a total of 9 tests on each type film for mechanical properties. Pieces of polyacrylic plastic (25.4 mm wide) were affixed to both ends of test strips with double stick tape. This resulted in test films of 25.4 mm × 102 mm. The affixed plastic helped in placement of the test area and also kept the jaws of the testing machine from cutting films. Tensile properties of films were determined by stretching samples at 20 mm/min until failure, a slower rate than designated in the ASTM method. However, faster elongation ruptured the films without meaningful information. The tests were performed on an Instron Universal Testing Machine (Instron Corp., Canton, MA) at 25°C and 35% relative humidity. Elastic modulus, yield strength, tensile strength at break and elongation at break were calculated (ASTM D882-91, 1991).

Statistical analyses

Statview 4.0 was used for statistical analyses (Abacus Concepts, Berkeley, CA). Analyses of variance and Fisher PLSD multiple comparisons were performed.

RESULTS & DISCUSSION

Protein determination

Since protein was the major structural element of the films, the film properties mainly related to treatments of the protein. When lactose was extracted from NDM with 95% ethanol, a powder containing ≈20% lactose was obtained. The amount of NDM which could be processed in a single batch was restricted by the rate of filtration. Protein content of batches of EER powder was low and variable (63.8 ± 4.2%).

The product from UF was more consistent, as separation was determined by the size of membrane pores. Completeness of separation was also controlled by diafiltration volume and number of passes. The 10,000 NMWL membrane gave a pore size below the smallest milk proteins, yet passed lactose and minerals. Diafiltering the retenate with four times the retentate volume resulted in a protein powder with ≈12% lactose. Batch size was restricted by equipment, but results were consistent. Protein content of the powder from ultrafiltration was 82.5 ± 0.2%. The commercial powders had higher protein (TMP 1100: 89%, TMP 1230: 87%, TMP 1350: 85%) than did the protein concentrates obtained by UF or ethanol extraction. Although removal of lactose by ethanol extraction and ultrafiltration was incomplete, no lactose crystallization occurred and films were homogeneous.

Mechanical properties

The commercial TMP products yielded more ductile (greater elongation) films than the EER or the UF-TMP (Table 1). Neither EER films nor UF-TMP films had true yield points, rupturing before yield strength could be determined (force derivative equal to zero). UF-TMP film had the highest elastic modulus, and the highest tensile strength at break. TMP 1230 film had the highest yield strength. TMP 1350 film was most ductile, with total elongation ≈39%. The commercial TMP films had tensile strengths at break similar to low density polyethylene (LDPE) and elongations similar to polyvinylidene chloride (PVDC) and cellophane.

Water vapor permeability

Water vapor transmissions for these films were typical of protein-based films (Table 2). All had WVP between 70 and 86 g·mm/KPa·m^2·day. UF-TMP film, the only different one, formed the best barrier to water vapor. Because of the hydrophilic nature of proteins, such films have WVP much greater than standard packaging films (Table 2). Most of the films dissolved rapidly when placed in contact with water. Only films made from EER remained intact up to 1 hr in cool (20°C) water.

Table 2—Water vapor permeability of total milk protein films

Film Type	Thickness (μm)	RH* (%)	Permeability (g-mm/kPa-day-m²)
UF-TMP[z]	71	65	70.3[a]
EER[y]	69	61	81.0[b]
TMP 1100	80	65	80.9[b]
TMP 1230	74	63	80.1[b]
TMP 1350	80	63	86.1[b]
Sodium Caseinate[x]	83	81	36.7
Whey Protein[w]	121	65	119.8
Gluten[v]	400	85	53.3
LDPE[u]	—	90	0.08
Cellophane[t]	—	90	7.27

* RH humidity at the internal surface of the film; 0% RH at outer surface of film.
[z] Total milk protein obtained by ultrafiltration.
[y] Total milk protein concentrate obtained by lactose extraction with ethanol.
[x] Avena-Bustillos and Krochta (1993).
[w] McHugh et al. (1994).
[v] Park and Chinnan (1990).
[u] Smith (1986).
[t] Taylor (1986).
[a,b] Films different at confidence level p=0.001.

CONCLUSIONS

ALL PROTEIN SOLUTIONS TESTED were heated to 100°C for 30 min. Heating them to higher temperatures may yield stronger, less brittle films due to further dissociation of micelles and additional intermolcular bonds. Insoluble films may be obtained from milk protein solutions heated to 135°C (Singh and Fox, 1989). Adding lipids to whey protein films can reduce WVP (McHugh and Krochta, 1994), and this approach needs study with TMP.

Caution is recommended when comparing results among films, especially when differing techniques or conditions are employed. The methods we used as guidelines, ASTM E96-80 for water vapor permeability test and ASTM D882-91 for mechanical properties, were designed for synthetic plastic films. Milk protein films are very different from materials for which the tests were originally designed. Films from TMP are less flexible, not as strong, and more permeable to water vapor than most synthetic plastic films.

TMP films in general were not adequate moisture barriers and type of separation treatment affected WVP. Protein from NDM by UF was relatively pure and yielded films with best moisture barrier characteristics of the TMP films studied. Residual lactose and variability make ethanol extraction to obtain TMP for edible films impractical. Denaturing effects by ethanol on milk protein films may reduce film water solubility.

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Grease Resistance of Corn Zein Coated Paper

T. A. TREZZA and P. J. VERGANO

ABSTRACT

The grease resistance of corn zein coated paper was measured as related to coating level, plasticizer addition and time. Overall grease resistance ws normalized by time and expressed as percent area stained/hr (%AS · hr^{-1}). For a 2.2 kg coating · $ream^{-1}$ of paper, %AS · hr^{-1} was greater than both unplasticized and plasticized 4.4 and 6.6 kg · $ream^{-1}$ coating levels ($p<0.001$). There was no difference in %AS · hr^{-1} among 4.4 and 6.6 kg · $ream^{-1}$ zein coated and commercial polyethylene laminated paper (p:ME0.098). Plasticizer increased ($p<0.001$) and creasing had no effect ($p=0.71$) on %AS · hr^{-1}. Electron microscopy showed discontinuities in zein coating at the 2.2 kg · $ream^{-1}$ level. The 4.4 and 6.6 kg · $ream^{-1}$ levels showed uniform coatings.

Key Words: corn, zein, paper coating, grease resistance

INTRODUCTION

NATURAL POLYMERS, such as proteins, cellulose derivatives, starches and other gums have potential as packaging materials. Research on such edible film materials has been mostly related to using them for auxiliary packaging, such as moisture barriers between dough and toppings in foods (pizza and pies). They have also been studied for preventing moisture loss in fresh fruits and vegetables, maintaining crispness of prepared ice cream cones during storage or preventing lipid migration to the nut center of chocolate coated candies (Kester and Fennema, 1989; Gennadios, 1991; Nelson and Fennema, 1991; Rico-Pena and Torres, 1990). Corn zein protein coatings are used as oxygen and moisture barriers for nuts, candies and other foods (Andres, 1984). Corn zein films and coatings have relative insolubility in water, and they form strong, glossy films resistant to grease and oxygen permeation. Gennadios et al. (1993) reported zein films had oxygen permeability coefficients, under dry conditions (0% relative humidity), much lower than many commercial polymer films including low and high density polyethylene, polypropylene, and nylon-6. Aydt et al. (1991) reported zein films cast from a commercial zein solution had lower bursting strength but greater tear strength than commercial cellophane films.

Little work has been reported on natural polymer films as primary packaging materials, such as bags, wraps and carton coatings. This is due to their usual susceptibility to drying, brittleness and generally lower mechanical and barrier properties, and degradation over time. Edible cellulose film pouches have been applied as ingredients carriers in the baking industry (Watson, 1970). Packaging materials of corn zein and other natural polymers have potential environmental advantages over conventional polymer films. Zein coatings on paper or paperboard also have potential advantages for some applications. They minimize effects of drying and brittleness because such coatings can be used in thinner sheets than free films. For foods with extremely short shelf lives, (e.g. quick-service restaurant sandwiches), edible film coated paper could meet packaging requirements. This type package would then be suitable for recycling would also provide alternative uses for corn. One type of quick-service sandwich packaging is composed of a polyethylene layer between two paper layers. The polyethylene content prevents effective separation, recycling and composting of the materials. Zein coatings do not interfere with paper recycling, and do not require separating protein and paper layers (Narayan, 1993).

Grease resistance is important for packaging products containing fats or oils. Little quantitative data are available on the grease barrier properties of packaging materials. Plastics may be classified as Excellent, Good, Poor or No barrier to grease (Modern Plastics Encyclopedia, 1986). Polyethylene, polypropylene and most plastics are generally classed as good grease barriers. Corn zein reportedly produced virtually grease proof films and coatings when used directly on nuts, chocolates and other candies (Cosler, 1954, 1959; Andres, 1984). Our objective was to determine grease resistance of corn zein protein coated paper and its potential use as a quick-service restaurant package.

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MATERIALS & METHODS

NOATING SOLUTIONS WERE PREPARED by dissolving corn zein powder (Freeman Inc., Tuckahoe, NY) into a 95% (v/v) aqueous ethyl alcohol solution, with or without glycerol (Aldrich Chemical Company, Inc., Milwaukee, WI) added as a plasticizer. Unplasticized coating contained 15% (wt/wt) corn zein. Plasticized solutions contained 12% zein and 3% glycerol. After dissolving the zein, plasticizer was added (if used), and the solution gently mixed at room temperature (≈23°C) for a minimum of 30 min.

Sample coated papers were made as follows: Uncoated paper used for wrapping quick-service sandwiches, with a basis weight of 6.6 kg · $ream^{-1}$, cut 31 cm square, was obtained from the manufacturer. Appropriate coating levels were calculated on the basis of zein + glycerol per ream of paper (278 m^2). The appropriate amount of 15% solution was then brushed across the paper using soft-bristled brushes. Three coating levels were used, 2.2, 4.4 and 6.6 kg of coating solids per ream of paper. The papers then were allowed to dry on the bench top for 30–60 min. Ambient conditions ranged from 23–25°C and 60–68% relative humidity. Free film samples were prepared according to Park (1991).

Testing

Grease resistance of coated papers was determined using a modified TAPPI test T 507, "Grease Resistance of Flexible Packaging Material" (TAPPI, 1991). Coated samples were cut 100 mm square, and placed between one clean 100 mm square sheet and one fat saturated 75 mm square sheet of S/P® Brand bibulous blotting paper (Baxter Healthcare, McGaw Park, IL) (Fig. 1). One mL of melted beef kidney fat (Clemson University Meat Science Laboratory) was applied to uniformly saturate the smaller blotter sheet. The fat had been dyed red using Oil Red O (Sigma Chemical, St. Louis, MO) to ease detection of grease spots. The saturated sheet was placed in contact with the zein coated side of the paper sample. Stacks of the 3-layer system were separated by aluminum foil sheets cut 125 mm square and all stacks set on a 6 mm thick glass plate. A rectangular steel plate (12.5 cm × 10.0 cm) with a mass of 430g was placed onto stacks of up to 10 3-piece layers. The assembly was then placed in an oven at 60°C.

Every hour for 4 hr, samples were removed, and the amount of grease which passed through the coated samples to the clean blotters was measured. When grease ran over the edge of the clean blotters, those sheets were discarded. This occurred rarely, and was probably due to not perfectly centering the saturated blotter over the zein coated sample.

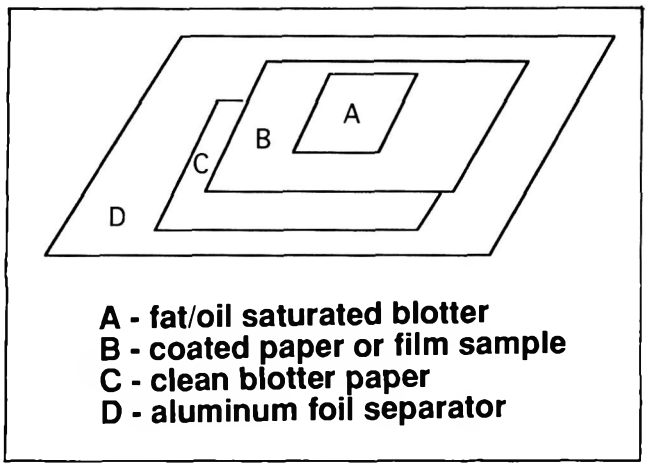

Fig. 1—Schematic of TAPPI test method T 507, *Grease Resistance of Flexible Packaging Material,* used for testing grease resistance of corn zein coated paper.

The percentage area of clean blotters stained with grease was determined according to a "point-counting method" (Howard and Cohen 1947; Underwood 1971, 1970). This method is commonly used for determining percentage of microconstituents in an alloy or metal. In metallurgy, point-counting is normally done from photomicrographs of metal cross-sections. In our study, for ease of handling, stained sheets were labeled and photocopied 1.75 times original size. A clear plastic sheet with a 6.5 mm square grid was placed over the same sheet (Fig 2). Fraction area stained ws calculated by determining the number of grid crosshatch points on stained portions, and dividing that number by the total number of crosshatches across the blotter sheet (729 for this experiment). The grid was randomly moved to completely cover stained portions and counting done 3 times to ensure validity of point values. This procedure was repeated for zein films prepared according to Park (1991) at Clemson University, and also for commercially prepared polyethylene laminated paper supplied by a local quick-service restaurant.

To determine durability of coatings, some samples were creased according to TAPPI T 465, "Static Creasing of Paper for Water Vapor Transmission Tests." This method was chosen because it is a more severe creasing treatment than other methods for this property. Free zein film samples were not creased due to their brittleness.

Statistics

Statistical analysis was performed using the JMP™ statistical software package (Version 1.0.1.©1989. SAS Institute, Inc., Cary, NC). Overall analysis of variance was performed, as well as linear contrasts between coating level, plasticizer and creasing effects (Ott, 1988). Fisher's LSD (Ott, 1988) was used for determining differences between percentage area stained (%AS) of coating level, plasticizer and time variable interactions.

RESULTS & DISCUSSION

ZEIN FILMS were highly impermeable to grease penetration for the 4 hrs tested (Table 1). Increasing percent area per hour stained indicated decreased grease resistance. Creasing did not affect percent area stained for plasticized or unplasticized zein coated samples ($p=0.71$). No difference ($p \geq 0.098$) occurred between percent area stained for the 4.4 and 6.6 kg · ream^{-1} zein coated papers. At 2.2 kg · ream^{-1} coating level, a significant increase occurred in percent area stained ($p < 0.001$) compared to higher coating levels. Grease resistance of polyethylene laminated paper was not different than the 4.4 or 6.6 kg · ream^{-1} zein coated papers ($p>0.1$). This was not a direct comparison because the zein coated papers were not heat sealed to a second sheet of paper, as were the commercial polyethylene laminated samples. Heat sealing would probably influence grease resistance. However, the results provide some relative comparison of grease resistance of zein coated paper with polyethylene laminates.

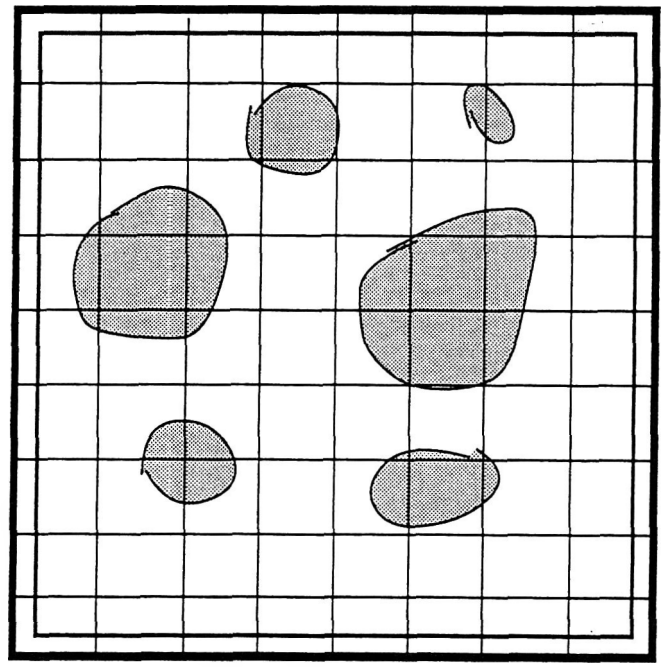

Fig. 2—Determination of percentage area stained for grease resistance testing.

Table 1—Percentage area stained/hr from beef fat on corn zein coated paper and polyethylene laminated paper as related to coating levels.

Coating level (kg/ream)	Number of samples	Percent area stained per hr	Standard deviation
Zein			
2.2	60	3.1[a]	3.4
4.4	88	0.56[b]	0.76
6.6	72	0.44[b]	0.72
Corn zein film	21	0.037[b]	0.056
Polyethylene 2.6	47	0.86[b]	1.7

[a,b] Same letter indicates no difference at $p \leq 0.05$ using Fischer's Least Significant Difference Test.

As expected, percent area stained increased as time increased (Table 2). For the 4.4 and 6.6 kg · ream^{-1} coating levels, both plasticized and unplasticized, the increase over time was almost linear. However, for the 2.2 kg · ream^{-1} coating level, the effect was exponential. Linear contrasts showed plasticizer significantly decreased grease resistance ($p<0.001$). The 4.4 and 6.6 kg · ream^{-1} unplasticized coating levels showed grease resistance equal to the polyethylene laminated reference (Fig. 3). The relative grease barriers of these coating levels, with plasticizer added, decreased (Fig. 4). Standard deviations of each coating treatment by hour measure were very high. After 2 hr testing, all treatments showed zero %AS. Even after 4 hr of testing, samples from the two higher coating levels, plasticized or not, showed <1%AS. This indicated coating uniformity was a factor in grease resistance of corn zein coated papers. In the absence of pinholes, cracks or other discontinuities, zein coated paper was a good grease barrier. Improving coating integrity and uniformity should make it more resistant to beef fat permeation.

A photomicrograph of uncoated, 6.6 kg · ream^{-1} paper showed the 2-dimensional web-like structure of the paper. An electron micrograph of zein coated paper (Fig. 6), showed nonuniformities in the coatings at the 2.2 kg · ream^{-1} coating level. Comparison of Fig. 5 and 6 shows portions in the coated samples were paper fibers and the fiber network were distinct. This helps explain the low grease resistance of that coating level, since uncoated portions would have virtually no resistance to grease permeation.

Table 2—Coating level, plasticizer and time related to area stained of blotter paper[a]

Coating or film[b]	Material Type	Test Time (hr) 1[d] n	x	s	2 n	x	s	3 n	x	s	4 n	x	s	Overall[e] n	xt	s
2.2 kg/ream	Unplasticized	7	0.50	0.62	8	2.0	1.2	8	7.3	3.0	4	11	7.3	27	1.6[f]	1.3
2.2 kg/ream	Plasticized	8	1.4	1.7	8	8.8	9.1	9	11	7.4	7	34	18	33	4.3[g]	4.0
4.4 kg/ream	Unplasticized	11	0.037	0.12	18	1.3	1.8	13	2.0	3.0	8	1.9	1.5	50	0.50[h]	0.78
4.4 kg/ream	Plasticized	8	0.060	0.19	11	0.69	0.28	10	2.7	1.9	9	4.8	4.0	38	0.64[h]	0.72
6.6 kg/ream	Unplasticized	8	0.029	0.080	10	0.46	0.53	10	0.8	1.0	9	1.2	0.71	37	0.22[h]	0.26
6.6 kg/ream	Plasticized	8	0.13	0.21	10	0.92	0.67	9	2.4	2.3	8	5.5	6.3	35	0.68[h]	0.95
Zein film	Plasticized	4	0.034	0.069	6	0.091	0.11	4	0.17	0.20	7	0.079	0.21	21	0.037[h]	0.056
Polyethylene	laminated[c]	10	0.59	1.8	10	0.64	1.0	11	2.0	3.5	16	6.0	9.5	47	0.86[f,h]	1.8

[a] TAPPI test T 507 for corn zein films, zein coated paper and polyethylene laminated paper at 60°C.
[b] Coating level in kilograms of solids (zein + plasticizer, if used) per ream of paper.
[c] Polyethylene laminated at approximately 2.6 kg/ream, from a commercial supplier.
[d] n = number of samples; x = mean % area stained; s = standard deviation.
[e] Overall is mean percent area stained per hour. Means with the same letter are not different at $p \leq 0.05$.

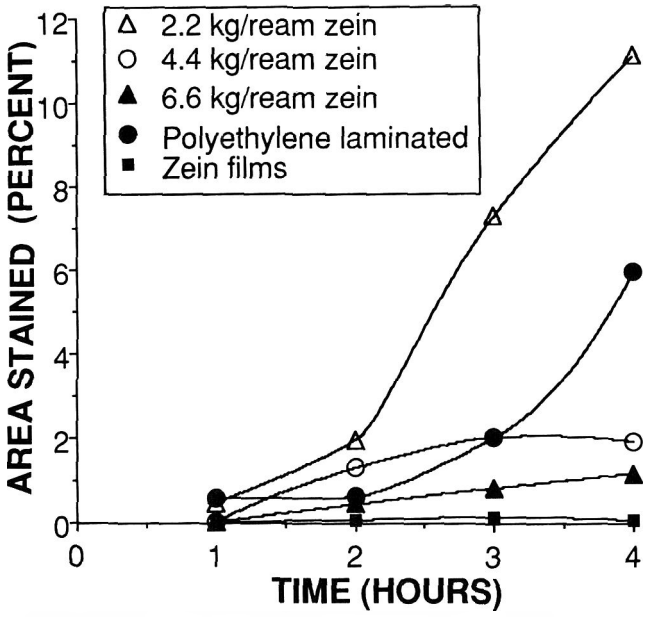

Fig. 3—Test time as related to area stained of different film types.

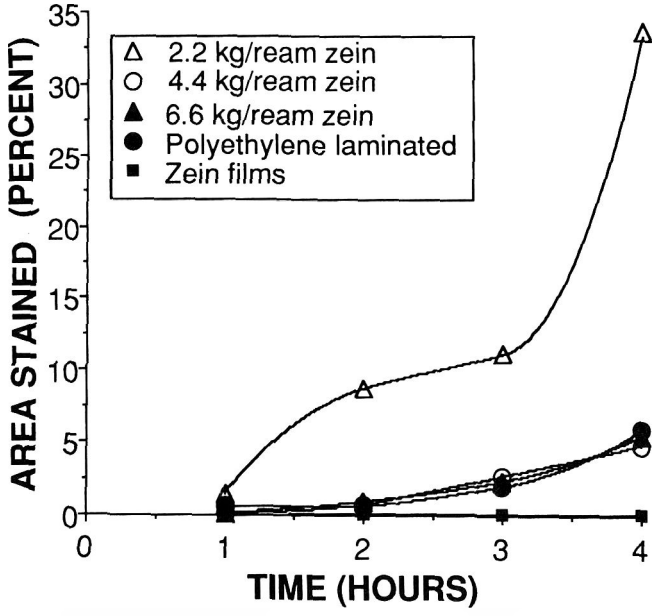

Fig. 4—Test time as related to area stained of differnt film types.

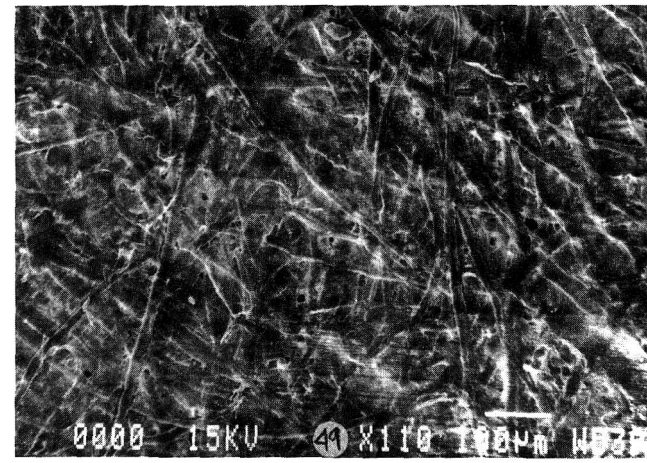

Fig. 5—Scanning electron micrograph of uncoated 6.6 kg/ream paper showing typical fiber network (× 110). White bar 100 micrometers.

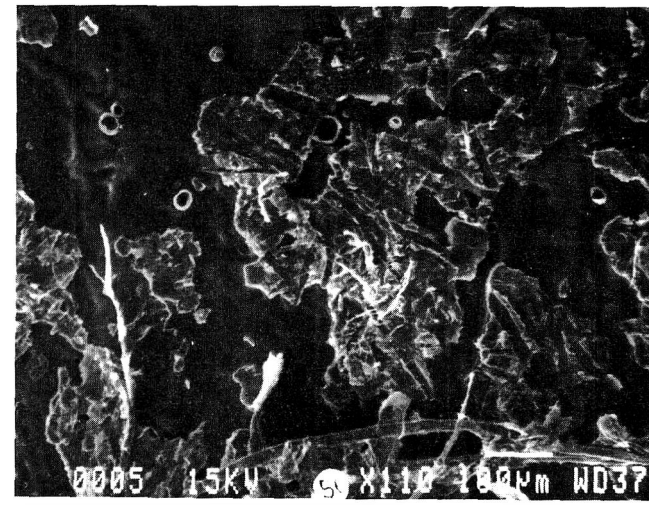

Fig. 6—Scanning electron micrograph of paper coated with 2.2 kg/ream corn zein, indicating nonuniform coating with paper fibers showing through zein layer (× 110). White bar 100 micrometers.

A uniform coating (Fig. 7) was typical of the 4.4 kg · ream^{-1} level. Paper fibers were covered showing few uncoated portions and a limited paper fiber network. Coating at the 6.6 kg · ream^{-1} level (Fig. 8) indicated that, as coating level increased, uniformity of the coating also increased. Even with uniform

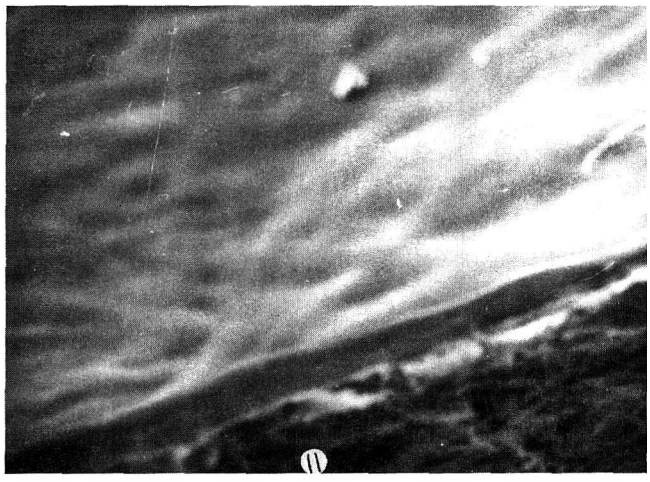

Fig. 7—Scanning electron micrograph of paper coated with 4.4 kg/ream corn zein, indicating a more uniform coating and less evidence of paper fibers at the paper/zein surface (× 200).

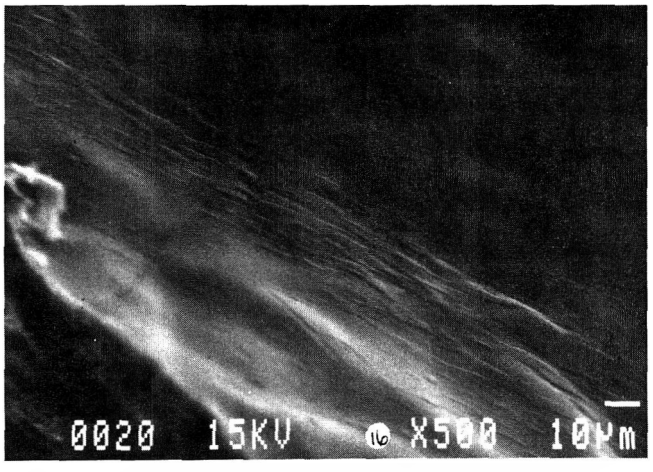

Fig. 8—Scanning electron micrograph of paper coated with 6.6 kg/ream corn zein, indicating increased surface uniformity compared to the 4.4 kg/ream coating level (× 500). White bar 10 micrometers.

coatings, grease would probably permeate through pinholes, cracks, thin portions and other small discontinuities in the coatings. Several features were observed that appeared to be pinhols or cracks, but could not be unambiguously identified. Defects in the coatings might explain why some grease still passed through the material at higher coating levels. Grease resistance was not decreased by creasing samples ($p=0.71$) for any treatments. This showed increased flexibility of paper coatings over free standing films of corn zein.

CONCLUSIONS

ZEIN COATED PAPERS were not as effective barriers to hot beef fat as were free films; however, they were equal to polyethylene laminates used for quick-service restaurant sandwich packaging. At 2.2 kg · ream^{-1} zein coating levels, grease resistance was low due to non-uniformity of coating. At 4.4 and 6.6 kg · ream^{-1} zein coated papers would be adequate grease barriers for quick-service sandwich packaging for 1 to 2 hr. Coating uniformity and quality are necessary for good grease resistance. High variability in grease resistance resulted from non-uniformity of applied coatings. Creasing samples did not diminish grease resistance. Even for unplasticized zein coatings, brittleness was decreased by applying films onto paper. The effects of heat sealing of zein coating and storage require further research.

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Fatty Acid Concentration Effect on Tensile Strength, Elongation, and Water Vapor Permeability of Laminated Edible Films

J. W. PARK, R. F. TESTIN, H. J. PARK, P. J. VERGANO, and C. L. WELLER

ABSTRACT

Water vapor permeability (WVP), tensile strength (TS), and elongation (E) were investigated in laminated methyl cellulose/corn zein-fatty acid films. They were prepared by casting corn zein-fatty acid solutions onto methyl cellulose films. WVP decreased as chain length and concentration of fatty acids increased. The TS of laminated edible film containing palmitic acid decreased as palmitic acid increased. The TS of films containing stearic-palmitic acid blends showed similar trends but there were no significant differences among blends. The TS of the film containing lauric acid was maximum at 30% lauric acid concentration. The E values for films containing fatty acids varied inversely with TS.

Key Words: edible films, corn zein, methyl cellulose, fatty acids, water vapor permeability

INTRODUCTION

MATERIALS THAT CAN BE USED to make edible films include polysaccharides, proteins, and lipids. Research on edible films was reviewed by Kester and Fennema (1986), Guilbert (1986) and Gennadios and Weller (1990). Hydrophilic edible films have oxygen and carbon dioxide barrier properties at low relative humidities and lipid edible films have water vapor barrier properties but less effective mechanical properties. Investigators have emphasized research on improving water vapor barrier properties (Gennadios et al., 1993; Greener and Fennema, 1989a, b; Hagemaier and Shaw, 1990; Kamper and Fennema, 1984a, b; Kester and Fennema, 1989; Krochta et al., 1988; Martin-Polo et al., 1992a, b; Park et al., 1993; Rico-Pena and Torres, 1990; Schultz et al., 1949).

Waxes and fatty acids were found to be effective in limiting water vapor transfer (Greener and Fennema, 1989a, b; Kamper and Fennema, 1984a, b; Kester and Fennema, 1989; Schultz et al. 1949). However, such materials lack film-forming characteristics due to ineffective mechanical properties. Films have been made by combining polysaccharides with lipids using emulsion or coating techniques (Greener and Fennema, 1989a, b; Hagenmaier and Shaw, 1990; Kamper and Fennema, 1984a, b; Kester and Fennema, 1989a, b; Martin-Polo et al., 1992; Vojdani and Torres, 1990). Edible films composed of hydroxypropyl methylcellulose and various lipid layers were tested for resistance to water vapor permeability by a coating technique and an emulsion technique (Kamper and Fennema, 1984a). Results indicated that, as degree of saturation of lipids increased, water vapor transmission rate (WVTR) decreased. Paraffin and beeswaxes impart more effective barrier properties than polyunsaturated corn oil for films prepared using emulsion techniques.

Water vapor permeability for a bilayer film of stearic and/or palmitic acids and hydroxypropyl methylcellulose (formed by emulsion) was measured as related to film composition, temperature and relative humidity (Kamper and Fennema 1984b). Such film was suggested to be effective at RH <90% and temperatures of −19°C to 40°C.

The effects of film composition and physical stress on water vapor permeability of edible bilayer films were reported by Kester and Fennema (1989b) and Greener and Fennema (1989a, b). Both studies found that wax-laminated film had effective water vapor barrier properties. They reported that "Wax-M" film (beeswax applied molten to methyl cellulose base film) maintained water vapor barrier properties more than "Wax-S" film (beeswax in ethanol applied to methyl cellulose based film) after mechanical abuse.

Moisture permeability and puncture properties of edible films made with fatty acid and hydroxypropyl methylcellulose were reported by Hagenmaier and Shaw (1990). The water vapor permeability of an edible film made with stearic acid content of about 40–50% was the lowest and puncture properties of HPMC-fatty acid films were not related to chain length of the fatty acid.

The influence of the preparation technique on moisture barrier properties of hydrophobic films was reported by Martin-Polo et al. (1992a). They showed that an emulsion and coating method resulted in the best water vapor barriers. All such results showed that water vapor permeability was decreased, but mechanical properties (tensile strength and elongation) were not reported.

Our objective was to determine the effects of concentrations and types of fatty acids on mechanical properties and water vapor permeability of laminated edible films.

MATERIALS & METHODS

Materials

Methylcellulose (MW, 20,000) and polyethylene glycol (MW, 400) were obtained from Aldrich Chemical Company, Inc., Milwaukee, WI. Lauric acid (C_{12}, 99–100%), and stearic-palmitic acid blend (54:46) were obtained from Mallinckrodt Inc., Paris, KY. Other materials were corn zein (Freeman Inc., Tuckahoe, NY), palmitic acid (J.Y. Baker Chemical Co., Philipsburg, NY) and glycerin (Baxter Healthcare Co., McGaw Park, IL). Ethanol (95%, reagent) was obtained from Fisher Scientific, Pittsburgh, PA.

Preparation of laminated edible films

Methyl cellulose film solution was prepared by dissolving 9g methyl cellulose in a mixed solvent of ethanol (200 mL) and water (100 mL). One gram of polyethylene glycol (MW, 400) was added. This solution was mixed at 6,000 rpm for 5 min (Virtis Co., Inc., Gardiner, NY). The solution was stirred and heated until it reached ≈85°C. Seventy mL of solution was poured onto a glass plate with 27cm × 27cm rim of paper and dried overnight at room temperature (≈23°C).

Solutions of corn zein and fatty acid for calculated permeability or lamination were prepared as follows: corn zein (25g) was added to 105.5 mL ethanol. The solution was stirred until dissolved. Fatty acids

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Table 1—Effect of fatty acids on tensile strength (TS) and percentage elongation at break (E) of laminated edible films with various fatty acid concentrations

Fatty acid (%, g fatty acid/g corn zein)	TS(MPa)			E(%)		
	Lauric acid	Palmitic acid	S-PA blend*	Lauric acid	Palmitic acid	S-PA blend
0**	33.00±4.39 [a]	33.00±4.39 [a]	33.00±4.39 [a]	28.41±13.04 [c]	28.41±13.04 [d]	28.41±13.04 [c]
10	18.31±2.61 [d]	24.92±2.61 [b]	19.80±1.79 [b]	68.38± 4.53 [a]	37.35±11.62 [c]	61.89± 7.27 [b]
20	16.25±1.74 [d]	21.28±3.74 [c]	19.81±2.25 [b]	72.64± 3.83 [a]	50.22± 7.52 [b]	60.89± 9.59 [b]
30	23.43±4.40 [d]	18.49±2.59 [d]	18.55±1.91 [b]	59.57± 7.09 [b]	52.62± 7.16 [b]	61.77± 7.09 [b]
40	20.96±3.23 [c]	17.76±2.06 [d]	18.68±1.67 [b]	57.40± 4.48 [b]	65.21± 5.62 [a]	69.03± 8.43 [a]

* S-PA blend : Stearic-palmitic acid blend.
** Control : laminated edible film which was composed of methyl cellulose and corn zein using coating technique.
[a-d] Means with different superscripts and significantly different ($p < 0.05$), n = 20.

(lauric, palmitic and stearic-palmitic acid blend) were than added at various concentrations (10, 20, 30 and 40%, w/w (g fatty acid/g corn zein)). 5.48 g of polyethylene glycol (MW, 400) and 6.08g of glycerin were added as described by Park et al. (1994). This solution was stirred and heated to ≈85°C. In preparing films for calculated permeability, 25–30 mL of hot solution were poured onto a glass plate (27 cm × 27 cm). In preparaing films for lamination, the hot solution was cooled to 40–50°C and 25–30 mL were poured onto a dried methyl cellulose film. In either case, plates were left at room temperature (≈23°C) for 10 min, then dried at 80–85°C for 30 min. The plates were left overnight at room temperature.

Thickness measurements

Before making laminated films, dried methyl cellulose films were removed from glass plates and mean thickness was determined. The film was then discarded. Methyl cellulose films were made again in the same manner for preparation of laminations. A hand-held micrometer (B.C. Ames Co., Waltham, MA) was used to measure film thickness. Five measurements were made on each test film and mean thickness was calculated. This procedure was repeated after casting and drying the corn zein-fatty acid solutions to determine thickness of laminated films.

Mechanical properties

Fifteen or more specimens, 6 cm × 2.54 cm, were cut from each sample prepared on glass plates. These samples were conditioned at 25°C and 50% RH for 48 hr in an environmental chamber (Model 317332, Hotpack, Corp., Philadelphia, PA). An Instron (Model 4210, Instron Engineering Corp., Canton, MA) was used to measure tensile strength [TS (MPa)] and percentage elongation at break [E (%)] according to ASTM Standard Method D 882-88 (ASTM, 1989). Initial grip separation was set at 50 mm and cross-head speed was set at 500 mm/min.

Water vapor permeability

Water vapor permeability (WVP) was calculated from Eq. (1) (Karel et al., 1959):

$$WVP = WVTR \times (L/p) \quad (1)$$

where, WVTR [ng /(m²·sec)] is the water vapor transmission rate of films measured at 25°C and 50% RH gradient; L (m) is the mean thickness of film specimens; and p (Pa) is the difference in partial water vapor pressure between two sides of film specimens.

WVTR was measured using a variation of the ASTM Standard Method E 96 (ASTM, 1987), known as the "cup methods." Cups and lids used for testing were made from poly(methylmethacrylate) (Piedmont Plastics, Inc., Greenville, SC). These cups and lids were originally designed by Dr. J.M. Krochta (Dept. of Food Science & Technology, Univ. of California, Davis). The cups had an i.d. of 4.6 cm, an o.d. of 8.7 cm, and a depth of 2.1 cm. The lids had an o.d. of 8.7 cm, a hole of 4.6 cm (to expose the test film), and a thickness of 0.5 cm. The cup was filled to a depth of 0.602 cm with distilled water and covered with a film (the lower part was the mixture film of corn zein and fatty acid and the upper was methyl cellulose) to be tested. Four screws, symmetrically placed around the cup perimeter, were tightened to securely hold film specimens between the lid and the cups. Silicone lubricant was used to seal film specimens between the lip and the cup. After film specimens were mounted, the assembly was weighed and placed in a chamber conditioned at 25°C, 50% RH (Model 317332, Hotpack, Corp., Philadelphia, PA). Weight loss of cups with time was measured and plotted. The slope of the straight line was calculated with linear regression. WVTR was estimated by dividing this slope by the open area of the cup (16.61 cm²).

Three WVP values were determined: the first was the measured value using Eq. (1), the second was the WVTR value corrected for resistance of the stagnant air gap (1.498 cm) between underside of film samples mounted on cups and surface of water inside the cups (Krochta, 1992; Gennadios et al., 1994), and the last was the calculated value using Eq. (2):

$$X_t/P_t = X_1/P_1 + X_2/P_2 \quad (2)$$

where X is thickness (m), P is permeability (ng·m/m²·sec·Pa), the subscript t denotes laminated edible films, and subscripts 1 and 2 denote methyl cellulose layer and corn zein-fatty acid layer, respectively (Crank and Park, 1968).

Statistical analysis

Three samples were prepared for WVP and 15 or more for mechanical properties tests. Analysis of variance (ANOVA) procedures were used to analyze data. Duncan's Multiple Range Test ($p < 0.05$) was used to detect differences among film property mean values at various fatty acid concentrations.

RESULTS & DISCUSSION

Film casting

Bilayer edible films from cellulose ether and fatty acids using the emulsion technique of Kamper and Fennema (1984a, b) and Vojdani and Torres (1990) exhibited distortion, cracking, and pinholes during oven drying and fatty acid losses upon removal from the glass plate (Vojdani and Torres, 1990). We used methyl cellulose to provide the laminated edible film with tensile strength. The bilayer film made from corn zein and fatty acid was used to impart a water vapor barrier to the laminated edible film. These films had none of the defects reported for the cellulose ether/fatty acid bilayer.

Tensile strength

Tensile strength of laminated edible film containing palmitic acid decreased as concentration of palmitic acid increased (Table 1). Tensile strength of laminated film composed of stearic-palmitic acid blends showed no significant differences among them as concentrations of stearic-palmitic acid blends increased. The TS of laminated film containing lauric acid did not follow this trend and was maximum at 30% lauric acid.

In comparing TS at different fatty acid concentrations, significant differences occurred among laminated films. Tensile strength of laminated edible films made with palmitic acid was highest at 10% fatty acid while for films made with lauric acid it was lowest at 20% fatty acid. Tensile strength of films with lauric acid was highest at 30–40% fatty acid concentration. These results did not agree with results reported by Hagenmaier and Shaw (1990), in which the TS of HPMC-fatty acid tested films by puncture was not related to fatty acid chain length. These differences could be results of different TS test methods and different film forming techniques. All TS values for films containing fatty acids were lower than that of the control (corn zein with no fatty acid).

Table 2—Effect of fatty acids on WVP of laminated edible films with various fatty acid concentrations

Fatty acid (%, g fatty acid/g corn zein)	WVP (ng·m/m²·sec·Pa)					
	Lauric acid		Palmitic acid		S-PA blend	
	MV[c]	CV[d]	MV	CV	MV	CV
0[e]	0.409[a] ± 0.018	0.689[b] ± 0.021 (40.6%)[f]	0.409[a] ± 0.018	0.689[b] ± 0.021 (40.6%)	0.409[a] ± 0.018	0.689[b] ± 0.021 (40.6%)
10	0.379[a] ± 0.029	0.710[b] ± 0.120 (46.6%)	0.142[a] ± 0.004	0.169[b] ± 0.005 (16.0%)	0.100[a] ± 0.027	0.134[a] ± 0.041 (25.4%)
20	0.142[a] ± 0.082	0.235[a] ± 0.184 (39.6%)	0.058[a] ± 0.008	0.062[a] ± 0.008 (6.5%)	0.029[a] ± 0.010	0.035[a] ± 0.012 (17.1%)
30	0.071[a] ± 0.047	0.078[a] ± 0.054 (9.0%)	0.030[a] ± 0.004	0.031[a] ± 0.004 (3.2%)	0.017[a] ± 0.004	0.021[a] ± 0.005 (19.0%)
40	0.069[a] ± 0.012	0.073[a] ± 0.013 (5.5%)	0.019[a] ± 0.003	0.019[a] ± 0.003 (0.0%)	0.022[a] ± 0.010	0.026[a] ± 0.012 (15.4%)

[a,b] Means with different superscripts are significantly different ($p < 0.05$), n = 3.
[c] MV : measured water vapor permeability value.
[d] CV : corrected water vapor permeability value.
[e] Control : laminated edible film which was composed of methyl cellulose and corn zein using coating technique.
[f] % error : [(CV − MV)/CV] × 100.

Table 3—Water vapor permeabilities of films of methyl cellulose/corn zein/fatty acids at 25°C and 50% RH (used to calculated CWV (Fig. 1) based on Eq. (2))

		WVP (ng·m/m²·sec·Pa)[a]
Methyl cellulose		0.280 ± 0.004
Corn zein		0.562 ± 0.022
Corn zein-lauric acid	10%[b]	0.412 ± 0.035
	20%[b]	0.366 ± 0.031
	30%[b]	0.350 ± 0.019
	40%[b]	0.329 ± 0.031
Corn zein-palmitic acid	10%[c]	0.372 ± 0.043
	20%[c]	0.262 ± 0.009
	30%[c]	0.232 ± 0.023
	40%[c]	0.232 ± 0.013
Corn zein-SP acid blend	10%[d]	0.315 ± 0.014
	20%[d]	0.232 ± 0.016
	30%[d]	0.215 ± 0.007
	40%[d]	0.196 ± 0.014

[a] WVP of methyl cellulose is P_1 and the other WVPs are P_2 based on Eq. (2).
[b] %, g lauric acid/g corn zein.
[c] %, g palmitic acid/g corn zein.
[d] %, g stearic-palmitic acid blend/g corn zein.

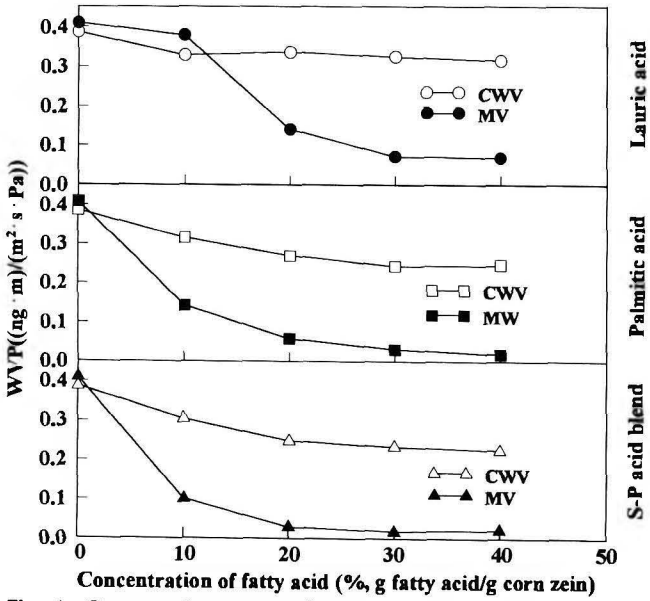

Fig. 1—Changes in measured and calculated values of water vapor permeability as related to fatty acid content. CWV—Calculated WVP value based on Eq. (2); MW—Measured WVP value; S-P acid blend—Stearic-palmitic acid blend; WVP—Water vapor permeability.

Percentage elongation at break

Means and standard deviations of E for the films (Table 1) showed that trends for E values of films containing fatty acid were opposite those for TS. Generally, as the film structure softened, TS decreased and E increased. Differences were significant ($p < 0.05$) in E, but some had broad standard deviations because of differences in film thickness. For example, the standard deviation was 11.62 at 10% palmitic acid, > 30% standard deviation. The E in general increased as film thickness increased, so comparisons of E should include film thickness. The thickness of our film samples was kept constant (3.2 ± 0.7 mil) to minimize effects of this variable.

Water vapor permeability

The effect of fatty acids on WVP of laminated edible films at various fatty acid concentrations was evaluated in terms of MV (measured value) and CV (corrected value) (Table 2). Differences occurred throughout the range of measured values. However no significant differences occurred between MV and CV in the range of 10% to 40% fatty acid concentration except for lauric and palmitic acid at 10% ($p < 0.05$).

Results showed that WVP decreased as chain length and concentration of fatty acid increased. These results were similar to those of Vojdani and Torres (1990) for effects of fatty acid concentration and chain length on potassium sorbate permeability. However, no significant differences occurred between WVP of laminated films composed of palmitic acid and stearic-palmitic acid blend at 30–40% fatty acid ($p < 0.05$).

Some trials to decrease WVP of bilayer edible films by adding fatty acids have been reported (Hagenmaier and Shaw, 1990; Kamper and Fennema, 1984a, b; Kester and Fennema, 1989). For example, the WVP of edible film reported by Hagenmaier and Shaw was 0.000359 (ng·m/m²·sec·Pa) at 85–0% RH gradients. The WVPs for Kamper and Fennema's (C_{18}-C_{16} HPMC) were 0.02133 and 0.00380 (ng·m/m²·sec·Pa) at 97–64% and 85–65% RH gradients, respectively. For Kester and Fennema's film (C_{18}-C_{16} MC/HPMC), the WVPs were 0.00296 and 0.01563 (ng·m/m²·sec·Pa) at 97–0% and 97–65% RH gradients, respectively.

The measured WVP (0.022 ng·m/m²·sec·Pa) of our film containing 40% stearic-palmitic acid blend was similar to that reported by Kamper and Fennema (1984b) and higher than that of others (Hagenmaier and Shaw, 1990; Kamper and Fennema, 1984a; Kester and Fennema, 1989). Such differences could be due to different RH gradients and fatty acid concentrations.

Measured WVP values (MV) were compared with calculated WVP values (CWV) for laminated edible films. WVP data of methyl cellulose, corn zein and edible films made with corn zein and fatty acid were used (Table 3). Results showed

that all MV values were lower than CWV values except for the control and the laminated edible film at 10% lauric acid (Fig. 1). No significant differences occurred between MV and CWV of controls and laminated edible films at 10% lauric acid concentration ($p < 0.05$). MV may have been lower than CWV because fatty acids were migrating from the corn zein-fatty acid layer to the methyl cellulose layer.

CONCLUSION

LAMINATED EDIBLE FILMS made with methyl cellulose and corn zein-fatty acid were water vapor barriers and had effective mechanical properties. Unlike others using the emulsion technique which exhibited distortion, cracking and pinhole formation, our laminated edible films exhibited none of those defects due to the methyl cellulose layer. Since the corn zein-fatty acid layer is insoluble in water, such films may be a useful wrap for high moisture foods.

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