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Characteristics of 95% Lean Beef German Sausages Varying in Phosphate and Added Water

T.L. FREDERICK, M.F. MILLER, K.S. TINNEY, L.R. BYE, and C.B. RAMSEY

ABSTRACT

Beef German sausages (95% lean) with added water levels (0, 10, 20, 35%) and phosphate levels (0.5 and 1.0%), were compared to a control (20% fat). Controls and sausages with 35% added water tended to be scored higher in juiciness and tenderness than other sausages. Aerobic plate counts increased to 2 wk storage and then were almost constant to 6 wk when 0.5% phosphate had been added, but counts increased up to 6 wk storage when 1% phosphate had been added. Most characteristics were similar to control sausages when the formula had 35% added water and either 0.5 or 1% phosphate.

Key Words: beef, German sausage, phosphate, flavor, low fat

INTRODUCTION

THE AMERICAN HEART ASSOCIATION recommended that consumers decrease total dietary fat intake to decrease serum cholesterol concentrations. This and other factors influenced trends toward increased consumption of low-fat (<20%) ground beef compared to that of regular (≈30% fat) ground beef (Hoelscher et al., 1987). Ground meat products (e.g., sausages) may experience similar changes if the concentrations of fat were lower.

Regulations for precooked, processed meat products allow a maximum of 40% fat and added water in final, cooked products (USDA, 1973). Sausages generally contain 30 to 40% fat. Reduced fat content in such products may be advantageous. The major problem in acceptability of low-fat processed meat products is the decline in palatability with fat reduction (Ahmed et al., 1990). Several studies have shown significantly lower sensory scores for tenderness and juiciness in ground beef (Berry and Leddy, 1984; Kregel et al., 1986) and ground pork (Reitmeier and Prusa, 1987) formulated with <20% fat compared to those with >20%. Ahmed, et al. (1990), reported that the reduction of fat from 35 to 15%, with a concurrent increase in added water, resulted in no differences in sensory evaluations for low-fat fresh pork sausage. Alternative processing methods may lead to low-fat ground meat products with more acceptable palatability. The most feasible approach would be replacement of fat with added water. Our objective was to evaluate effects of added water and phosphate on sensory, physical and microbiological characteristics of 95% lean beef sausage.

MATERIALS & METHODS

Products and processing

Beef top rounds of USDA Select quality grade were trimmed practically free of subcutaneous and intermuscular fat to provide 95% lean meat. German sausages with 5% fat, two phosphate levels (0.5 and 1.0%) and four water levels (0, 10, 20 and 35%) were prepared following a 2 × 4 factorial design. Proximate analysis was conducted to confirm the 5% fat level (AOAC, 1990). Controls were prepared using a commercial beef, German sausage formulation that contained 20% fat and 10% added water. Controls were used to compare experimental samples with industry products. Meat was ground through a 13-mm and then a 3.3-mm plate by a Hollymatic meat grinder Model GMG 180A (Hollymatic Corp., Park Forest, IL).

Beef (4.54 kg) and ice were weighed and combined with German Smoked Sausage Seasoning (Blend CP-9000-R, A.C. Legg Co., Birmingham, AL). The phosphate and 1% sodium chloride were added prior to mixing. Blends were mixed for 10 min in a Toledo Scale 5340 mixer (Toledo Scale Corp., Toledo, OH) and stuffed into natural pork casings using an E.F. Zuber vacuum stuffer (E.F. Zuber Engineering & Sales, Minneapolis, MN). Sausage was linked using a hand-linker, each link was 15 cm in length. Sausages were weighed before being cooked to internal temperature 71°C in a Vortron Automatic Smokehouse (Vortron, Inc., Beltoit, WI) using a 4-phase, 150-min processing schedule: (1) 30 min at a wet bulb (wb) temperature of 18°C and a dry bulb (db) temperature of 52°C; (2) 90 min at wb 43°C and db 60°C with smoke; (3) 15 min at a wb 59°C and db 71°C with smoke; and (4) 15 min at wb 79°C db 79°C with smoke until the internal temperature reached 71°C. Sausages were removed from the smokehouse and weighed before vacuum packaging in Cryovac®8540 barrier bags, and frozen at −34°C until analyzed (≈8 wk).

Proximate analysis and cooking yield

Analyses were performed in duplicate for moisture, fat (chloroform-methanol extractable component) and protein (Kjeldahl nitrogen) of cooked sausages by AOAC (1990) procedures. The cooking yield, to account for loss in the smokehouse, was determined with the equation: Cooking yield = (wt cooked sausage)/(wt raw sausage)* 100.

Cooking

Sausages for sensory measurements were prepared by thawing in a refrigerator 2°C for 24 hr before cooking. Weights were recorded prior to cooking using a Mettler PC4400 balance (Mettler Instrument Corp., Highstown, NJ). Links were cooked in Farberware skillets (Model No. B3000, Kidde, Inc., Bronx, NY) to internal temperature 71°C measured with an Omega DP80 potentiometer (Omega Engineering, Inc., Stanford, CT). Sausages were cooked ≈ 15 min while being rotated continuously to avoid scorching. After cooking, links were weighed again and sliced into 0.6-cm thick slices. Percentage cooking loss was determined by difference between thawed and cooked weights divided by thawed weights.

Trained sensory panel

Eight panelists were trained in four sessions in which they were served samples from a variety of treatments to familiarize them with the range of sensory characteristics to be judged (Cross et al., 1978). Sausage samples were kept warm until served in aluminum poaching pans. The pans had sand in bottom compartments and had been warmed in the oven at 93.3°C. Panelists sat in booths in an isolated room free of distractions. Red lighting was used to avoid visual bias (AMSA, 1978). Panelists were instructed to drink apple juice and water at room temperature (≈23°C) to cleanse the palate between samples. Training sessions were concluded when panelists were in close agreement (i.e., individual raw scores did not vary more than ±1 from mean scores and all panelists agreed they understood and could use the scoring systems).

Samples were scored on a 1- to 8-point rating scale for juiciness, springiness, tenderness, cohesiveness, texture, and flavor (8 = extremely juicy, springy, tender, cohesive, coarse, and flavorful and 1 = extremely dry, nonspringy, tough, noncohesive, fine, unflavorful.

Author Frederick is with Plantation Foods, 2510 E. Lakeshore Dr., Waco, TX 76705. Authors Miller, Tinney, Bye, and Ramsey are with the Dept. of Animal Science & Food Technology, Texas Tech Univ., Box 42162, Lubbock,TX 79409-2162.

Table 1—Proximate composition of 95% lean German sausage with varying water and phosphate levels (means)

Levels[a]	Moisture (%)	Fat (%)	Protein (%)	Cooking yield (%)
Control	65.1	18.2	15.7	96.1[b]
0:0.5	67.2	4.4	27.3	95.2[e]
10:0.5	70.2	4.3	24.3	95.1[e]
20:0.5	71.1	4.3	23.4	95.0[e]
35:0.5	75.7	4.2	18.8	93.9[d]
0:1.0	65.8	4.4	28.7	95.3[e]
10:1.0	69.8	4.2	24.7	94.3[d]
20:1.0	70.2	4.3	24.3	93.9[d]
35:1.0	74.5	4.2	20.0	93.3[d]

[a] Added water (%):phospate (%) in the formulation with 5% fat. Control formulated to contain 20% fat and 10% added water.
[b,d] Means with common superscripts are not different (P > 0.05).

Table 2—Sensory panel ratings[a] of 95% lean beef German sausage as influenced by water and phosphate levels (means)

Levels	Tenderness	Juiciness	Springiness	Cohesiveness	Texture	Flavor
Control	7.3[b]	7.5[b]	5.1[g]	4.5[g]	5.1[cd]	5.9[bc]
0:0.5	4.4[g]	4.7[fg]	7.2[b]	6.5[b]	5.9[b]	5.7[c]
10:0.5	5.2[d]	5.2[ef]	6.7[bcd]	6.0[bc]	5.5[bcd]	5.7[c]
20:0.5	5.5[cd]	5.7[de]	6.6[cde]	5.9[c]	5.8[b]	5.9[bc]
35:0.5	6.8[b]	7.6[b]	5.7[f]	5.1[d]	5.3[bcd]	6.2[bc]
0:1.0	4.8[g]	4.0[g]	7.1[bc]	6.0[bc]	5.7[bc]	5.9[bc]
10:1.0	6.1[c]	6.4[c]	6.2[e]	5.7[c]	5.5[bcd]	6.3[b]
20:1.0	5.9[c]	6.2[cd]	6.6[de]	5.9[c]	5.6[bcd]	6.2[bc]
35:1.0	7.1[b]	7.4[b]	5.2[g]	5.0[de]	5.0[d]	6.0[bc]

[a] Means based on an 8-point scale. 8 = extremely juicy, springy, tender, cohesive, coarse, flavorful; and 1 = extremely dry, nonspringy, tough, noncohesive, fine, unflavorful. Levels shown as water: phosphate levels.
[b,c,d,e,f,g] Means in a column with common superscripts are not different (P > 0.05).

Thiobarbituric acid (TBA) determination

Sausage (10g) was homogenized with 50 mL distilled water in an Osterizer Galaxie blender for 2 min. The blended sample was transferred into an 800-mL Kjeldahl flask by washing with 47.5 mL of distilled water. Then 2.5 mL of HCl solution (one part concentrated HCl to two parts distilled water) was added to the solution to lower pH to 1.5. Exactly 50 mL distillate was collected using a Lab Con Co. Kjeldahl rack. Then, 5 mL distillate was pipetted into a test tube along with 5 mL TBA reagent (2.88g 2-thiobarbituric acid, MW = 144.15 g/mole, in 1 L of 90% glacial acetic acid). A blank was prepared by placing 5 mL distilled water and 5 mL TBA reagent in a test tube. Tubes were placed in a boiling water bath for 35 min to allow color development. Absorbance was measured with a Spectronic 20 colorimeter at 535 nm (Tarladgis et al., 1960).

Microbiological analysis

Sausage samples were homogenized in 180 mL Butterfield's phosphate buffer (Pertel and Kazanas, 1984) for 2 min with an Osterizer blender. Samples were evaluated for total aerobic plate counts (APCs) by placing 10 mL of homogenate in the appropriate 10-fold dilutions and placing 1 mL dilutions on sterile petri dishes. APC agar (Difco, Detroit, MI) was poured in the plates and swirled to evenly distribute samples. Plates were incubated at 37°C in a Forma Scientific (Model 3546, Marietta, OH) Incubator for 48 h before being counted and reported as the $\log_{10}$ CFU/cm^2 for each sample for each storage time (0, 2, 4, and 6 wk).

Instrumental texture determination

Two 2.5-cm long by 2.5-cm diameter sections per treatment were removed from the sausage samples. Sausage sections were compressed to 50% of their original height with an Instron Universal Testing Machine (Model No. 1122, Instron Corp., Canton, MA). Measurements were obtained for hardness (peak force, kg needed for first compression) and springiness (distance in cm the sample recovered after first compression).

Statistical analysis

Sensory and instrumental texture measurements were analyzed in a two (phosphate level) by four (water levels) factorial design. ANOVA was conducted using the General Linear Models (GLM) procedure for SAS Institute, Inc. (1990). When main effects or interactions were significant, means were separated by the Least Significant Difference procedure (Steel and Torrie, 1980). The predetermined level of probability was 5%.

Table 3—Instrumental texture measures and TBA values of 95% lean beef German sausage as related to water, and phosphate levels and storage

Levels	Hardness[a] (kg)	Springiness[b] (cm)	TBA value (mg malonaldehyde/kg sample) Storage time (wk) 0	2	4	6	8
Control	13.45[c]	0.76[e]	0.39	0.26	3.25	0.19	0.13
0:0.5	13.64[c]	1.14[c]	0.21	0.20	0.07	0.23	0.07
10:0.5	14.11[c]	0.76[e]	0.09	0.34	0.17	0.28	0.14
20:0.5	11.12[ef]	0.95[cd]	0.02	0.11	0.32	0.12	0.32
35:0.5	11.56[e]	0.76[e]		0.21	0.23	0.09	0.14
0:1.0	13.65[c]	1.14[c]	0.61	0.51	0.12	0.12	0.18
10:1.0	10.60[f]	0.83[de]	0.42	0.32	0.12	0.05	0.09
20:1.0	12.59[d]	0.89[de]	0.36	0.16	0.11	0.25	0.10
35:1.0	11.68[e]	0.89[de]	0.28	0.13	0.18	0.25	0.18

[a] Hardness = Peak force needed for the first compression, kg.
[b] Springiness = Distance the sample recovers after the first compression, cm.
[c,d,e,f] Means in the same column with common superscripts are not different (P > 0.05).

RESULTS & DISCUSSION

Proximate analysis and cooking yield

Proximate composition of the cooked, 95% lean, German sausage (Table 1) showed moisture percentage tended to increase and crude protein to decrease as added water increased with 0.5 and 1.0% added phosphate. Fat content was more than four times greater in the control than in sausages with added water and phosphate. Yield of cooked product was highest ($P < 0.05$) for control sausage (96.1%). When 20% or less added water and 0.5% phosphate were used. Cooking yields were higher than for those sausages to which at least 10% water and 1.0% phosphate had been added. Phosphate level did not affect cooking yields when no water had been added.

Sensory evaluations

Scores for the sensory attributes of tenderness, and juiciness did not differ ($P > 0.05$) among controls and sausages to which 35% water had been added (Table 2). Sausages with 35% water were usually scored higher than all others. Among sausages treatments containing 0.5% phosphate, tenderness, juiciness, and flavor tended to increase and springiness and cohesiveness to decrease, as added water increased. No such patterns were evident in sausages to which 1.0% phosphate had been added. Phosphate level did not affect any sensory characteristic when no water was added. When 10% water had been added, 1% phosphate produced higher tenderness, juiciness, and flavor and lower springiness scores than did 0.5% phosphate. However, none of these traits differed between phosphate levels when 20% water had been added. When 35% water had been added, only springiness was affected by phosphate level. The 0.5% level produced more springy sausages than the 1% level. Only slight differences in flavor scores were found. The sausages formulated with 10% added water and 1% phosphate had higher flavor scores than those with 10% or less added water and 0.5% phosphate. Sausages to which 35% water and either 0.5 or 1% phosphate had been added were rated equal or higher than controls to which 10% water but no phosphate were added.

Instrumental texture determinations

Means for instrumental texture analyses (Table 3) showed controls, sausages containing ≤ 10% added water and 0.5% phosphate, and those with 1% phosphate but no added water were harder than other sausages ($P < 0.05$). The highest level of water tended to decrease hardness. These data generally

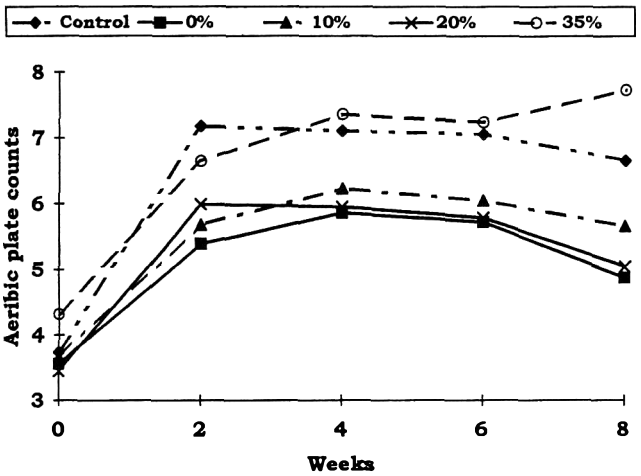

Fig. 1—Changes in aerobic plate counts ($log_{10}CFU/cm^2$) of vacuum packaged 95% lean beef German sausage (0.5% added phosphate and varying levels of added water) with storage at −34°C over 8 wk.

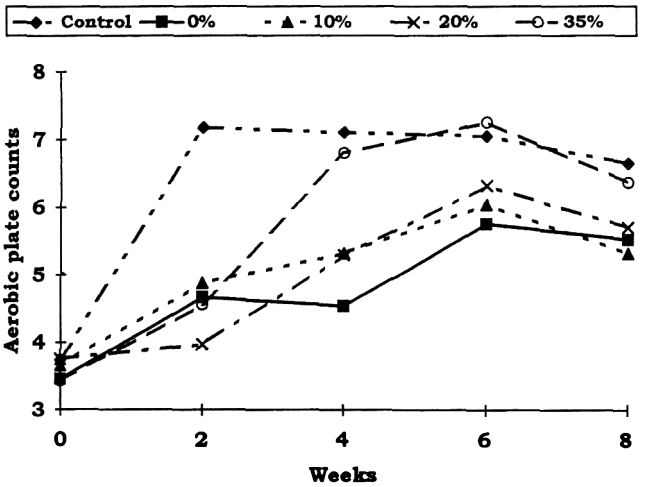

Fig. 2—Changes in aerobic plate counts ($log_{10}CFU/cm^2$) of vacuum packages 95% lean beef German sausage (1.0% added phosphate and varying levels of added water) with storage at 37°C over 8 wk.

confirmed results of Ziegler et al. (1987) who tested several types of dried and nondried sausages ranging in moisture from 27.7 to 62.4% and reported hardness increased as moisture decreased. Sausages formulated without added water were most springy ($P < 0.05$). When 1% phosphate was used, added water level did not affect springiness. When 0.5% phosphate had been added, the 20% level of added water resulted in more springiness than 10 or 35% added water.

TBA values

No trends were apparent in TBA values (Table 3) for any treatment at any storage time. At wk 4, the control was rancid, because the vacuum package leaked. However, other TBA values were within acceptable ranges (<1.0 malonaldehyde/1000g meat) for oxidative rancidity (Ockerman, 1976). Thus, cure ingredients, antioxidants, or removal of oxygen by vacuum packaging prevented oxidative rancidity in these low- fat, water added sausages.

Aerobic plate counts

At the beginning of storage no differences were found in APCs among treatments ($P > 0.05$, Fig. 1). APCs greatly increased in controls and all treatments containing 0.5% phosphate after 2 wk storage. Between 2 and 6 wk storage, little change in APCs occurred, but a slight decline was found from 6 to 8 wk storage in all sausages except those to which 35% water had been added. The control and sausages with 35% added water were higher than others in APCs after 2 wk storage. Egbert et al. (1992) reported no differences between low-fat all beef ground beef and a low-fat carrageenan based ground beef product. Additionally, the addition of water to the carrageenan based product had little effect on microbial growth (Egbert et al., 1992). A different pattern of storage time effects on APCs was found when 1% phosphate had been added (Fig. 2). In the control log counts increased sharply the first 2 wk of storage. Other sausages increased in APCs to 6 wk storage and then all declined to 8 wk storage. After 4 wk, the control and sausage to which 35% water had been added were higher in APCs than other sausages but did not differ from each other.

CONCLUSIONS

SOME NEGATIVE SENSORY and physical characteristics associated with low-fat sausages can be minimized by addition of 35% water and phosphate. Sensory panel ratings for tenderness and juiciness did not differ between controls (20% fat, 10% water) and sausages formulated with 5% fat and 35% added water at two phosphate levels. Instrumental measures of hardness and springiness for the sausages with 35% added water equalled or exceeded those of controls. The higher level of phosphate was associated with a slower increase in APCs during 6 wk storage. The 35% level of added water resulted in APCs similar to controls and higher than other levels of added water. Beef sausage with 5% fat can be produced and still maintain the integrity and acceptable sensory characteristics of a 20% fat level when water is used to replace the fat.

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Assessment of Sensory Quality of Meat Sausages Using Near Infrared Spectroscopy

MARIT RISBERG ELLEKJAER, TOMAS ISAKSSON and RAGNHILD SOLHEIM

ABSTRACT

Near infrared reflectance (NIR) and transmittance (NIT) spectroscopy were studied as potential methods for determination of sensory quality of sausages. Sausage formulations (57) with varying fat (6.3–30.1%), carbohydrate (2.6–9.7%) and NaCl (1.4–2.2%) were produced. Sausages were evaluated by a trained sensory panel using three color, four odor, six flavor, and five texture attributes. Sensory panel performance was evaluated to ensure accuracy and reliability. NIR and NIT analyses were performed both on batter and end-products. The main variation among sausages was described by differences in color attributes, odors and flavors of meat and smoke, juiciness and greasiness. NIT analysis of sausages gave the best description of sensory variations. However, both NIR and NIT spectra of batter described the main color and texture variations among sausages with a relative prediction (RAP) of 0.87 to 0.93 for color attributes and 0.83 to 0.91 for juiciness and greasiness. These rapid methods should be useful during production and for end-product control to help ensure consistent quality.

Key Words: sensory, sausages, infrared spectroscopy, multivariate analysis

INTRODUCTION

MEASUREMENT OF QUALITY CHARACTERISTICS by sensory methods is relatively time consuming, and not suitable for on-line control of food processes. For such uses it would be advantageous to predict sensory quality by rapid and dependable instruments. Near infrared reflectance (NIR) and transmittance (NIT) spectroscopy are both rapid and nondestructive, and can be calibrated to measure several components at the same time. These methods use the wavelengths region from 700 to 2500 nm and the spectrum is a result of specific light absorbance from C-H, O-H, N-H and C=O overtones and combination bands at different wavelengths. Near infrared spectroscopy has mostly been used for compositional analysis of foods (Osborn and Fearn, 1986; Williams and Norris, 1987), including meat and meat products (Martens et al., 1981; Lanza, 1983; Ellekjær et al., 1993). Applications for other food quality parameters have been reported (Iwamoto and Kawano, 1992). Prediction of sensory quality based on near infrared spectroscopy has been reported on peas (Martens and Martens, 1986; Kjølstad et al., 1990) and green and black china teas (Yan et al., 1990).

NIR and NIT spectroscopy are indirect methods which prior to analysis must be calibrated with a reference method. The accuracy of the NIR and NIT predicted property may therefore be affected by errors in the reference method. It is thus crucial to ensure the accuracy and the reliability of reference data.

Our main objective was to examine the potential of NIR and NIT spectroscopy as rapid methods to determine sensory quality of sausages. This was done by (1) examining the performance of the sensory panel to ensure the validity of reference data; (2) identifying main sources of variation in sensory quality and relating those to main NIR and NIT predicted sensory quality (3) evaluating the prediction ability of NIR and NIT spectra of batters for sensory attributes and comparing those with the accuracy of NIR and NIT spectra of sausages.

Authors Ellekjaer and Isaksson are with MATFORSK - Norwegian Food Research Institute, Osloveien 1, N-1430 ÅS, Norway. Author Solheim is with the Dept. of Food Science, Cornell Univ., Ithaca, NY 14853.

Table 1—Identification of sausage samples, and variation of fat, salt (NaCl) and starch content of sausages according to a complete factorial design

Id	Code*	Id	Code*	Id	Code*	Id	Code*
1	1.1.1	19	1.1.2	37	1.1.3	55	4.2.2
2	2.1.1	20	2.1.2	38	2.1.3	56	4.2.2
3	3.1.1	21	3.1.2	39	3.1.3	57	4.2.2
4	5.1.1	22	5.1.2	40	5.1.3		
5	6.1.1	23	6.1.2	41	6.1.3		
6	7.1.1	24	7.1.2	42	7.1.3		
7	1.2.1	25	1.2.2	43	1.2.3		
8	2.2.1	26	2.2.2	44	2.2.3		
9	3.2.1	27	3.2.2	45	3.2.3		
10	5.2.1	28	5.2.2	46	5.2.3		
11	6.2.1	29	6.2.2	47	6.2.3		
12	7.2.1	30	7.2.2	48	7.2.3		
13	1.3.1	31	1.3.2	49	1.3.3		
14	2.3.1	32	2.3.2	50	2.3.3		
15	3.3.1	33	3.3.2	51	3.3.3		
16	5.3.1	34	5.3.2	52	5.3.3		
17	6.3.1	35	6.3.2	53	6.3.3		
18	7.3.1	36	7.3.2	54	7.3.3		

* The first number represents the fat level: 1 = 8%, 2 = 12%, 3 = 16%, 4 = 18%, 5 = 20%, 6 = 24%, 7 = 28%; the second number represents the NaCl level: 1 = 1.3%, 2 = 1.6%, 3 = 1.9%; the third number represents the starch level: 1 = 1.5%, 2 = 4.5%, 3 = 7.5%. e.g. 1.1.1 = 8% fat, 1.3% NaCl, 1.5% starch.

MATERIALS & METHODS

Experimental design

Fat, NaCl and starch contents of sausages were varied according to a complete factorial design (see Table 1). The fat content varied at six levels(8, 12, 16, 20, 24 and 28%), the sodium chloride (NaCl) concentration at three levels (1.3, 1.6 and 1.9%), and the starch content at three levels (1.5, 4.5 and 7.5%). In addition, sausages containing 18% fat, 1.6% NaCl and 4.5% starch were produced in three replicates. Each sausage formulation contained about 11% protein.

Sausage production and sample preparation

Fresh beef meat (19.6% protein, 9.2% fat, 69.7% water) and pork fat (5.2% protein, 75.1% fat, 20.0% water) were ground separately and stored at −40°C until production. Batches enough for one day's production were thawed for 20 hr at 4°C. Thawed ground beef meat, thawed ground pork fat, potato starch (17% water, 83% carbohydrate), NaCl (with 0.3% sodium nitrite) and ice-water were used for production of sausages. In addition, 2.5% skimmed milk powder (37% protein, 1.1% fat, 53% carbohydrate and 4.0% water), 2.5% spice and 0.4% ascorbate were added to each batch. The batters were processed in a Kilia Vaccu 2000S Express 30-L chopper (Fritz Reimers, GmbH u. Co. KG, Kiel) until the temperature reached 15°C. NIR and NIT analysis was performed on batter samples collected from the vacuum chopper. The batters were stuffed into plastic casings with a vacuum stopper (Handtmann, VF 12 Albert Handtmann Maschinenfabrik GmbH & Co, KG, Biberach/Riss, Germany). The thermal processing was to 72°C internal end-point temperature and consisted of the following steps (air temperatures): Heating 15 min at 50°C, drying 10 min at 65°C, smoking 20 min. at 65°C, drying 5 min at 70°C, smoking 20 min at 70°C, drying 5 min at 70°C and heating 22 min at 78°C. Sausages were stored at −40°C for 2–4 wk and thawed overnight at 4°C before they were analyzed. All sausages were produced by the same procedure in randomized order. The sausages were homogenized for 30 sec in a rotating knife homogenizer (Moulinex Masterchef 20, Nieune, France) prior to NIR, NIT and chemical analyses.

Sensory analysis

Sausages were analyzed by conventional profiling using nine trained assessors. They were selected and trained according to guidelines in ISO 6658-1985 and ISO/DIN 8586-1.1989. Evaluations were performed in a laboratory designed as described in ISO 8589-1988. Assessors developed a vocabulary by describing differences between

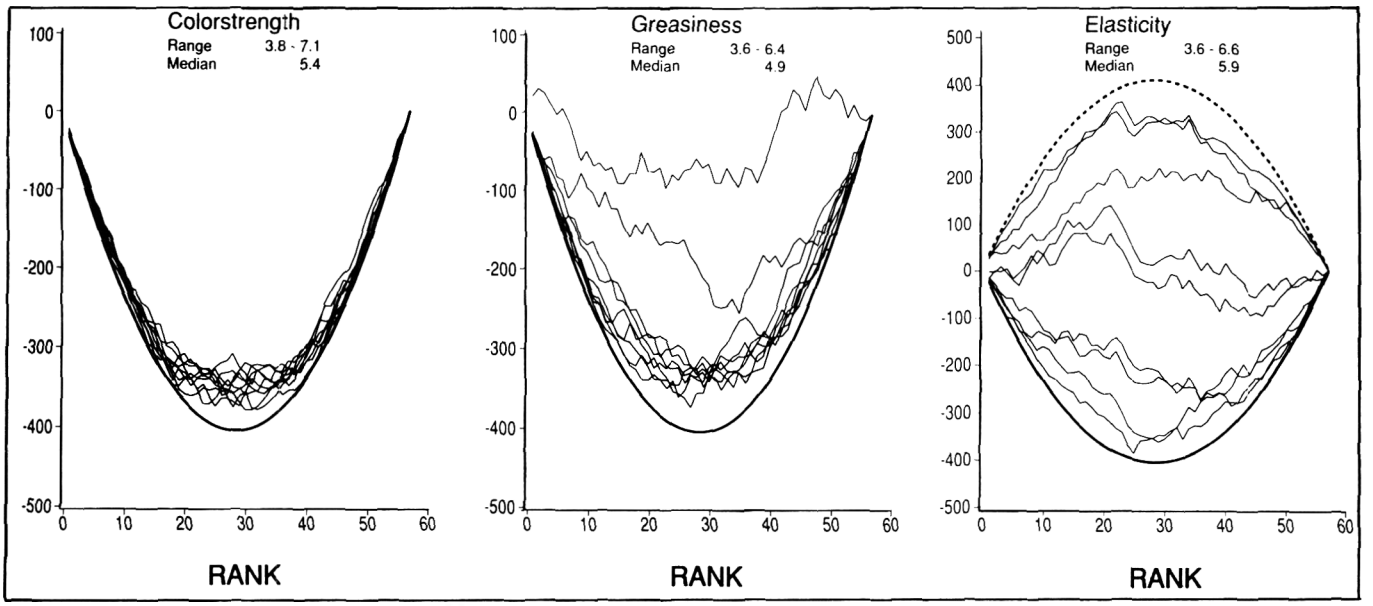

Fig. 1—Egg-shell plot of the ranking of 57 sausages by nine trained assessors with respect to selected attributes. Ranking based on average responses over three replicates for each assessor. Ranges and the medians were calculated from raw data.

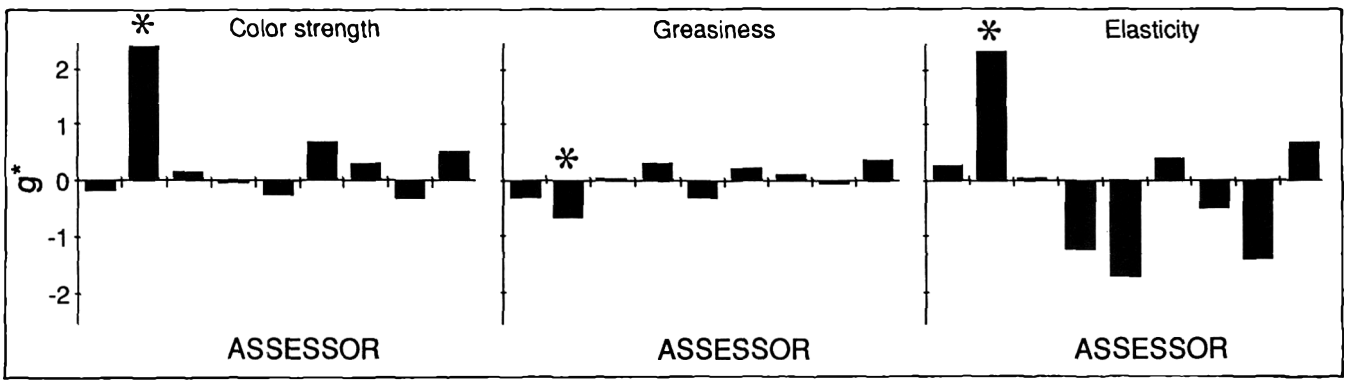

Fig. 2—Differences in use of scale and ranking of samples for each assessor and attribute. A g*-value above 0 or below 0 indicate the assessor uses respectively, a smaller or greater portion of the scale than average panelists. An assessor with different ranking of samples compared to average panelists has a g*-value < −1. *assessor whose data were deleted before averaging.

extreme samples and agreed on a consensus list of attributes for profiling. These were whiteness, color, color strength, odor intensity, odor of meat, odor of smoke, off-odor, flavor intensity, meat flavor, saltiness, flavor of smoke, spiciness, off-flavor, firmness, juiciness, greasiness, graininess and elasticity. Assessors were trained in definition of sensory attributes prior to profiling and in use of rating anchors by pre-testing extreme samples.

Sausages were heated in water bath to a core temperature of 72°C prior to testing. One sausage was served each assessor for each replicate. Three replicates were performed for each sausage formulation. The samples were served in randomized order within each replicate and with respect to each assessor. Samples (4-5) were evaluated in each session lasting about 45 min. Color attributes were evaluated on fresh lengthwise sections on a white table with 30% black (800 lux) as background. Color attributes were defined using the NCS system for description of colors (SSI *Color Atlas*, 1979). Low intensity in color was defined as yellow/red color and high intensity as red/blue color. Afterwards assessors evaluated smell, flavor and texture attributes for the same sample. They were instructed to use water (35°C) and unsalted crackers for palate cleaning between the samples. They were also instructed to expectorate when finished.

Each assessor evaluated the samples at their individual rate on a computerized system for direct recording of data (SENSTEC, Tecator AB, Höganäs, Sweden). A continuous nonstructured scale was used for evaluation. The left side of the scale corresponded to the lowest intensity of each attribute (value 1.0) and the right side corresponded to the highest intensity (value 9.0). Average response over replicates and assessors was used in multivariate analyses.

Table 2—The range, total standard deviation (S_{tot}) of average sensory data and standard error of sensory data (S_{ref})

Sensory attribute	Range	S_{tot}	S_{ref}
Whiteness	3.1—6.3	0.86	0.14
Color	4.3—7.6	0.88	0.15
Color strength	3.8—7.1	0.93	0.15
Odor intensity	6.4—7.8	0.33	0.15
Odor of meat	2.1—5.4	0.69	0.21
Odor of smoke	3.0—7.4	1.31	0.23
Off-odor	1.1-7.1	1.42	0.30
Flavor intensity	6.3—7.4	0.32	0.14
Meatflavor	2.4—5.8	0.74	0.21
Saltiness	4.7—7.2	0.63	0.18
Flavor of smoke	3.2—7.4	1.19	0.22
Spiciness	4.7—6.9	0.52	0.18
Off-flavor	1.0—6.6	1.27	0.27
Firmness	4.1—7.8	0.75	0.12
Juiciness	2.7—7.4	1.34	0.18
Greasiness	3.6—6.4	0.58	0.15

Chemical analyses

Sausages were analyzed by chemical methods in triplicate; airdrying for 14 hr at 105°C for water, ethylene tetrachloride extraction for fat (Foslet, A/S N. Foss Electric, Hillerød, Denmark), semi-automatic Kjeldahl for protein (Kjeltec, Tecator AB, Höganäs, Sweden), glucose for carbohydrates (YSI Model 2000 Glucose and L-Lactate Analyzer,

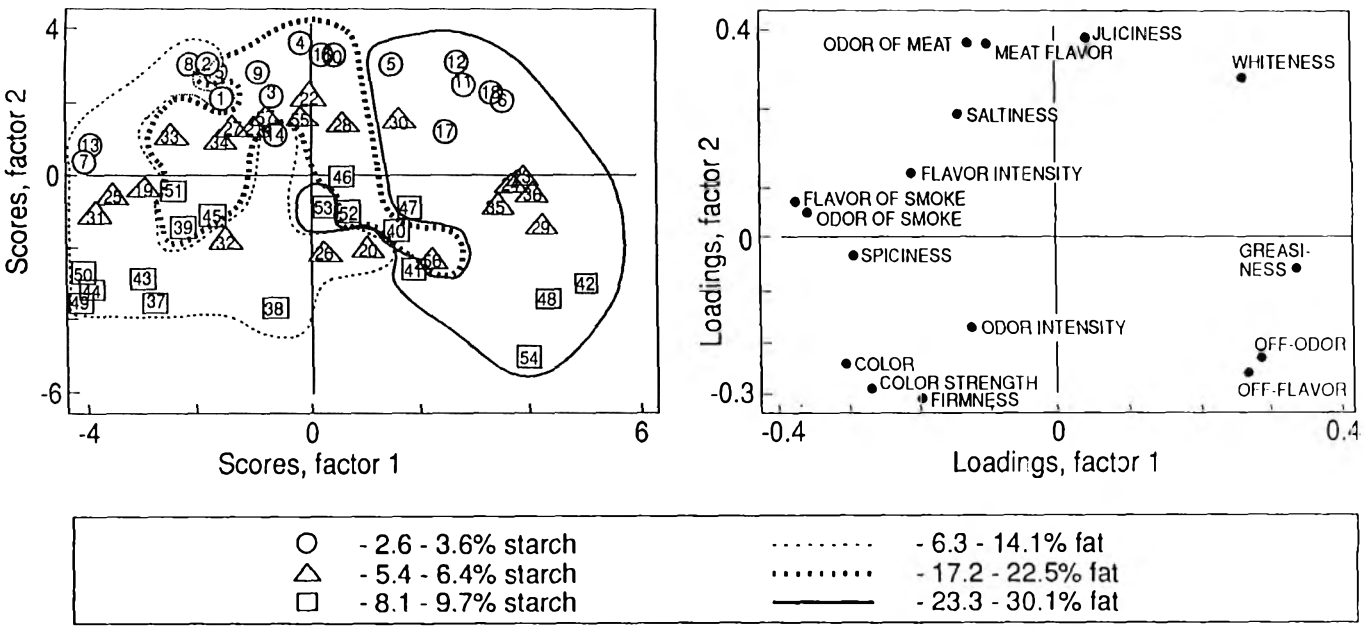

Fig. 3—Principal component analysis (PCA) of sensory data for sausages. PCA scores and loadings for factors 1 and 2.

Table 3—Results from principal component analysis (PCA) used to describe sensory quality of 57 sausage samples

	% Explained variances after PCA-factor*		
Sensory variable	1	2	3
Whiteness	34.8	89.5	92.2
Color	57.3	92.0	92.0
Color strength	38.8	88.4	90.8
Odor intensity	0.0	0.0	44.0
Odor of meat	0.0	75.6	88.5
Odor of smoke	84.0	84.2	94.3
Off-odor	43.5	77.8	94.4
Flavor intensity	16.9	15.8	92.4
Meatflavor	0.0	70.6	88.0
Saltiness	0.0	24.9	63.7
Flavor of smoke	89.2	91.6	91.1
Spiciness	49.4	45.3	43.1
Off-flavor	34.8	77.6	95.0
Firmness	10.3	68.4	69.1
Juiciness	0.0	69.1	80.9
Greasiness	73.4	74.3	77.6
Total	37.7	69.4	83.4

* PCA was performed on averages of eight assessors and three sensory replicates.

YSI Inc., Yellow Springs, Ohio) and chloride-ions for NaCl (Corning 926 Chloride Analyzer, Corning Limited, Halstead, GB).

Near infrared spectroscoy

NIR and NIT spectroscopy were performed on both sausage batters and sausages. Batters were analyzed the same day they were produced. Sausages were frozen, thawed and homogenized as described, before NIR and NIT analyses. The NIR spectra were measured on a Technicon 500 (Technicon Industrial Systems, NY) from 1100 to 2500 nm in an open sample cup at 4 nm intervals. Each sample was measured in five repacked replicates at about 20°C, and the mean of the spectra (log 1/Reflectance) was used in regression. The NIT analyses were performed on a Tecator 1225 Infratec Food and Feed analyzer (Tecator AB, Höganäs, Sweden), which operated from 850 nm to 1050 nm at 2 nm interals. Each sample was distributed into 5 stainless steel pipes (diameter 35 mm and height 15 mm for sausage batters and 17 mm for sausages), and placed on a horizontally rotating samples cup. One spectrum from each pipe was measured at about 20°C. Average spectra (log 1/Transmittance) for each sample were used in regressions.

Data analysis

The variation between assessors in ranking of samples was evaluated by "eggshell plots" (Hirst and Naes, 1994; Naes et al., 1994). Significance levels of differences between samples (except center-points) for each assessor and sensory attribute according to fat, starch and NaCl level were calculated by analysis of variance (ANOVA). A cross-classification model with main effects, first and second order interactions was applied. In addition, a statistical tool was used to detect and interpret variations between assessors in use of scale and in understanding attributes (Naes and Solheim, 1991). A stretching and shrinking constant, G*, for each assessor and attribute was calculated. These scores were used as indicators of agreement/disagreement among the assessors.

ANOVA was also performed on average responses for each sample (except centerpoints). A cross-classification model was applied with effects of fat, NaCl and starch level and the first order interactions included. Principal component analysis (PCA) (Martens and Naes, 1989) was performed on average responses over assessors and sensory replicates for each sample to study the main tendencies of variation among sausages.

Multiplicative scatter corrected (MSC) (Geladi et al., 1985) spectra were used to predict sensory quality. The multivariate methods partial least squares (PLS1 and PLS2) (Martens and Naes, 1989) were used for predicting sensory attributes (Y-data) by use of NIR or NIT spectra (X-data). The difference between PLS1 and PLS2 is that PLS1 makes one model for each sensory attribute, whereas PLS2 makes one model for all sensory attributes. The sensory attributes were standardized to equal variance prior to PCA and PLS2.

The calibration models (PCA, PLS1 and PLS2) were validated using full cross-validation where each sample was used to test the model estimated by all other samples. The accuracy of models was expressed by root mean square of prediction (RMSEP, Martens and Naes, 1989). RMSEP is defined for the sensory attribute y_j:

$$\text{RMSEP} = \sqrt{\frac{1}{I}\sum_{i=1}^{I}(\hat{y}_i - y_i)^2}$$

where y_i is the known $\hat{y}_i$ is the predicted value for sample number i, and I is the number of cross-validations (which in this case, equals the number of samples).

In order to compare the predictive ability of the calibration models for different sensory attributes, the relative ability of prediction (RAP, Martens and Naes, 1989) was used. This is a background variation compensated value where a value of 1.0 represents a perfect prediction. RAP is defined for the sensory attribute y_j by:

$$\text{RAP} = (S_{tot}^2 - \text{RMSEP}^2)/(S_{tot}^2 - S_{ref}^2)$$

where S_{tot} is the total standard deviation of the sensory attribute y_j. S_{ref} is the standard error of the reference method which here indicates the uncertainty of the analysis due to assessors. S_{ref} is defined for the sensory attribute y_j by:

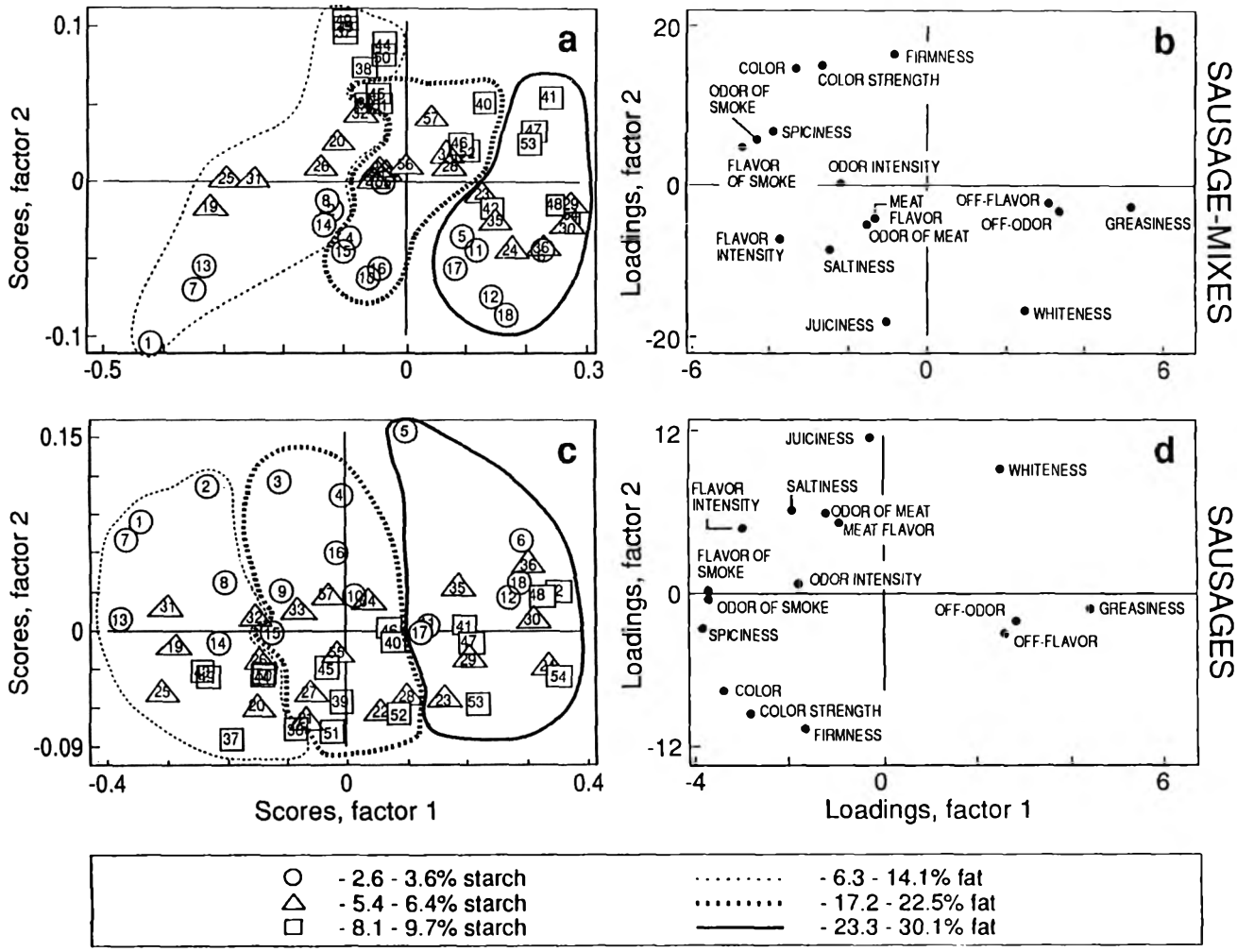

Fig. 4—Relationship of NIT spectra to sensory data using partial least squares (PLS2). Same symbols as Fig. 3. a,b = scores and sensory loadings for batter; c,d = scores and sensory loadings for sausages.

$$S_{ref} = \sqrt{MSE/A^*R}$$

where MSE is the mean square error derived from two-way analysis of variance with samples and assessors (A) as class-variables and with interactions included. R represents the number of sensory replicates.

Difference spectra were calculated in order to relate prediction ability of NIR and NIT for different sensory attributes with changes in spectra caused by varying fat and starch content. Calculation of average difference spectra for starch was done by averaging spectra with similar starch levels, and thereafter calculating difference spectra between high and low starch content and high and medium starch content. The same procedure was carried out when calculating average difference spectra for fat. Difference spectra were calculated for both batters and sausages.

A procedure in the IML language for matrix operations in the SAS system was used for testing sensory panelist performance (Næs and Solheim, 1991). ANOVA was performed by the GLM procedure in the SAS system (SAS Institute, Cary, NC). The MSC, PCA, PLS1 and PLS2 were performed by the Unscrambler-Ex Version 4.0 software package (Camo a/s, Trondheim, Norway).

RESULTS & DISCUSSION

Sensory panelists performance

Eggshell plots (Fig. 1) were used to evaluate how assessors differed in ranking the 57 samples. The baseline (solid smooth line) represented a consensus ranking for the panel. The other lines represented the ranking of samples (average over three replicates) performed by individual assessors. The closer an assessors' line was to the consensus the better the agreement between that assessors ranking and consensus. When an assessor differed in ranking of samples from the consensus this is shown by a different shape of the line. Ranking lines with high negative correlations with the consensus would lie within the mirror image of the baseline. If an assessors' curve was constant in an interval that assessor ranked all corresponding samples equally (Næs et al., 1994). The assessors agreed upon the ranking of the 57 sausage samples with respect to color attributes (Fig. 1), odor intensity, odor of meat, odor of smoke, off-odor, meat flavor, saltiness, flavor of smoke, spiciness and juiciness. Assessors diverged in ranking the samples with respect to flavor intensity, off-flavor, firmness, greasiness, graininess and elasticity. This is shown for greasiness and elasticity (Fig. 1). The narrow range used to describe differences with respect to flavor intensity among the 57 sausage productions explained the small differences in ranking between assessors with respect to this attribute. The difference between minimum value and median was 0.8 in intensity score, and the difference between median and maximum value was 0.7 in intensity score. One assessor ranked only the 10 samples lowest in firmness and the 15 samples highest in firmness in the same order as the rest of the panel. That assessor also ranked samples differently than the rest of the panel with respect to greasiness. For graininess and elasticity some assessors gave opposite rankings (Fig. 1).

ANOVA was performed on individual data to compare assessors' abilities to perceive differences between samples varying in fat, starch and NaCl levels separately. The significance was set at 0.01, as we expected a large variation between samples in sensory attributes, due to great variations in fat, starch and NaCl content. All assessors, except one, found highly significant differences between samples due to fat level with re-

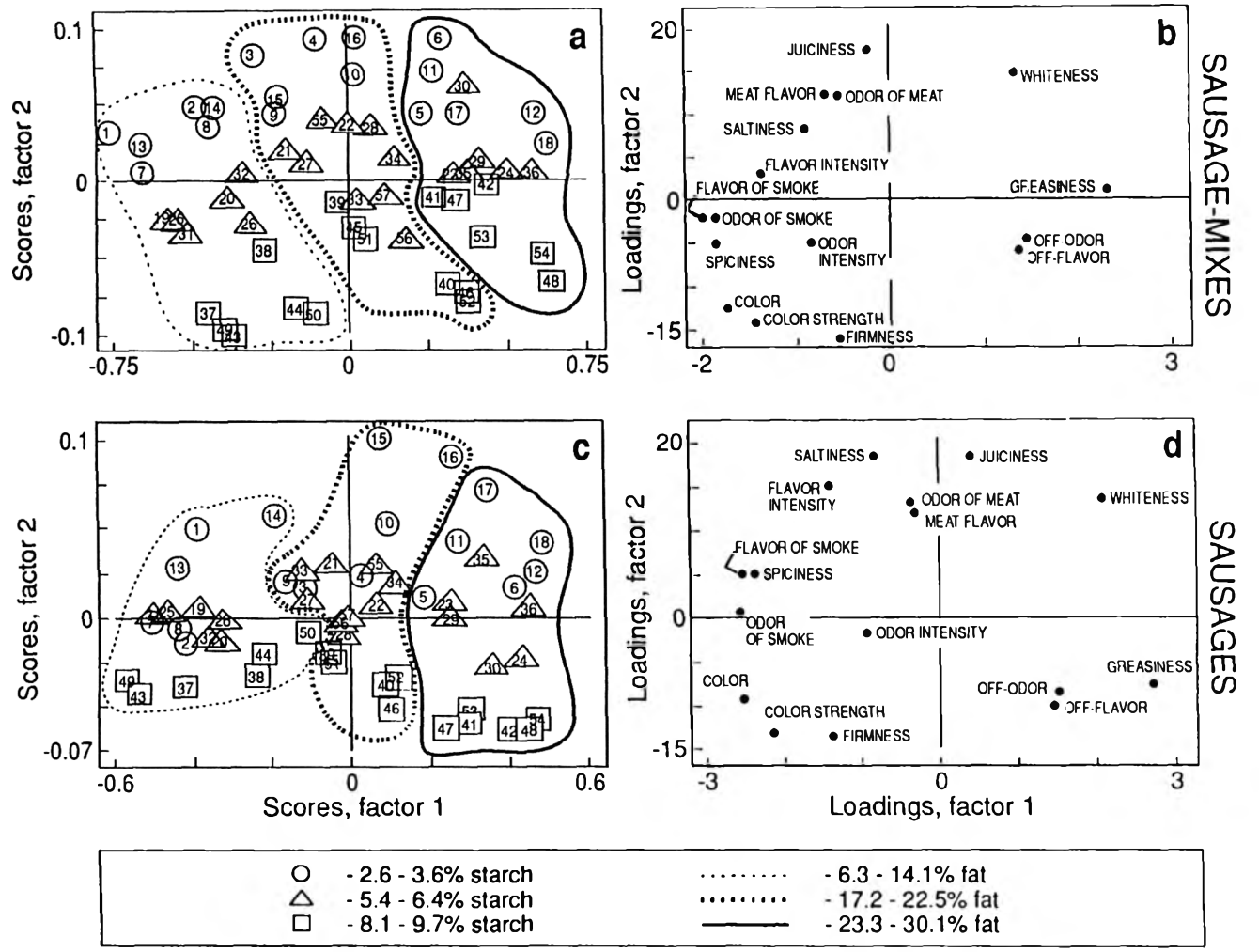

Fig. 5—Relationship of NIR spectra to sensory data using partial least squares (PLS2). Same symbols as Fig. 3. a,b = scores and sensory loadings for batter; c,d = scores and sensory loadings for sausages.

Table 4—Explained variance for sensory attributes of sausages with 1 and 2 factors in the model[a]

	Batter				Sausages			
	NIR		NIT		NIR		NIT	
Sensory variable	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2	Factor 1	Factor 2
Whiteness	24.6	78.2	16.0	75.8	41.2	64.7	27.5	51.4
Color	43.0	79.9	30.0	85.2	60.6	70.0	46.9	64.6
Color strength	28.3	77.8	19.5	77.5	45.4	65.2	31.9	57.0
Odor intensity	4.6	7.3	7.4	7.4	3.8	3.8	5.4	5.4
Odor of meat	2.8	36.0	3.9	6.7	0	21.2	1.3	8.5
Odor of smoke	53.3	53.3	52.9	59.5	49.1	49.1	59.6	59.6
Off-odor	30.4	32.9	27.4	28.5	22.5	29.6	34.0	34.0
Flavor intensity	24.9	24.9	39.3	48.0	19.4	43.6	35.1	39.1
Meat flavor	1.0	34.3	2.3	3.3	0	12.4	0	3.8
Saltiness	8.5	22.4	15.4	28.1	3.2	45.7	14.7	22.2
Flavor of smoke	61.5	61.5	63.2	67.6	58.8	60.6	71.3	71.3
Spiciness	47.8	54.4	42.0	53.2	61.6	63.7	60.6	61.8
Off-flavor	26.0	31.4	24.0	24.0	18.5	27.7	29.1	29.6
Firmness	0	58.0	0	65.4	13.2	33.5	3.8	33.8
Juiciness	0	66.2	0	72.9	0	36.8	0	34.1
Greasiness	76.1	76.1	76.0	76.6	72.3	79.0	84.8	85.1
TOTAL	26.5	49.4	25.6	48.3	28.6	43.3	31.3	41.0

[a] Partial least square (PLS2) regression was performed on NIR- and NIT-spectra of batters or sausages with regard to sensory data of the sausages.

spect to greasiness. That assessor had low F-values in general and was among those who found most non significant differences regarding starch, NaCl and fat levels.

The statistical tool (g*) described in Næs and Solheim (1991) was applied to detect differences between assessors in use of the scale. The assessor who diverged from the rest of the panel in the ranking of samples according to greasiness and firmness (Fig. 1) differed from the mean of the panel in 11 of the 18 attributes. That assessor used a small portion of the scale ($g^* > 0$) for the attributes whiteness, color, color strength, odor intensity, odor of meat, flavor of smoke, firmness and elasticity. This is shown for color strength (Fig. 2). However, that same assessor used a large portion of the scale for greasiness ($g^* < 0$) (Fig. 2). That assessor gave more often than any other assessor the comment boar taint or/and rancid for samples. That assessor had quite low thresholds for boar

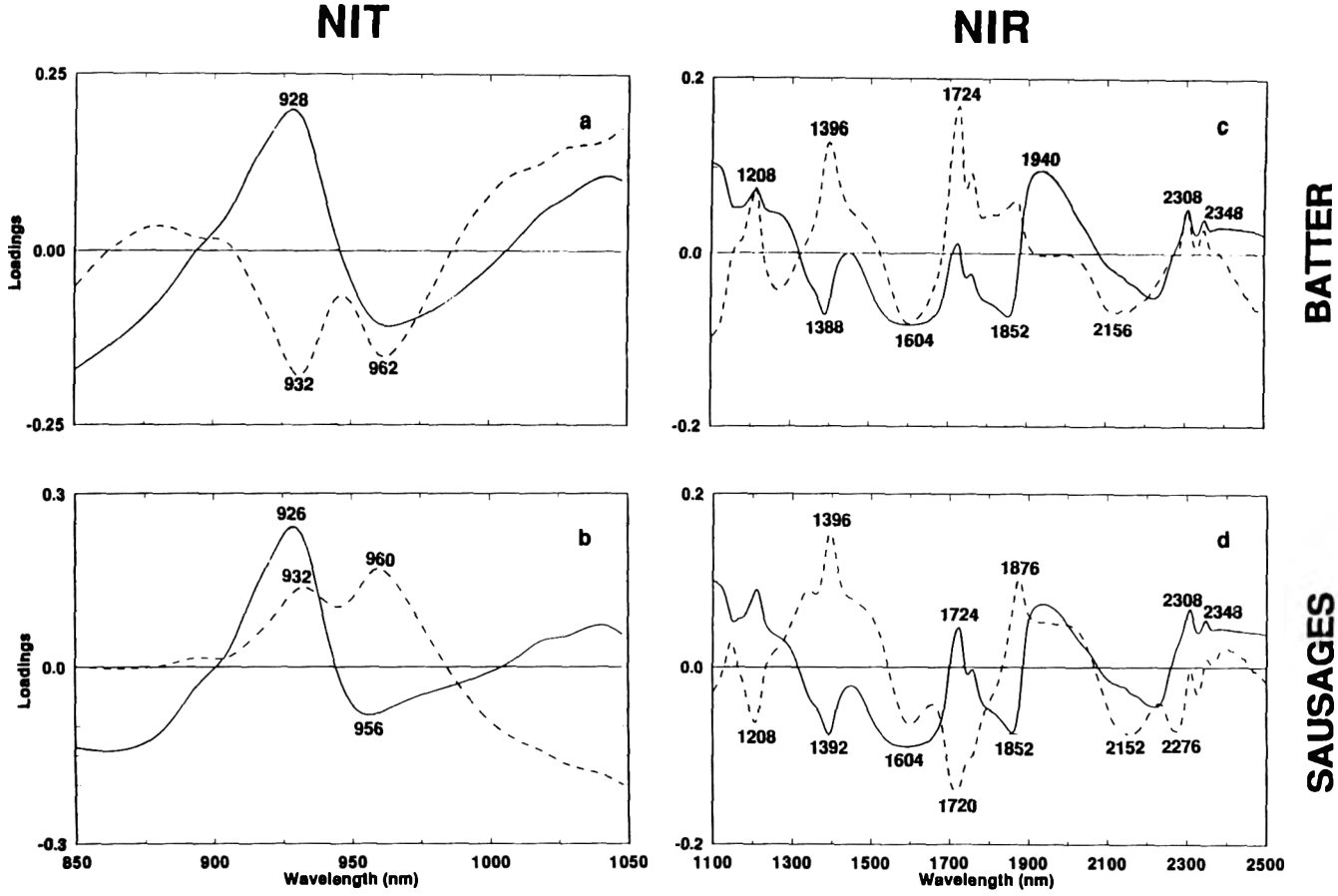

Fig. 6—Relationship of spectral loadings and sensory data (after PLS2). a = NIT for batters; b = NIT for sausages; c = NIR for batters; d = NIR for sausages. —, loadings factor 1; ---- loadings factor 2.

taints (Rødbotten et al., 1990). Greasiness was assumed to be of great importance for describing differences between sausages because of the variations in fat contents (8-28%). The data from that assessor with respect to greasiness should, because of the misunderstanding of the term, be deleted before further analysis. For simplicity all data from that assessor were deleted before calculating S_{ref} and before averaging data for ANOVA, PCA and PLS.

There was complete disagreement between panelists with respect to elasticity (Fig. 2). The samples were also ranked oppositely ($g^* < -1$) with respect to graininess by both an outlier assessor and another assessor compared to the mean of the panel. This was in confirmation of the data in the eggshell plot. Because the terms graininess and elasticity were not used reliably by assessors they were eliminated before performing ANOVA, PCA and PLS.

Sensory quality affected by fat, NaCl and starch content

The ranges, S_{tot} (total standard deviation) and S_{ref} (standard error of reference method) for the different sensory attributes were compared (Table 2). The scores and factor loadings for factor 1 and 2 (Fig. 3) and percent described variances by the three first PCA factors (Table 3) showed that three factors described about 83.4% of the total variance. The validity of these 3 factors was confirmed by cross validation. Factor 1 described 37.7% of variation in the sensory data and scores showed a relationship among sausage formulations correlated with fat level. Sausages with low fat level had high intensities of odor of smoke and flavor intensity. Sausages with high fat level had high intensities of greasiness and whiteness. All attributes showed significant differences between fat levels.

Scores for factor 2 (31.7% of total variation) described a systematic variation from low to high starch level. Sausages with low starch had high intensities of odor of meat, meat flavor and juiciness. Those with high starch content had high intensities of firmness. The differences between sausage formulations with respect to these attributes were significantly affected by starch level. High intensity of off-odor and off-flavor, corresponding to sausages with high fat and high starch level, was described in factor 1 and 2. The disagreement among assessors with respect to odor of smoke, off-odor, flavor of smoke and off-flavor indicated by S_{ref} (Table 2) was due to variations in use of the scale.

The first two factors also described the variation in color. Sausages with low fat and high starch level had high intensities of color and color strength and low intensities of whiteness. Significant interactions occurred between fat and starch for the whiteness, color strength, odor intensity, odor of smoke, off-odor, meat flavor, saltiness, spiciness, off-flavor and juiciness. The sample (Id. 56, Table 1) had very high intensity with respect to off-odor and off-flavor. No logical reason was found for this high intensity, but the assessors were trained to identify androstenone and skatole (boar taints) in a project performed 4 mo prior to these analyses. Those of the assessors who gave high scores for those attributes were among assessors who had low thresholds for androstenon and skatole (Rødbotten et al., 1990). This verified the characteristics given on Id. 56. The variation due to NaCl levels was described in factor 3 (13.7% of the total variation). That factor described the variation between sausages due to significant interactions between fat and starch with respect to odor intensity, flavor intensity, saltiness and juiciness. Significant differences occurred between sausages with respect to flavor intensity, meat flavor, saltiness, spiciness and firmness due to level of NaCl. Interactions be-

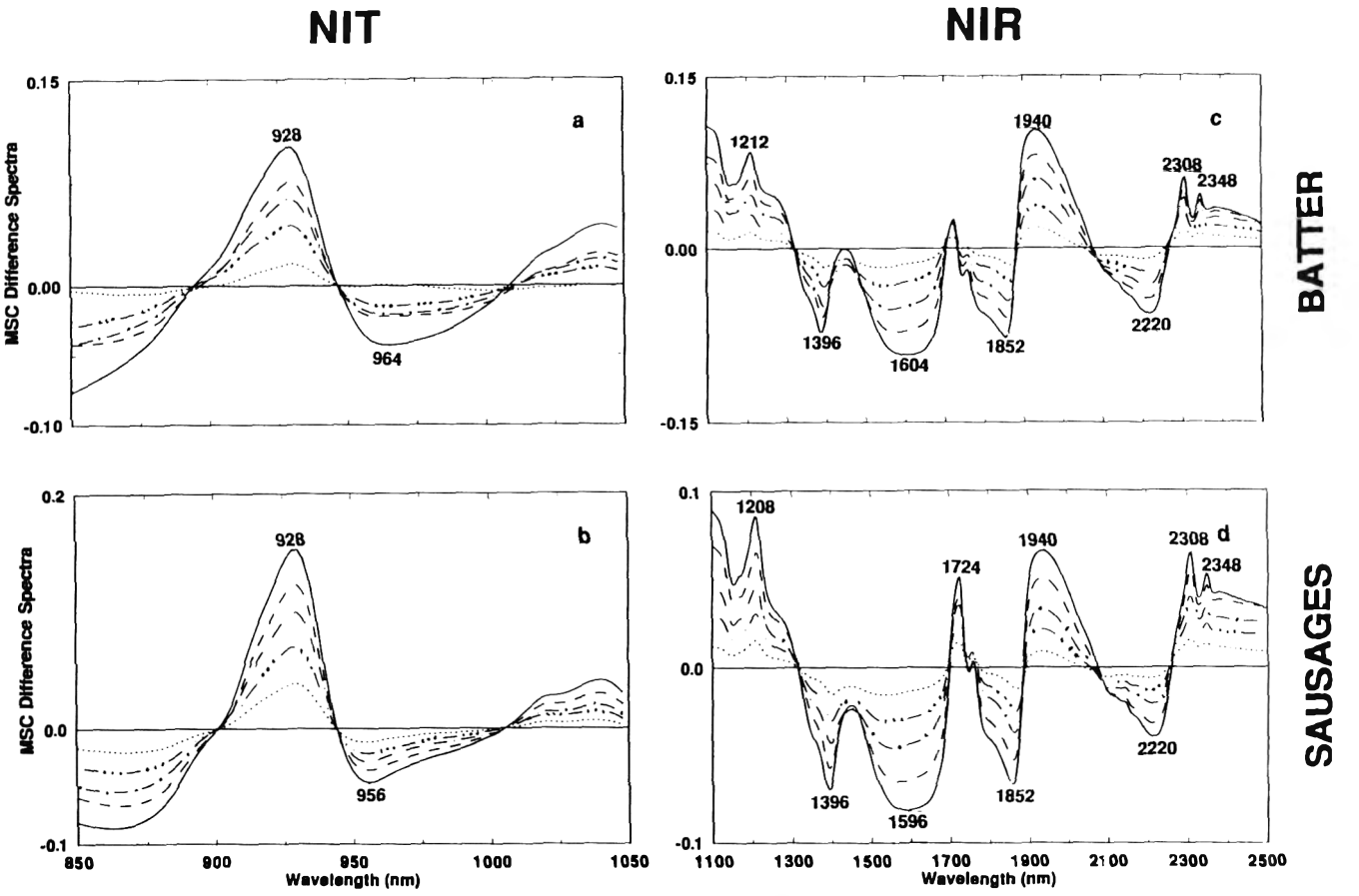

Fig. 7—Average NIT and NIR difference spectra for batters and sausages with different fat content. Calculation of difference spectra described in the test: — "28% fat" sausages–"8% fat" sausages; — — "28% fat" sausages–"12% fat" sausages; — • — "28% fat" sausages–"16% fat" sausages; —••• "28% fat" sausages—"20% fat" sausages; ••••• "28% fat" sausages–"24% fat" sausages; (a) Average fat-difference spectra for NIT data of batters. (b) Average fat-difference spectra for NIT data of sausages. (c) Average fat-difference spectra for NIR data of batters. (d) Average fat-difference spectra for NIR data of sausages.

tween fat and NaCl or starch and NaCl did not affect the variation between sausages with respect to any attributes.

Sensory quality of sausages described by NIR and NIT methods

PLS2 was performed on the NIR and NIT spectra of both batter and sausages with regard to the sensory data, which consisted of 16 attributes. The NIR analyses on the batter or sausages explained about 63.0% of total variation in the sensory data. This was achieved by using 12 and 8 factors in the PLS model, respectively. The NIT spectra of batters explained 61.2% of total variation in sensory data. The NIT spectra of sausages explained 74.3% of sensory variation. This was achieved by using a PLS model of 6 and 11 factors, respectively.

Sample scores and loadings of sensory data for factors 1 and 2 after PLS2 of the spectra of batters or sausages as related to the sensory data were compared (Fig. 4 for NIT, Fig. 5 for NIR). The explained variance of sensory data for the same factors were recorded (Table 4). The NIT and NIR data explained 41.0–49.4% of the variation in sensory data using only the first two factors. This is quite effective considering that these two factors mainly described the variation between sausages regarding the three color attributes, odor and flavor of smoke, spiciness and greasiness, and that variations in odor intensity, odor of meat, meat flavor, off-odor and off-flavor of the sausages was not reliably described (Table 4). Variation among samples at the first factor correlated with the fat level (Fig. 4 and 5). Sausages with high fat content had high intensities of greasiness and low intensities of odor and flavor of smoke. Variation among samples at the second PLS factor was related to variation in starch content samples. Sausages with low starch level had high intensities of juiciness and low intensities of firmness. The three color attributes (whiteness, color and color strength) were related to variations among samples at both factor 1 and 2. Sausages with high fat content and low starch content had high intensities of whiteness and low intensities of color and color strength. This was in accordance with the main variation in sensory attributes as shown by PCA of the sensory data (Fig. 3). However, the variation between sausages with respect to fat and starch level was more pronounced when combining the NIT or NIT spectra with sensory data (Fig. 4 and 5) compared to considering only sensory data (Fig. 3). This was expected since the main chemical variations among samples were related to variations in fat (6.3–30.1%) and carbohydrate (2.6–9.7%). Such macronutrients have been precisely predicted by NIR and NIT (Osborne and Fearn, 1986; Murray and Williams, 1987).

Loadings for the first and second factors of the NIT and NIR spectra of batters and sausages (Fig. 6) were studied to improve understanding of these methods in predicting main sensory attributes. These loadings correspond to scores and sensory loadings (Fig. 4 and 5) for NIT and NIR data, respectively. The loading spectra were compared with average difference spectra of batter and sausages for fat (Fig. 7) and starch (Fig. 8). The same spectral pattern was revealed when comparing loading spectra of the first factor (Fig. 6) with the average fat-difference spectra (Fig. 7) for the four spectral plots. This demonstrated that the main predictive ability of greasiness, odor of smoke, flavor of smoke, off-odor, off-flavor and spiciness in addition to some predictive ability of the three color attributes, was related to changes in spectra caused by

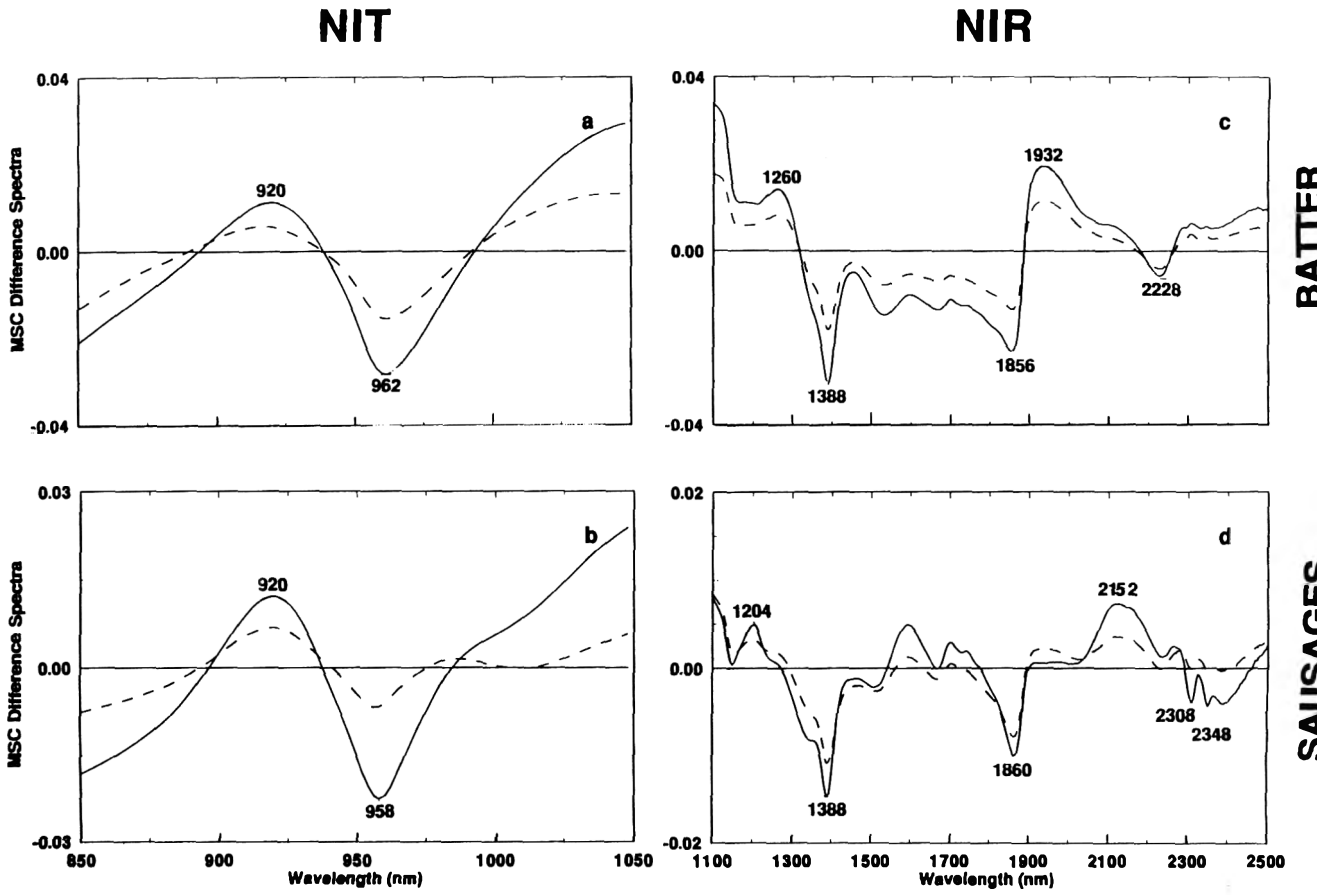

Fig. 8—Average NIT and NIR difference spectra for batters and sausages with different starch content. Calculation of difference spectra described in the text: — "7.5% starch" sausages–"1.5% starch" sausages; — — "7.5% starch" sausages–"4.5% starch" sausages. (a) Average starch-difference spectra for NIT data of batters. (b) Average starch-difference spectra for NIT data of sausages. (c) Average starch-difference spectra for NIR data of batters. (d) Average starch-difference spectra for NIR data of sausages.

different fat contents of batters or sausages. Spectral loadings of the second factor for the four spectral data had high loadings at wavelengths related to systematic differences in both starch and fat content (Fig. 7 and 8). This indicated that the main predictive ability of firmness, juiciness and the three color attributes was related to changes in spectra caused by both varying fat and starch content. This was consistent with results from the ANOVA of sensory data.

The spectral loadings of the second factor for NIR data of batter and sausages were studied further (Fig. 6c and 6d). Spectral loadings at factor 2 were very similar except for peaks at 1208 and about 1720 nm. Those peaks are related to absorbance of CH_2-groups (Osborne and Fearn, 1986). The high positive or negative loadings at these wavelengths correlated with fat content of the batters and sausages with high starch (Fig. 5a and 5c). However, the dominating variation among samples at factor 2 seemed to correspond with starch content. The band at 2152 nm in the NIR loading spectra was related to CH-groups (Murray and Williams, 1987), and might contain information about different starch content of samples since batters and sausages high in starch content had high intensity at this band. In addition, a systematic change in absorbance according to starch content was observed at the same wavelength in average difference spectra for sausages (Fig. 8d). However, this was not the case for the average starch difference spectra of batters (Fig. 8c). The dissimilarity in average difference spectra of batter and sausages for starch was probably related to gelatinization of potato starch during heat treatment of the sausages (Pomeranz, 1991).

Prediction ability of NIR and NIT for each attribute

The prediction results achieved when using all sensory data as Y-variables (PLS2) were compared with results from making a separate model for each attribute by use of PLS1. Almost the same predictions resulted when using the 2 methods. Only the prediction results for PLS2 of the various sensory attributes are presented.

The maximum predictive ability, expressed as RAP, was compared (Fig. 9) for the 16 sensory attributes when using NIR or NIT spectra of either batter or sausages for prediction. Generally, NIT analysis of sausages best predicted sensory attributes. With the NIT method, prediction ability improved for sausage analysis in comparison to batter. This was most notable for odor and flavor attributes. However, no systematic difference was seen in prediction ability related tc NIR analysis on the batter as compared with sausages. NIR and NIT analysis were performed on homogenized sausages. The samples differed in particle sizes because of difficulties with homogenizing some formulations which had a hard outer layer. This would affect reflected light from the samples and might have interfered with the spectra as related to different sensory attributes. The NIT method, which measured the transmitted light through 17 mm thick samples, was probably not as affected by different particle sizes. This might explain the improved prediction accuracy by NIT over NIR when performing analyses on sausages. NIR and NIT spectra of batter or sausages explained the main variations in three color attributes (whiteness, color and color strength). This was also the case for the 2 texture attributes; juiciness and greasiness, which also explained the main variation in texture attributes of sausages (Table 3).

NIT spectra of sausages gave the best prediction results for odor attributes; odor of meat and odor of smoke with a RAP of 0.70 and 0.83, respectively. However, none of the methods predicted odor intensity of sausages reasonably well. NIT spectra of sausages predicted off-flavors and off-odors with a

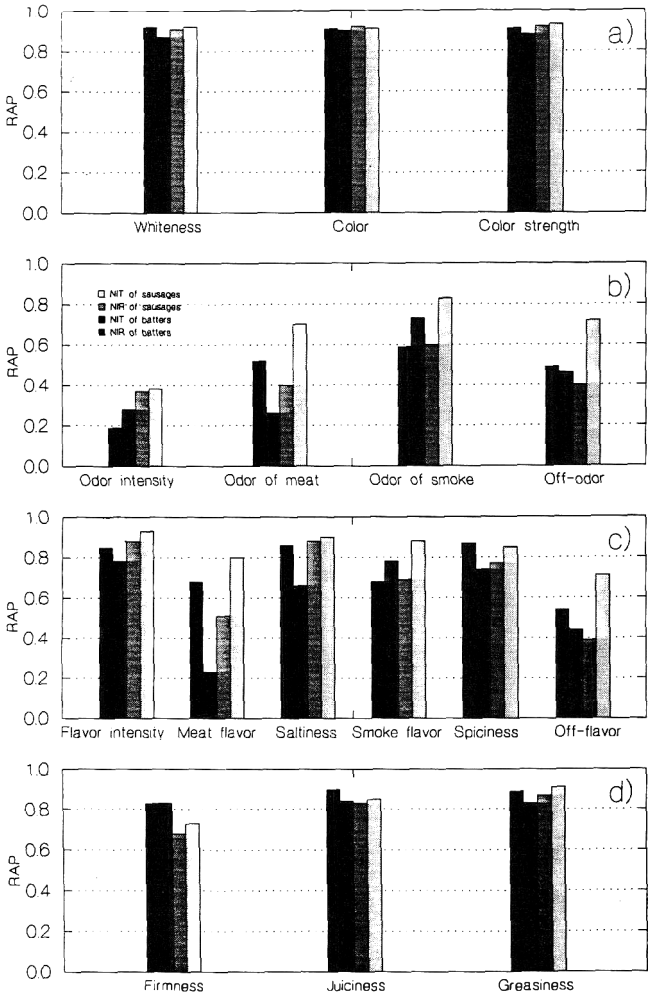

Fig. 9—Maximum relative ability of prediction (RAP; see text) of different sensory attributes with use of NIR or NIT spectra of batters or sausages. (a) RAP for the color attributes. (b) RAP for the odor attributes. (c) RAP for the flavor attributes. (d) RAP for the texture attributes.

RAP of 0.71 and 0.72, respectively. This was an improvement of 24% and 32% compared to the best RAP obtained among the other three datasets for these attributes. Variations in off-odor and off-flavor of sausages were only detected by NIT spectra. This explained why Id. 56 which had very high intensity of off-flavor and off-odor, was detected as an outlier by NIT data. This sample was removed from the NIT data of sausages prior to calibration. Odor and flavor sensations of foods are affected by fat content and texture properties. The predictive information in the NIT spectra about odor were therefore probably attributed to fat and starch contents of samples and not to various odor components, which were present in very low concentrations.

In general, flavor attributes of sausages were predicted with a better accuracy by NIR or NIT than were odor attributes. The RAP for flavor attributes showed that flavor intensity and saltiness were predicted with highest accuracy. NIR analysis of either batter or sausages and NIT analysis of sausages were almost equally reliable for these two attributes. The NIR or NIT spectra of the batter have also been used to predict NaCl content of sausages (Ellekjær et al., 1993). Thus it may be possible to analyze the batter during processing in order to predict both NaCl content and saltiness of the end-product. Both NaCl concentration and fat content affect perceived saltiness of products (Yackel and Cox, 1992). In our study, sausages low in fat, starch and NaCl had lower intensity of saltiness and flavor intensity than those high in fat or starch and low in salt. By performing NIR analysis of batters during processing, necessary corrections could be adjusted to produce sausages with the desired intensity of saltiness.

The main variation in sensory attributes was related to fat and starch contents of the sausages. NIR and NIT determined the main variation in color and texture attributes of sausages regardless of whether analysis was performed on batter or on sausages. This predictive ability was mainly related to changes in spectra caused by different fat and starch contents. NIR spectroscopy has potential as a rapid analyzing tool in sausage production to predict main texture, color and some odor and flavor attributes in addition to chemical composition of sausages. Further study is needed on the prediction ability of NIR and NIT when the variation in sensory quality is caused by variations in raw materials, chemical composition and processing parameters similar to variations in industrial production of sausages.

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Endpoint Temperature, Internal Cooked Color, and Expressible Juice Color Relationships in Ground Beef Patties

M.A. HAGUE, K.E. WARREN, M.C. HUNT, D.H. KROPF, C.L. KASTNER, S.L. STRODA, and D.E. JOHNSON

ABSTRACT

Ground beef patties from A- and E-maturity carcasses and imported trimmings were cooked to 55 to 77°C. Internal patty color became less red as endpoint temperature increased. However, some patties cooked to lower temperatures turned brown prematurely. Visual evaluation of internal patty color was not an accurate indicator of patty doneness. Raw material source had little effect on internal patty color. Expressible juices from imported trimmings were less red than those from A- and E-maturity carcasses. Expressible juice became less red and more yellow with increasing endpoint temperature; however, expressible juice never "ran clear." A more appropriate guideline would be "cook until juices lack redness."

Key Words: ground beef, endpoint temperature, internal color, expressible juice

INTRODUCTION

OVER 1.36 MILLION MT of ground beef are consumed annually in the United States. In 1991, cows (5.6 million) represented 17% (AMI, 1992) of domestic beef slaughter (excluding veal and calves) and yielded at least 1 million MT of boneless product. In addition, over 454 thousand MT of fresh beef for processing were imported (USDA, 1992). A large proportion of these raw materials probably was used for ground beef production.

Undercooked ground beef has been implicated as a vehicle for *Escherichia coli* O157:H7 (Neill, 1989), and in 1993, this organism in undercooked hamburgers from a fast food chain caused several deaths and over 400 confirmed cases of foodborne illness in four states. *E. coli* O157:H7 has been linked to foodborne illnesses including hemolytic uremic syndrome and hemorrhagic colitis. Because outbreaks of these diseases have occurred in nursing homes, fast food restaurants, and day-care facilities, Carter et al. (1987) suggested an increased susceptibility in the young and old to such infections. Doyle and Schoeni (1987) isolated *E. coli* O157:H7 in 5 of 17 (31%) ground beef samples obtained in the Calgary area, where a high incidence of infections had been reported. *E. coli* O157:H7 has been isolated from dairy cattle in North and South America, Western Europe, and places where outbreaks have occurred (Neill, 1989).

In response to such concerns, USDA-FSIS (1989) recommended that precooked patties for foodservice institutions be cooked to 71°C, and FDA (1993) stated that patties should be cooked to 68°C for at least 15 sec. However, because of the difficulty in measuring internal temperature of patties, USDA-FSIS (1989) suggested: "Heat all meat patties until they are hot, steaming, and juices run clear. The center of the patty should be grayish-brown with no evidence of pink color."

Authors Hunt, Kropf, Kastner, and Stroda are with the Dept. of Animal Sciences & Industry, Weber Hall, and author Johnson is with the Dept. of Statistics, Dickens Hall, Kansas State University, Manhattan, KS 66506-0201. Author Hague is with the Cryovac Division, W.R. Grace & Co.-Conn, 803 North Maple St., P.O. Box 338, Simpsonville, SC 29681. Author Warren is with Tyson Foods, 2210 Oaklawn Drive, Springdale AR, 72764. Address reprints requests to M.C. Hunt.

Table 1—Visual and instrumental measurements of internal color of cooked ground beef patties, related to endpoint temperature[f]

Trait	Endpoint temperature 55°C	60°C	66°C	71°C	77°C
Internal color					
Visual score[g]	3.0[a]	3.2[a]	3.6[b]	3.7[b]	4.0[c]
L* value	50.9[a]	51.9[b]	52.6[c]	52.6[c]	52.2[bc]
a* value	14.6[d]	13.9[c]	12.6[b]	11.4[a]	11.0[a]
b* value	18.4[d]	17.7[c]	16.7[b]	16.0[a]	15.9[a]
Saturation index	23.5[d]	22.5[c]	21.0[b]	19.7[a]	19.3[a]
Hue angle	51.8[a]	52.0[a]	53.2[b]	54.4[c]	55.3[d]
a*/b* ratio	0.79[c]	0.79[c]	0.75[b]	0.72[a]	0.69[a]

[a-e] Means within a row with a different superscript differ (P<0.05).
[f] Averaged over patties from three sources: A-maturity, E-maturity, and imported trimmings.
[g] Visual score: 1 = pink, 2 = moderately pink, 3 = slightly pink, 4 = brown.

Table 2—Visual and instrumental measurements of internal color of cooked ground beef patties related to raw material source[c]

Trait	Raw material source A-maturity	E-maturity	Imported
internal color			
Visual score[d]	3.5	3.4	3.6
L* value	52.2[b]	50.5[a]	53.5[b]
a* value	13.3	12.7	12.1
b* value	17.0	16.6	17.2
Saturation index	21.6	20.9	21.0
Hue angle	52.3[a]	52.8[a]	54.9[b]
a*/b* ratio	0.78[b]	0.77[b]	0.70[a]

[a-b] Means within a row with a different superscript letter differ (P<0.05).
[c] Averaged over five end-point temperatures.
[d] Visual score: 1 = pink, 2 = moderately pink, 3 = slightly pink, 4 = brown.

Typically, internal cooked color in meat changes from red to pink to tan as endpoint temperature increases. However, Marksberry (1990) observed the premature formation of cooked color in ground beef from old (D/E-maturity) carcasses when compared to young (A-maturity) carcasses. Patties made with meat from A-maturity carcasses were consistently more red than those from D/E-maturity carcasses. This was noted especially at lower temperatures (65 and 68°C), at which patties usually have a reddish-pink internal color. Patties made from D/E-maturity carcasses did not differ at five endpoint temperatures (65 to 77°C) for instrumental color measurements. Therefore, they concluded that color was not a reliable indicator of doneness for ground beef patties made with meat from older carcasses. Thus, ground beef made from D/E-maturity carcasses may appear to be adequately cooked when it is actually undercooked. This introduces concern for food safety. Our objectives were to investigate the influence of raw material source and endpoint temperature on internal cooked color and color of expressible juices from different maturity carcasses and imported trimmings and to determine interrelationships with cooking time, endpoint temperature, and USDA recommendations for ground beef cookery.

MATERIALS & METHODS

Raw materials

Ten knuckles (quadriceps muscles) from A-maturity carcasses were obtained commercially, and nine knuckles from E-maturity beef car-

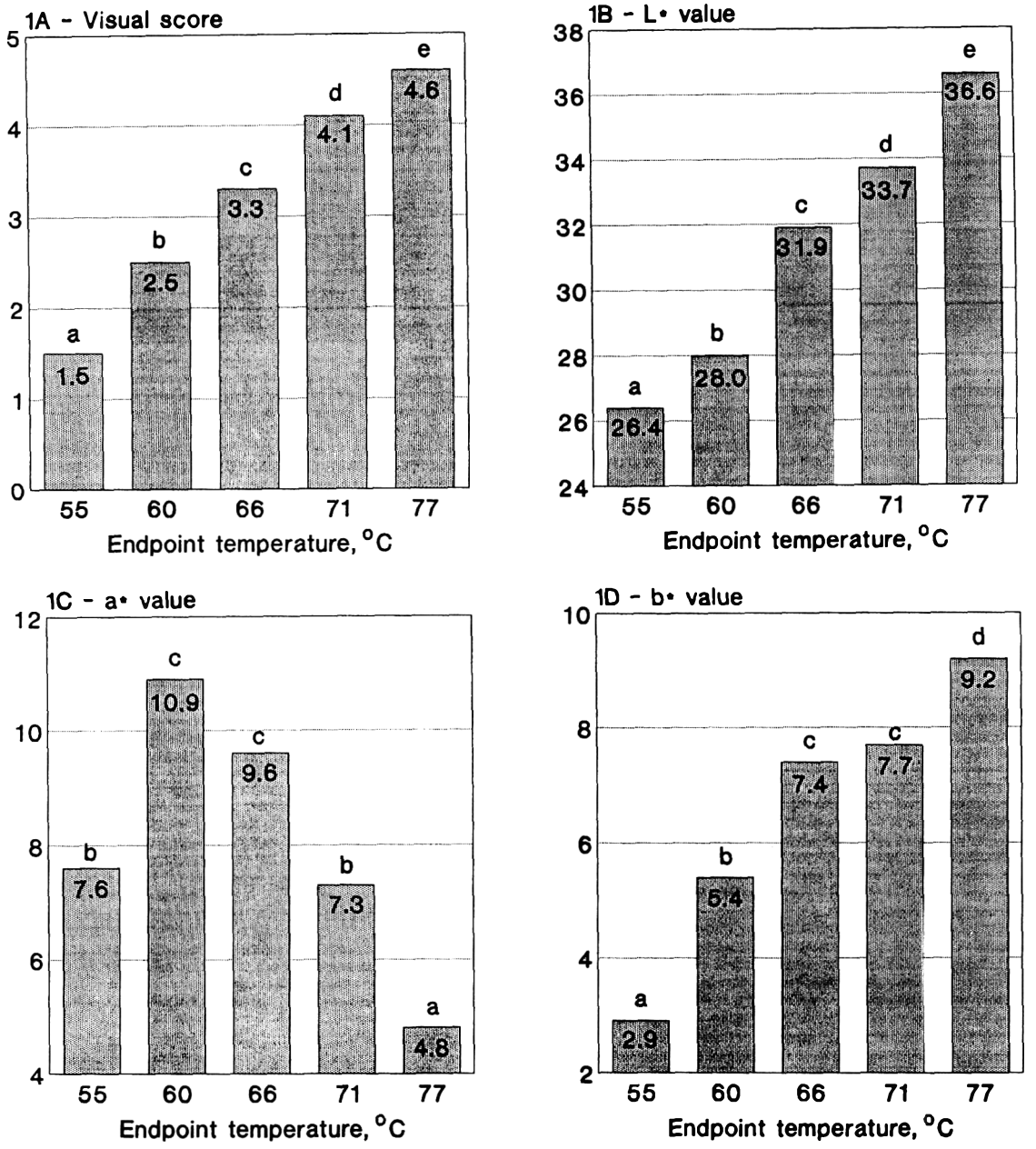


Fig. 1—Endpoint temperature effects on expressible juice color traits from cooked ground beef patties: (1A) Visual score; (1B) L* values; (1C) a* value; (1D) b* value. a-eMeans on bars with a different superscript differ ($P<0.05$).

casses were obtained from the Kansas State University Meat Laboratory. The cuts were trimmed of external and seam fat, vacuum packaged, and frozen intact before being made into patties. Five lots of frozen, imported beef trimmings from Australia and New Zealand were obtained commercially. Frozen beef trim (74% fat) from A-maturity carcasses was used when necessary to adjust fat to 15%.

Beef patties

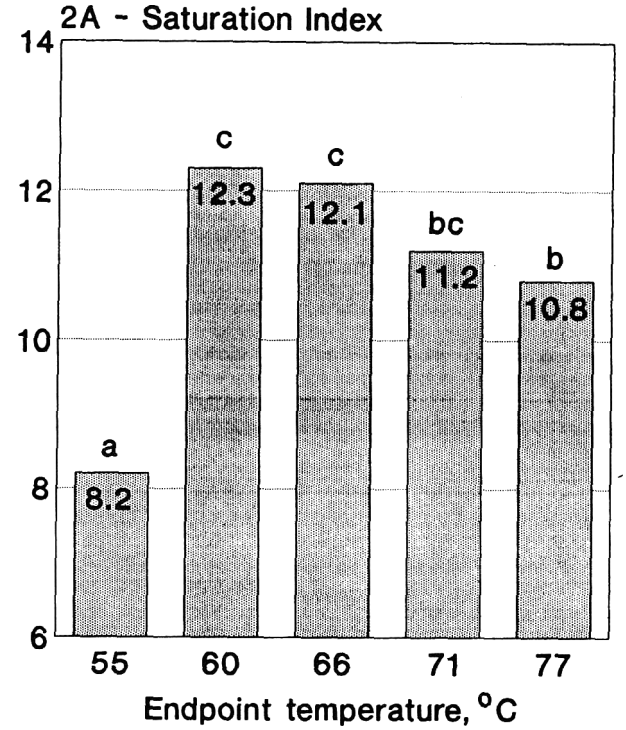
Lean and fat sources were thawed and ground through a 1.27 cm plate. Ten grams of each lean source were blended with 100 mL distilled, deionized water to determine pH. Each source was mixed thoroughly, and samples were removed for moisture (oven drying) and fat analysis (Foss-Let Fat Analyzer) using AOAC (1990) methods. Finely ground (0.32 cm plate) fat was added to adjust the amount to 15% and mixed (2 min) with the lean source in a Hobart mixer (Model 4732, Troy, MI). Batches were ground twice through a 0.32 cm plate and made into 113-g patties using a Hollymatic pattymaker (Jet Flow Super, Model 54, Countryside, IL). Patties were stacked in layers of two on metal trays and placed in a blast freezer (−40°C) for 15-20 min until crust frozen. Patties were bagged, vacuum packaged, and stored at −15° until needed (22 wk for patties from A- and E-maturity carcasses and 12 wk for patties of imported trim).

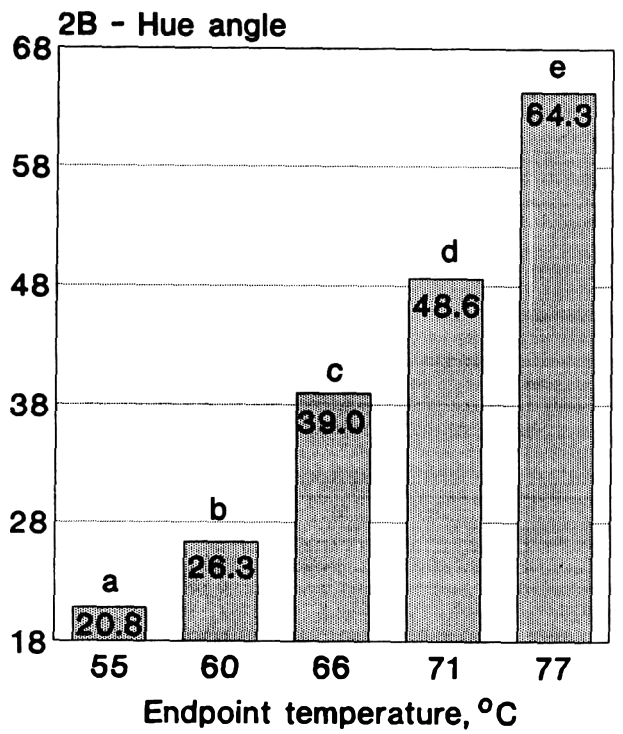
Cooking procedures

Patties were thawed at 2°C for 24 hr. Precooking temperatures of patties (known to affect cooking time) were 3.2 to 4.1°C. Patties were cooked in random order on a preheated (162.8°C) electric griddle. Temperatures of patties were measured using a 20-gauge hypodermic probe-type thermocouple connected to a Doric temperature recorder (Trendicator 410A, San Diego, CA). This device could be inserted and withdrawn intermittently during cooking to closely monitor endpoint temperatures in the center of the patty. Patties were turned at 30-sec intervals until the internal temperatures was 1–1.5°C below the desired final endpoint temperature (55, 60, 66, 71, or 77°C), which allowed for a postcooking temperature rise to the desired endpoint. Cooking time was determined for each patty. Patties were then removed from the griddle, blotted once on each side, and cooled for 5 min.

Color evaluation of internal patty

Patties were cut in half and then cut longitudinally to obtain four half-circle shaped slices. Internal color was determined visually by





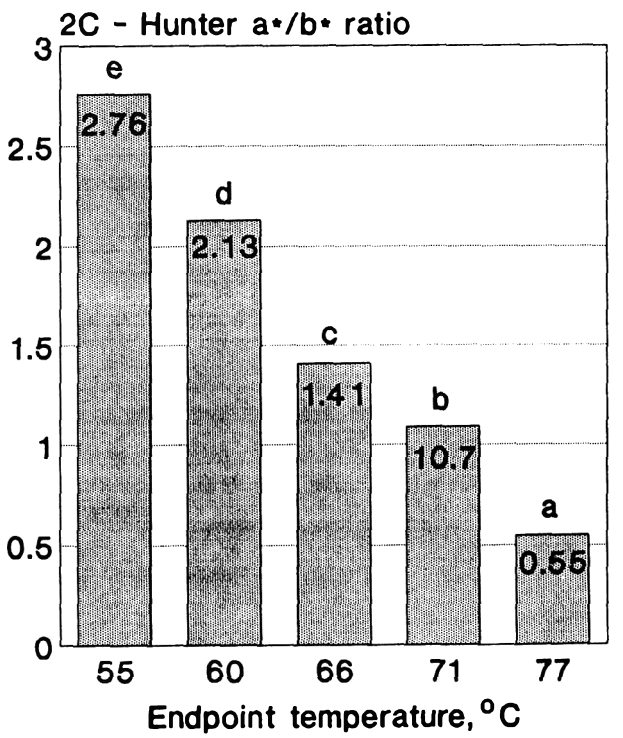


Fig. 2—Endpoint temperature effects on expressible juice color traits from cooked ground beef patties: (2A) Saturation index; (2B) Hue angle; (2C) a*/b* ratio. [a-e]Means on bars with a different superscript letter differ ($P<0.05$).

three experienced observers immediately following slicing using a 4-point scale to the nearest one-half point (1=pink; 2=moderately pink; 3=slightly pink; 4=brown). This color scale and that for expressible juice were constructed from preliminary observations of over 100 cooked patties. Instrumental measurements of internal color were determined immediately following slicing. A LabScan 6000 Spectrocolorimeter (2 cm diameter aperature, Hunterlab, Reston, VA) was used to measure L*a*b* values (Illuminant A), from which the ratio of a* to b*, saturation index, and hue angle were calculated (AMSA, 1991).

Expressible juice

Three half-circle slices were stacked on a white porcelain plate and pressed with a plastic, household, patty press (10.8 cm diam) to expel juices which were transferred to a clean test tube and allowed to set 30 min. During that time, the fat and colored aqueous portions separated in the tubes.

Color evaluation of expressible juices

Visual color of the expressible, internal juices (aqueous phase) was determined to the nearest one-half point using a 5-point scale (1=dark, dull red; 2=red, 3=pink; 4=pinkish tan; 5=yellow, no pink) by three experienced observers. A portion of the aqueous phase from each patty was pipetted into a clear, plastic, tissue culture well for instrumental color evaluation using a Minolta Chroma Meter (Model CR-200, Japan) for determination of L*a*b* values and the calculation of a*/b* ratio, saturation index, and hue angle.

Table 3—Visual and instrumental measurements of expressible juice color of cooked ground beef patties related to raw material source[a]

Trait	Raw material source		
	A-maturity	E-maturity	Imported
Expressible juice Visual score[d]	3.0	3.2	3.4
L* value	30.7	30.8	32.5
a* value	9.6[b]	8.3[b]	6.2[a]
b* value	6.4	6.3	6.7
Saturation index	11.9[b]	11.0[ab]	9.7[a]
Hue angle	34.2[a]	38.3[ab]	46.9[b]
a*/b* ratio	1.80	1.68	1.28

[a-b] Means within a row with a different superscript letter differ ($P<0.05$).
[c] Averaged over five end-point temperatures.
[d] Visual score: 1 = dark, dull red; 2 = red; 3 = pink; 4 = pinkish tan; 5 = yellow, no pink.

Microbial evaluation

Patties were selected randomly from three batches of each raw material source. Ten grams of each patty were removed aseptically, added to 90 mL of sterile phosphate buffer, and homogenized for 2 min using a Stomacher (Tekmar Co., Cincinnati, OH). Serial dilutions from 10^2 to 10^6 were plated on Plate Count Agar (Difco, Detroit, MI) and incubated at 32°C for 48 h to determine viable cell counts (FDA, 1978).

Statistical analysis

Data were analyzed using analysis of variance in the General Linear Model Procedure of the Statistical Analysis System (SAS Institute, Inc., 1988). A split-plot design was used to determine differences between sources (whole plot) and endpoint temperature (subplot) for various color measurements. Least square means procedures were used to separate means at $P<0.05$. Ten replications were done for A-maturity lean, nine for E-maturity lean, and five for imported trimmings. Simple correlations were calculated between selected variables.

RESULTS & DISCUSSION

RAW MATERIAL SOURCE and endpoint temperature did not interact for comparisons of internal and expressible juice color measurements; therefore, only the main effects will be discussed.

Internal color

Visual scores increased (less red) as endpoint temperature increased (Table 1). However, at 55°C, patties were only slightly pink internally, not predominately red as would be expected of patties cooked very rare. Patties cooked to 55 and 60°C were similar in visual scores. At 66°C, patties were evaluated as very slightly pink to tan and could not be distinguished ($P>0.05$) from those cooked to 71°C, the temperature on which the USDA (1992) cooking recommendations are based. These color development patterns could potentially allow consumption of patties that are undercooked by as much as 10 to 15°C, if the patty were cooked until the center was "grayish-brown" as suggesting by USDA guidelines.

As expected, L* values increased (became lighter) whereas a* and b* values decreased (less red and less yellow, respectively, $P<0.05$, Table 1) as endpoint temperature increased. These color changes agreed with results of Marksberry (1990) for ground beef from A-maturity carcasses and of Bowers et al. (1987) for whole muscle. Unlike visual scores, differences in a* and b* values were found in patties cooked to 66 and 71°C. However, values for patties cooked to 71 and 77°C were similar ($P>0.05$). Saturation index and a*/b* ratio decreased and hue angle increased as endpoint temperature increased, indicating that patties became less red and less intense in color ($P<0.05$, Table 1). Using these calculations, patties cooked to 66°C could be distinguished from those cooked to 71°C. However, patties cooked to 55 and 60°C had similar hue angle and a*/b* ratio, and those cooked to 71 and 77°C had similar saturation index and a*/b* ratio. Such minor differences in instrumental color between adjacent temperatures probably could not be applied practially or detected visually.

Instrumental measurements indicated that patties became less red at the higher endpoint temperatures. Although visual scores also indicated this change, patties cooked to the lower temperatures appeared considerably less red than expected. Because of this "premature" browning, undercooked patties may appear fully cooked. Marksberry (1990) also reported this same observance for internal cooked color of patties. Compounding the problem further, Shipp et al. (1992) detected *E. coli* O157:H7 in inoculated patties with 10% fat cooked to 60°C. They stated that low fat patties cooked to lower endpoint temperatures would be unsafe if they were contaminated with *E. coli* O157:H7 or other pathogens.

The pH ranged from 5.43 to 5.79, with averages of 5.53 for A-maturity lean, 5.56 for E-maturity, and 5.75 for imported trimmings. Average viable cell counts were: 3.4×10^6 for A-maturity patties, 7.7×10^5 for E-maturity patties, and 7.6×10^4 for imported trimmings. These data for raw materials and patties were typical and would not be expected to affect unusual internal color development. Visual scores, a*, b*, and saturation index values were similar ($P>0.05$) for patties from A- and E-maturity carcasses and imported trimmings (Table 2). Patties made from E-maturity carcasses had lower L* values (darker) than those from A-maturity carcasses and imported trimmings (Table 2), possible indicating increased pigment in older animal tissue. Patties made from imported trimmings had greater hue angle values and lower a*/b* ratios (indicates less red color) than those from A- and E-maturity carcasses (Table 2). This did not agree with Marksberry (1990), who reported that patties from D/E-maturity carcasses were significantly less red (especially at 65 and 68°C); less yellow (especially at 71, 74, and 77°C); and less intense in color than those from A-maturity carcasses.

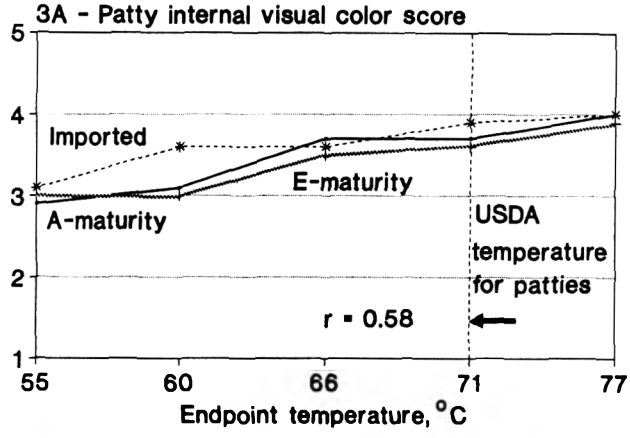
Data from our study indicated little difference in instrumental and visual measurements on patties from A- and E-maturity carcasses and imported trimmings. However, Marksberry (1990) reported large differences in a* and b* values, saturation index, and visual measurements between patties from A- and D/E-maturity carcasses. Note that our visual and instrumental measurements on patties from E-maturity carcasses and Marksberry's (1990) results for D/E-maturity carcasses were more similar than respective measurements on meat from A-maturity carcasses. Reasons for the dissimilarities between the two studies are unknown.

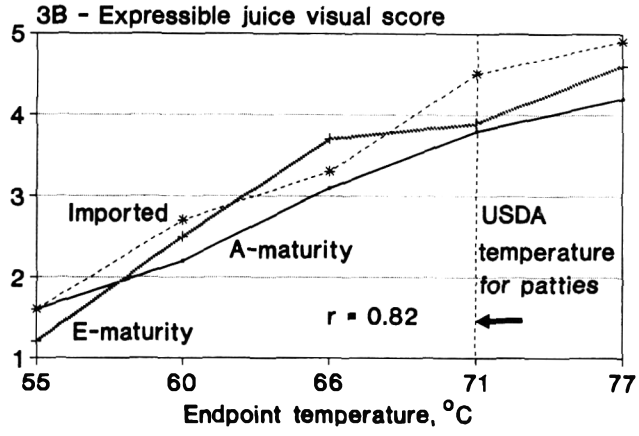
Expressible juice

Visual scores for expressible juice increased as endpoint temperature increased, indicating loss of redness and increase in yellowness (Fig. 1A). At 55°C, the expressible juice had a visual score of 1.5 (dark, dull red to red). The expressible juice was pinkish tan at 71°C, the temperature to which USDA (1992) recommends cooking patties. Even at 77°C, expressible juice was pinkish tan to yellow (visual score 4.5). Thus, the juices never ran "clear." As endpoint temperature increased, L*, b*, and hue angle values increased (Fig. 1B, 1D and 2B) and a*/b* ratio decreased (Fig. 2C). This supported the visual evaluation that juices changed from red to yellow with increasing endpoint temperature. A* values (Fig. 1C) and saturation index (Fig. 2A) increased from 55 to 60°C and then progressively decreased (less red) from 60 to 77°C. A possible explanation for increased redness from 55 to 60°C is that some pigments in the juice were undenatured and in a deoxygenated, purplish-red state at 55°C. If further heating to 60°C denatured some of the pigments the remaining pigment would be a lighter, brighter red.

Both visual and instrumental measurements showed expressible juice became less red and more yellow with increasing endpoint temperature. Patties cooked to lower endpoint tem-

—Text continued on page 470





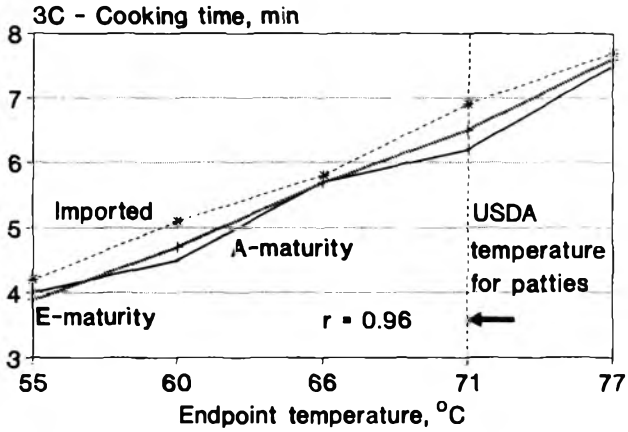
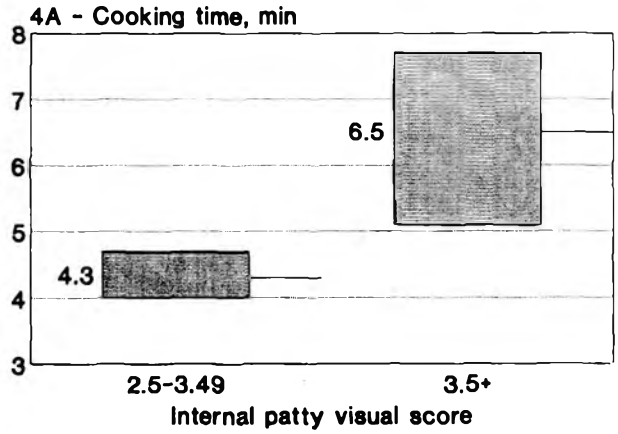
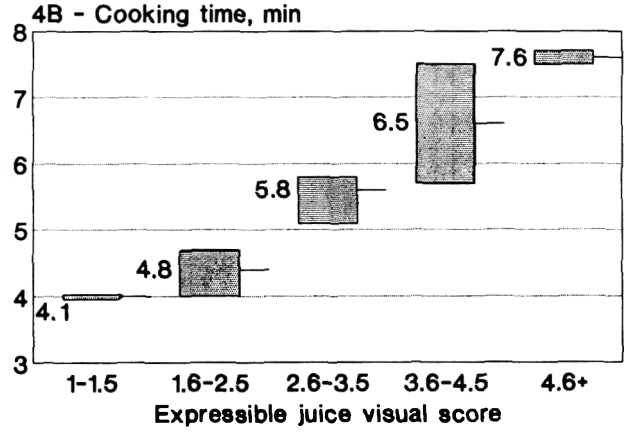


Fig. 3—Cooking times needed to reach endpoint temperature of patties from three sources: (3A) Internal color visual score by temperature relationship; (3B) Expressible juice visual score by temperature relationship; (3C) Time by temperature relationship. Dotted vertical line at 71°C is patty temperature recommended by USDA.





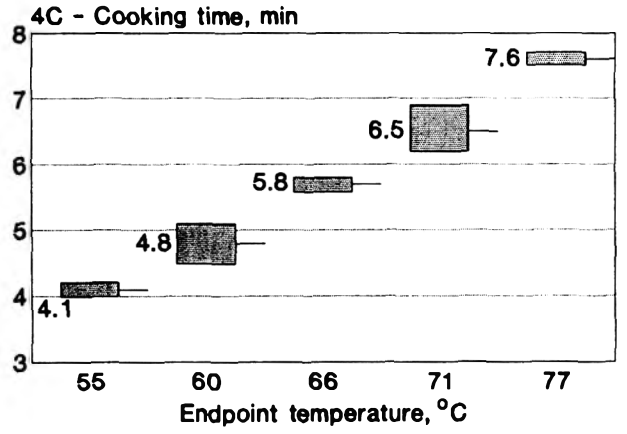


Fig. 4—Relationship between cooking time and color traits for patties from three sources: (4A) *Range of time* to cook patties to an internal color visual score endpoint (1.5–2.49=moderately pink, 2.5–3.49=slightly pink, 3.5+=brown); (4B) Range of time to cook patties to an expressible juice visual score endpoint (1–1.5=dark, dull red, 1.6–2.5=red, 2.6–3.5=pink, 3.6–4.5=pinkish tan, 4.6+=yellow); (4C) Range of time to cook patties to endpoint temperatures. Numerical mean for each group is represented by a line.

peratures (55, 60, and 66°C) could be distinguished from those cooked to 71°C. This indicated that expressible juice color could be used as an indicator of patty doneness and could potentially help prevent undercooking. However, expressible juices never "ran clear" and only at 77°C did they show an absence of pink. A more accurate instruction may be to cook patties until juices "lack pinkness." However, obtaining expressible juice to determine patty doneness without destroying the patty is a concern, and observation of juice color as it is released from the patty is extremely unreliable.

Expressible juice visual scores and L*, b*, and a*/b* values were similar ($P>0.05$) for patties made from A- and E-maturity carcasses and imported trimmings (Table 3). Expressible juice of patties from imported trimmings was less red (lower a*) than that of patties from A- and E-maturity carcasses (Table 3). Juice from imported trimmings had lower saturation index and higher hue angle values than that from patties of A-maturity carcasses (Table 3).

Application of results

The consumption of undercooked meat patties is a potential food safety problem, especially if they are contaminated with *E. coli* O157:H7. The USDA (1992) has suggested heating meat patties to minimum internal 71°C, at which *E. coli* O157:H7 has been destroyed (American Association of Food Hygiene Veterinarians, 1989; Shipp et al., 1992). This should be fully equivalent to the FDA (1993) recommendation of 68°C for 15 sec. Monitoring the internal temperature of the patty during cooking with an accurate temperature recording device is the best method to ensure it reaches 71°C. However, some food institutions and most general consumers would not have such methods available. Therefore, visual appraisal, of internal patty color or expressible juice color, or cooking for a specified time are often used.

Visual evaluation of internal patty color was not an accurate indicator of patty doneness because those cooked as low as 66°C could appear as brown as those cooked to 71°C. Only 34% of the variation in patty color was due to endpoint temperature. Visual evaluation of internal color alone should not be used to predict destruction of *E. coli* O157:H7 (Fig. 3A). Cooking times were only moderately correlated with internal a* values, saturation index, and visual scores ($r = -0.66$, -0.69, 0.56, respectively, data not shown). However, if internal color was related to cooking time (Fig. 4A), then some measure of safety was obtained. Cooking for 6.5 min, the mean time for internal color score of 3.5 and no evidence of pink color, would exceed the upper range (4.7 min) required to cook patties to a mean color score of 3.0 (slightly pink).

Visual evalution of expressible juice color could be more reliable in predicting destruction of *E. coli* O157:H7 (Fig. 3B), because 67% of color variation was related to temperature. Expressible juice from the patty should be tan to yellow with an absence of pinkness to ensure that the patty is safe for consumption. "Lack of redness in expressible juice" would be a more accurate guideline than cooking until "juices ran clear." Cooking times correlated more highly with expressible juice appearance (visual score = 0.79, L* value = 0.81, and a*/b* ratio = −0.78) than with internal doneness scores. Cooking to the mean time for each expressible juice color endpoint (Fig. 4B) would exceed the upper range of time for the next lower visual score category. Thus, juice appraisal could be used by consumers, if a good method for evaluation was readily available.

Cooking ground beef based only on time was related to endpoint temperature (Fig. 3C). Because cooking times correlated highly (0.96) with endpoint temperature, cooking by time was the best predictor of inactivation of *E. coli* O157:H7. Cooking to the mean time for each endpoint temperature would equal (Fig. 4C) or exceed the upper range of the adjacent lower endpoint temperature. Cooking for 6.5 min under our conditions should ensure safety, but palatability traits including juiciness might be affected adversely.

When using cooking times as an indicator of patty doneness, other factors should be considered. Cross and Berry (1980) reported that cooking times to obtain a similar approximate degree of doneness increased with increasing patty size. Marksberry (1990) observed shorter cooking times with less compacted patties. Troutt et al. (1992b) reported that patties with 5% fat required longer cooking times than those with 30% fat. Troutt et al. (1992a) reported no consistent relationships between cooking times and texture-modifying ingredients in low-fat ground beef patties. Other very important considerations include whether cooked from frozen or raw state, intial precooking patty temperature, cookery method, and cooking temperature.

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Restructuring Veal Steaks with Salt/Phosphate and Sodium Alginate/Calcium Lactate

S. RAHARJO, D.R. DEXTER, R.C. WORFEL, J.N. SOFOS, M.B. SOLOMON, G.W. SHULTS, and G.R. SCHMIDT

ABSTRACT

Quality characteristics of restructured steaks formulated with veal trimmings or veal leg meat and Na-alginate/Ca-lactate or salt (NaCl)/phosphate were evaluated. Trimmings restructured with salt/phosphate had higher ($P<0.05$) bind scores (measured instrumentally and by sensory) than veal trimmings restructured with Na-alginate/Ca-lactate. Salt/phosphate (0.3–0.5%) reduced ($P<0.05$) purge and increased binding force and sensory score of bind in restructured veal trimmings and leg meat compared to control and Na- alginate/Ca-lactate treatments. The use of Na-alginate/Ca-lactate as a binder for veal leg meat increased ($P<0.05$) binding force and sensory score of bind and decreased cook loss when used at 0.4%.

Key Words: restructured steaks, veal, NaCl, phosphate, Na-alginate, Ca-lactate

INTRODUCTION

MEAT RESTRUCTURING TECHNOLOGY enables control of product characteristics such as shape, color, texture, fat and moisture content, cook yield, bind strength, juiciness and flavor. Restructured meat products have commonly been prepared by flaked/formed or chunked/formed techniques along with use of salt and phosphate to facilitate binding between particles. The use of salt (NaCl) and phosphate in restructured meat products enhances cohesiveness, cook yield and flavor quality (Clarke et al., 1987; Mann et al., 1990; Wheeler et al., 1990; Craig et al., 1991). The addition of phosphate (up to 0.5%) also protects against lipid oxidation (Huffman et al., 1987; Arganosa et al., 1991; Stoick et al., 1991; Liu et al., 1992). The use of salt (0.5–1.5%), however, has been associated with discoloration (Chu et al., 1987) and rancidity development (Wheeler et al., 1990; Andersen and Skibsted, 1991) in raw restructured beef steaks.

Consumers concern about salt consumption (Kolari, 1980) has led to partial or complete replacement of salt with other components. Those such as alginate/calcium gel, are possible means of maintaining or improving quality of restructured meat products (Means and Schmidt, 1986; Means et al., 1987; Clarke et al., 1988; Ernst et al., 1989; Ensor et al., 1989, 1990; Trout et al., 1990). Alginate restructuring provides adequate binding of meat chunks in both raw and cooked states and does not significantly affect aroma and flavor of the cooked product (Ensor et al., 1989). Levels of sodium alginate and calcium lactate or NaCl and phosphate may be adjusted to correspond to the relative properties of the meat block. Thus, in our study veal trimmings (relatively low binding ability) were restructured with 0.5% NaCl and 0.5% phosphate or 0.5% Na-alginate and 0.5% Ca-lactate, while veal leg meat (relatively high binding ability) was restructured with the same components at 0.4% and 0.3% levels.

A demand exists for technology to use beef, pork, lamb, veal and poultry at relatively low cost in schools, hospitals and military feeding applications. Our objective was to evaluate use of Na-alginate/Ca-lactate, salt/phosphate and low value types of veal cuts (veal trimmings and boneless legs) for preparation of restructured veal steaks intended for such institutional markets.

Authors Raharjo, Dexter, Worfel, Sofos, and Schmidt are with the Dept. of Animal Sciences, Colorado State Univ., Fort Collins, CO 80523. Author Solomon is with the Meat Science Research Laboratory, USDA-ARS FQD1, Bldg. 201, BARC-EAST, 10300 Baltimore Ave., Beltsville, MD 20705-2350. Author Shults is with the Product Development & Engineering Branch, Food Engineering Directorate, U.S. Army Natick Research, Development and Engineering Center, Natick, MA 01760. Address inquiries to Dr. D.R. Dexter.

MATERIALS & METHODS

Sample preparation

Frozen boneless veal trimmings (from the entire carcass) and veal leg meat were obtained from Berliner & Marx, Inc. (Edison, NJ). Prior to use the meat was thawed at 4°C for 48 hr. Visible connective tissue and fat were removed which resulted in ≈52% yield (from trimmings) and 94% yield from legs. Restructured veal steaks were prepared in 2,270-g batches. Veal trimmings were restructured with: (a) 0.5% salt (NaCl)/0.5% Heller's soluble phosphate WJ-0052 (sodium tripolyphosphate and sodium hexametaphosphate) (Heller Seasonings and Ingredients, Inc., Bedford Park, IL); (b) 0.5% sodium alginate/0.5% encapsulated calcium lactate (Kelco, Chicago, IL); and (c) no additives (control). In another experiment, veal leg meat was restructured with: (a) 0.3% salt/0.3% phosphate; (b) 0.4% salt/0.4% phosphate; (c) 0.3% sodium alginate/0.3% encapsulated calcium lactate; (d) 0.4% sodium alginate/0.4% encapsulated calcium lactate; and (e) no additives (control). The raw meat materials were ground through a 2.5 cm plate (Hobart Mfg. Co., Troy, OH) without a cutting blade. This provided relatively large chunks of meat with irregular shape. Each batch (2,270-g/batch) of meat was mixed with dry ingredients (including the control, no additives) for 2 min in a mixer (model K45SS Kitchen-Aid Inc., St. Joseph, MI) at a speed setting of 2. Salt or sodium alginate was added and mixed for 1 min, then phosphate or encapsulated calcium lactate was added and mixed for another min. The mixtures were placed in plastic bags (15 cm × 51 cm, Koch, Kansas City, MO), sealed under vacuum (Multivac, Allgäu, Germany) and manually formed into logs (9 cm diam × 35 cm long). The meat logs were initially placed in a cooler at 4°C for 6 hr to provide adequate time for formation of alginate/calcium gels and subsequently stored in a freezer at approximately −20°C for 48 hr. Frozen meat logs were sawed into steaks (1.9 cm thick, ≈120g), which were individually vacuum-packaged, and stored at −20°C for about 1 wk for further evaluation.

Purge

Three frozen restructured veal steaks from each treatment were thawed at 4°C to allow meat juices to drain. After 24 hr, steaks were removed from the packages and their surfaces were manually wiped with a paper towel to remove visible meat exudate. The purge was calculated as the weight loss divided by the initial weight, expressed as a percentage.

Cook loss

Three frozen restructured beef steaks were tempered for 1 hr at 24–25°C prior to cooking. The steaks were cooked using an Open Hearth Broiler® (Farberware, Yonkers, NY) for 11 min on one side and another 10 min on the other side until an internal temperature of ≈71°C was reached. Cook loss was calculated as weight loss due to cooking divided by the initial weight of the raw steak, expressed as a percentage.

Table 1—Relationship of some functional properties (means ± standard deviation) of restructured veal steaks to b nder treatments

Treatment	Purge[x] (%)	Cook loss[y] (%)	Binding force[z] (N)
Veal trimmings			
Control (no additives)	7.7 ± 0.9[a*]	32.2 ± 1.2[a*]	18.9 ± 3.6[b*]
NaCl/phosphate (0.5%/0.5%)	2.0 ± 0.5[c]	24.7 ± 1.2[c]	31.1 ± 7.4[a]
NaAlginate/Ca-lactate (0.5%/0.5%)	4.0 ± 1.0[b]	27.4 ± 1.3[b]	17.3 ± 2.2[b]
Veal leg meat			
Control (no additives)	5.8 ± 0.7[a]	34.7 ± 2.3[a]	14.3 ± 3.3[c]
NaCl/phosphate (0.3%/0.3%)	1.9 ± 0.5[b]	34.3 ± 3.1[a]	22.8 ± 2.5[a]
NaCl/phosphate (0.4%/0.4%)	1.4 ± 0.2[b]	30.0 ± 5.1[ab]	24.9 ± 3.9[a]
Na-Alginate/Ca-lactate (0.3%/0.3%)	4.6 ± 0.9[a]	28.5 ± 3.3[ab]	16.3 ± 3.5[bc]
Na-Alginate/Ca-lactate (0.4%/0.4%)	5.1 ± 0.9[a]	27.1 ± 5.5[b]	20.9 ± 1.8[ab]

[x-y] Weight loss divided by initial weight of raw steak.
[z] Force (N) required to penetrate cooked steak with a 2-cm diameter steel ball.
[a-c] Means with common superscript within each type of meat and within the same column are not significantly (P>0.05) different.
[*] Significantly (P<0.05) different from its corresponding control values in veal leg meat.

Table 2—Sensory evaluations (mean ± standard deviation) of cooked restructured veal steaks with different binder treatments

Treatment	Juiciness	Bind	Flavor	Texture	Color
Veal trimmings					
Control (no additives)	4.6 ± 0.8[a]	2.1 ± 1.2[b*]	4.3 ± 0.9[ab]	3.8 ± 1.2[a]	4.1 ± 0.8[a]
NaCl/phosphate (0.5%/0.5%)	5.0 ± 0.9[a]	5.0 ± 0.6[a]	4.8 ± 0.6[a]	4.7 ± 0.7[a]	4.1 ± 1.1[a]
Na-Alginate/Ca-lactate (0.5%/0.5%)	4.3 ± 1.0[a]	3.0 ± 1.2[b]	3.6 ± 0.9[b]	4.0 ± 0.8[a]	4.4 ± 0.7[a]
Veal leg meat					
Control (no additives)	4.0 ± 1.3[a]	3.2 ± 1.0[b]	3.7 ± 1.1[a]	4.1 ± 0.8[a]	4.7 ± 0.6[a]
NaCl/phosphate (0.3%/0.3%)	4.7 ± 0.8[a]	4.7 ± 1.0[a]	4.5 ± 0.7[a]	4.7 ± 1.0[a]	4.7 ± 0.8[a]
NaCl/phosphate (0.4%/0.4%)	4.4 ± 0.9[a]	4.7 ± 1.1[a]	4.6 ± 1.0[a]	4.3 ± 0.6[a]	4.2 ± 0.8[a]
Na-Alginate/Ca-lactate (0.3%/0.3%)	4.6 ± 1.1[a]	3.7 ± 1.2[ab]	3.8 ± 1.1[a]	3.7 ± 1.1[a]	4.4 ± 0.8[a]
Na-Alginate/Ca-lactate (0.4%/.04%)	4.6 ± 1.2[a]	4.5 ± 0.9[a]	3.7 ± 0.9[a]	4.2 ± 1.1[a]	4.6 ± 0.5[a]

[x] Juiciness (1: very dry; 6: very juicy); bind (1: very weak bind; 6: very strong bind); flavor (1: very undesirable; 6: very desirable); texture (1: ground beef-like texture, 6: whole muscle steak-like texture); and color (1: very poor cooked color uniformity; 6: very good cooked color uniformity).
[a-b] Means with common superscript within each type of meat and within the same column are not significantly (P>0.05) different.
[*] Significantly (P<0.05) different from its corresponding control values in veal leg meat.

Binding force

An instrumental assessment of the degree to which particles of restructured steaks physically adhere within the cooked product was made using a test cell and a 2 cm diameter steel ball probe (modification of Field et al., 1984) on a J.J. Lloyd Model T5002 tensile tester (Pacific Scientific, Santa Ana, CA) at 24–25°C with the following settings: load cell 100 N, sensitivity 1.0, and cross-head speed 200 mm/min. Three steaks (those used for cook loss evaluation) from each treatment were tested. The peak height was measured (mm) and converted to Newtons.

Sensory evaluation

Six trained panelists evaluated the restructured steaks. Panelists were trained following the procedure of Means and Schmidt (1987). The steaks were cooked as described above and were served immediately to panelists after cooking. In every session, only three to five treatments were evaluated and each panelist was supplied with a plate, a steak knife, a fork, a glass of cold tap water and a disposal cup. Each panelist received ≈30g of restructured veal steak from each treatment. They were asked to rinse their mouth with cold tap water before evaluating each sample. Restructured veal steaks were cut with a knife and received a score of 1 for very weak bind between meat chunks (comparable to ground beef patty), up to a score of 6 for very strong bind (comparable with whole muscle steak). Juiciness (score 1: very dry to 6: very juicy), flavor (score 1: very undesirable to 6: very desirable), and texture (score 1: ground beef-like texture to 6: steak-like texture) were evaluated by chewing, tasting and swallowing the samples. Because this was a trained panel, panelists were instructed to base flavor desirability scores on the presence of any off-flavors associated with the restructured steak, instead of personal preference. Uniformity of the interior color of the cooked steak was also evaluated. Particle to particle variation (color) can exist in restructured meat products. A score of 1 indicated very little cooked color uniformity, while a score of 6 indicated very good cooked color uniformity.

Statistical analysis

Each experiment was replicated twice with meat from a separate box and triplicate measurements were conducted in each replicate. Purge, cook loss, binding force, and sensory evaluation data were evaluated as means along with standard deviations. Data collected from steaks restructured with veal trimmings were analyzed separately from those restructured with veal leg meat. However, variables measured for steaks from control (no additives) treatments were compared for statistically significant differences between meat sources. An analysis of variance was conducted for each variable measured in each of two replicates. Treatment means were separated by Tukey's test (Steel and Torrie, 1980) when a significant (P<0.05) F-test for main effects was found.

RESULTS & DISCUSSION

Purge, cook loss and binding force

Steaks formed with veal trimmings (Table 1) and no additives (control) had higher purge, lower cook loss and higher binding force scores than control (no additives) steaks restructured with veal leg meat (P<0.05). Although visible connective tissue was extensively removed from veal trimmings, as indicated by the 52% yield, the trimmed meat still had more visual connective tissue than the veal leg meat. Ensor et al. (1990) reported that steaks restructured from beef foreshank meat (4.83% native connective tissue, no additives) had no measurable bind after cooking due to collagen shrinkage at 60–75°C. Connective tissue (collagen matrices) in the muscle of younger animals (veal) is less crosslinked (less heat stable). This may have enabled its utilization in this product without detrimental effects on cooked product bind from collagen shrinkage (Bailey and Light, 1989). In addition, the veal trimming particles were relatively smaller and thinner than those from veal leg meat. No binding agent was added to controls. Thus we expected that steaks restructured from smaller meat pieces would have higher purge compared with those restructured from relatively larger meat pieces. In part, this may have been due to more cellular disruption in the smaller meat pieces and consequently more cytoplasmic fluid purging upon thawing.

When steaks were restructured from veal trimmings, the addition of 0.5% salt/0.5% phosphate reduced ($P<0.05$) purge and cook loss, and increased binding force compared to controls (Table 1). Addition of 0.5% Na-alginate/0.5% Ca-lactate significantly reduced ($P<0.05$) purge and cook loss as compared with controls. Our results were in confirmation of those of Shand et al. (1993) who found all-meat control treatments (beef rolls) had lowest cook yields, Na-alginate/Ca-lactate treatments were intermediate and salt/phosphate treatments had highest cook yields. Binding force of steaks restructured with Na-alginate/Ca-lactate, however, was not different ($P>0.05$) than that of controls (Table 1). That Na-alginate/Ca-lactate did not result in adequate binding may have been due to relatively large amounts of connective tissue in veal trimmings. However this problem did not occur when the veal trimmings were restructured with 0.5% salt/0.5% phosphate.

Steaks restructured from veal leg meat and (0.3 or 0.4%) salt/(0.3 or 0.4%) phosphate had less ($P<0.05$) purge and higher binding force than controls (Table 1). Addition of salt/phosphate, however, did not decrease ($P>0.05$) cooking loss. The levels of salt/phosphate added (0.3 or 0.4%) had similar ($P>0.05$) effects on purge, cook loss and binding force. Veal leg meat restructured with 0.3% Na-alginate/0.3% Ca-lactate was not different ($P>0.05$) with respect to purge, cook loss or binding force, from the control. Addition of 0.4% Na- alginate/0.4% Ca-lactate decreased ($P<0.05$) cook loss and increased binding force compared with controls. Addition of Na-alginate/Ca-lactate (0.3 or 0.4%) did not decrease ($P>0.05$) purge. Thus, to facilitate binding between meat particles in steaks restructured from veal leg meat, a minimum of 0.4% Na-alginate/0.4% Ca-lactate is needed. A similar degree of binding could be obtained by incorporating salt/phosphate at either 0.3%/0.3% or 0.4%/0.4%. Clark et al. (1988) evaluated Na-alginate/Ca-lactate levels of 0, 0.28, 0.57, 0.85, 1.13 and 1.42% in restructured beef. Cooked product bind and cook yields were increased ($P<0.05$) at a binder level of 0.57% and no further advantage was observed using higher levels.

Sensory evaluation

When steaks were formed with veal trimmings (Table 2), the addition of 0.5% salt/0.5% phosphate increased ($P<0.05$) the bind score (5.0 ± 0.6) compared with controls (2.1 ± 1.2). This is in agreement with results of binding force measurement determined instrumentally (Table 1). Use of salt/phosphate, however, did not increase ($P>0.05$) juiciness, flavor, texture or color scores compared with controls. Steaks restructured with 0.5% Na-alginate/0.5% Ca-lactate had similar ($P>0.05$) juiciness, bind, flavor, texture and color scores as controls. However, steaks restructured with Na-alginate/Ca-lactate had lower ($P<0.05$) flavor score (3.6 ± 0.9) than those restructured with salt/phosphate (4.8 ± 0.6). Some panelists reported off-flavors in veal trimmings restructured with Na-alginate/Ca-lactate. Such off-flavors may be related to pockets of Na-alginate and/or Ca-lactate which had not been sufficiently dispersed and hydrated during mixing. Means and Schmidt (1986) reported that percentage of alginate pockets and alginate pocket size increased as Na-alginate usage level increased. The relatively bland flavor of veal may be very sensitive to low levels of off-flavor produced by such additives.

Steaks restructured from veal leg meat with no additives had higher ($P<0.05$) binding scores (3.2 ± 1.0) than those from veal trimmings with no additives (2.1 ± 1.2) (Table 2). However, the type of veal cut had no effect ($P>0.05$) on juiciness, flavor, texture and color of steaks restructured with no additives. In steaks restructured from veal leg meat, addition of Na-alginate/Ca-lactate (0.3-0.4%) and salt/phosphate (0.3-0.4%) did not improve ($P>0.05$) juiciness, flavor, texture or color scores compared with controls. Addition of salt/phosphate (0.3 or 0.4%) and Na-alginate/Ca-lactate (0.4%) increased ($P<0.05$) the binding scores compared to controls. These results were in agreement with results of binding force measurements determined instrumentally (Table 1). Addition of 0.3% Na-alginate/0.3% Ca-lactate did not improve ($P>0.05$) the binding score compared with controls. Therefore, results indicate that 0.4% Na-alginate/0.4% Ca-lactate, 0.3% salt/0.3% phosphate or 0.4% salt/0.4% phosphate significantly improved bind without detrimental effects on juiciness, flavor, texture or color of restructured steaks using veal leg meat.

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Microstructure of Frankfurters Extended with Wheat Germ Proteins

RAVIN GNANASAMBANDAM and J.F. ZAYAS

ABSTRACT

Transmission electron microscopy was utilized to study the microsctructure of frankfurters extended with wheat germ protein flour (WGPF) at 3.5, 5.0, and 7.0%. Samples with 3.5% WGPF showed a denser matrix than the control. They also had a uniform interfacial protein film (IPF) with a slight increase in average thickness. At WGPF >3.5%, no further effects on IPF were observed. At 7.0% WGPF, an increase in added water, and the resultant dilution of meat and fat components, resulted in fewer fat globules, some with incomplete IPFs. Proteins in the WGPF contributed to IPF formation up to a specific level. Nonprotein components also were important in batter stability by absorbing water.

Key Words: frankfurters, wheat germ proteins, microstructure, meat batters, electron microscopy

INTRODUCTION

BATTERS OF COMMUNITED MEAT PRODUCTS (CMP) are finely chopped mixtures of protein, water, fat, salt, and other ingredients that produce a uniform products upon cooking. The structure of CMP may be complex because of numerous components bound to one another by various interactive forces (Jones, 1984). The microstructure of CMP is influenced by the type of meat, fat, and other ingredients; levels of salt and water; the process of comminution; and the heat treatment. The size of fat globules and the pattern of their distribution indicate the stability of a meat batter. Major factors that contribute to fat stabilization are biophysical properties of the interfacial protein film (IPF), gelation properties of the protein matrix, and physical characteristics of fat such as melting properties (Gordon and Barbut, 1992). THe interaction between fat droplets, protein matrix, and water strongly influences the stability of the system. The influence of protein coating on the lipid-protein interactions is important for stability.

Considerable size variation of fat globules can occur in CMP. Some of them may be over 20 μm in diameter differentiating the system from a true emulsion. Some of the structural changes in meat batter may be understood in terms of changes associated with true emulsions. However, interpretation of microstructure should not be restricted to one of the two proposed theories: emulsion theory and physical entrapment theory. Emulsion theory is based on formation of an IPF between fat globule and surrounding matrix and is usually considered mainly in regards to lipid-protein interaction. Another theory explains functionality of meat batters as related to physical entrapment of fat phase in a protein-water matrix. Electron microscopy can facilitate understanding of the microstructure of CMP and scanning electron microscopy (SEM) and transmission electron microscopy (TEM) are best suited to assessing the morphology of fat globules and changes at the fat protein interface (Lee, 1985).

Several protein sources of nonmeat origin are widely utilized in the manufacture of CMP to improve functionality, increase nutritional quality, and decrease cost. Wheat germ is a good source of minerals, vitamins and high-quality protein. Hemicellulos, cellulose, starch and sugar are major carbohydrate fractions of wheat germ. Fraser and Holmes (1957) reported that wheat germ contains about 3.9% pentosans. Several studies reported beneficial effects of wheat germ as an additive in foods (Vitti et al., 1979; Zaitsev and Rhomets et al., 1983; Turnbough and Baldwin, 1986). Defatted wheat germ has a protein content of 30-35%. In the U.S., incorporation of nonmeat additives in CMP is restricted to a maximum of 3.5%. Functional advantages of nonmeat additives have been demonstrated by several physical methods of analyses (Wills and Kabirullah, 1981; Mittal and Usborn, 1985; Parks and Carpenter, 1988; Zayas and Lin, 1988; Hung and Zayas, 1992; Wang and Zayas, 1992). However, information available on the microstructure of CMP extended with nonmeat cereal proteins is limited. Our objective was to investigate the effects of wheat germ proteinflour (WGPF) on the microstructure of CMP.

The authors are affiliated with the Dept. of Foods & Nutrition, Kansas State Univ., Manhattan, KS 66506-2602.

MATERIALS & METHODS

WGPF (defatted wheat germ flour R-80) was from Vitamins, Inc., (Chicago, IL.). Meat (beef trim from fore and hind quarters and boneless picnic ham, 50/50) was ground through a 9.4-mm plate, mixed, reground through a 4.7-mm plate, and after freezing at −25°C stored at −20°C in sealed vacuum packages. Meat was thawed at 4°C for 18 hr before processing. WGPF was added to the meat as powder at 3.5%, 5.0%, and 7.0%. Higher levels of water were added to experimental formulations at 2% water for every 1% protein additive. Thus, while control formulations had water added at 25%, treatment formulations had 32%, 35% and 39% added water. Formulations also contained salt (2.84%), Prague powder (6.25% sodium nitrite) (0.34%), commercial spice (0.5%), and ascorbic acid (0.1%). A Hobart bowl mixer was used to mix meat with salt and 1/3 of the water for 2 min. Prague powder and ascorbic acid were added individually with another 1/3 of the water and finally the spice and WGPF were added with the remaining water. Mixing time was kept constant at 2 min for every addition. The mix was comminuted through a Mincemaster emulsion mill (Griffith GL-86, Griffith Design and Equipment Co., Chicago, IL) with a 1.7-mm plate. The temperature of the batter did not exceed 8-10°C, controlled using crushed ice with water. Batters were stuffed under vacuum (VEMAG, Robot Reiser Co., Canton, MA) into 24-mm cellulose casings and formed into links of 11 cm. Frankfurters were cooked in a commercial smokehouse (Maurer and Sohne, Reichenau, Germany) on a schedule of 48°C/10 min, 55°C/30 min, 55°C smoking (natural hickory saw dust smoke)/3 min, and 80°C until an internal temperature of 70°C was reached. The frankfurters were chilled in a 5 min cold water shower, peeled manually, vacuum packaged (Super-Vac, Smith Equipment Co., Clifton, NJ), and stored in a refrigerator at 3–4°C for 4-6 hr before analysis.

Viscosity

All batters were incubated in 100-mL beakers at room temperature for 30 min before measurements were made. The viscosity of meat batters (100g) was measured with an RV-8 viscometer (Viscometers UK Ltd., Longon, England). Spindle No. 7 at 10 rpm was used and readings (in cp) were taken after 30 sec shearing time. Averages of 5 measurements are reported.

Thermal stability

Methods of Haq et al. (1972) and Saffle et al. (1967) with slight modifications (Lin and Zayas, 1987) were used to test batter stability.

Table 1—Viscosity and thermal stability of meat batters containing wheat germ protein flour (WGPF)

	Water added (%)[c]	Viscosity ×10[3] cPs	Cooking loss (%)	Water separated (%)	Fat separated (%)
Control	28	2.08[a]	11.21[a]	10.13[a]	1.73[a]
WGPF%					
3.5	32	1.99[a]	8.09[b]	8.35[ab]	1.58[a]
5.0	35	2.00[a]	7.80[b]	6.82[b]	1.53[a]
7.0	39	2.08[a]	7.53[b]	8.22[ab]	0.85[b]

[a,b] Values in same column with different superscripts significantly different (P <0.05).
[c] Water added in meat batter formulation.

Table 2—Proximate composition and yield of frankfurters containing wheat germ protein flour (WGPF)

	Added water (%)[d]	Protein (%)	Fat (%)	Moisture (%)	Processing yield (%)	Total yield (%)
Control	25	14.98[a]	16.25[a]	65.27[bc]	91.09[a]	120.42[a]
WGPF%						
3.5	32	14.90[a]	15.23[ab]	65.85[a]	92.54[a]	137.98[b]
5.0	35	14.87[a]	14.23[ab]	65.45[ab]	91.94[a]	141.44[b]
7.0	39	14.87[a]	13.58[b]	64.87[c]	91.35[a]	151.82[b]

[a,b,c] Values in same column with different superscripts significantly different (P <0.05).
[d] Water added in meat batter formulations.
[e] Yield after smokehouse processing.
[f] Yield calculated when meat, fat, and additives equal 100%.

A 30-g sample was placed in a screw-cap centrifuge tube (27.5 mm × 110 mm) and heated for 30 min in a 70°C water bath. The sample was centrifuged at 4,000 rpm (1600 × *g*) for 1 min at room temperature. Separated exudate was drained into a 25-mL graduated cylinder. After 5 min, separated water and fat were measured and expressed as a percentage of meat batte.r Total weight loss in meat batters after cookking was expressed as cooking losses.

Proximate composition and yield

Proximate analyses of frankfurters were performed according to AOAC (1984) to determine protein, fat, moisture, and ash content.

Processing yields of the products were calculated from differences in weights between stuffed, raw frankfurters and those after processing in the smokehouse. Total yield was calculated as the weight obtained based on original meat, fat, and additives at 100%.

Electron microscopy

Procedures of Schiff and Gennaro (1979) and Mollenhauer (1964) were followed in the preparation of samples. Small blocks (<1 mm^2) were cut from the cores of the frankfurters in an excess of fixing solution (3% glutaraldehyde buffer with 0.1M piperazine-N-N'bis 20 ethanol sulfonic acid (PIPES) at pH 7.4 and 1% tannic acid. Samples were fixed in the same buffer at 4°C overnight. Samples were washed 3 × for 30 min each in sucrose-PIPES buffer (0.1M/0.1M). Post-fixation was done in 1% osmium tetroxide-PIPES buffer for 3 hr. Samples were washed 4 × for 10 min each with distilled water at 4°C. Uranyl acetate (1%) was used to stain the samples overnight, and they were rapidly dehydrated in increasing concentrations of acetone for 15 min each (acetone series 30, 50, 70, 85, 95, and 100%) in an ice bath. After dehydration, the samples were warmed to room temperature. The dehydrated samples were infiltrated through an acetone:Klomparens mix [Mollenhauer and Spurr's resin, 1:1 (Spurr, A.J., 1969)] (Klomparens, 1986) in a series 2:1, 1:1, 1:2 for a minimum of 4 hr each and then through 100% Klomparens mix for 12 hr. Samples were embedded in a flat BEEM mold and cured at 70°C for 48 hr. Gray to silver (50–70 nm) sections were cut with glass knives on a Reichert Om-2 ultramicrotome (Amer. Optical Corp., Buffalo, NY) and counterstained with 5% uranyl acetate in 50% ethyl alcohol and lead citrate (Reynolds, 1963) for 10 min each. A Philips 201 transmission electron microscope operating at 60 kV was used to examine the sections, and representative micrographs of the sections were recorded at magnifications in the range of X 17,750 to X 71,000 on plates.

Statistical analysis

Analysis of variance (SAS Institute, Inc., 1986) was conducted, and the least square means procedure was usd to differentiate treatment means if they were found to be significant (P <0.05).

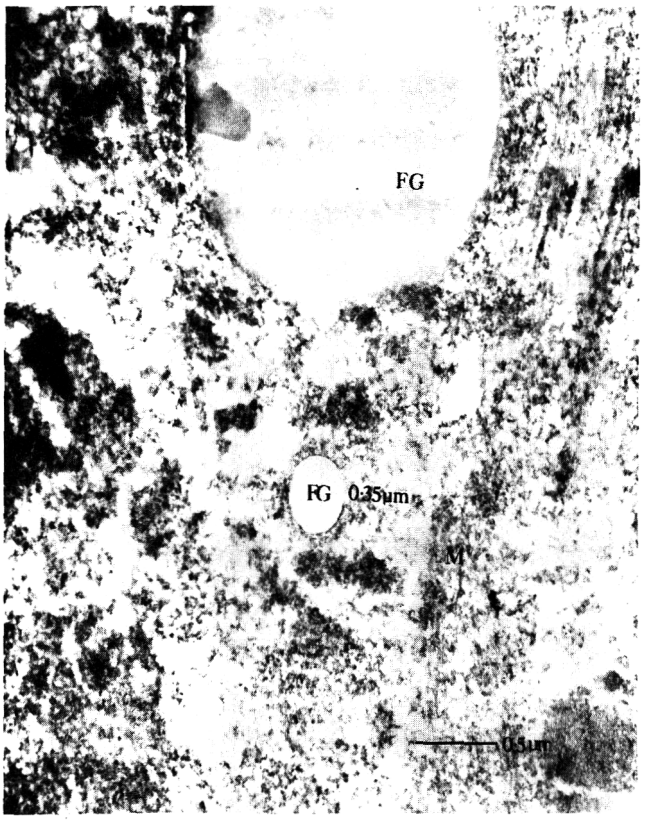

Fig. 1—Frankfurter without additive; M = matrix, FG = fat globule.

RESULTS & DISCUSSION

VISCOSITY AND THERMAL STABILITY of meat batters (Table 1) showed viscosities were not different for treatments as compared to controls. An increase in added water may have caused a dilution and hence no increase in viscosity although there was an increase in WGPF. Batters containing WGPF were more thermally stable as observed by a decrease in cooking loss. This decrease was significant between the control and 3.5% WGPF but no further benefit was observed upon addition of WGPF up to 7.0%. A similar trend was observed for percent water separated, which was significantly lower in batters with WGPF at the 5.0% level. Batters with 7.0% WGPF had the least separated fat, probably because of dilution. Samples with 3.5% WGPF had higher moisture than controls of samples with 7.0% WGPF (Table 2). Samples with 7.0% WGPF had less moisture than the 3.5% and 5% samples. Heat treatment of batters with 7.0% WGPF resulted in loss of moisture, perhaps indicating an inability of the batter to hold the high level of added water (39%). Processing losses for samples with WGPF were not different from those for controls, but all WGPF samples had higher total yields than controls.

Micrographs of control samples (Fig. 1) showed a uniform matrix of protein (dark stain) holding fat globules. The fat globules were slightly oval to elongated in shape. The size of fat globules varied from small globules in clusters to a much larger size (0.35 µm) (Fig. 1). A higher magnification (Fig. 2) showed membranes of varying thickness surrounding fat globules. The average thickness of the membrane was 0.02 µm. Several researchers have reported that salt-soluble meat proteins were involved in formation of the IPF between fat globules and the surrounding matrix (Swift et al., 1961; Swasdee et al., 1982). This film formation was observed during chopping and comminution (before heart treatment). Heat treatment during cooking stabilizes the film (Beas et al., 1988; Gordon and Barbut, 1991).

Frankfurters with 3.5% WGPF had a denser protein matrix (Fig. 3). Size and shape of fat globules were similar to con-

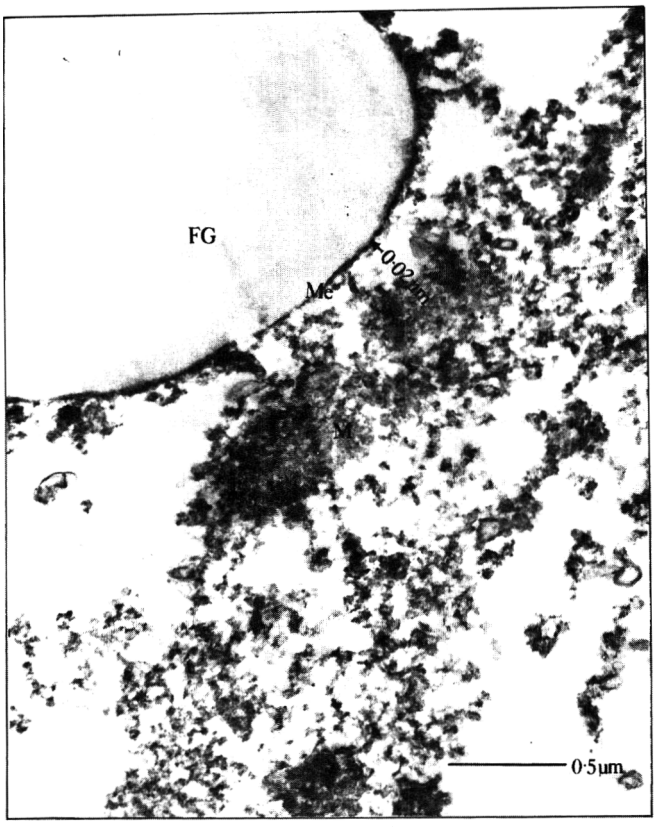

Fig. 2—Frankfurter without additive; M = matrix, Me = membrane, FG = fat globule.

Fig. 3—Frankfurter containing wheat germ protein flour at 3.5%; FG = fat globule, Me = membrane, M = matrix.

trols. The thickness of the membrane covering the fat globules at the center of the field was more uniform and averaged 0.03 μm. The sample with 5.0% WGPF (Fig. 4) did not differ from

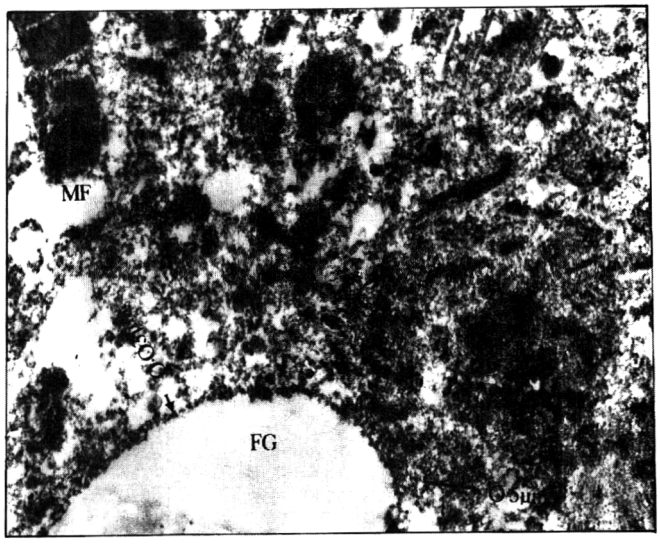

Fig. 4—Frankfurter containing wheat germ protein flour at 5.0%; FG = fat globule, MF = muscle fiber.

3.5% WGPF in matrix or average membrane thickness (0.03 μm.). A disrupted muscle fiber (top of the micrograph) suggests the wide variations in structure of frankfurters. Theno and Schmidt (1978) reported that the structure of commercial frankfurters varied from a fine homogeneous matrix to a coarse structure with large fat globules and intact muscle fibers. Fat globules also varied in size, similar to reports of Borchert et al. (1967) and Lin and Zayas (1987).

An increase in thickness of IPF in samples containing 3.5% and 5.0% WGPF supported the hypothesis that protein additives may contribute to IPF formation. These findings were similar to results reported by Lin and Zayas (1987), who reported an improved IPF with addition of 2% corn germ protein flour to meat batters. Gordon and Barbut (1990) suggested that IPF had more than one distinct layer contributing to its structure: a thin internal layer coating the surface of the fat globule bound to another layer of similar density. These layers are bound to a very thick and diffuse protein coat (Gordon and Barbut, 1992). Hydrophobic groups of myosin heads have been thought to be responsible for the monomolecular layer surrounding the fat globule (Jones, 1984). Protein-protein interactions that occur subsequent to monolayer formation are also important for IPF and fat globule stabilization. WGPF has a protein content of 30-33%, mostly albumins and globulins (Pomeranz et al., 1970). These proteins may contribute hydrophobicity at the fat globule interface and participate in subsequent protein-protein interactions beyond the initial monomolecular hydrophobic layer. Hansen (1960) also suggested that salt-soluble proteins may be attracted to and concentrated at the surface of fat globules before heat treatment. Thus, the stability provided by protein additives might commence during communition, well before heating. However, protein mediated IPF formation alone did not explain the batter-stabilizing effect of WGPF.

Samples containing 7.0% WGPF had a matrix similar to the other samples (Fig. 5). However, the IPF was not uniform and ranged from 0.015 μm to 0.025 μm. These samples also had fewer fat globules. The formation of weak spots in the membrane surrounding a large fat globule (>2.0 μm) was evident. Weak spots in the protein film resulted in the formation of pores that allowed the release of fat into several smaller globules during thermal expansion. This would lessen stresses on the overall integrity of the membrane and help maintain the stability of the system (Gordon and Barbut, 1992).

Samples containing WGPF were more stable to thermal treatment as indicated by decreased cooking losses and a comparable capacity to bind water. However, the addition of

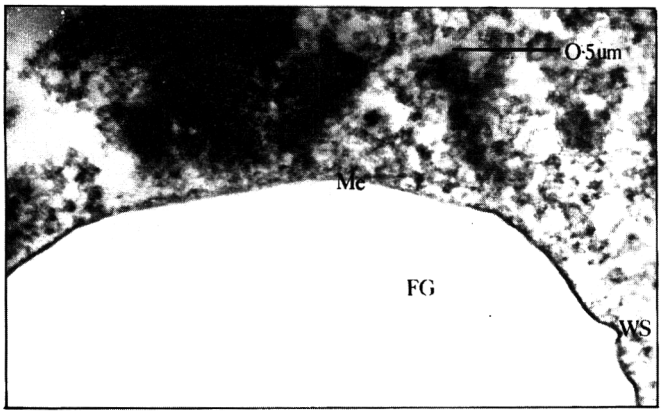

Fig. 5—Frankfurter containing wheat germ protein flour at 7.0%; FG = fat globule, Me = membrane, WS = weak spot.

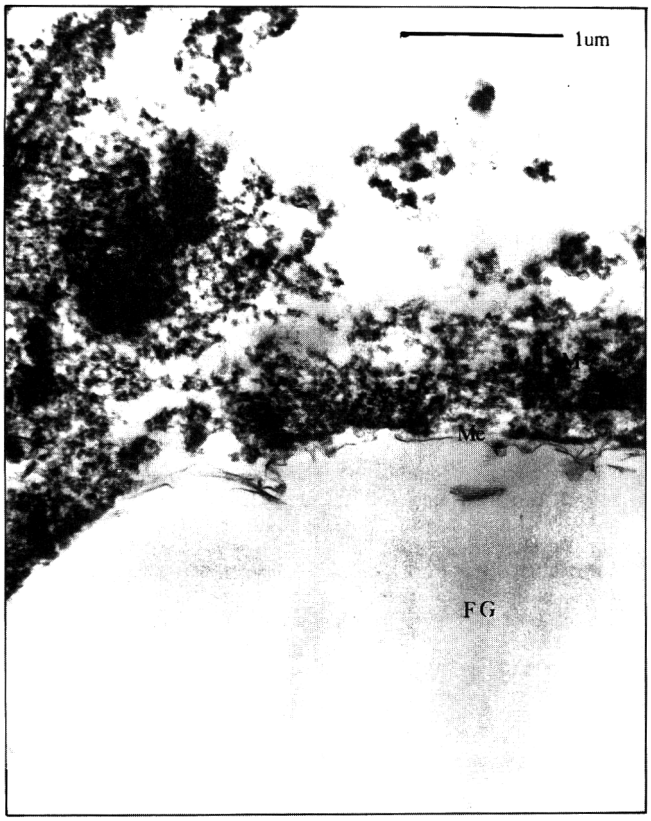

Fig. 6—Frankfurter containing wheat germ protein flour at 7.0%; FG = fat globule, Me = membrane.

WGPF above 3.5% was not effective, probably because of much higher added water. This was also evident from the amounts of water separated. 5.0% WGPF had lower values than controls while 7.0% WGPF was not different from controls. All samples had a similar protein content (Table 2) and a proportional increase in IPF formation upon addition of WGPF was not evident. Thus those factors did not account for the batter-stabilizing effect of WGPF. Physical restriction of fat globules by the protein matrix may be another mechanism of fat globule stabilization.

Nonprotein components such as polysaccharides might contribute to the functionality of WGPF by absorbing and retaining excess water in meat batters. An incomplete protein membrane around the fat globule was observed in the sample with 7.0% WGPF (Fig. 6). Dilution of the meat protein component by the additive and excess added water might be reasons the protein films were not complete. But the globules did not coalesce when heated, probably because the matrix (darkly stained area) physically restricted the fat globule movement. Lee et al. (1981) reported that physical properties of the protein matrix and incorporated fat affected emulsion stability. Interaction of soluble proteins of meat and nonmeat origin in a communited meat system containing nonmeat additives might contribute to such a matrix (Comer et al., 1986). Water absorption and gelation may also be more important than emulsification in the stability of comminuted meat systems (Comer and Allan-Wojtas, 1988). The ability of WGPF to bind added water might have resulted in a matrix that physically restricted the fat globules despite dilution of the meat protein components that are important for formation of a monomolecular hydrophobic layer around fat globules (Jones, 1984).

CONCLUSIOSN

MICROGRAPHS INDICATED that addition of WGPF at low levels increased meat batter stability. Increased membrane thickness around fat globules may have been due to functions of the protein additive in formation of the IPF. However, this effect was not linear with increasing levels of additive. The nonprotein components up to a specific level, could influence the continuous matrix surrounding fat globules. Hence, dilution of meat proteins by added water and additives could be compensated for up to a specific level. Absorption of excess moisture by nonmeat additive may contribute to structural integrity of the matrix. Soluble proteins of meat and nonmeat origin may interact in CMP with nonmeat additives. Nonprotein components such as carbohydrates with good water-binding potential also contribute to cohesiveness. Complex interactions between fat globules and protein matrix and physical restriction of the fat globules by the matrix probably influence meat batter stabilization.

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Conditions for Extraction and Concentration of Beef Fat Volatiles with Supercritical Carbon Dioxide

J.A. MERKLE and D.K. LARICK

ABSTRACT

Beef fat was fractionated using supercritical carbon dioxide at 40°C and pressures from 10.3–27.6 MPa. Fractions were analyzed for volatile content using purge and trap gas chromatography. One hundred six volatiles were identified. The concentrations of straight or branched-chain hydrocarbons, aldehydes, ketones, olefins, enals, lactones, and total volatiles were significantly influenced by extraction conditons. The control contained the least volatiles in each class analyzed. Total volatiles were concentrated over controls by 10–100 fold depending on treatment, with lowest pressure extraction conditions yielding highest concentrations of volatiles.

Key Words: beef, volatiles, supercritical fluids, flavor

INTRODUCTION

MARKET FOR ANIMAL FATS have been adversely affected due to the health implications of saturated fats and cholesterol. Animal fats, however, impart a desired texture and flavor to foods, especially those deep fat-fried. Substitution by plant oils has resulted in a loss of such desirable flavor attributes.

Annual edible beef tallow production in the US exceeds 500 million kilograms. Decreased use has resulted in large excesses. Extraction of volatiles as potential flavor compounds from beef tallow would help utilize this product. Furthermore, development of an economical, natural, meat flavoring would be useful to the food industry.

Researchers have reported that flavors and fragrances extracted using supercritical fluids were of higher quality and more true to the source than those from other methods including solvent extraction, steam distillation, hydrodiffusion, accelerated distillation, vacuum distillation, and molecular distillation (Calame and Steiner, 1982; Meyer-Warnod, 1984; Vollbrecht, 1982). Use of carbon dioxide eliminates toxic solvent residues and energy required to remove residual solvents. Supercritical extraction is advantageous for extraction of unstable and heat sensitive compounds because of the low temperatures of operation.

Researchers have reported that supercritical fluid extraction is useful for analysis and identification of flavor compounds in food products (Caragay, 1981; Chen and Ho, 1988; Hawthorne et al., 1988; Miles and Quimby, 1990; Del Valle and Aguilera, 1989). Supercritical fluids are used commercially to extract oleoresins from spices and essential oils from hops (Gardner, 1982; Vollbrecht, 1982). Zosel (1979) reported a method to deodorize fats and oils using supercritical fluids. Um et al. (1992) reported on the volatile compounds of heated pork fat extracted utilizing supercritical fluid extraction. de Haan et al. (1989, 1990) have shown that milk fat flavors could be concentrated 20–50 times with supercritical fluids and reported that theoretical concentrations of 500–1000 times the original may be possible.

Our objective was to determine the effects of different extraction and separation conditions on the concentrations of volatiles compounds in beef fat extracts.

Author Merkle is with the Dept. of Food Science & Nutrition, Univ. of Minnesota, 1334 Eckles Ave., St. Paul, MN 55108. Author Larick is with the Dept. of Food Science, Box 7624, North Carolina State Univ., Raleigh, NC 27695-7624. Address inquiries to Dr. D.L. Larick.

MATERIALS & METHODS

TWELVE Angus and Angus-Hereford cross steers were fed a controlled diet of Tifleaf pearl Millet for 101 days with a preceding clover-fescue pasture for 3 mos. Subcutaneous fat was collected from the loins of each animal and ground once through a 0.95 cm plate. Samples (50g) were weighed and vacuum-packaged in low oxygen permeability bags (Cryovac Corp., Duncan, SC) and overwrapped with polyethylene coated freezer paper. Samples were stored at −10°C and were allowed to thaw overnight at 4°C prior to use.

Supercritical fluid extraction

A Superpressure model 46-1341-2 supercritical fluid extractor (Newport Scientific, Jessup, MD) with a 69.0 MPa double end diaphragm compressor was used to fractionate the beef fat (Fig. 1). A 50-g sample, immobilized between two plugs of glass wool, was loaded into a 845 mL (internal volume) stainless steel extraction vessel. A one- or two-stage separation from supercritical carbon dioxide followed the single extraction step. For two stage separations, a 500 mL stainless steel separation vessel was used between the extraction vessel and the collection vessel. The temperature of extraction and in-line separation vessels were maintained at 40°C, using an internal thermocouple and thermostatic control and heaters wrapped around the outside of the vessels. All stainless steel transfer lines were insulated to prevent heat loss and precipitation of solubilized material due to temperature drop. The unit was physically cleaned and flushed with supercritical carbon dioxide between extractions to eliminate any potential for sample carryover.

The gas was depressurized at room temperature (≈23°C) and atmospheric conditions into a 500 mL Pyrex glass flask. Flow was maintained at 15 L/min through the flow indicator and the extraction was terminated after 500L of carbon dioxide gas had been used (Fig. 1). The expanding gas quickly cooled the flask and the extracted material collected in the bottom of the flask. Samples were heated in a water bath at 60°C to melt the extract and transferred with a Pasteur pipette into 16 mm × 125 mm Pyrex tubes fitted with teflon-lined caps. The tubes were nitrogen-flushed and placed into freezing at −10°C until analyzed.

Dynamic headpsace analysis

Samples were removed from the freezer and melted in an 80°C water bath. Extract (300 mg) was weighted into another tube and 1035 ng internal standard (2,3,4-trimethylpentane) was added. Volatile concentration (ppm) was determined relative to the reponse of the internal standard. A response factor of one was utilized for each volatile. The tube was sealed with a teflon-lined cap and vortexed to ensure proper mixing. Samples (100 mg) were loaded into a 9 mm × 85 mm glass tube between two plugs of loosely packed (pesticide grade) glass wool (Supelco, Inc., Bellefonte, PA). The tubes and glass wool were heated at 290°C for at least 24 hr to remove any trace volatile compounds and further conditioned by purging with helium for 90 min at 150°C before loading the sample. Volatiles were stripped from the fat using an External Closed Inlet Device (Scientific Instrument Service, River Ridge, LA), previously described by Suzuki and Bailey (1985). Maintained temperatures were: inlet 150 °C, valve 160°C and carrier line 170°C. Volatiles were flushed onto a 30m DB-5, 0.32 mm diameter capillary column (J & W Scientific, Folsom, CA) with a film thickness of 1.0 micron, for 5 min. The chromatograph (Varian model 3700, Palo Alto, CA) oven was maintained at −30°C during sampling and a 1 min hold was used to allow cryofocussing of the compounds. The chromatograph oven was ramp heated following the 1 min hold from −30° to 290°C at 4°C/min. The injector was held at 310°C and detector at 320°C. Helium carrier gas was used at a head pressure of ≈1kg/cm², split ratio of 20.16:1, and a column flow of 4.6 mL/min at −30°C. Data were collected and analyzed from a flame ionization detector

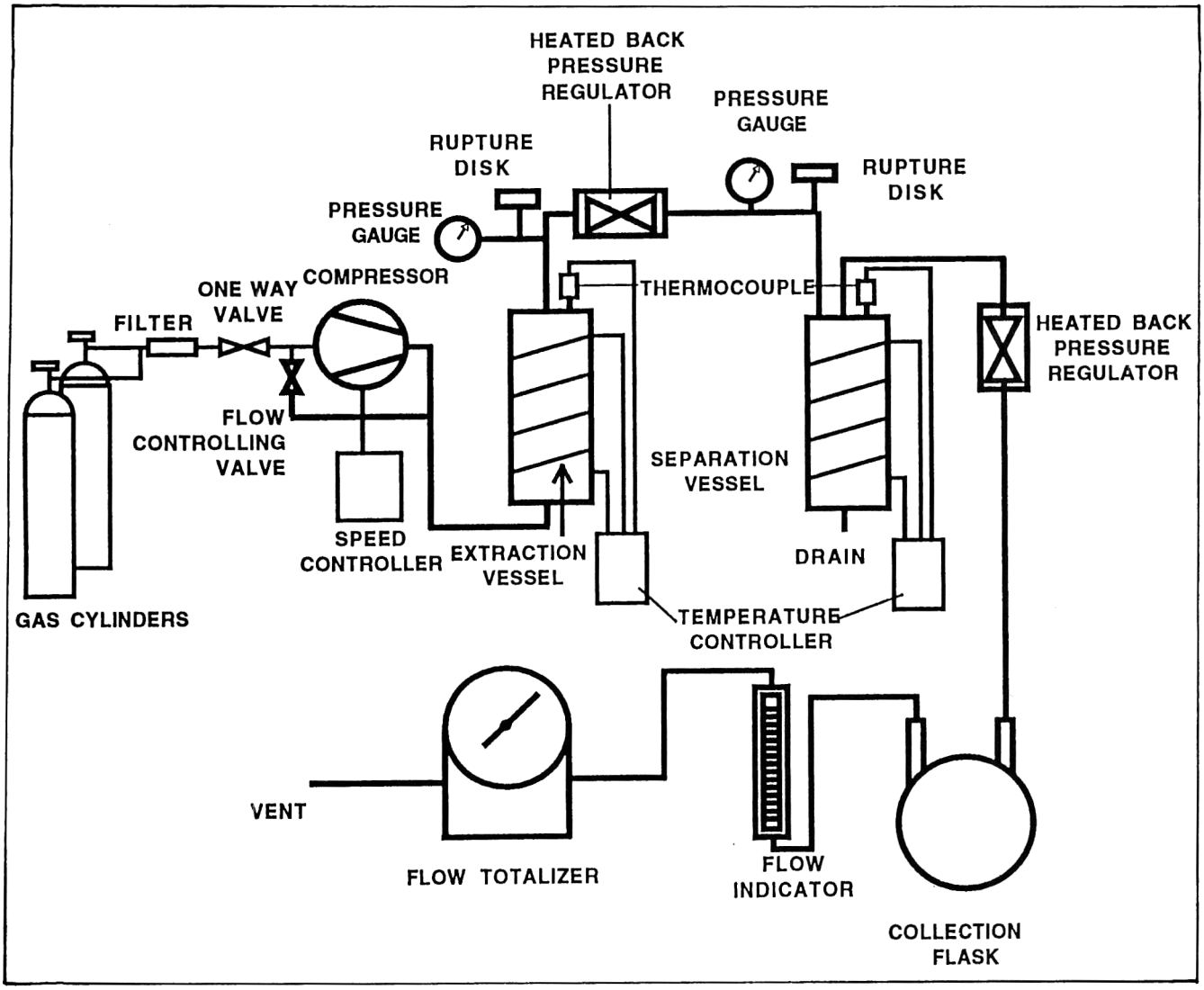

Fig. 1—Supercritical fluid extractor.

(FID) using the Maxima 820 Chromatography Workstation (Millipore, Waters Chromatography Division, Milford, MA).

Likens-Nickerson extraction

A modified Likens-Nickerson extraction (Likens and Nickerson, 1964) was used to extract volatile compounds from beef fat for mass spectrometry. Finely chopped subcutaneous fat (100g) was weighed into a 500 mL round bottom Pyrex flash containing boiling chips and 150 mL deionized, distilled water. Methylene chloride (75 mL) was added to a 100 mL round bottom Pyrex flask and the flask was connected to the other arm of the extractor. The two flasks were allowed to co-extract for 6 hr. Following extraction, the flasks were allowed to cool to room temperature. The methylene chloride remaining in the side arm of the extractor was drained and combined with that in the flask while the fat and water mixture was discarded. The methylene chloride was evaporated to 2 mL using a Buchi rotary evaporator (Rinco Instrument Co., Inc., Greenville, IL). The sample was transferred to a conical, graduated 5 mL Pyrex tube using an additional three 1 mL aliquots to rinse the flask. The methylene chloride was concentrated to a final volume of 0.1 mL under a gentle stream of nitrogen.

Mass spectrometry

Volatile identification was performed with a Hewlett Packard 5897 gas chromatograph/mass spectrometer (GC/MS) using electron ionization (EI) with an ionization potential of 70 eV. Many compounds gave very weak or no molecular ions under EI conditions. Therefore, samples were also analyzed using methane, positive chemical ionization (CI) with a scanning range of 40–500 atomic mass units. The capillary column and chromatographic conditions were identical to those outlined above for dynamic headspace analysis.

Sampling procedures to prepare extracts for injection or for direct sampling of volatiles included direct injection, Likens-Nickerson liquid-liquid extraction, and two different purge and trap procedures. For direct injection, 1 μL of liquified beef fat was injected directly onto the column. A modified Likens-Nickerson extraction apparatus was also utilized to concentrate beef volatiles for direct injection. A 1 μL direct injection was used with both electron ionization at 70 eV and chemical ionization using methane. A Tekmar LSC-3 headspace concentrator (Tekmar Corp, Cincinnati, OH) was used with 1 g extracted material in the sample tube. The tube was heated with heat tape to 110°C and purged for 10 min. An External Closed Inlet Device (Scientific Instrument Service, River Ridge, LA) was used with a 250 mg sample. The sample was heated to 150°C and sampled for 8 min. Peaks were identified based on library (NIH/EPA, 1978) search of the EI results and molecular weights based on CI results.

Experimental design

A range of extraction pressures followed by one or two stage separation steps were used (Table 1). Extractions were performed in duplicate. The analysis of variance was calculated for each data set using the general linear model proceudre (SAS Institute, Inc., 1985). Waller-Duncan k-ratio t-tests were calculated for significantly different main

Table 1—Summary of volatiles from purge and trap analysis of supercritical fluid extracted beef fat

		Concentration (ppm)									
EV p[a]	SV p[a]	HC[b]**	Aldehyde*	Ketone**	Alcohol	Olefin**	Enal**	Lactone**	Acid	BHC[b]**	Total**
Control		3.50^{i}	1.47^{g}	0.16^{g}	0.18	3.77^{h}	0.02^{g}	0.07^{f}	0.20	2.76^{ijk}	19.8^{g}
27.6	20.7	21.25^{hi}	9.42^{fg}	4.30^{fg}	1.77	44.99^{gh}	1.54^{g}	5.48^{f}	6.74	10.31^{ijk}	158.8^{fg}
27.6	17.2	32.07^{gh}	9.85^{defg}	6.20^{efg}	2.98	69.61^{gh}	2.80^{efg}	7.66^{ef}	15.63	14.57^{hij}	223.8^{fg}
20.7	17.2	33.29^{gh}	8.66^{defg}	5.25^{efg}	3.38	71.15^{gh}	3.09^{efg}	5.24^{f}	12.20	13.50^{hij}	209.6^{fg}
27.6	13.8	29.97^{ghi}	12.76^{def}	6.88^{efg}	2.29	105.36^{fgh}	2.03^{fg}	7.37^{ef}	6.29	12.03^{ij}	253.6^{fg}
20.7	13.8	55.22^{fg}	9.71^{defg}	8.45^{efg}	2.54	185.67^{efgh}	3.82^{efg}	11.76^{ef}	17.11	21.65^{fgh}	386.0^{fg}
17.2	13.8	51.41^{fg}	7.29^{efg}	7.31^{efg}	1.77	209.99^{efgh}	4.88^{efg}	12.36^{ef}	8.45	18.84^{ghi}	380.2^{fg}
27.6	10.3	52.46^{fg}	5.09^{fg}	6.79^{efg}	1.06	350.13^{ef}	6.10^{ef}	22.37^{de}	39.75	16.94^{ghij}	556.0^{efg}
20.7	10.3	93.26^{e}	14.31^{de}	11.22^{ef}	2.46	356.54^{ef}	6.93^{e}	23.15^{de}	30.56	35.53^{e}	659.6^{de}
17.2	10.3	72.37^{ef}	9.72^{def}	10.22^{ef}	2.23	268.54^{efg}	6.64^{ef}	15.64^{ef}	14.76	28.55^{ef}	486.0^{fg}
13.8	10.3	139.09^{d}	14.93^{d}	14.41^{e}	6.47	746.50^{d}	12.74^{d}	39.15^{d}	37.38	46.92^{d}	1146.8^{de}
20.7	0[c]	20.14^{hi}	7.30^{fg}	3.07^{fg}	1.70	58.32^{gh}	1.65^{g}	4.03^{f}	6.96	9.60^{ijk}	146.1^{fg}
17.2	0	28.94^{hi}	5.97^{fg}	5.05^{efg}	1.44	123.10^{efgh}	2.29^{fg}	7.38^{ef}	8.45	12.17^{ij}	235.9^{fg}
13.8	0	42.09^{gh}	4.11^{fg}	14.32^{e}	1.23	265.80^{efg}	4.52^{efg}	13.38^{ef}	29.63	10.96^{ijk}	432.6^{fg}
10.3	0	125.23^{d}	10.34^{def}	88.13^{d}	2.82	709.82^{d}	15.02^{d}	23.75^{de}	51.74	24.18^{fg}	1198.6^{d}

[a] EV p = Extraction vessel pressure (MPa); SV p = Separation vessel pressure (MPa).
[b] HC = Hydrocarbon; BHC = Branched chain hydrocarbon.
[c] Separation vessel pressures of 0 indicate that the separation vessel was not used - one stage extraction.
[d-k] Means in the same column with the same letter do not differ significantly at level indicated at top of column.
* $P \leq 0.05$
** $P \leq 0.01$

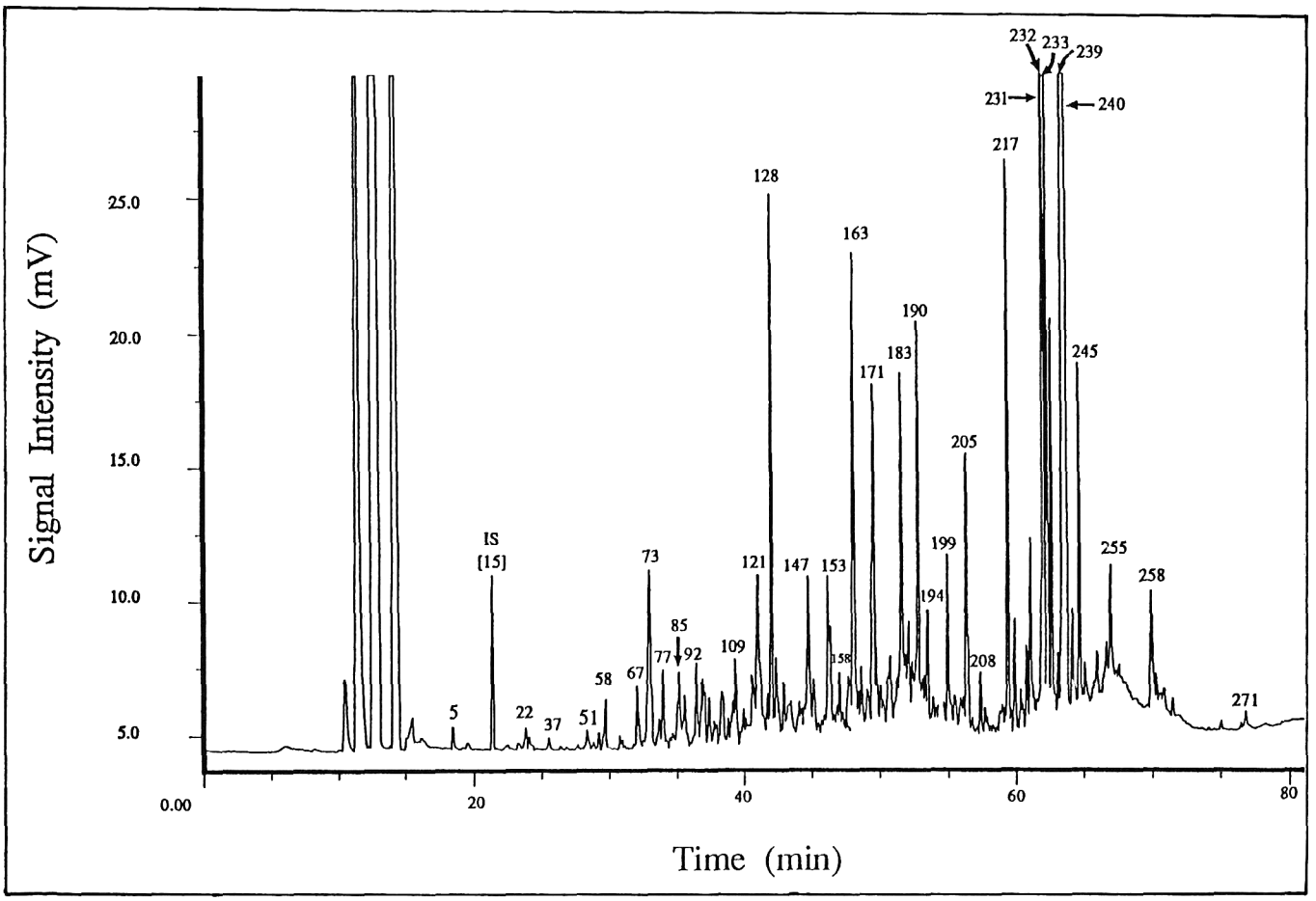

Fig. 2—Purge and trap chromatogram of supercritically extracted beef fat.

effect means ($P \leq 0.05$). Values were considered significantly different at the $P \leq 0.05$ level.

RESULTS & DISCUSSION

A TYPICAL PURGE AND TRAP chromatogram of beef fat volatiles is presented (Fig. 2). In all, 274 compounds were quantitated, 106 of which were identified. Many peaks were not identified, but those identified, accounted for a major percentage of total volatiles (ppm) in the sample. These data were summarized (Tables 1 and 2) into the following classes of compounds: 37 hydrocarbons (of which 23 were branched), 13 aldehydes, 11 ketones, 3 alcohols, 19 olefins, 4 enals, 6 lactones, and 4 acids. As a class, olefins constituted the largest concentration.

Total volatiles (ppm) increased as extraction pressure decreased. The lowest extraction/separation pressures (10.3 MPa and 27.6 MPA/10.3 MPa) resulted in about a 60-fold increase over controls in total volatiles. Extractions with separation at atmospheric conditions resulted in a significant increase in total volatiles from the highest extraction pressure, 20.7 MPa, to

Table 2—Volatiles (peak no. from Fig 2) identified in supercritical fluid extracted beef fat by class

Acids
Tetradecanoic (227)
Pentadecanoic (245)
Hexadecanoic (254)
Octadecanoic (268)
Alcohols
Octanol (74)
Dihydrophytol (259)
Phytol (263)
Aldehyde
Pentanal (7)
Hexanal (30)
Heptanal (57)
Benzaldehyde (68)
Octanal (76)
Nonanal (104)
Decanal (132)
Undecanal (154)
Dodecanal (173)
Tridecanal (193)
Tetradecanal (208)
Pentadecanal (223)
Hexadecanal (237)
Olefins
Benzene (5)
Toluene (19)
1-octene (28)
2-octene (32)
3-methyl-2-heptene (35)
Xylene (54)
Ethylbenzene (55)
Dimethylbenzene (56)
Limonene (82)
Trimethylbenzene (84)
1-decene (88)
Methylnonene (96)
Naphthalene (134)
3-methyl-3-undecene (137)
Tridecene (153)
Phyt-1-ene (231)
Neophytadiene (239)
Phyt-2-ene (240)
Phytadiene (247)
Enals
2-nonenal (133)
2-undecenal (158)
E,E-2,4-decadienal (169)
2-octadecenal (258)
Hydrocarbons
Heptane (8)
Cyclohexane (11)
Octane (31)
Cycloctane (38)
Nonane (58)
Decane (77)
Undecane (103)
Dodecane (131)
Tridecane (153)
Tetradecane (171)
Pentadecane (190)
Hexadecane (205)
Heptadecane (217)
Octadecane (232)
+ Branched Hydrocarbons
Ketones
2-heptanone (59)
2-octanone (75)
2-nonanone (105)
2-decanone (130)
2-undecanone (152)
2-dodecanone (172)
2-tridecanone (192)
2-tetradecanone (206)
2-pentadecanone (220)
2-hexadecanone (235)
2-heptadecanone (257)
Branched Hydrocarbons
Methylcyclohexane (12)
3,4-dimethylhexane (20)
4-methylheptane (21)
2,2,4-trimethylhexane (25)
2,3,5-trimethylhexane (41)
3,5-dimethylheptane (48)
2,3-dimethylheptane (49)
1,3,5-trimethylcyclohexane (51)
2,3-dimethylheptane (52)
2,3,6-trimethylheptane (62)
2,6,6-trimethyloctane (80)
Trimethyloctane (83)
3,6-dimethyldecane (106)
2,6-dimethyldecane (113)
2,2,5-trimethyldecane (115)
3-methyl-5-propylnonane (117)
3,6-dimethylundecane (120)
4-ethyl-2,2,6,6-tetramethylheptane (121)
3-methyldodecane (136)
Heptylcyclohexane (183)
Trimethyldodecane (189)
Dimethyltetradecane (194)
Phytane (233)
Lactones
γ-heptalactone (26)
δ-decalactone (163)
γ-dodecalactone (219)
δ-dodecalactone (221)
δ-tetradecalactone (255)
δ-hexadecalactone (267)

the lowest, 10.3 MPa. Extractions where an intermediate separation vessel was used showed similar results with increasing total volatile concentrations as the intermediate vessel pressure decreased. The concentration of total volatiles in extracts with an intermediate separation pressure was lower than the corresponding extracts collected via a single separation at atmospheric conditions. The concentration of total volatiles in extracts with intermediate separation approached that of the atmospheric separation extracts as difference between stages (pressure drop between extraction and separation vessel) decreased. This was likely due to a precipitation which occurred as the lipid-rich supercritical carbon dioxide dropped in density from the higher pressure extraction vessel to the lower pressure separation vessel. This pressure drop resulted in a precipitation of material which was no longer soluble in the less dense fluid. As this density difference increased, the precipitation of fatty acids and triglycerides may have entrapped smaller molecules and forced them out of solution. Previous work has shown that the solubility of triglycerides and fatty acids increased as pressure increased (Merkle and Larick, 1993a, 1993b). It is reasonable to expect that, since volatiles were in much lower concentration, their extraction would be hindered by the much more prevalent fatty acids and triglycerides. de Haan et al. (1990) have shown similar results for extraction of flavor from butterfat. Modelling extraction of δ-dodecalactone, they demonstrated that the solubility of δ-dodecalactone increased as extraction pressure increased. However, in the extraction from butterfat, little gain was observed from higher pressure extraction conditions. Extraction of each compound is dependent on its initial concentration, vapor pressure, and solubility. In that case, the compound was totally extracted early and, therefore, little concentration was seen. Expressed on a ppm basis, this compound would decrease as pressures increased, due to an increase in quantities of other components.

Olefin concentration increased from 10- to 190-fold over the control (100 mg of the original fat). The lowest extraction pressures resulted in the highest concentrations of olefinic compounds. Extractions with separation at atmospheric conditions resulted in a logarithmic increase in concentration of olefinic compounds as pressure was decreased. Extractions with intermediate separation steps showed similar results. Concentrations of volatiles increased as pressure, of the intermediate separation step, was decreased. Olefins identified (Table 2) included 8 alkenes, 4 diterpenes, and 7 aromatics. The diterpenes comprised up to 99% of the volatiles in this class. The high solubility of these compounds would be expected based on their structure.

Alkenes are probably formed from alkyl-free radicals, (from thermally decomposed hydroperoxides) reacting with hydrogen radicals (Selke et al., 1975). The alkenes identified most likely originated from unsaturated fatty acid oxidation. Aromatic volatile compounds are formed from the breakdown of aromatic amino acids (Kato et al., 1971; Krishnamurthy and Chang, 1967). Kato et al. (1971) heated phenylalanine at 300°C. Thermal breakdown products included toluene, benzaldehyde and vinylbenzene. Toluene, ethylbenzene, and m-xylene decreased beef fat aroma and intensity and increased undesirable milky-oily aroma (Varner et al., 1988).

Diterpenes have been isolated from feed (Boardman, 1966; Body, 1977; Ferretti and Flanagan, 1977), animal tissues (Ferretti and Flanagan, 1977; Lorenz et al., 1983), and animal products (Body, 1977; Flanagan and Ferretti, 1973; Patton and Benson, 1966). Urbach and Stark (1975) reported that levels of diterpenes were dependent on the diet. Levels of phyt-1-ene and neophytadiene were found to decrease with days off fresh pasture while phyt-2-ene remained the same. Urbach and Stark (1975) proposed that the precursor for these compounds was neophytadiene which they isolated from pasture grass. Body (1977) theorized that rumen microbes produced phytenes from dietary phytol.

Maruri and Larick (1992) have shown that the off-flavors of forage-fed beef were associated with the lipid portion. Several researchers have implicated individuals diterpenes as the source of grassy off-flavors in beef (Larick et al., 1987; Maruri and Larick, 1992). Feretti and Flanagan (1977) studied various tissues of rabbits. They found that adipose was the only tissue which had a similar overall composition of diterpenes to that in the feed. They demonstrated that diterpenes were deposited

in the fat of animals. From their data, a correlation between diet and levels of diterpenes was clear. From our results, these compounds were highly soluble in supercritical carbon dioxide. If these compounds are responsible for off-flavors, avoiding fat higher in such compounds, (typically from forage fed beef) as a flavor source would be advisable.

The hydrocarbons identified (Table 2) in the fractions included n-alkanes (C7-C18), cyclic hydrocarbons, and branched hydrocarbons. Concentrations increased from 6-40 fold over controls as extraction pressures (separation at atmospheric conditions) decreased from 20.7 MPa to 10.3 MPa. Of the extractions with an intermediate separation pressure, the 13.8/10.3 MPa extraction resulted in highest concentrations of hydrocarbons. Alkanes have been shown to be miscible with carbon dioxide for carbon lengths of 12 and less. Above 12 solubility decreases but C18 is soluble at low concentrations (Dandge et al., 1985; Hyatt, 1984). Branched alkanes show greater solubility than their straight chain counterparts (Dandge et al., 1985) and are miscible in carbon dioxide up to 30 carbon atoms. This is thought to result from decreased methylene groups along the hydrocarbon chain which results in diminished intermolecular interactions and thus lower solubility. This results in the increased solubility of such compounds in dense carbon dioxide (Dandge et al., 1985). A similar phenomenon is also shown by the lower melting point of branched versus straight chain hydrocarbons. In lipids the hydrocarbons are theorized to result from oxidation of fatty acids forming alkyl-free radicals which then react with hydrogen radicals (Frankel et al., 1981; Selke et al., 1975). Branched hydrocarbons may arise from oxidation of branched chain fatty acids, breakdown of diterpenes, or storage of dietary ingredients and metabolites in fat depots.

The ketones identified (Table 2) consisted of the 2-n-alkanones from C7-C17. These compounds were concentrated from 30- to 550-fold over the control. This was the only class of compounds in which the lowest pressure atmospheric separation (10.3 MPa) and lowest intermediate separation pressure (13.8MPa/10.3MPa) extractions did not show similar results. The reason for enhanced extraction of ketones at 10.3 MPa and atmospheric separation is unknown.

The formation of ketones in the autoxidation of oleic and linoleic acid was shown by Mookherjee et al., 1965. They theorized that ketones may be formed by: (1) addition of water across the double bond next to a keto group formed by a hydroperoxide; (2) formation of 2-alkanones by decarboxylation of β-keto acids; or (3) oxidative scission in the neighborhood of double bond to form vinyl ketones and production of 2-alkanones by oxidation of the vinyl ketone.

Lactone concentrations increased over controls 75- to 500-fold. Lactone concentration increased as extraction pressure decreased. Lactones impart a favorable flavor attribute and are especially important in the flavor of butter. Lactone formation results from thermal decomposition products of trilinolein (Selke et al., 1977, 1980; Thompson et al., 1978), tristearin (Selke et al., 1975, 1977), and esterified olein-stearin (Selke et al., 1977). Thermal degradation of oleic acid has also yielded lactones (Watanabe and Sato, 1969, 1971). The mechanisms for formation include the production of corresponding γ- or δ-hydroxy fatty acids into lactones (Parle and Perkins, 1992; Watanabe and Sato, 1971). Also lower saturated fatty acids, aldehydes and ketones formed from oxidative degradation of fats are converted to lactones (Watanabe and Sato, 1971). Stark et al. (1978) and Urbach (1990) demonstrated the biosynthetic production of lactones from unsaturated fatty acids, especially linoleic and oleic, via formation of 9-hydroxy fatty acids followed by β-oxidation.

Aldehydes identified included n-alkanals (C5-C16), and benzaldehyde. Aldehyde concentration increased 6- to 10-fold over the control depending on treatment. In general, aldehyde concentration increased as pressure decreased. Enal concentration was extremely low in the control while extraction resulted in 70-750 fold increases. Enal concentration increased as pressure decreased. Aldehydes and enals are formed by the thermal decomposition of monohydroperoxides, initial products of thermally oxidized fats. Enals result from the corresponding unsaturated fatty acid decomposition. Based on the sharp increase in concentration, supercritical fluid extraction conditions may be favorable for enal production.

Other compounds found in the fractions included acids and alcohols. Acids (Table 2) are most likely due to thermal degradation of triglyceride esters (Krishnamurthy and Chang, 1967). Alcohols (Table 2) are formed by the reaction of alkyl-free radicals with hydride radicals. The concentration of such classes was not significantly affected by extraction conditions. These compounds are more polar than the others and thus would be less soluble in carbon dioxide.

The concentration of volatiles was enhanced by lower extraction pressures. This may be due to several factors. Volatiles solubilization may be suppressed by the presence of other volatiles, fatty acids, and triglycerides in the sample. As extraction pressure is increased, the solubility of fatty acids and triglycerides is increased (Merkle and Larick, 1993, 1994). However, the melting point of the volatiles is much lower than the other lipid components. Previously, this was reported to predict the solubility of isomers of similar compounds in carbon dioxide (Krukonis and Kurnik, 1985). The partial pressure of the fatty acids and lipids is also much lower than that of the volatiles extracted.

CONCLUSIONS

SUPERCRITICAL CARBON DIOXIDE extracts and concentrates volatiles from beef fat. For most classes of compounds, lower extraction pressures resulted in greater concentration of volatiles (ppm). The extraction of each compound is dependent on its initial concentration, vapor pressure, and solubility. Compounds in low concentration and of high solubility will quickly be exhaustively extracted at high pressures while at lower pressures, lower solubilities of volatile compounds and other lipid components result in greater selectivity. Pressures near the critical point should yield the best recovery and concentration of volatiles. Some fractionation of volatiles is possible because ketones, lactones, and enals tend to be concentrated to a much greater extent (over 500-fold) than other classes such as hydrocarbons (6- to 40-fold) an aldehydes (6- to 10-fold).

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Konjac Flour Gel as Fat Substitute in Low-fat Prerigor Fresh Pork Sausage

W. N. OSBURN and J. T. KEETON

ABSTRACT

Prerigor trimmings from four lean cuts of pork carcasses (n = 3) were used to manufacture three replications of low-fat (10%) fresh pork sausage containing konjac flour gel, at 0, 10 or 20% levels and compared to a 40% fat control. Treatment sausages showed equal or improved cooked yields, slightly higher shear force (kg/g) and sensory textural attributes, but rated slightly lower in juiciness. As konjac flour gel levels increased, shear force and sensory textural attributes became more like the control. Storage time had minimal effect on quality and shelf life. Acceptable low-fat, prerigor pork sausage can be produced with 10–20% incorporation of konjac flour gel.

Key Words: konjac flour, low fat, fat substitute, pork sausage

INTRODUCTION

REDUCED-FAT MEAT PRODUCTS may have less desirable flavor and textural attributes than traditional products. The physicochemical and textural attributes of various low-fat ground and emulsified products have been studied. Attempts were made to retain sensory and textural attributes through fat reduction by replacing fat with water (Claus et al., 1989; Ahmed et al., 1990), monounsaturated oils, (Marquez et al., 1989; Park et al., 1990), carbohydrate based fat substitutes (Skrede, 1989; Berry and Wergin, 1990), protein-based fat substitutes (Ziprin et al., 1981; Reitmeier and Prusa, 1991), and modification of fatty acids through diet (Rhee et al., 1990; Shackelford et al., 1990).

Hydrocolloids (gums) have been used to replace fat in processed foods. They provide viscosity and a lubricating mouthfeel that simulates and mimics the sensory properties of fats (Glicksman, 1991). Effectiveness of hydrocolloids has been reported for binding water in low-fat meat emulsions (Wallingford and Labuza 1983; Foegeding and Ramsey, 1986), modifying the texture of ground beef patties (Huffman and Egbert, 1990), and enhancing the textural attributes of low-fat (15%), high moisture and high protein frankfurters (Lin et al., 1988).

Many meat processors utilize prerigor pork trimmings to manufacture fresh pork sausage. Konjac flour, in the form of a hydrocolloid gel, may make possible the production of low-fat (10%) fresh pork sausage on existing process lines, with sensory properties similar to regular (40% fat) pork sausage. Our objectives were to determine the physical, chemical, sensory, textural and microbial characteristics of low-fat pork sausage containing varying levels of konjac flour gel and to evaluate the feasibility of its incorporation in a prerigor pork sausage system.

MATERIALS & METHODS

Formulation and processing

Market hogs (≈ 100 kg each, n = 3) were humanely slaughtered on three separate days (1 hog/day) at the Rosenthal Meat Science and Technology Center at Texas A&M Univ., College Station, TX. Prerigor raw sausage materials were obtained within 1 hr postmortem by fabricating each carcass into the boston butt, picnic shoulder, loin and ham. All skin, subcutaneous fat, bone and necessary seam fat were removed to produce a 10% fat meat block for each sausage treatment. Lean trimmings, excess subcutaneous fat, and konjac flour gel were each coarse ground separately through a 1.27 cm plate. Fat and lean trimming subsamples were analyzed for fat content using a CEM Automatic Volatility Computer (Model AVC-80) and a CEM Automatic Extraction System (CEM Corp., Indian Trail, NC), and formulated to contain 10 or 40% fat by the Pearson square method. Four 9.09 kg batches from each pork carcass were formulated to contain 40% fat (40F control), or 10% fat with either 0% (0KF), 10% (10KF), or 20% (20KF) finely ground (0.32 cm) Nutricol® KC5G konjac flour gel. Appropriate amounts of fresh pork sausage seasoning (Old Plantation Pork Sausage Seasoning, Blend #6, A.C. Legg, Inc., Birmingham, AL) were added to the hot-boned, coarse ground (0.48 cm) materials along with 3% added water in the 40F and 0KF sausages, and appropriate percentages of ground konjac flour gel in the 10KF and 20KF treatments. Due to the small batch sizes, each treatment was hand mixed (while wearing disposable gloves) for 5 min prior to grinding through a 0.48 cm plate. Each batch was vacuum stuffed into 0.5 kg plastic chubs, sealed with a metal clip, color coded according to treatment, and crust frozen to −1°C in a −20°C blast freezer then placed in a 2°C cooler. Samples were stored for 0, 7, 14 or 21 days and then were removed for pH determination, proximate analysis, sensory evaluation, Allo-Kramer shear, microbial screening and color evaluation. This entire procedure was replicated three times (1 hog = 1 replication).

Konjac flour gel

Nutricol® konjac flour KC5G was selected based on its likelihood of incorporation as a fat replacement in a prerigor sausage system, and its ability to absorb coloring agents, thus enabling direct incorporation of seasonings into the formulation (Stone, 1991). A proto-type study indicated problems with absorption of water soluble pigments by konjac flour gel particles causing a "blood clot" appearance on the exposed cut surface of the raw sausage patty. Dilution of cooked pork flavor due to partial replacement of meat with konjac flour gel was also observed. To correct these problems, caramel coloring (Warner-Jenkinson, Chicago, IL) and pork sausage seasoning was incorporated into the konjac flour prior to gel formation to mask the "blood clot" appearance, aid in producing a "browned" appearance when pan-frying, and offset the dilution of cooked pork flavor. A final grind of 0.32 cm was utilized to reduce the visibility of konjac flour gel particles on the exposed sausage patty surface.

Nutricol® konjac flour (FMC Marine Colloids Div. Philadelphia, PA) KC5G was formulated into a gel consisting of 9,808.5g H_2O, 1,008.0g of 0.8% sodium citrate solution, 283.5g of Nutricol® KC5G konjac flour, 227.0g of fresh pork sausage seasoning and 14.18g (0.125%) caramel coloring. The total konjac flour gel batch weight was 11,360g (11.36 kg).

Konjac flour gel was manufactured by placing water in a steam jacketed kettle and bringing it to a boil, slowly adding Nutricol® konjac flour, sodium citrate, caramel coloring, and seasoning while mixing with a high speed brine agitator. The hot konjac flour gel was poured into containers, cooled at room temperature (≈23°C) for 60 min, and chilled to 2°C. Konjac flour gel was then ground to the desired particle size (0.32 cm) and appropriate amounts added to the meat block during mixing. Konjac flour is generally recognized as safe (GRAS) as defined in the Federal Food, Drug and Cosmetic Act and implementing regulations in Title 21 of the Code of Federal Regulations.

Proximate composition

Representative samples (≈454g) of all treatment combinations were homogenized in a Cuisinart DLC-8 Plus Food Processor. Three, 4–5

Author Keeton is with the Animal Science Department, Texas A&M Univ, College Station, TX 77843. Author Osburn is currently affiliated with the Department of Animal Science, University of Nebraska-Lincoln, Nebraska 68583-0908. Address inquiries to Dr. J.T. Keeton.

g subsamples were randomly selected from each raw and cooked ground sample and analyzed for percentage moisture (oven air-drying method), fat (ether extractable component), ash (muffle furnace) and protein (Kjeldahl nitrogen) following AOAC (1990) procedures. All determinations were performed in triplicate. Samples for analysis were collected from each treatment combination on Day 0.

Caloric content

Total calorie estimates (Kcal) for raw and cooked sausages were calculated on the basis of a 100g portion using Atwater values for fat (9 Kcal/g), protein (4.02 Kcal/g) and carbohydrate (3.87 Kcal/g). Calories contributed by konjac flour (carbohydrate) were based on 2.5% konjac flour in the gel and adjusted to the level of incorporation (0, 10 or 20%). Since analysis of percent carbohydrates in the meat samples was not performed, the caloric values were estimates and not actual values.

Cooking procedure and yield determination

Sausage patties for sensory evaluation, Allo-Kramer shear and cooked proximate analysis were prepared by slicing tempered sausage chubs (0°C) into 1.27 cm thick patties equilibrated to 2°C prior to cooking. Teflon coated electric skillets (The West Bend Company, West Bend, WI) were calibrated to 148.9°C, and a copper/iron thermocouple placed into the geometric center of randomly selected patties. Internal temperature was monitored with an Omega digital temperature recorder to a "well done" endpoint of 80°C (assuming most consumers cook pork sausage patties to this temperature). Patties were cooked following the procedure outlined by Ahmed et al. (1990) with the exception of a higher endpoint temperature. Six sausage patties per treatment were weighed on a Mettler PC8000 balance (Mettler Instrument Corp., Highstown, NJ), cooked as described, drained for 1 min and blotted dry prior to reweighing for cook yield determinations. Cook yield was calculated as follows:

$$\text{Cook yield} = (\text{cooked product wt of 6 patties/raw product wt of 6 patties}) \times 100\%$$

Percent cooking loss was determined by difference between thawed and cooked weight divided by thawed weight.

pH analysis

Samples (25g) from each treatment were taken on each day of storage after the sausage was crust frozen to −2°C (≈2.0–2.5 hr postmortem). Samples were blended for 30 sec in a Virtis homogenizer (The Virtis Company, Gardiner, NY) with 50 mL distilled and deionized water. A combination electrode, standardized between pH 4.0 and 7.0 and attached to an Orion pH meter (Model 610, Orion Research Inc., Cambridge, MA), was inserted into the stirred slurry. After equilibration for 30 sec, pH was recorded (±0.01 pH unit). Means of 2 pH readings/treatment were calculated.

Determination of lipid oxidation

The TBA distillation method of Tarladgis et al. (1960) was followed with addition of 5 mL of a 0.5% solution of propyl gallate and EDTA for each 10 g sample in the blending process (Rhee, 1978). A 60-g sample was blended with 90 mL distilled water and 30 mL propyl gallate-EDTA solution in a Kitchen Aid food processor (Model KFP 400; Hobart Corp., Troy, OH). Slurry (30g representing 10g meat) was used for distillation. Distilled samples were reacted with TBA reagent and color measured at 540 nm using a Beckman DU-7 spectrophotometer. TBA values reported (mg malonaldehyde/kg sample) were means of duplicate distillation and color determinations.

Sensory evaluation of texture and flavor

An experienced five-member descriptive attribute texture/flavor profile panel was employed for sensory analysis and trained previously according to procedures outlined by Cross et al. (1978). Four product familiarization sessions were held at the Texas A&M University Sensory Testing Facility. During training, panelists were served samples from a preliminary study which included all treatment combinations utilized in this study. Konjac flour (Nutricol® KC5G) gel was available to panelists to evaluate its flavor and textural attributes. Aromatics, mouthfeel and basic tastes of each sausage sample were identified and defined. "Starchy flavor," due to its presence in the konjac flour gel, was identified and added to the ballot. Panelists were familiarized with texture references and had previous experience evaluating similar pork sausage products. Textural properties evaluated included juiciness, gumminess, cohesiveness, springiness, chewiness, fracturability, denseness and hardness. Samples were evaluated for aromatics, mouthfeel and basic tastes using the Spectrum™ universal intensity scale (Meilgaard et al., 1987) with 0 = absent and 15 = extremely intense. Evaluation of textural properties was modeled after this scale. Chewiness was defined as the total number of chews required before the sample was ready to swallow and therefore, could exceed the upper scale numeric limit (15).

Panel members were served 6 randomized samples per test session with a 10 min interval between samples and a 15 min break after the third sample to reduce fatigue. Warm-up sessions were conducted prior to each session. Two sessions per day were conducted. Sensory evaluation was replicated twice for a total of 16 test sessions. Sensory testing was performed by panelists in booths separated by partitions with red incandescent lighting.

Sausage patties (1.25 cm thick) were cooked to 80°C, sliced into six wedge-shaped pieces and immediately served warm (32°C) to panelists. Each panel member received six randomly selected wedges for flavor and texture evaluation for each sausage treatment. Distilled water and unsalted crackers were provided to cleanse the palate between samples. Expectorant cups were available and panelists were instructed not to swallow samples to minimize taste fatigue. Scores were averaged for each attribute for each treatment.

Color evaluation by panel

Fresh sausage patties were evaluated for lean and fat color, and the amount of visible konjac flour gel particles by a seven-member panel at the end of each storage period. Panelists were graduate students and technicians from the Meat Science section who were not members of the sensory panel. Sausage patties were randomly assigned positions on a black fiber board background and evaluated under white fluorescent light (1600 lumens/m^2). Each panel member evaluated lean color using an 8 point scale (1 = greyish-white; 8 = dark red) and fat color (1 = extremely dark brown or green; 8 = white). To estimate the influence of added konjac flour gel on the color of raw sausage patties, panelists evaluated the percent konjac flour gel particles visible on the exposed surface of the raw sausage patties. Detection of visible konjac flour gel particles was rated on an 8-point scale with 1 = abundant (>30%); 8 = none (0%). Training sessions were held in which panelists evaluated several sausage products containing 0–40% caramel colored konjac flour gel. Color standards (National Pork Producers Council, 1988) were used as reference scales for lean color. Reference scales for fat color were developed from fat samples with varying degrees of whiteness. Warm-up sessons held prior to each color determination session included sausage products containing 0–40% konjac flour gel and samples of the ground konjac flour gel utilized in the study. One evaluation session per day was conducted with 4 samples/session evaluated in duplicate. Sensory color determination means were reported as the average of 2 means/treatment/storage period.

Allo-Kramer shear

Allo-Kramer shear force (kg/g) was determined by shearing sausage strips (2.5 cm wide) removed from the center of sausage patties cooked to 80°C. Six strips/treatment were equilibrated to 25°C and sheared using a multi-bladed Allo-Kramer shearing device attached to an Instron Model 1011 Universal Testing Machine (Instron Corp., Canton, MA). The full scale load was set at 100 kg with the crosshead speed set at 200 mm/min. Peak shear force was recorded and divided by sample weight of each 2.5 cm strip to calculate shear force in kg force/g sample. Data were reported as the average of 6 readings/treatment.

Color evaluation

A Hunter Model JB-1201M Spectrocolorimeter (Hunter Associates Laboratory, Reston, VA) was used to determine Hunter L, a and b values on raw sausage patties where L = reflectance of light, a = redness and b = yellowness. All measurements were taken at the end of each storage period. Four 1.27 cm slices were obtained from each sausage treatment and two measurements were made on each of four patties. The center of one exposed interior surface was measured, and a measurement made on the opposite side. The colorimeter was standardized using a white blank (Illuminant D65 10°; x = 79.31, y =

Table 1—Treatment means for pre- and post-cooking[a] chemical composition of low-fat prerigor pork sausage patties with or without konjac flour gel

		Treatments[b]				Contrasts[c]		
						C1	C2	C3
Measure (%)	SEM[d]	Control 40F	0KF	10KF	20KF	0KF vs control	10KF vs control	20KF vs control
Raw								
Moisture	0.18	48.96	69.06	70.63	73.38	0.001	0.001	0.001
Fat	0.28	37.12	9.99	9.08	8.14	0.001	0.001	0.001
Protein	0.11	13.23	19.47	17.85	16.14	0.001	0.001	0.001
Ash	0.01	1.99	2.31	2.39	2.42	0.001	0.001	0.001
Carbohydrate[e]		1.02	1.02	1.02	1.02			
Kcal[f]		391.00	171.87	157.20	141.90			
Cooked[g]								
Moisture	0.26	38.55	59.19	61.23	62.30	0.001	0.001	0.001
Fat	0.26	43.67	13.72	13.11	12.77	0.001	0.001	0.001
Protein	0.14	19.75	26.91	25.80	23.78	0.001	0.001	0.001
Ash	0.02	2.42	2.67	2.79	2.95	0.001	0.001	0.001
Carbohydrate[e]		1.02	1.02	1.02	1.02			
Kcal[f]		476.38	235.61	225.66	214.48			
Difference[h]								
Moisture	0.19	−12.41	−9.86	−9.41	−11.08	0.001	0.001	0.022
Fat	0.21	6.55	3.73	4.03	4.63	0.001	0.001	0.003
Protein	0.16	6.52	7.44	7.95	7.64	0.046	0.003	0.017
Ash	0.02	0.53	0.36	0.40	0.43	0.272	0.646	0.132
Kcal[f]		+85.38	+63.74	+68.46	+72.58			

[a] Proximate analysis conducted on samples taken at Day 0.
[b] 40F = 40% fat control; 0KF, 10KF and 20KF = 0, 10 and 20% konjac flour gel.
[c] P-values for predetermined contrast comparisons.
[d] Standard error of the mean.
[e] Estimate of calories contributed by the carbohydrate composition of konjac flour at 2.5% konjac flour in the gel.
[f] Estimated Kcal based on Atwater values reported on a 100 g per serving basis.
[g] Cooked to an internal temperature of 80°C.
[h] Difference due to cooking = cook analysis − raw analysis.

84.16 and z = 88.22) with a 30 mm aperture. Data were reported as means of eight measurements from each treatment and control sausage.

Microbiological analysis

On each sampling day, two 10-g samples were excised from the interior of randomly selected sausage chubs with a sterile scalpel and forceps from each treatment, placed in a Stomacher bag containing 99 mL of sterile 0.1% peptone (Difco) diluent and pummeled for 1 min in a Stomacher-400 (Tekmar Company, Cincinnati, OH). Aerobic plate counts (APC) were determined by plating 1 mL (0.25 mL per plate, 4 plates total) of the sample homogenate, and 0.1 mL of appropriate decimal dilutions on prepoured and dried tryptic soy agar (TSA, Difco) plates. The dilutions of sample homogenate were spread over the surface of the plates with a sterile bend glass rod. Plates were incubated for 48 hr at 25°C. On each sampling day, the number of colonies on a countable plate was divided by 2 and then reported as the $\log_{10}$ number of colony forming units (CFU) per gram. Duplicates of each treatment combination were tested.

Statistical analysis

Data were analyzed by General Linear Models (GLM) procedure of the Statistical Analysis System (SAS Institute, Inc., 1988) as a factorially arranged 4 × 4 randomized complete block design with treatment (fat and added konjac flour gel) and days of storage (0, 7, 14 and 21) as main effects. Predetermined contrast comparisons were made by comparing all treatments to the control sausage. When the main effect of days of storage was significant, means were separated using Tukey's studentized range test (HSD). A predetermined significance level of $P < 0.05$ was used for these comparisons. All treatment combinations were replicated three times, utilizing batch (sausage produced from 1 hog/day) as a blocking factor.

RESULTS & DISCUSSION

Proximate composition

All treatment sausages were different ($P < 0.001$) from the 40F control, exhibiting a higher percentage for raw moisture, protein and ash and lower percentage fat content (Table 1). Similar, but proportionally different results were observed for cooked proximate analysis. Cooking decreased the overall percent moisture of all treatments and the control, while increasing percent fat, protein and ash. Higher percent moisture in the raw 0KF, 10KF and 20KF treatments could be attributed to higher lean content. The 10KF and 20KF treatments were higher in percent moisture than the 0KF treatment due to addition of konjac flour gel.

The 40F control was lower (≈3%) in raw fat than the targeted fat level of 40%, while the 0KF treatment was very close (9.99%) to the targeted raw fat content (10%). The 10KF and 20KF treatments were slightly lower (≈10 and 20%, respectively), than the 10% target. This was caused by a dilution effect of the added konjac flour gel, since the meat block, prior to konjac flour gel addition, would be ≈9.99% (10KF) and 9.77% (20KF), respectively. Percent protein for all raw samples treated was higher than the control due to the higher lean and lower fat content of treated sausages. The raw 0KF treatment had higher protein content than the 10KF and 20KF treatments, due to the higher amount of lean. Percents ash for all raw sample treatments were higher than the control, with the 10KF and 20KF samples having the highest values. Addition of seasoning to the konjac flour gel during formulation may explain this variation.

Cooking to an internal temperature of 80°C resulted in increases in percent fat, protein and ash and a decrease in percent moisture. For raw vs cooked ash, differences were not significant ($P > 0.05$). The 0KF and 10KF treatments appeared similar on a raw basis for percent moisture, fat and ash, and remained proportionally similar on a cooked basis. However, the 20KF treatment, though different from the control in percent raw moisture, appeared to lose almost as much moisture as the 40F control.

Caloric content

As fat content decreased or level of konjac flour gel increased, in both raw and cooked patties, total calories declined (Table 1). Percent reductions in calorie content of raw treatment patties from the 40F control were 56.04, 59.79 and 63.71% for the 0KF, 10KF and 20KF sausages, respectively.

Table 2—Treatment effects on shelf-life, yield, shear force and color attributes of low-fat prerigor pork sausage patties with or without konjac flour gel

		Treatments[a]				Contrasts[b]		
						C1	C2	C3
Analysis	SEM[c]	Control 40F	0KF	10KF	20KF	0KF vs control	10KF vs control	20KF vs control
Shelf-life attributes								
pH	0.01	6.12	5.98	6.02	6.07	0.002	0.022	0.237
TBA[d]	0.01	0.18	0.12	0.11	0.14	0.004	0.001	0.035
Log (Log_{10} CFU/g)	0.04	4.30	4.22	4.27	4.27	0.660	0.710	1.000
Yield/shear force								
Yield[e] (%)	0.31	71.80	75.79	75.14	72.52	0.001	0.006	0.427
Cook loss[f] (%)	0.31	27.49	24.05	24.86	28.20	0.001	0.006	0.427
Cook diff.[g] (g)	0.68	56.35	51.98	53.29	59.65	0.031	0.124	0.098
Shear force (kg/g)	0.03	1.51	1.98	1.83	1.65	0.001	0.001	0.111
Hunter color[h]								
L	0.20	61.08	46.96	46.54	44.16	0.001	0.001	0.001
a	0.12	11.12	13.86	13.43	13.15	0.001	0.001	0.001
b	0.15	16.32	15.22	15.10	14.69	0.016	0.008	0.001
Color panel[i]								
Lean	0.03	5.80	6.37	6.27	6.47	0.001	0.001	0.001
Fat	0.03	6.46	6.60	6.70	6.76	0.158	0.013	0.002
Visible konjac	0.05	7.29	7.38	6.16	5.61	0.553	0.001	0.001

[a] 40F = 40% fat control; 0KF, 10KF and 20KF = 0, 10 and 20% konjac flour gel.
[b] P values for predetermined linear contrasts.
[c] Standard error of the mean.
[d] Mg malonaldehyde/kg sample.
[e] Percent yield = [(Raw weight − cooked weight) ÷ raw weight] × 100.
[f] Cook loss = Percent cook loss: 100 − percent yield.
[g] Cook diff. = Raw weight − cooked weight difference in g.
[h] Hunter Lab: L = reflectance (whiteness); a = redness; b = yellowness.
[i] Lean and fat color, visible konjac flour gel particles based on an eight point scale: lean color; (1 = greyish-white; 8 = dark red); fat color; (1 = extremely dark brown or green; 8 = white); visible konjac; (1 = abundant: >30%; 8 = none: 0%).

Table 3—Influence of days of storage on shelf-life, yield, shear force and color attributes of low-fat prerigor pork sausage patties with or without konjac flour gel

		Days of storage			
Analysis	SEM[d]	0	7	14	21
Shelf-life attributes					
pH	0.01	6.28[a]	5.84[c]	6.03[b]	6.02[b]
TBA[e]	0.01	0.19[a]	0.14[ab]	0.10[b]	0.12[b]
Log (Log_{10} CFU/g)	0.04	3.98[b]	4.04[b]	4.08[b]	4.98[a]
Yield/shear force					
Yield[f] (%)	0.31	73.51[a]	74.19[a]	73.19[a]	74.37[a]
Cook loss[g] (%)	0.31	26.50[a]	26.42[a]	26.42[a]	25.87[a]
Cook diff.[h] (g)	0.68	55.90[a]	54.14[a]	55.64[a]	55.58[a]
Shear force (kg/g)	0.03	1.82[a]	1.74[a]	1.73[a]	1.69[a]
Hunter color[i]					
L	0.20	48.55[b]	49.84[ab]	49.82[ab]	50.53[a]
a	0.12	12.53[a]	13.19[a]	12.90[a]	12.94[a]
b	0.15	17.23[a]	15.58[b]	14.38[c]	14.13[c]
Color panel[j]					
Lean	0.03	6.08[b]	6.29[ab]	6.38[a]	6.17[ab]
Fat	0.03	6.66[ab]	6.68[ab]	6.73[a]	6.46[b]
Visible konjac[k]	0.05	6.38[b]	6.58[ab]	6.68[ab]	6.80[a]

[abc] Means with a common superscript are not significantly different (P > 0.05).
[d] Standard error of the mean.
[e] Mg malonaldehyde/kg sample.
[f] Percent yield = [(Raw weight − cocked weight) ÷ raw weight] × 100.
[g] Cook loss = Percent cook loss: 100 − percent yield.
[h] Cook diff = Raw weight − cooked weight difference in g.
[i] Hunter Lab: L = reflectance (whiteness); a = redness; b = yellowness.
[j] Lean and fat color and visible konjac flour particles based on an eight point scale: lean color; (1 = greyish-white; 8 = dark red); fat color; (1 = extremely dark brown or green; 8 = white); visible konjac; (1 = abundant: >30%; 8 = none: 0%).

Percent reductions in calorie content of cooked treatment patties from the 40F control were 50.54, 52.63 and 54.98% for the 0KF, 10KF and 20KF sausages, respectively. Reductions in raw and cooked percentages for total fat averaged ≈75% and 69% respectively, for all treatments. Reductions in cooked caloric content were also noted for low-fat (10%) ground beef patties made with 0.5% carrageenan (22–23% or 55–60 Kcal/100 g serving) used as a water binder (Huffman and Egbert, 1990).

Under current regulations, the allowable moisture for fresh pork sausage is 4 times the protein concentration plus 3% (CFR, 1990). Since the 20KF sausage contains 16.14% raw protein and 73.38% raw moisture, only 67.56% would be allowed under the current guideline. The 20KF sausage exceeded the maximum limit by almost 6%, and would need to be reformulated to conform to existing regulations.

Although incremental addition of konjac flour gel decreased total calories, this reduction was not as great as that effected by reducing total fat from the sausage product. However, the real value of incorporating konjac flour gel into a ground meat system is to reduce fat while preserving sensory and textural attributes usually contributed by the fat.

pH, TBA and microbiological analyses

The pH for 0KF and 10KF treatments was slightly lower than the control, but the 20KF treatment was not different (Table 2). Lower pH values for the 0KF and 10KF treatments may have been due to variations in available glycogen antemortem, rates of postmortem glycolysis between animals or time required to crust freeze samples. The pH differences were small between the 20KF and 40F sausages, and may have been a consequence of inherent tissue variations between or within muscle groups.

The pH at Day 0 was highest in comparison to storage Days 7, 14 and 21 (Table 3). A decline in pH to 5.84 occurred by Day 7 with a gradual rise in pH by Day 14 and an apparent leveling through Day 21. Day 7 had the lowest pH values, possibly due to anaerobic glycolysis and sufficient generation of carbon dioxide to produce a modified atmosphere in chub packed sausages. The slight rise in pH at Day 14 and maintenance at that level through Day 21 may have been due to dissipation of the CO_2 within the chub pack, or buffering of the naturally occurring phosphates. Lin et al. (1979) reported a mean pH value of 5.82 for prerigor pork sausage stuffed in fibrous casings and stored at 2°C over a 28-day period.

Raw sausage TBA values were significantly different between treatment and control sausages and over days of storage

Table 4—Mean sensory panel scores for aromatic, mouthfeel, taste, and texture attributes[a] of low-fat prerigor pork sausage patties with or without konjac flour gel

		Treatments[b]				Contrasts[c]		
						C1	C2	C3
Attribute	SEM[d]	Control 40F	0 KF	10 KF	20 KF	0 KF vs control	10 KF vs control	20 KF vs control
Aromatics								
Pork flavor	0.03	5.27	5.48	5.58	5.33	0.014	0.001	0.486
Cook pork flavor	0.04	3.47	2.54	2.54	2.51	0.001	0.001	0.001
Livery	0.01	0.02	0.01	0.00	0.00	0.382	0.083	0.083
Starchy	0.03	2.32	1.64	1.72	1.76	0.001	0.001	0.001
Spice complex	0.31	2.50	2.75	2.92	2.84	0.010	0.001	0.001
Mouthfeel								
Metallic	0.01	1.11	1.11	1.11	1.13	0.898	0.991	0.585
Astringent	0.01	1.06	1.06	1.07	1.10	0.907	0.736	0.153
Taste								
Salt	0.07	5.01	4.94	5.18	5.35	0.692	0.351	0.066
Sweet	0.01	1.01	1.01	0.98	0.98	1.000	0.233	0.233
Sour	0.01	0.02	0.02	0.00	0.01	1.000	0.196	0.516
Bitter	0.01	0.00	0.05	0.02	0.03	0.006	0.372	0.077
Mouth coating	0.02	1.44	1.28	1.29	1.30	0.018	0.032	0.044
Spice aftertaste	0.04	1.57	1.81	1.85	1.79	0.023	0.008	0.032
Texture								
Springiness	0.02	2.15	2.91	2.84	2.65	0.001	0.001	0.001
Cohesiveness	0.02	2.23	2.91	2.86	2.69	0.001	0.001	0.001
Chewiness[e]	0.11	20.39	22.76	22.76	21.97	0.001	0.001	0.001
Hardness	0.02	2.17	2.84	2.75	2.61	0.001	0.001	0.001
Density	0.02	2.65	3.08	3.03	2.88	0.001	0.001	0.001
Fracturability	0.02	2.10	2.41	2.42	2.28	0.001	0.001	0.001
Juiciness	0.06	6.33	5.58	5.84	5.82	0.001	0.002	0.001

[a] Based on the Spectrum™ universal scale: 0 = absent, 15 = extremely strong
[b] 40F = 40% fat control; 0KF, 10KF and 20KF = 0, 10 and 20% konjac flour gel.
[c] P values of predetermined contrast comparisons.
[d] SEM = Standard error of the mean.
[e] Chewiness scores are based on the total number of chews required before the sample is ready to swallow.

(Tables 2 and 3). However, none of the TBA values would indicate concern for off-aromas or flavors. The 40F control had the highest TBA value and the 10KF had the lowest. Apparently, konjac flour gel does not contribute to lipid oxidation. The lower fat levels may also influence TBA by limiting the substrate available for lipid oxidation. Bentley et al. (1987) reported that hot-boned sausage stored at −1°C had lower TBA values than cold-boned sausage stored at the same temperature. In addition, they observed a significant increase in TBA values from 28 to 56 days of storage.

Aerobic plate counts (APC's) were not different between controls and treatment sausages (Table 2). APC's (Table 3) remained constant through Day 14 but increased by Day 21. The total number of microorganisms at Day 21 were slightly less than 5.0 $\log_{10}$ CFU/g which is below the typical spoilage level of ≈7.0 $\log_{10}$ CFU/g. The combination of pH and salt may account for retarding microbial growth. Bentley et al. (1987) reported total bacteria counts of prerigor sausage were higher than chilled sausage at Day 0 and that microbial growth of hot-boned pork over time decreased shelf-life. Lin et al. (1979), however, reported no difference in numbers of psychrotropic bacteria in prerigor sausage vs chilled sausage. No significant treatment × day interactions existed for pH, TBA or microbiological analyses.

Cook yield and shear force values

Cook yield data (Tables 2 and 3) indicate higher processing yields with the 0KF and 10KF treatments. Cooked yield for 20KF was not different from the control and was lower than the 0KF and 10KF sausage treatments. Most of the weight loss in all sausage patties can be attributed to moisture loss during cooking as shown by cooked moisture proximate analysis (Table 1). Days of storage (Table 3) had no effect on percent yield or shrink of treatment or control sausages.

Allo-Kramer shear values (Table 2) indicated significantly less shear force (kg/g of sample) was required for a 2.5 cm strip of the 40F control than the 0KF or 10KF sausages. The 0KF sausage had the highest shear value but the 20KF and 40F control were not different. As konjac flour gel level increased, shear force decreased, confirming studies by Ahmed et al. (1990) who reported that shear force decreased with increasing fat levels (15, 25 and 35%) and added water (3 and 13%) in pork sausage patties.

Color

Hunter Lab values were significantly different than the control for reflectance (L), redness (a) and yellowness (b). "L" and "b" values for the 40F control were higher and the "a" value lower for all treatment sausages. The 0KF and 10KF sausages had similar L, a, and b values while the 20KF values were slightly lower (Table 2). Hunter "L" values increased slightly after 21 days but less than 2% from Day 0 to Day 21 (Table 3). This could be due to pigment fading as a result of oxidation of myoglobin to metmyoglobin. Yellowness (b) values decreased at Day 7 and again at Day 14 but did not change after 21 days (Table 3). Previous studies showed that Hunter "L" and "b" values increased in chilled fresh pork sausage varying from 15 to 40% fat, while "a" values decreased as fat level increased (Reagan et al., 1983; Ahmed et al., 1990). Additions of konjac flour gel tended to decrease Hunter "L" values for reflectance and increase "a" values when compared to the 40F control. The addition of caramel coloring may have been more important in this observation than the konjac flour gel due to the darkening effect of caramel.

Color panel scores (Table 2) for treatments were higher than the control sausage for lean color; the control had moderately pink color and all treatments had bright pink lean color. No treatment was rated as having undesirable dark red (8) or greyish-white (1) lean color. Scores for fat color showed no differences between the 0KF and control sausages whereas the 10KF and 20KF sausages were significantly more white than controls. All treatment and control sausages were rated as having moderately creamy white fat color. Shackelford et al. (1990) stated that percent fat was the only factor affecting

visual appearance when rated by a consumer panel, with higher fat (35%) sausages rated lower than lower fat (15 and 25%) sausages.

Visible gelled konjac flour particle scores revealed that panelists detected no difference between the 40F control and 0KF treatment sausages. Panelists, however, detected the added konjac flour in the 10KF and 20KF sausages, rating them as having more konjac flour gel than the control. Panelists rated the 40F control and 10KF sausages as having trace amounts of visible gelled particles and 10KF and 20 KF sausages as having slight and small amounts, respectively. Although panelists detected added konjac flour gel on exposed surfaces of 10KF and 20KF patties, the konjac flour gel was perceived to be less than the amount actually present.

Sensory attributes

The effect of panelists was not significant ($P > 0.05$). No differences were found between control and treatment comparisons for liver, metallic, astringent, salt, sweet, and sour sensory attributes (Table 4). Higher pork flavor scores indicated that the 0KF and 10KF treatments were different from the control, but the 20KF treatment was not different. The 40F control was higher than all treatments for cooked pork fat flavor. All treatments were rated significantly lower than the control for starchy flavor. This unexpected observation may be partially due to the greater fat content of the 40F control, which panelists may have perceived as "starchy". All treatments were higher than the control for spice complex with the 10KF and 20KF treatments having higher scores possibly due to addition of spices in the konjac flour gel formulation. All treatment sausages had significantly higher sensory scores than the control for springiness, cohesiveness, chewiness, hardness, denseness, and fracturability, but were lower in juiciness. The 40F control was less springy and cohesive, required less force to fracture and to deform, was less dense and required fewer chews.

Although treatment sausages were higher than the control for all sensory textural attributes, the addition of konjac flour in the 10KF and 20KF treatments resulted in decreasing textural attribute scores and tended to lessen the force necessary to shear. This was magnified as the konjac flour gel level increased from 0 to 20%. This indicated that addition of konjac flour gel may have enhanced textural properties over the 0KF sausage by making the 10KF and 20KF sausages more like the 40F control. Higher springiness and cohesiveness and perhaps lower juiciness scores might be expected in treatment sausages due to a higher protein content with more myofibrillar proteins available for binding. No differences due to days of storage were found for any sensory attribute (Data not shown).

CONCLUSIONS

LOW-FAT, PRERIGOR FRESH PORK SAUSAGE manufactured with 10 to 20% levels of added konjac flour gel, had substantially less fat and calories from fat while maintaining sensory and textural attributes similar to higher-fat sausages. With some modifications, addition of konjac flour gel could be utilized in current processing systems to manufacture prerigor fresh pork sausage. The major benefit would be reduction of textural differences between low-fat and higher-fat sausages.

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Mention of a trade name, proprietary product or specific equipment is necessary to report factually on this research project; however, Texas A&M University neither guarantees nor warrants use of a specific product, and the use of such a product implies no preference of the product to the exclusion of other suitable products.

Time-Temperature Indicator using Phospholipid-Phospholipase System and Application to Storage of Frozen Pork

S.H. YOON, C.H. LEE, D.Y. KIM, J.W. KIM, and K.H. PARK

ABSTRACT

A novel time-temperature indicator (TTI) using phospholipid-phospholipase was developed to monitor quality change of frozen foods during storage. The TTI was more reliable than the lipase system and monitored quality changes of frozen pork during storage. The TTI was designed for reactions at sub-zero temperatures because the substrate emulsion would be stable at such temperatures. The TTI contained glycerol and sorbitol as anti-freeze reagents and was designed to show distinctive color changes according to pH by mixing bromothymol blue, neutral red, and methyl red. The TTI had an activation energy of 32.12 kcal/mol, suitable for many food reactions.

Key Words: pork, time-temperature indicator, phospholipid, phospholipase

INTRODUCTION

BOTH TEMPERATURE and time are important for quality control of frozen foods during storage and transportation. Singh and Wells (1985) indicated that the major physicochemical changes that occur during frozen storage are a result of oxidation of lipids, denaturation of proteins, discoloration, sublimation of ice, and recrystallization of ice crystals. Exposure to high temperature and/or fluctuation of storage temperature could produce cumulative adverse effects on frozen food quality. The rate of food quality deterioration increases logarithmically with increase of storage temperature and such reactions are irreversible. International Institute of Refrigeration recommends −18°C as the maximum temperature for storage of frozen foods. Londahl (1982), however, reported that more than 45% of the frozen products (on average) were stored above −15°C during in-transit spot checks conducted in five European countries.

The knowledge of temperature fluctuation was used as a factor in creating a device for consumers to determine storage life of a product. Various types of TTI have been developed and are based on mechanical, chemical, microbial, or enzymatic irreversible changes for visualization of the degree of food deterioration (Schoen and Byrne, 1972; Kramer and Farquhar, 1976; Singh and Wells, 1985; Wells and Singh, 1985; Mistry and Kosikowski, 1983; Shellhammer and Singh, 1991; Fu et al., 1991). Commercial TTIs have been studied for correlation with quality changes of frozen and chilled foods.

The I-POINT[R] Time-Temperature Monitor (I-POINT Technologies, Malmo, Sweden), a commercial indicator, would respond continuously over all time-temperature history. This type TTI is easy to use and low cost to manufacture compared to other TTIs. Such indicator consists of two inner transparent plastic pouches. One pouch contains a pH indicator and enzyme solution and the other contains a substrate solution. After activating the indicator by breaking the seal and mixing solutions of the two pouches, enzymatic hydrolysis of the substrate causes a change in pH of the solution. The pH change is monitored by a pH indicator and the color of the solution changes gradually. The hydrolysis reaction is accelerated by increase in reaction time and/or temperature.

Various lipid hydrolyzing enzymes such as lipase could be used for this kind of TTI (Taoukis et al., 1991), but stable substrate emulsion is necessary to produce a reproducible reaction rate. The emulsion of triglyceride, a substrate for lipase, may be unstable. It is practically impossible to obtain uniform droplet size in the emulsion and it cannot be stored over very long periods, especially at low temperatures. Therefore, the device using an unstable emulsion system is not reliable to predict enzymatic reactions to an acceptable accuracy and reproducibility. In order to improve the stability of substrate emulsion, many attempts have been made to adsorb substrate onto inorganic carriers of silica or polyvinyl chloride, which have large surface areas. This helped provide increased enzymatic activity and reproducibility of reactions (Agerhem and Nilsson, 1981). However, phospholipid, a substrate of phospholipase, with both hydrophilic and lipophilic portions in the molecule maintains a stable emulsion system. Commercial preparations of phospholipids derived from soy bean oil are used extensively in manufactured foods.

Our objective was to develop a full-history TTI using a phospholipid-phospholipase system. Our goal was to improve the stability of the substrate emulsion of a TTI at sub-zero temperature, thereby making it possible to monitor quality changes of frozen foods during storage. The device was evaluated for reproducibility of the enzyme reaction and was applied to predict quality changes in frozen pork with storage time and temperature.

MATERIALS & METHODS

Enzymes and reagents

Lipase (Sigma Chemicals, St. Louis, MO) from *Candida cylindracea* was used after fractionation with 50% ammonium sulfate and dialysis vs 0.05M phosphate buffer. Phospholipase A_2 (Sigma Chemicals) separated from porcine pancreas was used. Bromothymol blue (BTB) solution was made by dissolving 0.1g BTB in 16 mL 0.01N NaOH solution and then diluted with 250 mL distilled water. Methyl red (MR) and Neutral red (NR) solutions were made by dissolving 0.2g of MR and 0.1g of NR in 50% and 60% (v/v) ethanol, respectively, to a final volume of 100 mL.

Preparation of TTIs

Two TTIs were prepared, one based on lipase-triglyceride system and the other based on phospholipase-phospholipid system. The TTIs were composed of an enzyme, substrate emulsion, salts, antifreeze agents, pH indicators, and emulsion stabilizer. The substrate emulsion for lipase was prepared by sonicating the mixture of 1.25 mL olive oil and 23.75 mL 10% (w/v) gum arabic solution (Fisher, Desmembrator Model 300) for 1.5 min (Park et al., 1975). The substrate emulsion for phospholipase was prepared by sonicating 25 mL of 3% (w/v) soya lecithin for 3 min (Dennis, 1973) (See Table 1 for composition).

The authors are affiliated with the Dept. of Food Science & Technology and Research Center for New Bio-Materials in Agriculture, College of Agriculture & Life Sciences, Seoul National Univ., Suwon, 441-744, Korea. Address inquiries to Dr. K.H. Park.

Table 1—Composition of time-temperature indicators

	Reagents	Amount
Buffer	0.1M Tris (pH8.0)	1.25 mL
	0.15M $CaCl_2$	3.75 mL[a]
		7.5 mL[b]
	3M NaCl	10 mL
Substrate	triglyceride[a] or	
	lecithin[b]	20 mL
Antifreeze	glycerol	15 mL
agent	sorbitol	45.8 g
pH-Indicator	bromothymol blue	8 mL
mixture	methyl red	4 mL[a]
		2 mL[b]
	neutral red	0.1 mL
Emulsifier	sodium deoxycholate	0.5 g

[a] TTI using lipase.
[b] TTI using phospholipase.

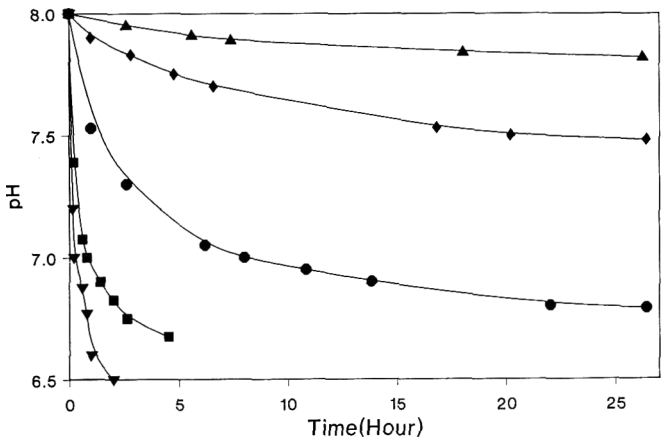

Fig. 1—Temperature dependence of pH decrease caused by lipid hydrolysis in the phospholipid-phospholipase system. The curve marked with ▼ represents the reaction at 20°C, ■ at 10°C, ● at 0°C, ♦ at −10°C, and ▲ at −15°C.

Lipid hydrolysis

The hydrolysis reaction was initiated by adding a pre-determined amount of enzyme into the reaction solution, pH 8.0. The amount of free fatty acid liberated by enzymatic hydrolysis was determined by the pH-stat method (Roberts et al., 1978). One unit of enzyme activity was defined as the amount of enzyme which produced 1 μmole of free fatty acid/min.

Prediction of time for color change of TTIs

Assuming that production of free fatty acid by the enzyme followed a first order reaction, it could be expressed by the following equation (1):

$$\frac{d[A]}{dt} = -k_a \cdot [A] \qquad [1]$$

where, [A] is the concentration of the substrate (mM), k_a is the reaction rate constant (mM/min), t is the reaction time (min). The concentration of the substrate was derived from the pH of the reaction system, empirically based on 2.5 mM of fatty acid decreasing the pH of the solution from 8 to 7.

Measurement of the quality change of frozen pork

The quality change of ground frozen pork during storage time was measured using the thiobarbituric acid (TBA) test (Turner et al., 1954). Five milliliters of 20% TCA in 2M phosphoric acid and 10 mL 0.01M TBA solution in 90% acetic acid were added to 5g meat in a 50 mL centrifuge tube. After heat treatment in boiling water for 30 min, the tube was kept in an ice bath for 10 min. Solid fat was removed and 15 mL isoamyl alcohol-pyridine mixture (2:1) was added. After vigorous shaking for 2 min, the mixture was centrifuged at 6,000 rpm for 15 min and the absorbance of the organic solvent extract layer was measured at 532 nm against the solvent blank. The absorbance at 532 nm was taken as the TBA value.

Table 2—Reaction rate constants of lipid hydrolysis reactions by the two lipases at various temperatures

Temp	Phospholipase		Lipase	
(°C)	k_a (1/min)	SD[a]	k_a (1/min)	SD
30	0.0012919	0.0001459	0.00195278	0.000228
20	0.0006749	7.942E-05	0.00134049	0.000282
10	0.0002048	3.875E-05	0.00079098	0.00021
0	3.706E-05	8.561E-06	0.0003010	0.000116
−5	1.498E-06	4.081E-06	0.00024044	5.79E-05
−10	2.416E-06	4.528E-07	2.3691E-05	6.58E-06
−15	8.772E-07	1.769E-07	7.6187E-07	1.56E-07
−18	5.524E-08	1.779E-08	9.5927E-08	1.2E-08

[a] SD; standard deviation.

RESULTS & DISCUSSION

TTIs using enzymatic reactions

TTIs to be used for monitoring frozen food quality should employ a reaction system that is not frozen at sub-zero temperatures. Generally, salts or polyhydroxyl alcohols are used to lower the freezing point of solutions. Glycerol and sorbitol were chosen to be included because they are very soluble and can lower the freezing point efficiently. They also stimulated activity of the enzymes. When glycerol and sorbitol were added together, activity of the enzymes was much higher than when no glycerol, or glycerol alone, was added. This may have been due to sorbitol action as an emulsifier to enlarge the surface of the interphase between olive oil and water so the space where enzymes could act on substrate was increased. However, when glycerol was added at higher concentrations (> 30%, v/v), glycerol was hydrolyzed to release fatty acids. When 15% (v/v) glycerol was added, 8 μmole or 10 μmole fatty acids were released in presence of lipase or phospholipase, respectively (negligible for the entire reaction). As much as 2.5 mM fatty acid was required to lower pH of the reaction mixture by 1. Therefore, 15% (v/v) glycerol was added to the mixtures. At that concentration the freezing point of the mixture was calculated to be −21°C, from molar concentrations of added salts and sorbitol. The TTI reaction mixture was confirmed as not frozen at temperatures as low as −30°C.

In order to make the most appropriate pH indicator mixture, BTB, NR, and MR were mixed at various ratios over pH ranges of 5.75 to 8.0. The most distinctive color change was detected when BTB, NR, and MR were mixed at the ratio of 8:2:0.1 (v/v).

Rate constant and reproducibility of phospholipase system

pH change of the TTI was monitored between 30°C and −15°C (Fig. 1). The pH of the reaction at 0°C and above dropped sharply during the first hour and then slowed considerably. This could be due to the limiting amount of substrate in the reaction. The reactions at 0°C, −10°C, and −15°C were monitored an additional 20 hr. The pH of the reaction did not decrease further during the time at −15°C. On the other hand, pH of the reaction dropped gradually until it reached pH 7.5 after 20 hr at −10°C or 6.8 at 0°C. The reaction rate constant, k_a, was determined for each temperature by using equation (1), assuming the reactions were first order (Table 2). The reaction rate constant was largely dependent on temperature and was used to predict the time for color change.

Standard deviations of the initial rate constants for lipase and phospholipase systems were determined (95% confidence) from experiments repeated six times at each temperature using the pH-stat test (Table 2). For each reaction, 7.4 units of lipase and 4.8 units of phospholipase were used. Standard deviations of the reaction rate constant of phospholipase were less than

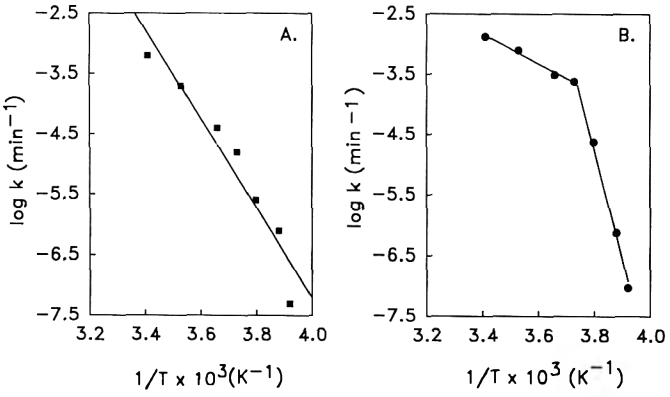

Fig. 2—Arrhenius plot of reaction rates in the range 20 to −18°C. (A) phospholipid-phospholipase system; (B) triglyceride-lipase system.

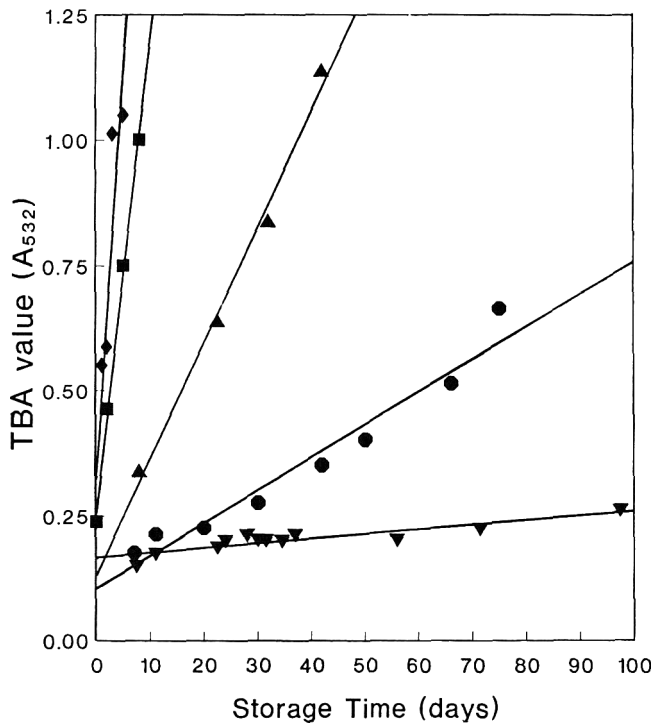

Fig. 3—Effect of temperature on increase in TBA values of ground pork during storage. The curve marked with ♦ represents TBA values of ground pork stored at 5°C, ■ at 0°C, ▲ at −5°C, ● at −10°C, and ▼ at −15°C.

those of lipase at all reaction temperatures (especially −10°C and −15°C). The relationship between reaction rate constant and temperature was interpreted by Arrhenius plot (Duggleby, 1981). The plot of the lipase reaction was not a straight line (Fig. 2, Panel B). The curve was very steep below 0°C, probably due to the increase of viscosity of the reaction solution. The calculated activation energy of lipase was 11.1 ± 0.78 kcal/mole above −5°C and 82.7 ± 3.91 kcal/mole below −5°C. The TTI showed a good linear fit (R^2 = 0.999) and a reasonable activation energy at temperatures > −5°C. However, the activation energy of the TTI < −5°C was too large to use for monitoring food deterioration, although it showed good linear fit (R^2 = 0.998).

The Arrhenius relationship of the phospholipase reaction and temperature was a straight line with a good linear fit (R^2 = 0.980; Fig. 2, Panel A). The activation energy of the phospholipase reaction was 32.12 ± 3.44 kcal/mole. Taoukis et al. (1991) analyzed various types of TTI and reported a TTI must have an activation energy of 25–30 kcal/mole, suitable for

Table 3—Reaction rate constants of TBA increments of frozen pork as related to storage temperature

Temp (°C)	k_b (TBA value/day)	Standard deviation
5	2.2×10^{-1}	2.6×10^{-2}
0	1.2×10^{-1}	1.9×10^{-2}
−5	1.7×10^{-2}	5.5×10^{-3}
−10	3.6×10^{-3}	1.2×10^{-3}
−15	1.6×10^{-3}	6.7×10^{-4}

many food reactions. The activation energy of the TTI was within 10% above the range. We concluded that the reproducibility of the reaction system of phospholipase was better than that of lipase and that it was more suitable to employ the phospholipase system for a TTI for monitoring food quality change during storage at very low temperature.

The time required for the TTI solution to reach pH 7.5 at various temperatures was plotted semi-logarithmically (data not shown). We could predict the time to reach a specific pH (or color) at specific storage temperature from the graph. This type TTI may be applied to other frozen foods by adjusting the amount of enzyme. However, the reaction rate of the enzyme in a TTI and the deterioration rate of food may not correlate with each other. Therefore, it would be important to select an enzymatic or chemical reaction which correlates with change of food quality.

Rate of deterioration during storage

In order to test the correlation between the TTI and frozen food quality change, ground pork was stored at various temperatures between 5°C and −15°C for 100 days. The quality loss of frozen pork was expressed by TBA value. When frozen pork was stored in a refrigerator at each reaction temperature, the TBA value increased linearly with storage time (Fig. 3). Therefore, we could express the change of TBA value by the following equation:

$$TBA_t = TBA_i + k_b \cdot t \qquad (2)$$

where TBA_i and TBA_t were the TBA value at 0 time and at a given storage time, k_b was the rate constant of the TBA increment (TBA value/day), and t was storage time (day). The rate constants of the TBA increments (k_b) are listed in Table 3. The TBA increments were dependent on storage temperature as well as storage time. From the Arrhenius plot of k_b vs storage temperature, the activation energy of frozen pork deterioration was 35.5 ± 6.05 kcal/mole. Taoukis and Labuza (1989) proposed that if activation energies of the food and the TTI differed by < 10 kcal/mole, then the two effective temperatures differed by < 1°C, resulting in an error of quality estimation of < 15%, which in many cases would be acceptable. The difference between activation energies of the TTI using phospholipase and frozen pork would be within the acceptable range when deviations in activation energy of pork were taken into account. This would result in an error of quality estimation < 15%. In order to monitor change in food quality accurately, a TTI activation energy should be closely matched to that of food quality factor. To meet such requirement, the activation energy of the TTI might be adjusted close to the food quality factor by changing composition of antifreezing reagents in the TTI, immobilizing the enzyme, or by fixing its contents on a strip of matrix.

Correlation between TTI and quality changes during storage

The TTI with pH between 8.0 and 7.5 would indicate that frozen pork had the TBA value in an acceptable range (0–0.5), pH between 7.5 and 7.0 uncertain (0.5–1.0), and pH between 7.0 and 6.5 unacceptable (1.0–1.5). Turner et al. (1954) have

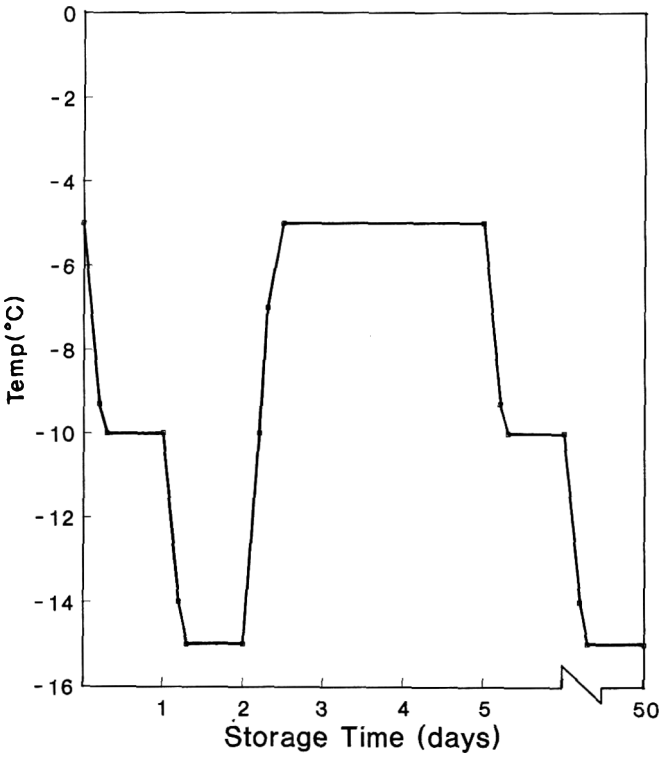

Fig. 4—Storage temperature variation profile for fluctuating temperature exposure.

Table 4—Predicted (P) and experimental (E) pH and TBA values

Time (day)	pH		TBA	
	P	E	P	E
0	8.0	8.0	0.16	0.16
5	7.3			
10	7.0	7.5	0.5	0.53
15	6.9		0.65	
20	6.7			
25	6.6	7.0	0.98	0.89
30	6.5		1.14	
35	6.4			
40	6.3			
45	6.3	6.5	1.39	1.38
50	6.3			

determined the rancidity in frozen pork by using the correlation between TBA and taste tests. Pork with TBA < 0.5 was judged as acceptable in taste, between 0.5 and 1 borderline and > 1.0 as unacceptable in taste by a panel. The amount of enzyme in the TTI was adjusted so that the pH of the reaction mixture reached 7.5, 7.0, and 6.5 when the TBA value of frozen pork became 0.5, 1.0, and 1.5, respectively, depending on storage time and temperature. Thus, the storage life of frozen ground pork could be estimated from the color of the TTI.

To examine the correlation between TTI and frozen food quality loss, the TTI and frozen pork were stored in a freezer programmed with various temperatures (Fig. 4). Changes of the TTI reaction color and TBA values of frozen pork under the fluctuating temperature conditions were compared with respective pH and TBA values predicted using the data of k_a, and k_b (Table 4). The predicted data were derived by a simulation program (not shown). After 10 days the pH of the TTI of frozen pork reached 7.5 and the TBA 0.53 (acceptable range). After 25 days they reached pH 7.0, TBA 0.89 (uncertain range) and after 45 days, pH 6.5, TBA 1.38 (unacceptable). These values correlated well with predicted data from the simulation program. The color of the TTI at each time point was clearly different from the others, enough to estimate pH of the TTI (data not shown).

CONCLUSIONS

ACTIVATION ENERGIES of ground frozen pork and TTI differed by 3.4 kcal/mole, which could result in an error of quality estimation in the acceptable range. The pH of the TTI and the TBA value of frozen pork fell into three ranges to indicate usability of frozen pork. These values were predicted using a simulation program under fluctuating temperature conditions and correlated well with the empirical data. The color change of the TTI with pH change correlated well with TBA value change of frozen pork. The TTI using the phospholipid-phospholipase system could be applied to other frozen foods.

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Sensitive Catalase Test for End-point Temperature of Heated Chicken Meat

C.Y.W. ANG, F. LIU, W.E. TOWNSEND, and D.Y.C. FUNG

ABSTRACT

Our improved method enables detection of catalase-related activities as a biochemical marker for estimating cooking end-point temperature (EPT). The broad range of inactivation temperature in chicken tissues was first determined to be > 68°C and < 72°C. Then samples were heated to EPTs from 69 to 71.5°C at 0.5°C intervals. The catalase activity at 23 and 37°C was followed up to 120 min. The probability of obtaining a positive result with an EPT of 69°C was ≥0.99 after 45 min when incubated at 23°C. This probability decreased to ≤0.03 as EPT increased to 71°C. Higher incubation temperatures (37°C) increased the probability of positive results.

Key Words: chicken meat, end-point temperature, catalase test

INTRODUCTION

TO INSURE THE INACTIVATION of harmful microorganisms in fully cooked animal products, USDA regulations require that they be heat-processed to specific internal end-point temperatures (EPT). Poultry rolls and other uncured poultry meat products must be heated to a minimum EPT of 71.1°C, cured/smoked poultry products to 68.3°C and roast beef to 62.8°C (USDA-FSIS, 1985a,b). Several analytical methods are used by the Food Safety & Inspection Service (FSIS) to verify EPTs of heat-processed meat products (Townsend and Blankenship, 1989). For example, a coagulation test is used to monitor maximum internal temperature of beef and pork products heat-processed at < 65°C (USDA-FSIS, 1986a). A residual acid phosphatase activity method (USDA-FSIS, 1986b) is used to determine maximum internal temperature (68.8°C) of imported canned hams, picnic hams and luncheon meat. The "bovine catalase test" was developed by Eye (1982) for detection of under-processing of rare roast beef and canned beef (62.8°C). No official method is available for determining the 71.1°C EPT specifically required for poultry products. A rapid, simple method to indicate whether poultry products had been processed to the required temperature is needed. Much research effort has been focused on development of new and/or improved methods for assessing the extent of heat treatments of meat and poultry (Townsend and Blankenship, 1989). However, no methods have been precise enough to meet FSIS criteria (detect EPT within ±0.5°C of the legal requirement for each product) (Ellis, personal communication, 1990). The enzymatic system seemed to provide the best potential. A slight change in product EPT may cause a slight change in enzyme structure associated with the enzymatic reaction. Thus the determination of enzymatic activity could indicate changes in EPT.

Catalase is widely distributed in biological systems, reacting with H_2O_2 to produce H_2O and O_2. The simplest procedure to test catalase activity is based on oxygen liberated upon reaction with H_2O_2 (Guibault, 1976). Eye (1982) reported that catalase was inactivated at 62.8°C for rare cooked roast beef. However, Spanier et al. (1990) reported that 50% of the catalase activity was retained even after heating diluted, filtered beef homogenate to 74°C. Melo et al. (1988) investigated the catalase activity in heat-treated pork as an indicator of the African swine fever virus. Preliminary data at Kansas State University indicated that heating ground beef to 65°C for 2 min destroyed "bovine catalase" but not bacterial catalase (Fung and Kustywati, personal communication, 1992). Our objective was to modify the "bovine catalase test" to develop a sensitive, improved method to assure adequacy of heat treatment of poultry meat.

Author Townsend is with the USDA-ARS, R.B. Russell Research Center, P.O. Box 5677, Athens, GA 30613. Author Liu is with the Dept. of Food Science & Technology, Univ. of Georgia, Athens, GA 30602. Author Fung is with the Dept. of Animal Sciences & Industry, Kansas State Univ., Manhattan, KS 66506. Author Ang, formerly with the USDA-ARS, Athens, is currently affiliated with the U.S. Dept. of Health & Human Services, Food & Drug Administration, National Center for Toxicological Research, Jefferson, AR 72079. Direct inquiries to Dr. C.Y.W. Ang.

MATERIALS & METHODS

Sample preparation

Fresh broiler breasts and leg parts (thigh and drumstick) were purchased from a supermarket in Athens, Ga. Muscle tissues were freed from skin, bones and fatty material and ground through a 1-cm plate. Lean ground tissue was weighed into 16×150 mm test tubes unless otherwise specified. A thermocouple was inserted into the center of the sample in each tube. All samples were cooled to 1 to 4°C by holding at −10°C for ≈10 min before heat treatment.

Determining broad-range inactivation temperatures

The first objective was to determine the broad temperature range of catalase inactivation in chicken tissues (65–75°C). Samples were heated in a water bath (Model 220P refrigerated circulating bath with temperature control at ±0.01°C, NESLAB Instruments, Inc., Newington, NH) to specific EPTs ranging from 65–75°C at 1°C intervals. Various conditions were tested: (1) 2.5-g samples in 5-mL sealed vials (1.5 cm o.d. × 3.5 cm h.) heated at the target EPTs (total heating time ranged from 9 min 50 sec to 15 min 20 sec); (2) 15-g samples heated at 1°C or 2°C above the EPTs (heating time ranged from 6 min 45 sec to 9 min 40 sec); and (3) 10-g samples heated at 1°C above the EPTs (heating time ranged from 5 min 35 sec to 7 min 30 sec). For each trial, two tubes were heated to a specific EPT. Immediately after heating, the tube was removed from the water bath and rapidly chilled in an ice-water bath. Several trials were run for each treatment (Table 1).

Table 1—Catalase activity after 30 min incubation at 23 ± 2°C for chicken tissues previously heated to various internal end-point temperatures (EPTs)

	Breast		Leg	
EPT,°C	N[a]	Positive[b]	N	Positive
65–68	25	+++25	12	+++12
69	16	++15	8	++8
70	15	+6	6	+2
71	20	+2	6	0.5[c]
72–75	28	0[d]	12	0[d]

[a] N = Total number of trials. Each was an independent heating treatment and/or storage time. One tube heated per trial. Duplicate catalase tests were made for each trial.

[b] +++ denotes strong positive reactions—solid phase floated on top of the liquid or bubbles formed; ++ denotes medium positive reactions—some liquid in the bottom of the tube, no bubbles; + denotes weak positive reaction (a light separation of solid and liquid phases).

[c] One of the duplicate tests was positive.

[d] All solids were in the bottom of the test tube.

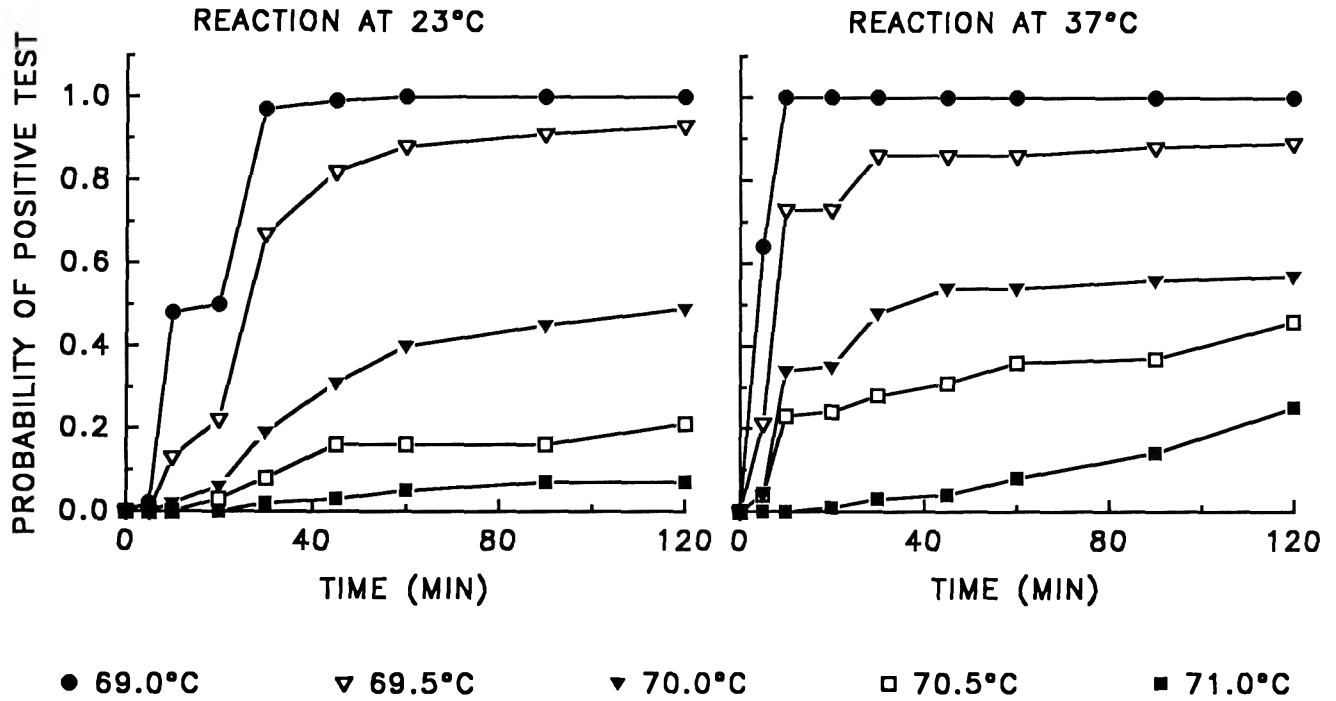

Fig. 1—Influence of reaction time on probability of positive catalase activity for chicken breast meat previously heated to end-point temperatures between 69 and 71°C.

Samples were placed in capped glass jars and stored at −20°C until the catalase reaction test was conducted (within 1 mo).

Determining EPT within ± 0.5°C

The second objective was to refine the catalase test so that EPTs in the range 69.0 to 71.5°C could be determined within ±0.5°C. Samples were prepared in a similar manner as in phase I, except sample weights and target EPTs were different. Ten-gram samples were placed in each tube and heated in a water bath preset at 1°C above target EPTs of 69, 69.5, 70, 70.5, 71 and 71.5°C. The heating time ranged from 5 to 6 min. Two tubes were heated in each trial to each specific EPT. The number of trials for each heating treatment ranged from 19 to 49 depending on the specific EPT and tissue. Each trial represented an independent heating treatment and/or storage time after heating.

Enzymatic reactions

Frozen cooked samples were thawed at 4°C for 2 hr and blended in a food processor for 30 sec under nitrogen. Duplicate samples (2g each) from each heating tube were weighed into 19×150 mm test tubes and 4 mL of 3% H_2O_2 was added. The contents of the tube were mixed with a plastic stirring rod and incubated at room temperature (23 ± 2°C) or at 37°C. Observations were made at 5, 10, 20, 30, 45, 90 and 120

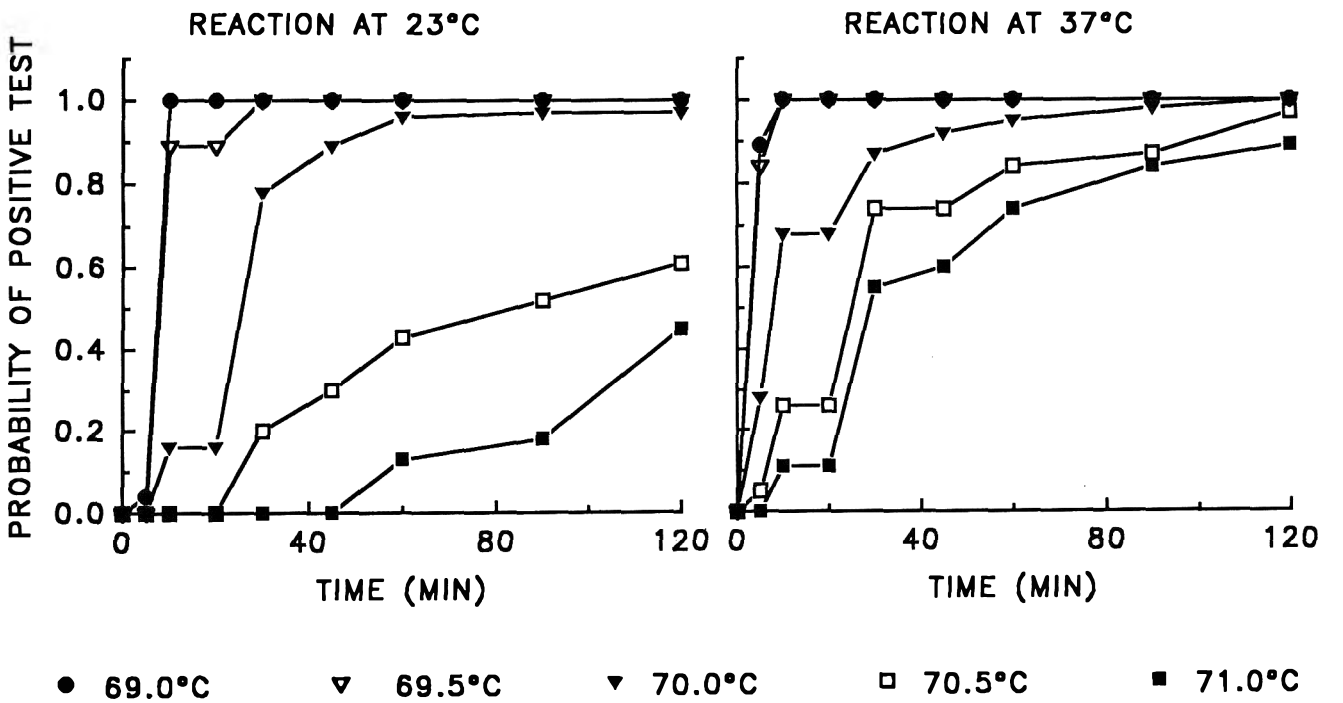

Fig. 2—Influence of reaction time on probability of positive catalase activity for chicken leg meat previously heated to end-point temperatures between 69 and 71°C.

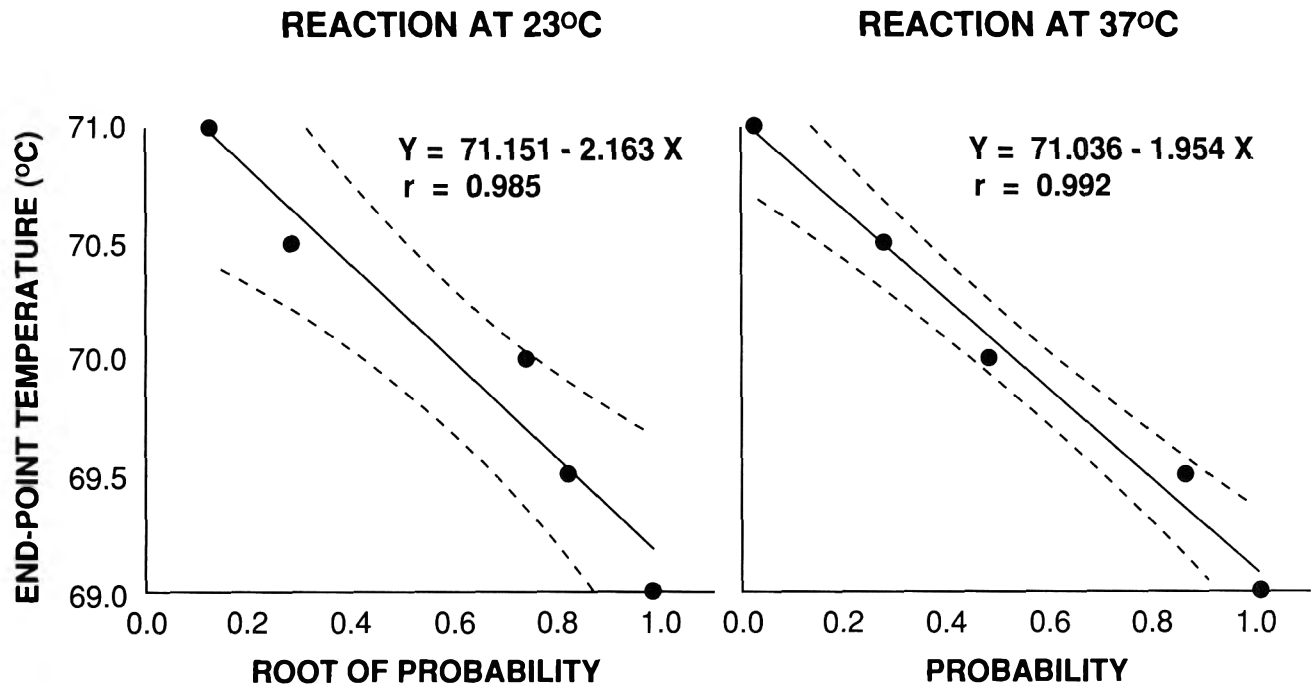

Fig. 3—Regression curves for predicting end-point temperatures of previously heated chicken breast meat by catalase reaction (_____ : regression curve; •: observed values; curved - - -: ± 95% confidence limits; y = Y axis; x = X axis; r = correlation coefficient between end-point temperature and probability).

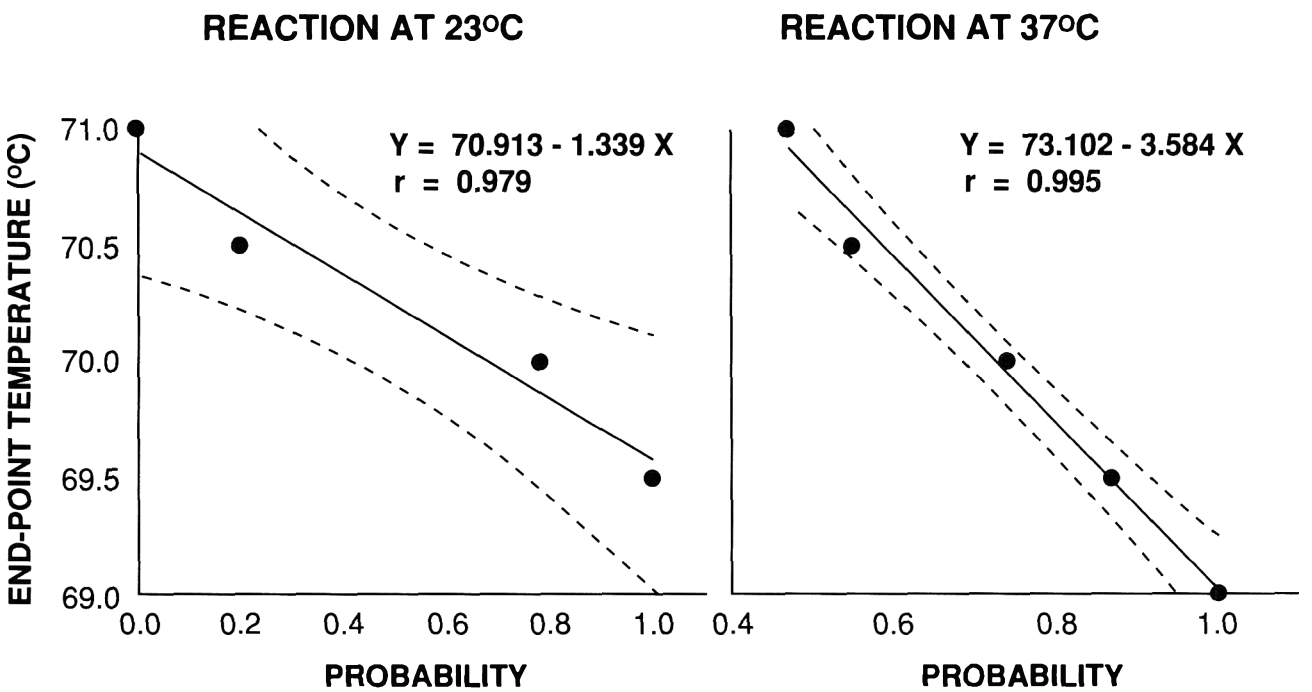

Fig. 4—Regression curves for predicting end-point temperatures of previously heated chicken leg meat by catalase reaction test. (_____ : regression curve; •: observed values; curved - - -: ± 95% confidence limits: y = Y axis; x = X axis; r = correlation coefficient between end-point temperature and probability).

min after addition of H_2O_2. The formation of gas bubbles, flotation or separation of the solid phase indicated a positive catalase reaction. The precipitation of all solids in the bottom of the tube indicated negative catalase activity.

Note that the decomposition of H_2O_2 can also be catalyzed by peroxidase under certain conditions (Whitaker, 1972; Guibault, 1976). Whitaker (1972) indicated that catalase was not as stable as peroxidase and could lose its activity rapidly at 35°C while peroxidase was stable to 65-70°C at pH near 7. In our study, no attempt was made to distinguish between catalase and peroxidase activity. Reactions between H_2O_2 and the naturally occurring enzymes are referred to here as the catalase activity.

Statistical analysis

The probability (fraction) of positive enzyme reaction for each specific EPT and tissue was calculated by dividing the number of positive tests by the total number of tests in all trials. The total number of positive tests included all strong or weak positive responses. Duplicate

enzymatic tests were conducted for each heated sample. The regression analysis, 95% confidence limits and correlation coefficients were computed by the Statistical Analysis System (SAS Institute, Inc., 1985).

RESULTS & DISCUSSION

Broad-range inactivation temperatures

Several preliminary tests were conducted on raw tissues and those heated to EPTs < 65°C. Gas bubbles were generated in the reaction tubes. Eye (1982) reported generation of gas bubbles for rare roast beef. Samples heated above 65°C generated fewer gas bubbles. Ezymatic activity was very high for samples heated < 65°C. Thus, the first study was narrowed to EPTs of 65 to 75°C. Muscles heated to EPT of ≤ 68°C always resulted in positive reactions and muscles with EPT of ≥ 72°C always resulted in negative responses (Table 1). The total volume of sample mixture (2 g) and H_2O_2 (4 mL) was >6 mL for samples heated to ≤69°C. The increase in volume was the result of numerous small bubbles which raised the solid phase to the top of the liquid phase. When tissues were heated to 72°C and above, no bubbles were generated and all solids settled to the bottom of the tube.

Eye (1982) reported the generation of gas or foam for positive catalase reactions and indicated no activities once the internal temperature of roast beef reached 62.8°C. However, the lack of gas or foam would not prove the lack of enzyme activity. In our study, when the reaction was allowed to continue for longer times (up to 120 min), the flotation of the solid phase was observed. Thus, though foaming or bubbling was not visible the enzyme reaction was detected. The data indicate substantial enzymatic activity occurred at temperatures higher than those reported by Eye (1982). Spanier et al. (1990) also noted some residual catalase activity as beef homogenate was heated to 74°C.

Samples included several lots of chicken breasts and legs, some frozen, stored and thawed before being ground for heating treatments. Some samples were stored frozen for up to 17 days after heating before conducting enzyme tests. Sample sizes in heating tubes varied from 2.5 to 15g. Regardless of sample lots and heating techniques, results of tests were consistent for EPT of 68°C or below (positive) and for 72°C or above (negative). Variability in enzyme responses occurred from 69 to 71°C, indicating that the broad-range inactivation temperatures could be > 68° but < 72°C. As EPT increased from 69 to 71°C, the probability of positive reactions decreased.

Determination of EPT within 0.5°C

The effects of EPT on detection of enzyme activity were compared (Fig. 1 and Fig. 2) for breast and leg meat. For each specific EPT, the probability of a positive response increased with reaction time; longer time increased probability of positive results. For a given reaction time, the probability of resulting in a positive test was less as EPTs increased from 69 to 71.5°C.

For breast tissue with eyzyme incubation temperature held at 23°C, the increase in positive responses was fast during the first 30 min of reaction. The increase was slower after 45 min. More enzyme activity was noted as incubation temperature increased to 37°C (Fig. 1). The effect of EPT on catalase activity of leg meat (Fig. 2) followed the same trend as for breast meat, except that positive reactions developed at a much faster rate for leg meat (higher probabilities).

At 23°C, the probability of obtaining a positive result for EPT of 69°C was ≥0.99 and for 71°C was ≤0.03 for breast meat. The higher reaction temperatures (37°C) offered faster responses or higher probability of a positive test than the reaction at 23°C. The probability of a positive test at 37°C for leg meat preheated to EPT of 71.5°C could be as high as 0.47. Thus, leg meat samples are recommended to be incubated at 23°C for the catalase reaction test to avoid variations.

The relationships between the EPT's in the range 69 to 71°C and the probability of a positive reaction at 23°C and 37°C were compared for the breast meat (Fig. 3) and leg meat (Fig. 4). The EPTs between 69 and 71°C can be estimated within ± 0.5°C for previously heated chicken tissues by using the prediction curves or regression equations. The best fit models are presented. Note that for the best fit regression at 23°C (Fig. 3) was between the EPT and the root of probability instead of the original probability values.

In several trials, thermocouples were placed both in sample centers and the outer portion which came in contact with the inside wall of the test tube. The sample temperature of the outer portion was ≈ 0.6°C higher than the center temperature. Thus, when heated tissues were blended and aliquots were taken for catalase reactions, the actual temperature range of the test sample would have been from the center EPT to 0.6°C greater.

In commercial processing some temperature gradient would always occur in a heated product. Though the geometric center was heated to a specific EPT, the test sample from the geometric center would include materials from surrounding areas which may have been at higher temperatures. The temperature range of 0.6°C we found might be low in practical situations. Samples should be taken within a temperature range as narrow as possible for a precise result.

CONCLUSION

A SENSITIVE, IMPROVED PROCEDURE was developed for determining EPTs within ±0.5°C between 69 and 71°C for previously heated chicken tissues. The method provides a rapid and simple means for verifying adequacy of heat-processing of poultry products.

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Listeria monocytogenes Inhibition in Refrigerated Vacuum Packaged Turkey Bologna by Chemical Additives

HEIDI J. WEDERQUIST, JOHN N. SOFOS, and GLENN R. SCHMIDT

ABSTRACT

Sliced cooked turkey bologna with various additive formulations was surface-inoculated with *Listeria monocytogenes* (2.06–2.75 log CFU/g), vacuum packaged, and stored at 4°C. Sodium acetate was most inhibitory against growth of *L. monocytogenes*, followed by sodium lactate and potassium sorbate, while sodium bicarbonate allowed a maximum net growth of 6.78 log CFU/g, not significantly different ($p>0.05$) from the control (6.43 log CFU/g). Addition of 0.5% sodium acetate, 2.0% sodium lactate, or 0.26% potassium sorbate may significantly ($p<0.05$) decrease growth of *L. monocytogenes* in refrigerated turkey bologna surface-inoculated after thermal processing and slicing.

Key Words: *Listeria monocytogenes*, turkey bologna, antibacterial additives, vacuum packaging

INTRODUCTION

A MONITORING PROGRAM of ready-to-eat meat products in the U.S. revealed that 5–12% in 1987, and 10–13% of products sampled in 1988 were contaminated with *Listeria monocytogenes* (Wilson, 1989). Grau and Vandelinde (1992) in Australia, reported listeriae were detected in 93 of 175 samples of vacuum-packaged processed meats from retail stores. Glass and Doyle (1989) reported growth of *L. monocytogenes* at 4.4°C on ham, sliced chicken and turkey products, wieners, and fresh bratwurst. Processed meats with pH ≥6.0, enabled more growth of *L. monocytogenes* than meat products with a lower pH. Thus, turkey bologna and frankfurters of pH >6.0 may provide enhanced potential for the survival and growth of *L. monocytogenes*.

In 1989, a human case of listeriosis leading to death was traced to a poultry product (Barnes et al., 1989). A cancer patient died after developing listerial meningitis, and the source of the *L. monocytogenes* was reported as turkey frankfurters. The same strain and isoenzyme type of *L. monocytogenes* was found in unopened packages of the same brand of frankfurters at a nearby retail store. Later the case strain of *L. monocytogenes* was reported to be present in the frankfurter processing plant (Wenger et al., 1990). The ability of *L. monocytogenes* to survive and grow in vacuum packaged, ready-to-eat meat and poultry products such as turkey frankfurters and bologna is of great concern to the food industry.

Incorporation of chemical preservatives in meat products may be important for control of *L. monocytogenes* when used in combination with other processing techniques. Sodium acetate, sodium bicarbonate, sodium lactate, and potassium sorbate (all "generally recognized as safe," GRAS) are regulated in the United States by the Food & Drug Administration. Use of these additives in meat and poultry products is subject to regulation by the USDA.

Sodium lactate is approved and used in formulation of meats and poultry products to extend shelf life and enhance flavor. Bacus and Bontenbal (1991) reported growth inhibition of *L. monocytogenes* and increased shelf life in both cured and uncured meat and poultry products with addition of sodium lactate. Potassium sorbate, though widely used in the food industry as a preservative, is approved for meats only as a 10% solution for dipping dry sausages to retard mold growth during storage (Sofos, 1989). El Shenawy and Marth (1988) demonstrated that the effectiveness of potassium sorbate in inhibiting *L. monocytogenes* was most pronounced at low pH and temperatures. Ryser and Marth (1988) reported the elimination of four strains of the pathogen from cold-pack cheese-food of pH 5.45 stored for 130 days. Sodium bicarbonate was reported to have antimicrobial properties against pathogenic periodontal bacteria (Newbrun et al., 1984), and was effective in extending shelf life of cod fillets dipped in an 8% solution (Curran et al., 1990). Bechtel et al. (1985) reported that addition of sodium bicarbonate had no effect on total aerobic plate counts of frankfurters formulated with sodium bicarbonate, compared to a sodium chloride control. Sodium acetate extended vacuum packaged beef steak shelf life in combination with other additives (Unda et al., 1990). Roskey and Lachica (1992) reported effective control of *L. monocytogenes* in meat and broth supplemented with sodium acetate, and indicated that 0.5% of the additive would be a useful and appropriate antimicrobial agent for beef and luncheon meat. Our objective was to evaluate four additives for their potential to control growth of *L. monocytogenes* in turkey bologna which was surface-inoculated after processing, vacuum packaged, and refrigerated.

Authors Sofos and Schmidt are with the Dept. of Animal Sciences and Dept. of Food Science & Human Nutrition, Animal Sciences and Dept. of Colorado State Univ., Fort Collins, CO 80523. Author Wederquist, formerly with the Dept. of Animal Sciences, Colorado State Univ. is presently affiliated with Bil Mar Foods, Zeeland, MI. Address inquiries to Dr. John N. Sofos.

MATERIALS & METHODS

Mechanically deboned turkey meat

Fresh mechanically deboned turkey meat (MDTM), from turkey frames, was purchased from a local supplier (Longmont Foods, Longmont, CO). The initial microflora of the MDTM was $\approx 10^3$ CFU/g total aerobic bacteria, and no *L. monocytogenes* were isolated from the raw meat supply. The meat was refrigerated and used within 24 hr of deboning to prepare six turkey bologna formulations using the selected test additives.

Test additives

Sodium acetate, anhydrous powder (Matheson Coleman and Bell, Norwood, OH), potassium sorbate, granular, food grade (Monsanto, St. Louis, MO), sodium bicarbonate, powder (Mallinkrodt, Paris, KY), and sodium lactate, natural 60% SP liquid (Purac, Inc., Arlington Heights, IL), were used in their respective amounts (Table 1) to prepare formulations of turkey bologna.

Turkey bologna

Raw MDTM and chemical additives were weighed for six 9.08-kg batches of bologna, each representing a different treatment (Table 1). MDTM and ingredients were emulsified in a 35-L bowl chopper (Meissner, represented by RMF Kansas City, MO) at high speed for 5 min (3000 rpm blade speed, 18 rpm bowl speed). The final temperature after mixing of the batches in the chopper ranged from 4.4 to 7.2°C.

Turkey bologna emulsion from each 9.08-kg batch was extruded into 7.62-cm diameter fibrous cellulose casings (Koch, Kansas City, MO), weighed, and labeled to determine cook yields. The bologna was cooked in a single truck smokehouse (Vortron, Beloit, WI) to a final

Table 1—Mechanically deboned turkey meat (MDTM) bologna emulsion ingredients, including test additives, (grams per batch)

Ingredients	Treatment					
	c[a]	1	2	3	4	5
MDTM	7464	7418	7373	7160	7441	7001
Water	908	908	908	908	908	908
Dextrose	181.6	181.6	181.6	181.6	181.6	181.6
Corn Syr. Solids[b]	181.6	181.6	181.6	181.6	181.6	181.6
Salt	181.6	181.6	181.6	181.6	181.6	181.6
Dry Mustard	81.7	81.7	81.7	81.7	81.7	81.7
Phosphate[c]	36.3	36.3	36.3	36.3	36.3	36.3
Sodium nitrite	1.416	1.416	1.416	1.416	1.416	1.416
Na erythorbate	4.54	4.54	4.54	4.54	4.54	4.54
Paprika	22.7	22 7	22.7	22.7	22.7	22.7
Onion powder	4.54	4.54	4.54	4.54	4.54	4.54
Garlic powder	4.54	4 54	4.54	4.54	4.54	4.54
Coriander	4.54	4 54	4.54	4.54	4.54	4.54
White pepper	4.54	4 54	4.54	4.54	4.54	4.54
Sodium acetate	—	45.4	—	—	—	45.4
Na bicarbonate	—	—	90.8	—	—	90.8
Sodium lactate	—	—	—	303.3	—	303.3
Potassium sorbate	—	—	—	—	23.61	23.61

[a] Control treatment (no test additives).
[b] Corn syrup solids (42 D.E.).
[c] Hellers soluble phosphate (50% STPP + 50% SHMP).

internal temperature of 70.0°C. After cooking, the bologna was showered with cool tap water in the smokehouse for ≈5 min and then stored at 4°C to chill completely. Bologna was weighed after 12 hr of chilling to determine cooking yields (between 92% and 93% for all six formulations).

The bologna was placed in a freezer for 10 min before casing removal (peeling) and slicing 4–5 mm thick with a Hobart slicer (Hobart Manufacturing Company, Troy, OH) to an average slice weight of ≈30g. The control bologna and those formulated with 2.0% sodium lactate, 0.5% sodium acetate and 0.26% potassium sorbate, peeled and sliced cleanly. However, formulations containing 1.0% sodium bicarbonate and the combination of additives adhered to casings and did not slice as cleanly. Slices were packaged in plastic bags and transferred to the microbiology laboratory for inoculation, packaging, storage, and testing.

Inoculum

Bologna slices were surface-inoculated with a mixture of seven *L. monocytogenes* strains: Scott A serotype 4b (human isolate); Na-16 serotype 1/2a (cooked chicken roll isolate) and Na-12 serotype 4b (cooked, sliced turkey isolate), all three provided by Dr. R.V. Lachica, U.S. Army Natick Research, Development, and Engineering Center (Natick, MA); and strain #11 serotype 1/2 (chicken meat isolate), strain #503 serotype 1/2b (frankfurter isolate), strain #163 serotype 4 (meat isolate), and strain #65 serotype 1 (pork and beef salami isolate), all provided by Dr. J.M. Farber, Bureau of Microbiological Hazards (Ottawa, Ontario, Canada). Stock cultures were maintained in tryptic soy broth (TSB) (Difco Laboratories, Detroit, MI) at 4°C with monthly transfers to maintain viable cells. Inocula for each *L. monocytogenes* culture were grown in TSB incubated at 10°C for 96 hr, after which maximum optical density was reached. Each culture was then centrifuged at 5420 × *g* (10°C), for 15 min (model J2-21 Beckman Instruments, Inc., Fullerton, CA), and resulting pellets were resuspended in 25 mL of sterile phosphate buffer (pH 7.2 ± 0.1) (BBL Microbiology Systems, Cockeysville, MD). The seven 25 mL volumes of *L. monocytogenes* cell suspensions were then mixed in a sterile capped flask before surface-inoculating the bologna slices. The cell suspension was serially diluted in sterile phosphate buffer and plated on tryptic soy agar (TSA, Difco) to determine initial concentration of cells. The composite cell suspension was used to inoculate bologna slices at a level of 2.0 log *L. monocytogenes*.

Inoculation of bologna slices

Two bologna slices at a time were placed together on top of a square piece of polyvinylchloride (PVC) foodservice film wrap, and 0.1 mL of inoculum was dispensed into the center between the slices and spread evenly across the surface with a sterile bent glass rod. The two slices were then wrapped in the PVC film and inserted into 15.2 × 20.3 cm vacuum bags (Koch, Kansas City, MO), vacuum packaged (Multivac, Germany) at 120 mm Hg and stored at 4°C for up to 98 days. Duplicate samples of each treatment were plated immediately (0-time), and then at 7-day intervals for 98 days to enumerate *L. monocytogenes* and to record pH changes. The initial inoculum of the slices ranged from 2.06 to 2.75 log CFU of the pathogen/g product.

Microbiological analysis

Bologna (50g) were blended with 450 mL of sterile phosphate buffer in a Stomacher Lab-Blender 400 (Tekmar Company, Cincinnati, OH) and serial dilutions were made for plating on TSA (Difco). Colonies of *L. monocytogenes* cultured on TSA were identified using a simplified Henry's oblique trans-illumination technique (SHOT) described by Lachica (1990a). Colonies were counted as *L. monocytogenes* if they were opaque blue, smooth and low convex with entire margins measuring 1 to 2.5 mm diameter. Representative colonies typical of *L. monocytogenes* were picked for confirmation, including β-hemolysis (+); carbohydrate fermentation (acid production; positive for l-rhamnose, and negative for d-xylose); tumbling motility on a wet mount under a microscope (+); catalase test (+); KOH viscosity test (−); motility at 21°C in motility agar medium (+) (Lachica, 1990b); as well as Gram staining reaction (+).

pH measurement

For measurement of pH, duplicate 10-g samples of each bologna formulation were blended with 90 mL of deionized water for 2 min in a Stomacher Lab-Blender 400 following the method of Sebranek (1978). A Corning pH meter (model 125, Corning, Inc., Medfield, MA) was used in conjunction with a Corning combination electrode (Corning Glass Works, Medfield, MA) to measure the pH of the turkey bologna slurries.

Water activity

The water activity (a_w) of each turkey bologna treatment was measured using a Rotronic Hygroskop DT water activity system (Rotronic Instrument Corporation, Huntington, NY) in combination with a circulating waterbath (MWG Lauda RM6, Brinkmann Instruments, Westbury, NY) to maintain constant temperature. Each formulation was tested in duplicate to determine if the curing ingredients or chemical additives had an effect on availability of moisture. Samples were finely ground and filled into plastic sample cups to within 0.3-cm from the top of the cup, and immediately placed in the sensor for testing. Each sample was measured for 45 min to obtain an accurate reading, and a desiccant (anhydrous $CaSO_4$) was used for 10 min between samples to allow the sensor to purge any additives or vapors from the previous sample.

Proximate analysis

Moisture, fat, and protein were determined in duplicate for the cooked mechanically deboned turkey meat bologna treatments. Moisture was determined by drying the samples in a vacuum oven (Napco, Precision Scientific, Chicago, IL) at 60° to 65°C for 12 hr. Fat content was determined on the dried samples by Soxhlet extraction for 4 hr with petroleum ether. Protein content was estimated using Kjeldahl nitrogen determination with the Kjeltec System (Tecator, Höganäs, Sweden), including digestion system, distilling unit, and burette (Multi-Bürett, Metrohm).

Statistical analysis

Analysis of variance was used to assess differences in pH and bacterial numbers. Duncan's procedure was used to compare means of logarithmic growth between additive treatments. The whole experiment was replicated twice, at different times and with different batches of turkey meat.

RESULTS & DISCUSSION

pH of treatments

Based on initial pH (Fig. 1), the six treatments fell into two groups. The first group included turkey bologna formulated with (a) sodium bicarbonate (pH 7.59) and (b) the combination of all four additives (pH 7.63). Initial pH values of the second group, which included the control, sodium acetate, sodium lac-

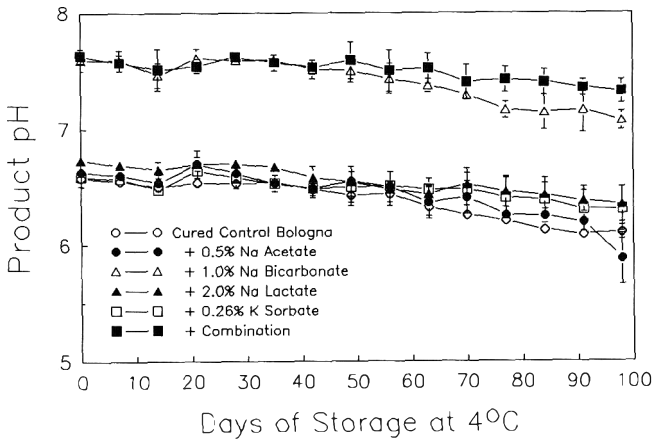

Fig. 1—Changes in pH for turkey bologna formulations, inoculated, and stored vacuum packaged at 4°C for 98 days.

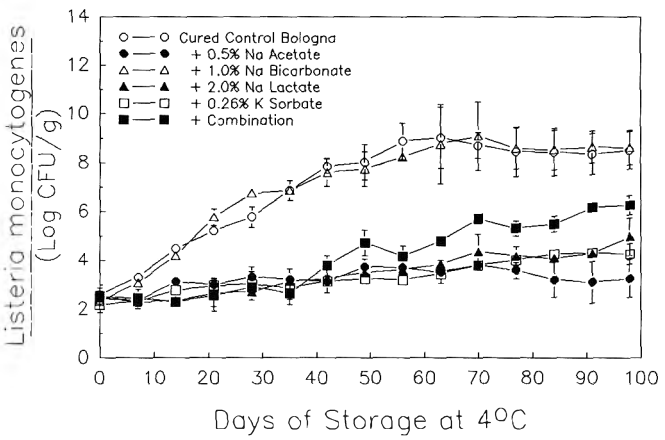

Fig. 2—Growth of *L. monocytogenes* on cooked, surface-inoculated turkey bologna formulations as detected by plating on trypticase soy agar during 98 days storage at 4°C in vacuum packages.

tate and potassium sorbate treatments were 6.58, 6.63, 6.73 and 6.59, respectively. *Listeria monocytogenes* can grow at pH values 5.6–9.6, with optimal growth at pH 7.0 or slightly higher (Seeliger and Jones, 1987). However, in microbiological media, the organism was reported to grow at pH as low as 4.4 (Ryser and Marth, 1991). With storage of the bologna products, the pH of all treatments gradually decreased, but even after 98 days storage, they exceeded 5.8 (5.88–7.32). Therefore, pH was probably not a factor influencing microbial growth, with the possible exception of the sodium bicarbonate and combination treatments. The pH of each turkey bologna treatment remained well within the range at which *L. monocytogenes* can grow. However, the elevated pH (>7.0) of the sodium bicarbonate treatment may have allowed for enhanced growth of *L. monocytogenes* compared to the other additives.

Water activity and composition

The turkey bologna formulated with sodium bicarbonate had the highest a_w, followed by the control a_w (Table 2). The average a_w values for turkey bologna formulated with potassium sorbate, sodium lactate, and the combined additive treatment were similar. Turkey bologna formulated with 0.5% sodium acetate had the lowest a_w of all treatments. *Listeria monocytogenes*, like most other bacterial species, exhibits optimal growth at a_w ≈0.97 (Petran and Zottola, 1989), yet, it also has the rather unique ability to proliferate at a_w as low as 0.92 (Ryser and Marth, 1991). The a_w values of the turkey bologna formulated with various chemical additives were all within the range of growth for *L. monocytogenes*. As expected, a direct relationship was observed between high bacterial counts and higher a_w values of specific treatments. The sodium bicarbonate treatment and the control treatment allowed the most bacterial growth, and also had highest a_w values. In contrast, the sodium acetate formulation had the lowest a_w and was most inhibitory to *L. monocytogenes*. The proximate compositions (Table 3) were similar among treatments.

Table 2—Average water activity (a_w) of cooked mechanically deboned turkey meat (MDTM) bologna formulated with various additives before storage

Treatment	Water Activity
Control bologna	0.962
+ Sodium acetate (0.5%)	0.945
+ Sodium bicarbonate (1.0%)	0.966
+ Sodium lactate (2.0%)	0.954
+ Potassium sorbate (0.26%)	0.958
+ Combined additives[a] (3.76%)	0.954

[a] Combination of 0.5% sodium acetate, 1.0% sodium bicarbonate, 2.0% sodium lactate, and 0.26% potasium sorbate.

Table 3—Average proximate composition of cooked mechanically deboned turkey meat (MDTM) bologna treatments formulated with various additives

Treatment	Moisture (%)	Fat (%)	Protein (%)
Control Bologna	64.52	14.49	15.70
+ Sodium acetate (0.5%)	63.71	13.79	15.02
+ Sodium bicarbonate (1.0%)	65.69	13.52	14.27
+ Sodium lactate (2.0%)	63.87	14.02	15.03
+ Potassium sorbate (0.26%)	64.10	14.13	14.70
+ Combined additives[a] (3.76%)	61.78	14.22	14.97

[a] Combination of 0.5% sodium acetate, 1.0% sodium bicarbonate, 2.0% sodium lactate, ad 0.26% potassium sorbate.

Microbial growth

Growth of *L. monocytogenes* inoculated in turkey bologna after slicing was rapid and quite extensive in the control reaching 9.02 log CFU/g in 63 days (Fig. 2), from an initial population of 2.54 log CFU/g (maximum net growth of 6.43 log CFU/g). When 0.5% sodium acetate was incorporated in the formulation however, the lag phase was prolonged, and growth was significantly ($p<0.05$) reduced with the pathogen reaching a net maximum growth of only 1.33 log CFU/g after storage at 4°C for 70 days. Potassium sorbate and sodium lactate were ($p>0.05$) similar in their effectiveness to inhibit the organism. Each extended the lag phase, and held the net maximum growth of *L. monocytogenes* to 2.13 log CFU/g at 91 days and 2.51 log CFU/g at 98 days. The combination of all four additives was slightly less inhibitory. However, the lag phase was extended to ≈35 days and growth of *L. monocytogenes* was limited to a net maximum of 3.74 log CFU/g at 98 days. Conversely, sodium bicarbonate, which raised the pH of the turkey bologna to 7.5 (Fig. 1) and did not affect a_w (Table 2), allowed extensive growth of *L. monocytogenes* which was not significantly ($p>0.05$) different from the control.

Expected shelf life of refrigerated cured meat products such as bologna and turkey frankfurters is ≈75 days. Therefore, high levels of *L. monocytogenes* could be reached well within the "sell-by" date during storage of such products in the retail meat case. Growth of *L. monocytogenes* in vacuum-packaged refrigerated turkey bologna may be significantly ($p<0.05$) reduced by incorporation of 0.5% sodium acetate, 2.0% sodium lactate, or 0.26% potassium sorbate. Research is needed on the effectiveness of other levels and combinations of such additives on microbiological and sensory properties of turkey bologna.

—Continued on page 516

Manitoba Whitefish (*Coregonus clupeaformis*) Potentials for Fabrication of Texturized Seafood Analogs

M.A.H. ISMOND and J.R. TONOGAI

ABSTRACT

Strong resilient fibers were formed from a protein extract isolated from Manitoba whitefish (*Coregonus clupeaformis*) upon pH adjustment to 8.0. Despite pH requirements, protein denaturation was not a prerequisite for fiber formation. However, rheological measurements appeared to indicate an increased requirement for viscous components of the extracts and the possibility of protein orientation in the direction of flow. By linearly aligning protein fibers in a 1:1 ratio into a whitefish surimi base containing 3% sucrose, 1.5% NaCl, 3.5% potato starch, and 25% crab flavoring, an analog with fibrous consistency was produced.

Key Words: whitefish, texture, seafood analog, surimi

INTRODUCTION

WHITEFISH are abundant in freshwaters in Manitoba and their marketability has been hindered by parasite infestation. Few studies have considered post-harvest product enhancement to increase the economic potential of such parasitized fish. In other fish related research, considerable interest has been focused on utilization of less marketable varieties, such as Alaska pollock, in commercial production of simulated seafood products. This post-harvest route may be attractive for whitefish utilization, but the methodology for analog manufacture is still developing. Such products have not all met equal consumer acceptance. Most such products have textural problems due to lack of a fibrous characteristic associated with seafood.

One method to texturize such analogs involves incorporation of fibers, nearer to the diameter of true seafood muscle fibers, into surimi. Manufacturers have tried to simulate fibrousness by folding or rolling of partially heat set, scored sheets of surimi (Lee, 1984), but such attempts have not duplicated the muscle texture in meat (Yang and Olsen, 1974). A new approach by Ismond et al. (1985) involved formation of fibers from a protein extract, isolated from minced, washed whitefish. That method utilized a combination of the extraction procedure of Liu et al. (1982) and the protein micellar mass method of Murray et al. (1978), providing the means for mildly extracting protein from fish muscle. In that method, semi-frozen fish, gently homogenized in 0.04M Na_2HPO_4 at pH 7.2 with 0.5M NaCl, yielded a precipitated mass of protein micelles after undergoing rapid ionic strength reduction upon addition of cold water. This protein extract could be formed into discrete fibers when pumped through a 0.8 mm orifice into a 95% ethanol coagulating bath adjusted to pH 4.5 with acetic acid.

The unfolding of various proteins into long polypeptide chains at alkaline pH values varies considerably and influences fiber formation. Most texturization has involved fabrication of meat-like products from plant proteins. The protein content of fish muscle is very different from the globular conformation of plant storage proteins. The muscle proteins naturally have elongated linear conformations which lead to fiber formation.

Our objective was to determine the changes in conformation that occur in whitefish muscle and their effects on fiber formation and to assess the effect of the fibers on texture of whitefish surimi formulated into analogs.

Authors Ismond and Tonogai are with the Dept. of Food Science, Univ. of Manitoba, Winnipeg, Manitoba, Canada R3T 2N2.

MATERIALS & METHODS

Minced fish

Commercial gutted parasitized whitefish (0.9–1.4 kg each) were purchased from the Freshwater Fish Marketing Corporation (Winnipeg, Manitoba). "Skin on" fish fillets were kept on ice until fed into a Baader Flesh Separator #694 (Nassau Nova Scotia Corp., NY), equipped with a 5 mm diameter perforated drum. The deboned flesh was washed six times with water (4°C; 4:1 w/w). Each cycle consisted of a 2 min agitation period and a 2 min rest period, after which blood, lipid and water soluble proteins were removed by decantation. During the final washing cycle, 0.1% (w/w) food grade NaCl was added to the water.

After washing, the flesh was contained in fine mesh polyester bags (about 4 meshes/cm) to allow the removal of most of the water upon kneading and draining. Further dewatering and removal of unwanted material (i.e., skin) were accomplished with the Baader Flesh Separator, equipped with 3 mm diameter perforated drum. The dewatered fish was packed into blocks and quick-frozen with a double plate Dole Freze Cel contact freezer #2735 (Dole Refrigerating Products Ltd., Oakville, Ontario) for 3 hr. The fish meat was subsequently stored at −40°C.

Preparation of protein extract

Using the standard protein extraction method (Ismond et al., 1985), 70g partially frozen fish was sliced into 400 mL food grade saline-phosphate buffer (0.04M Na_2HPO_4 with 0.5M NaCl adjusted to pH 7.2 with 1.0N NaOH, 4°C). Using the Silverson Standard Lab Homogenizer equipped with emulsor screen, the fish was homogenized 2 min followed by 2 min rests. This process was repeated three times to extract the most available muscle proteins without heat damage. Homogenization was carried out on ice.

The homogenate was then centrifuged at 20,000 × *g* for 15 min (4°C) using a Sorvall Refrigerated Centrifuge, Model RC2-B (DuPont Co., Wilmington, DE). The supernatant was decanted and diluted with an equal volume of distilled water (4°C). The suspension was stirred 5 min, diluted with distilled water to double the volume, stirred again 5 min and centrifuged to provide the protein precipitate.

Rheological properties of protein extract

Samples of pH adjusted protein extracts (7.2–9.0 at about 0.5 pH intervals with 1.0N NaOH) were monitored using a Bohlin VOR Rheometer (Bohlin Rheologi Inc., Edison, NJ), with a cone (5° angle) and plate geometry (30 mm diameter) and a torque element of 95.8 g·cm. Measurements were conducted at 4°C in a frequency range of 0.05–1.00 Hz. The dynamic rheological parameters used to evaluate the extracts were the storage modulus (G') and tan δ or loss tangent (tan δ = G"/G'). The loss tangent represents the ratio of energy lost to energy stored in a cyclic deformation. The viscosity aspects of the extracts were measured over a shear rate range of 1.48×10^{-1} to 14.8 sec^{-1}. The behavior and consistency indexes were calculated using the power law equation $\tau = m \cdot \gamma^n$ where τ = shear stress, m = consistency index, γ = shear rate, and n = flow behavior index.

Thermal properties of protein extract

Differential scanning calorimetry (DSC) of fish protein extracts was carried out using a DuPont 9900 Thermal Analyser equipped with a 910 DSC pressure cell (Westec Industrial Ltd., Mississauga, Ont.).

Table 1—Relationship of pH to loss tangent and frequency range

Frequency (Hz)	Loss tangent* pH 7.16	7.49	8.00	8.61	9.21
0.05	0.246 ± 0.017[a]	0.311 ± 0.024[b]	0.344 ± 0.015[c]	0.391 ± 0.020[d]	0.414 ± 0.038[d]
0.10	0.194 ± 0.013[a]	0.256 ± 0.026[b]	0.292 ± 0.011[c]	0.334 ± 0.019[d]	0.346 ± 0.03[cd]
0.20	0.168 ± 0.012[a]	0.223 ± 0.010[b]	0.258 ± 0.010[c]	0.294 ± 0.018[d]	0.310 ± 0.026[d]
0.50	0.145 ± 0.013[a]	0.191 ± 0.029[b]	0.227 ± 0.013[c]	0.260 ± 0.021[d]	0.282 ± 0.02[cd]
1.00	0.140 ± 0.016[a]	0.180 ± 0.030[b]	0.216 ± 0.021[c]	0.247 ± 0.021[d]	0.278 ± 0.03[cd]

* Mean horizontal row values followed by the same letter are not statistically significant (P≤0.05). Mean values based on seven determinations.

Table 2—Relationship of pH to consistency (m) and flow behavior (n) indexes of the protein extract

pH	m	n
7.16	11.19 ± 1.89[a]	0.380 ± 0.033[a]
7.49	15.25 ± 3.76[a]	0.339 ± 0.048[a]
8.00	8.95 ± 4.40[a]	0.203 ± 0.040[ab]
8.61	2.98 ± 0.84[b]	0.331 ± 0.094[ab]
9.21	4.44 ± 0.80[b]	0.174 ± 0.061[b]

[a,b] Mean vertical column values followed by the same letter are not significantly different (P<0.05). Mean values based on five determinations.

Lyophilized protein samples were rehydrated with saline phosphate buffer adjusted to pH 7.2 through 9.0 at ≈ 0.5 pH intervals, with 1.0N NaOH at 15% (w/w) solids. Sample (10 μL) was hermetically sealed in aluminum DSC pans. Plots of heat flow as a function of temperature, (thermal curves, Arntfield and Murray, 1981), were established at a heating rate of 10°C min^{-1} over a range of 24 to 100 °C under constant pressure (1,400 kPa with N_2). The thermal curves allowed determination of the denaturation temperature (Td) and heat required for denaturation (ΔH).

Texturized seafood analogue prototype

A Moyno pump (Robbins and Myers Inc., Springfield, OH) was driven by a 1.5 KW (2 hp) variable drive DC motor forcing the protein extract (2L) through a circular brass die housing 40 16G hypodermic needles (Paryniuk, 1987) forming fibers. At 4°C, the fibers were carried on a conveyor belt through the coagulating bath of 95% ethanol, pH 4.5 with acetic acid and into a water rinse bath containing tap water. Upon emergence from the rinse bath, a bundling device, composed of directed air streams, blew the fibers into a rope. The emerging fibers retained a substantial amount of water; as a result, they were blotted with absorbent paper.

Ingredients and cooking protocol for the crab leg analogs were established by open discussion among experienced but untrained panelists. A paste consisting of surimi (67%), prepared from whitefish and containing 3% sucrose, was mixed in a Kitchen Aid Mixer (Hobart Mfg. Co., Troy, OH) with 1.5% salt for 5 min, followed by addition of 3.5% potato starch and 25% Astral 1717 natural crab-flavoring (Mitsubishi Foods, White Plains, NY) and another 10 min stir (Tonogai, 1988). Using a 1:1 fiber to surimi ratio, the flavored paste and fibers were combined by linearly aligning the paste and fibers together (Tonogai, 1988). The fiber-paste combination was rolled into strips, increasing the contact between the two. The strips were cut into 5 cm lengths, lined onto cheese cloth and steam-boiled for about 10 min until a soft gel was formed. After steaming, obvious browning of fibers occurred. The crown color was masked by adding blue food dye (McCormicks, Ltd., Hunt Valley, MD) to the fish protein extract prior to fiber formation (i.e., 1 drop/400 g protein extract).

Ten panelists were asked to compare the crab leg analogs with Alaska King Crab Legs (Scott National, Winnipeg, Manitoba). In preparation, the frozen split crab legs were placed in boiling water for 5 min. Shells were removed and the meat cut into 2 cm lengths for evaluation. Characteristics assessed were flavor (sweetness, saltiness, crab flavor intensity and aftertaste) and texture (firmness and chewiness). In order to assess these characteristics a prototype of the unstructured line developed by Stone et al. (1974) for the Quantitative Description Analysis method was used.

Statistical Analysis

Differences in thermal parameters (Td, ΔH) and rheological measurements (tan δ, m and n) for different pH levels were evaluated by a multiple T-test.

RESULTS & DISCUSSION

Effect of pH on fiber formation

Of the five pH- adjusted extracts, only those with pH 8.0, 8.61 and 9.21 produced visually strong intact fibers. The strength of fibers was subjectively judged by their ability to remain intact during removal from the media with forceps. At pH 7.49 and 7.16, the fibers were weak or non-existent. For pH 8.0 and above, the fibers appeared to be about equally strong.

Thermal characterization of pH adjusted extracts

This study revealed the presence of only one protein endotherm at the various pH values over the 7.0–9.21 range. This was in comparison to three transitions (at 38, 51, and 65°C) reported by Wu et al. (1985) for actomyosin from Atlantic croaker. Several possibilities exist for differences in peak numbers. One obvious difference was pH of the samples. In the study by Wu et al. (1985), pH was not controlled and always fell within the range pH 6.6–6.9. In addition, inclusion of salt caused a downward shift of higher temperature peaks and complete disappearance of the 38°C peak. In our study, addition of 1.0N NaOH may have caused peak disappearance similar to that observed in presence of salt. Variations could easily have occurred from differences in sample preparation. Our whitefish pH adjusted extracts were lyophilized prior to analysis, while the croaker actomyosin had been concentrated by dialysis. In addition, peak losses may have resulted from the utilization of previously frozen fish. Frozen storage at −10°C caused partial myosin denaturation of cod muscle after 2 wk (Hastings et al., 1985).

No significant changes occurred in the Td and ΔH values for the thermal curves at the various pH values (P≤0.05), with a mean Td value of 67.09°C and ΔH of 2.66 J/g. The lack of changes, over the entire pH range, appears to indicate little, if any, protein unfolding with increases in pH, even up to pH 9.21.

Rheological characterization of pH adjusted extracts

Generally, a visual increase in viscosity occurred around pH 8.0, where strong fibers could be formed. Visual assessments of extracts proved to be a good indicator of resulting strength of fibers. The oscillatory measurements supported the visual changes observed in viscosity. Throughout the oscillatory frequency range (0.05–1.00 Hz) significant differences in calculated loss tangent (tan δ) and viscosity were evident as a function of pH (P≤0.05) (Table 1). This result possibly indicated increased linear orientation of the fish proteins which would favor formation of fibers.

Some differences in viscosity measurements of pH adjusted extracts were evident from consistency (m) and flow behavior (n) indexes (Table 2). A decrease in consistency and flow behavior indexes occurred with increasing pH. Postulating possible molecular changes in the extracts with pH, some explanations could be developed. The consistency index is affected by temperature and particle size and is an indicator of thickness of the sample. While a higher m value reflects a thicker sample, it would not necessarily be more viscous. The

Table 3—Characteristics of crab leg analog

	Summarized description
Appearance	Alignment of fibers necessary for fibrous appearance. Blue dye, added to protein, prior to fiber formation, whitened fibers.
Aroma	No distinct fishy odor. Cooked aroma evident but quickly disappeared. Little, if any, seafood aroma.
Flavor	Sweet, slightly salty, crab-like.
Texture	Fibers seemed to give elasticity and meatiness. Similar brittleness and chewiness to real crab.
Afertaste	Slightly sweet and salty. No offensive fishy taste.

decrease in consistency tends to reveal an increase in particle streamlining or "rafting" at the higher pH. Particle alignment or streamlining from a more random molecular array should lead to fiber formation and reflects the behavior of thinner materials.

The flow behavior index is the deviation from Newtonian flow such that n is always less than unity in pseudoplastic flow (Acton et al., 1983). The decrease in n, indicates the extract apparently had greater pseudoplasticity with increasing pH (Table 2).

Possible conformational changes with increased pH

Molecular unfolding appears essential for formation of fibers from plant proteins, but the muscle proteins, whose symmetry is naturally linear, do not appear to require unfolding as a prerequisite. Our results did not reveal extensive unfolding of protein polypeptide chains. The oscillatory data appeared to indicate an increased viscous component for fiber formation. If elongation of the protein was occurring, with minimal unfolding, the increase in particle streamlining could occur without causing significant decreases in Td and ΔH values. The general reduction of the consistency index could reflect increased fiber formation if protein orientation, in the direction of flow, was occurring.

Development of a seafood analog

Incorporation of fibers into an appropriately flavored and gelled surimi matrix could yield a prototype resembling seafood (Ismond et al., 1985). A crab leg analog was selected for study because many natural and artificial crab flavorings were available and both natural crab and surimi have a slightly sweet flavor.

The fibers obtained directly off the large scale fiber forming apparatus at pH 4.5 were strong, allowing ready incorporation into the surimi matrix.

Comparison with Alaskan king crab legs

Sensory analysis of the crab leg analogs led to a general description of characteristics (Table 3). Results describe products resulting from the combination of surimi pastes, fibers, flavors, and color. In general, incorporation of fibers in an undirectional and parallel manner into the surimi paste resulted in a product with the appearance and texture of crab.

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Substrate Specificity of Mackerel Flesh Lipopolygenase

PETER HARRIS and JANICE TALL

ABSTRACT

A lipoxygenase enzyme was found in mackerel flesh which oxidized linoleic and docosahexaenoic acids more efficiently than eicosapentaenoic or linolenic acids. Activity was inhibited by esculetin and was destroyed by boiling; 40% of activity was lost after incubation at 50°C for 10 min, and 80% after incubation at 60°C. Ultrafiltration of the crude enzyme extract with a molecular weight cut off of 10 KD showed the activity came predominantly from the higher molecular weight fraction. Results support the hypothesis that there are several different lipoxygenases located in different fish tissues.

Key Words: mackerel, fish, lipoxygenase, substrate, specificity

INTRODUCTION

FATTY FISH such as mackerel (*Scomber scombrus*) and herring (*Clupea harengus*) contain high levels of polyunsaturated fatty acids (Love, 1988) which makes them especially prone to oxidative rancidity even during frozen storage (Hwang, 1988). Rancidity is the result of lipid oxidation causing the development of products associated with off-flavors, thus rendering the fish unacceptable in quality. The rancidity mechanism is a chain reaction that takes place through several intermediates including hydroperoxides and peroxides, eventually producing aldehydes, alcohols and other breakdown products (Hamilton, 1989). Although lipid oxidation is highly favorable thermodynamically, the direct reaction between oxygen and even highly unsaturated lipids is kinetically hindered (Labuza, 1971). Hence, an activating reaction is necessary to initiate free radical chain reactions and lipoxygenase (LOX, linoleate: oxygen oxidoreductase, EC 1.13.11.12) has been identified as a potential initiator in fish (German and Kinsella, 1985; Mohri et al., 1990; Hsieh and Kinsella, 1986, 1989). LOX from many animal and plant sources readily oxidizes unsaturated fatty acids with a 1,4-pentadiene sequence (Mohri et al., 1990). Hydroperoxides formed are then reduced either enzymically (glutathione peroxidase) or non enzymically to hydroxyl analogs (Hsieh and Kinsella, 1989). Alternatively, they may decompose to give free radicals that can initiate chain reactions toward progressing autoxidation. Most work has investigated the presence of LOX in the gill and skin tissues of fish (German and Kinsella, 1985; Mohri et al., 1990; Hsieh and Kinsella, 1986, 1989). Hsieh et al. (1988a) reported no detectable LOX activity in the muscle tissues of several fresh water fish species. Since fish flesh is primarily used for products, understanding the mechanism of rancidity development in skinless fillets is of major importance. Josephson et al. (1984) provided evidence for involvement of LOX in producing volatiles associated with fresh fish aroma of both fresh and saltwater fish. Wang et al. (1991) found Lox activity was higher in light muscle than in the skin and dark muscle of lake herring.

Our objective was to identify the presence of LOX activity in mackerel flesh and to establish which fatty acids were preferred as a substrate.

The authors are affiliated with Unilever Research Laboratory, Colworth House, Sharnbrook, Bedford MK44 1LQ, UK.

MATERIALS & METHODS

Mackerel preparation

Four fresh mackerel (1 day on ice) were washed, headed, gutted, skinned and filleted. Fillets were then macerated in a Tefal mini mixer (Tefal UK), divided into 10g aliquots and frozen at −80°C until required.

Lipoxygenase preparation

Fish tissue (8g) was homogenized with 200 mL phosphate buffer (10 mM, pH 6.6). The homogenate was centrifuged for 15 min at 31,300 × *g* and 4°C and the resultant supernatant fraction was passed through a 0.2 μm filter to remove fat globules and bacterial contamination. This extract was used as crude enzyme source without further purification. Protein concentration was estimated by the Biuret method (Torten and Whitaker, 1964) using bovine serum albumin as standard.

The Rank oxygen electrode

The Rank oxygen electrode (Rank Brothers, Cambridge UK) consists of a platinum cathode and a silver anode immersed in the same solution of concentrated potassium chloride and separated from the reaction mixture by a Teflon membrane. Oxygen diffuses from the reaction chamber through the membrane into the electrode compartment which gives rise to a current (Jenkins, 1986). With this electrode, the substrate solution was used to calibrate 100% oxygen saturation.

Measurement of LOX activity

The LOX activity was determined by incubating 2 mL of crude enzyme preparation at 27°C with 3 mL substrate solution (10 mM phosphate buffer pH 6.6; 10 mg/mL substrate, 10 mg/mL Tween 20) in the Rank oxygen electrode. Oxygen uptake was monitored and recorded as percentage oxygen uptake/min. Substrates tested were: oleic acid (18:1n-9), α-linolenic acid (18:3n-3), γ-linolenic acid (18:3n-6), eicosapentaenoic acid (20:5n-3), arachidonic acid (20:4n-6), docosahexaenoic acid (22:6n-3), sunflower oil (purified on an aluminum column to remove any free fatty acids), Chilean fish oil (purified on an aluminum column), 1,3-dilinolein (1,3-di[(cis,cis)-9,12-octadecadienoly]-rac-glycerol), trilinolein (1,2,3-tri[(cis,cis)-9,12-octadecadienoyl]-glycerol), trilinolelaidin acid (1,2,3-tri[(trans,trans)-9,12-octadecadienoyl]-glycerol), lecithin (L-α-phosphatidyl choline type V-E from egg yolk). All substrates except sunflower oil and Chilean fish oil were purchased from Sigma and were 99% pure by GC.

Effect of esculetin on LOX activity

Enzymic activity was investigated in the presence of the LOX inhibitor esculetin (German and Kinsella, 1985; Josephson et al., 1987; Hseih et al., 1988b). Activity was determined using linoleic acid as a substrate in the presence of 12.5 μM and 957 μM esculetin.

Effect of heating on LOX activity

The effect of heat on enzyme activity was also investigated. Aliquots of mackerel extract were incubated 10 min at 40, 50, 60, and 70°C; another aliquot was boiled for 3 min. Each sample was assayed with linoleic acid and activities compared against an unheated sample.

Location of LOX activity in the crude enzyme extract

An additional experiment was carried out to establish whether the activity was due to high or low molecular weight soluble fractions of the crude enzyme extract. The crude 0.2 μm filtered enzyme extract was ultrafiltered using a molecular weight cut off membrane of 10 KD.

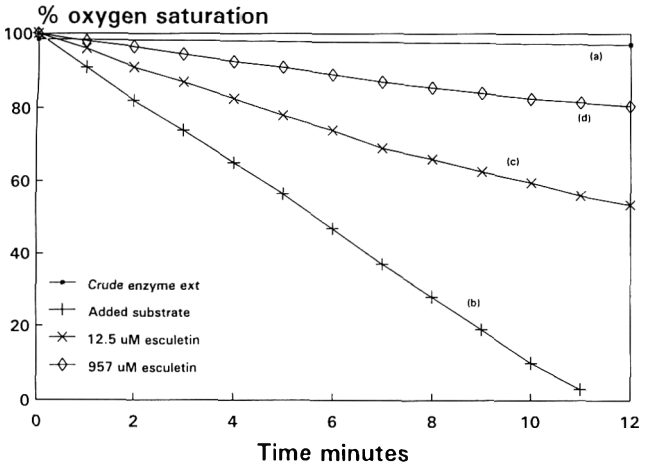

Fig. 1—Oxygen uptake activity as measured by oxygen electrode (a) Mackerel flesh crude enzyme extract, mean protein concentration 1.2 mg/mL; (b) Mackerel flesh crude enzyme extract (0.5 mg/mL protein) with 6 mg/mL linoleic acid; (c) Mackerel flesh crude enzyme extract (0.5 mg/mL protein) with 6 mg/mL linoleic acid and 12.5 μM esculetin; (d) Mackerel flesh crude enzyme extract (0.5 mg/mL protein) with 6 mg/mL linoleic acid and 957 μM esculetin. Averages of four replicates.

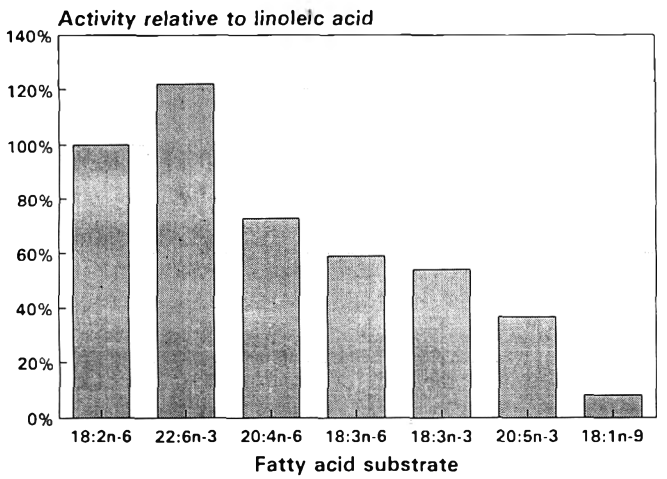

Fig. 2—Oxygen uptake activity relative to linoleic acid. All fatty acids were 6 mg/mL. The crude mackerel flesh enzyme contained 1.2 mg/mL protein. Each bar is an average of at least three repetitions. Key : 18:2n-6, Linoleic acid; 22:6n-3, Docosahexaenoic acid; 20:4n-6, Arachidonic acid; 18:3n-6, γ-Linolenic acid; 18:3n-3, α-Linolenic acid; 20:5n-3, Eicosapentaenoic acid; 18:1n-9, Oleic acid.

The retentate (above 10 KD) and the permeate (below 10 KD) fractions were assayed for LOX activity using linoleic acid as substrate. The protein concentration of each fraction was determined by the Lowry et al. (1951) method.

RESULTS

LOX assay

The rate of oxygen uptake (Fig. 1) with the extract on its own was very slow. It required about 6 hr before it increased significantly, presumably due to autoxidation. The addition of linoleic acid caused a sharp increase in oxygen uptake (≈ 10% oxygen uptake/min). However, the subsequent addition of esculetin (specific inhibitor of LOX) to a sample containing linoleic acid as substrate caused a reduction in oxygen uptake of 60% with 12.5μM esculetin and 80% when ≈ 1mM esculetin was added.

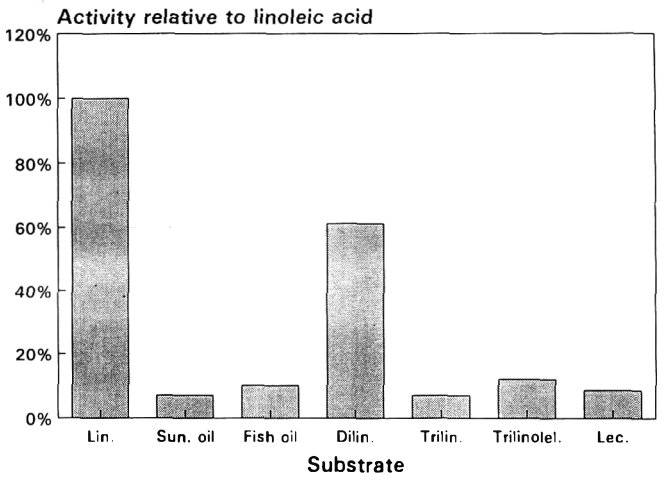

Fig. 3—Oxygen uptake activity relative to linoleic acid. All substrates were 6 mg/mL. The crude mackerel flesh enzyme contained 1.1–1.7 mg/mL protein. Each bar is an average of at least three repetitions. Key: Lin., linoleic acid. Sun. oil, sunflower oil. Fish oil, Chilean fish oil. Dilin., 1,3-dilinolein. Trilin., trilinolein. Trilinolel., trilinolaidin. Lec., 1α-phosphatidyl choline.

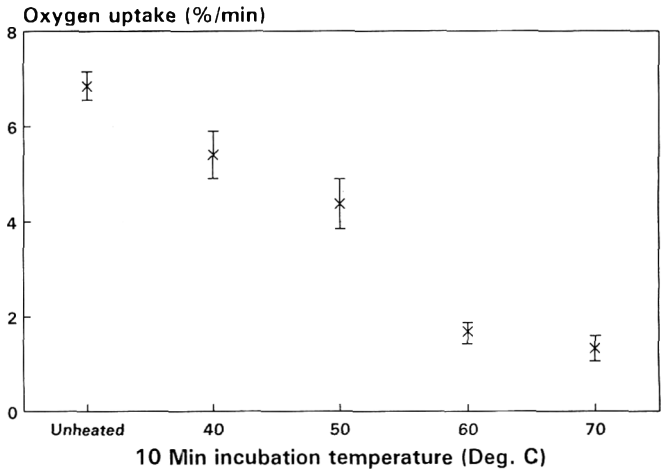

Fig. 4—Oxygen uptake activity of mackerel flesh crude enzyme extract with 6 mg/mL linoleic acid after 10 min incubation at ambient, 40, 50, 60 and 70°C. Each point represents the mean of five repetitions with a 95% confidence interval.

Table 1—Oxygen uptake activity of molecular weight fractions from mackerel flesh crude enzyme extract using linoleic acid as substrate

Fraction	Subst. conc (mg/mL)	Protein conc. (mg/mL)	Mean oxygen uptake (%/min)
Crude extract	6.00	1.256	3.40 ± 0.32
0.2μm filtrate	6.00	1.230	3.61 ± 0.17
Above 10 KD	6.00	0.868	3.53 ± 0.16
Below 10 KD	6.00	0.040	1.04 ± 0.2

Substrate specificity

The effects of different substrate fatty acids on LOX activity (Fig. 2) expressed as percentage of activity of linoleic acid, showed docosahexaenoic acid had the highest activity, and that of oleic acid was practically zero. This was expected since LOX requires the presence of a pentadiene group in the fatty acid. The effectiveness of lipids as substrates for LOX activity (Fig. 3) showed that triacylglycerols were not an effective substrate irrespective of fatty acid composition; neither was lecithin. Note that the diacylglycerol 1,3-dilinolein was reasonably effective. The boiled extract showed no LOX activity and lost

40% of its activity after incubation at 50°C for 10 min and 80% after incubation at either 60 or 70°C (Fig. 4).

Ultrafiltration of the crude extract

The activities of the low and high molecular weight fractions after ultrafiltration showed (Table 1) the major oxygen uptake activity was in the high molecular weight fraction (above 10 KD). However, activity also occurred in the < 10 KD fraction which had a higher specific activity (26%/min/mg protein) than the > 10KD fraction (4%/min/mg protein). This activity in the <10KD fraction was almost certainly due to nonenzymic metal-catalyzed oxidation similar to that reported by Decker and Hultin (1990) in the <5 KD fraction of mackerel muscle.

DISCUSSION

THE BODY of evidence that LOX was important in both onset of rancidity as well as contributing towards fresh fish aroma has grown. Two different LOX have been reported in fish tissue (Josephson et al., 1987). It has not been established whether other tissue in addition to skin and gills also express these enzymes. Possibly there are distinct tissue and cell type differences in enzyme distributions and different LOX may produce different volatiles from the same fatty acid precursors.

The substrate specificity of LOX from partially purified sardine skin was reported for linoleic and α-linolenic fatty acids (Mohri et al., 1990). That from rainbow trout skin appeared to be more specific for n-3 unsaturated fatty acids than the n-6 arachidonic acid. Linoleic acid was not tested (Hsieh and Kinsella, 1986). Trout gill showed high substrate preference for eicosapentaenoic acid, docosahexaenoic acid and arachidonic acid with little reactivity for linoleic acid (Hsieh et al., 1988a).

Of the fatty acids tested with mackerel flesh extract docosahexaenoic acid (22:6n-3) and linoleic acid (18:2n-6) were most reactive. Oleic acid showed very little activity which implicated the presence of a LOX enzyme which was only specific for fatty acids with a pentadiene group (Mohri et al., 1990). Further evidence for LOX activity was confirmed by the fact that activity was inhibited by esculetin (German and Kinsella, 1985; Josephson et al., 1987; Hsieh et al., 1988b). The enzyme from mackerel flesh did not seem specific for n-3 or n-6 fatty acids as activities for both α-linolenic and γ-linolenic acid were relatively equal. The substrate specificity of mackerel flesh LOX does not appear to be the same as other systems studied (Mohri et al., 1990; Hsieh and Kinsella, 1986; Hsieh et al., 1988a). This substantiates the hypothesis that LOX substrate specificity may vary with species or even with tissues.

Winkler et al. (1991) demonstrated the presence of an n-9 LOX in gill tissue of both marine and fresh water fish. This enzyme appeared to have a common stereospecificity to that of mammalian lung tissue LOX in that it would react with arachidonic acid containing double bonds at n-9 and n-12 positions to produce 12(S) hydroperoxy fatty acid isomers. Our studies with mackerel flesh extract demonstrated activity with linoleic acid that does not contain a double bond at the n-12 position. However, Winkler et al. (1991) reported moderate activity with linolenic acid which indicated the presence of another LOX.

German and Creveling (1990) showed that during purification of the n-9 an additional LOX with n-6 activity was present. They established that the n-9 LOX required double bonds at n-9 and n-12 whilst the n-6 LOX required double bonds at the n-6 and n-9 positions. The n-6 LOX was difficult to detect in the crude gill extract. They postulated that the extract exhibited an inhibitor for the n-6 LOX activity as the Kms of both LOX were the same. Possibly mackerel flesh tissue expresses the n-6 enzyme, hence its high reactivity with linoleic acid or the inhibitor is not present in mackerel flesh. Determination of the positional specificity of the resulting hydroperoxide could answer this question.

Instability of LOX has been reported in several previous works (German and Kinsella, 1985; Hsieh et al., 1988a; German and Creveling, 1990). This was attributed to accumulation of excess hydroperoxides. Stability was improved by adding glutathione which removes the hydroperoxides (German and Kinsella, 1986; Hsieh et al., 1988a), although low levels of hydroperoxides are required for LOX activity (Hsieh et al., 1988a). In the absence of glutathione 50% of the hydroperoxides were converted nonenzymically to trihydroxy derivatives whereas the n-9 monohydroxy derivative was produced when glutathione was present. With the mackerel flesh extract, the enzyme remained active thoughout the day despite the lack of additional glutathione.

Mohri et al. (1992) showed the most active substrates for partially purified sardine skin LOX were methyl linoleate and trilinolein. Our results showed the mackerel flesh extract had little activity with the triacylglycerides tested. However, 61% activity (relative to linoleic acid) was shown for the diacylglyceride-1,3-dilinolein. This may indicate that the fatty acid in the two position of the triacylglyceride sterically hinders the activity of the mackerel LOX on fatty acids in the 1 position.

Ultrafiltration of the crude enzyme extract (molecular weight cut off 10 KD) showed that the activity came predominantly from the high molecular weight fraction. However, some activity was observed when linoleic acid was assayed with the low molecular weight fraction. This may be due to low molecular weight initiators such as free iron since it has been shown that nonenzymic metal-catalyzed lipid oxidation occurs in the presence of reducing agents such as superoxide, ascorbate or cysteine (Kanner et al., 1987, Decker and Hultin 1990). This type of induction is prompted by a free radical mechanism which is not as substrate specific as LOX. This could account for the activity observed in such substrates as oleic acid and the triacylglycerides (sunflower and fish oil). Boiled extract from trout gill showed no activity. However, high activity of LOX was recovered at temperatures near freezing (Hsieh et al., 1988a). At 40°C, 80% activity was retained in trout gill extract which was comparable with our results for mackerel flesh extract. At 60°C and above minimal activity was recovered from trout gill tissue and 90% of activity was lost after the extract had been exposed to 60°C for 20 sec (Hsieh et al., 1988a). The mackerel extract lost 40% activity after incubation at 50°C for 10 min and 80% activity after incubations at 60°C and 70°C for 10 min. No activity was recovered from the boiled extract fraction. Wang et al (1991) also found that heat treatment inactivated LOX activity in lake herring, but nonenzymic oxidation appeared to be accelerated during subsequent storage after heat activation.

CONCLUSIONS

AN ENZYME with LOX-like activity exists in mackerel flesh. Further investigations are needed to establish whether its positional specificity is n-6 or n-9 or a combination. Elucidation of the enzymes positional specificity may substantiate the hypothesis that the variation in fresh fish flavor is a function of type and level of fatty acid present and also dependent on types of enzymes present.

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Grade Classification of Canned Pink Salmon with Static Headspace Volatile Patterns

BENOIT GIRARD and SHURYO NAKAI

ABSTRACT

Pacific pink salmon (*Oncorhynchus gorbuscha*) were subjected to refrigerated decomposition prior to canning. Data on 44 volatile compounds obtained by static headspace gas chromatography were analyzed using multivariate statistical methods. Principal component analysis and common factor analysis showed two dimensions were particularly important to delineate variation between quality grades A, B, and reject. Three types of discriminant analysis (DA) were carried out on PCA scores as well as three selected volatiles (ethanol, 3-methyl-1-butanol, and 2-methyl-1-propanal). Although all DA procduced misclassification rates below 7%, the nonparametric Epanechnikov kernal DA was more reliable followed by quadratic and linear DA. Use of 3-methyl-1-butanol and 2-methyl-1-propanal in addition to ethanol improved the detection of initial decomposition stages.

Key Words: canned salmon, static headspace, gas chromotography, principal component analysis, discriminant analysis

INTRODUCTION

POSTMORTEM DETERIORATION of fresh fish is complex and no single factor is responsible, but it involves a combination of several interrelated processes. Over time, undesirable odors and off-flavors eventually develop as well as softening of flesh, loss of cellular fluid, and discoloration. Aided by enzymatic activity, microbial spoilage is very important in chilled fish and shellfish (Wheaton and Lawson, 1985). Large numbers of bacteria are normally present in the surface slime, on the gills, and in the intestines of live fish. Rate of microbial growth depends on numbers and types present and the temperature. While fish are chilled, psychrotrophic *Pseudomonas* species generally tend to predominate, followed by *Achromobacter* and *Flavobacterium* species. At higher temperatures, *Micrococcus* and *Bacillus* appear to increase (Frazier and Westhoff, 1978).

The primary substrate for bacterial growth and the main source of spoilage products is the soluble material in the muscle. Since free carbohydrate is very low in fish, opportunistic microorganisms utilize the nonprotein nitrogen (NPN) fraction of muscle. Proteolysis becomes more vigorous in later stages of spoilage as amino acids become a limiting factor (Chung, 1968). Various products can be formed during decomposition, and are categorized as alcohols, ketones, fatty acids, aldehydes, sulfides, thiols, mercaptans, and others (Liston, 1982).

The search for chemical compounds useful to indicate spoilage has been the subject of many investigations. Research has mainly focussed on amines such as total volatile bases (Botta et al. 1984), trimethylamine/dimethylamine (Hebard et al., 1982), ammonia (Leblanc and Gill, 1984), and biogenic amines (Mietz and Karmas, 1978), nucleotides such as hypoxanthine (Jahns et al., 1976), and the K-value (Ehira and Uchiyama, 1987), and ethanol (Hollingworth et al., 1986). Since seafood decomposition results from various processes including autolytic degradation, chemical oxidation, and bacterial activity, measurement of several volatile compounds in one analysis may provide practical indices. Girard and Nakai (1991) optimized a static headspace gas chromatographic method (SHGC) for volatiles in canned salmon. The objective of this research was to explore the multivariate data generated by this SHGC method for classifying grades of canned pink salmon obtained through refrigerated decomposition.

Author Nakai is with the Dept. of Food Science, Univ. of British Columbia, 6650 N.W. Marine Drive, Vancouver, BC, Canada V6T 1W5. Author Girard is affiliated with Agriculture Canada, Research Station, 4200 Hwy 97, Summerland, BC, Canada V0H 1Z0. Address inquiries to Dr. B. Girard.

MATERIALS & METHODS

Collection and canning of salmon

Pacific pink salmon (*O. gorbuscha*) with good external appearance were taken from commercial boats at a local fish processor in Vancouver (British Columbia) and stored between layers of ice in a cold room at 2–5°C. The fish were not eviscerated before storage on ice. Every two days, six pink salmon were randomly sampled and the remaining fish were re-iced as needed. The 2 day sampling period was increased towards the end of storage to 3 or 4 days to extend decompositon. The experiment was repeated a second year: 42 and 54 fish were collected at the start and provided enough samples for 13 and 21 days of refrigerated storage during years 1 and 2, respectively.

The quality of each fish selected was assessed before canning with the help of experienced personnel from a local fish processor, using a grading guide (Table 1). The procedure evaluated condition of the eyes, gills, and flesh texture for external characteristics. Once opened and eviscerated, the integrity and odor of the belly cavity were the main criteria examined.

After quality assessment, the salmon were cleaned and eviscerated. Steaks of ≈ 3.8 cm thick were cut and 215g salmon flesh was placed in 307 × 115 two-piece cans with 2g sodium chloride. The cans were vacuum-sealed, retorted, and cooled according to practice of the commercial plant.

Static headspace gas chromatography (SHGC)

Two samples/fish (one each from the head and tail sides) were examined for headspace volatiles. Each can was opened and the liquor drained by tilting the can and its lid for 2 min. After flaking the white muscle 3–5 mm in size with a spatula, 10g were transferred in 20 mL headspace vials (Hewlett Packard, Avondale, PA) avoiding dark meat, skin, and bones. One millilitre of 3-hexanol working standard solution (84.8 ppm) was added to the vials, which were then sealed with teflon-faced silicone septums and aluminum caps.

The SHGC method was derived from Girard and Nakai (1991). A HP 5890 gas chromatograph (Hewlett Packard, Avondale, PA) equipped with a flame ionization detector (FID) was connected to a HP 19395A headspace sampler. A HP 3396A integrator recorded the chromatograms and also transmitted the output to an IBM-compatible personal computer. A program "FILE SERVER" allowed the host computer to serve as a remote external disk drive. Separation of volatiles was made on a HP ULTRA 2 fused silica capillary column (25m length × 0.32mm i.d. × 0.52 μm film thickness).

Standard curves for quantification were obtained for ethanol, 3-methyl-1-propanal, 3-methyl-1-butanol, and 3-haxanol by injecting 2 μL of five solutions diluted in the appropriate response range. A split ratio of 10:1 was used for manual injections.

Identification of volatiles was performed by connecting the headspace sampler to a Hewlett Packard 5985B GC-MS system. The same column (mentioned above for SHGC analysis) was used. Electron impact mass spectra were recorded at 70 eV with a source temperature of 200°C. Conditions of operation and separation as well as mass spectra acquisition and confirmation by retention time comparison with reference standards were described in Girard and Nakai (1991).

Table 1—Grading guide for whole raw Pacific salmon

Criteria	Grade A	Grade B	Reject
External characteristics			
Eyes	clear, bright, convex	slightly sunken and dull	dull, sunken, and cloudy
Gills color	bright red	pink	brown, grey
Gills odor	fresh, seaweed, or shellfish odor	neutral, slight but definite sour, faecal, or putrid odor	strong sour, putrid, or faecal odor
Texture	firm and resilient, flesh springs back when thumb depression is released	moderately soft, thumb indentations may slowly fill out	very soft, thumb indentations may remain in flesh
Internal characteristics			
Belly cavity	transparent, intact peritoneal lining	moderate reddening, some ribs may protrude	extensive reddening, liquefaction of belly walls
Odor of belly	fresh and characteristic odor	slight but definite sour, faecal, or putrid odor	strong sour, faecal, or putrid odor

Table 2—Volatile compounds from canned pink salmon of different quality grades

			Quality grade[b]		
No.	Compound name	ID[a]	A (n=107)	B (n=48)	R (n=34)
1	hydrogen sulfide	MS			
1	acetaldehyde	MS,RT	260992.1	290086.6	317692.5
2	methane thiol	MS	85986.2	88928.2	78525.4
3	ethanol	MS,RT	6222.7	40605.8	229484.7
4	butane	MS	75466.2	83628.1	6712.1
5	3-methyl-1-butene	MS,RT	4916.0	7232.7	8380.6
6	dimethyl sulfide	MS,RT	12509.7	11136.9	10993.1
7	2-methyl propanal	MS,RT	6564.0	9977.0	9821.1
8	hexane	MS,RT	4569.8	4774.4	4510.9
9	butanal	MS,RT	7917.8	10477.0	10681.1
10	2-methyl furan	MS	3487.0	4859.3	5100.0
11	benzene	MS,RT	2300.4	4930.2	6514.7
12	2-methyl butanal	MS,RT	2247.5	5352.7	5043.2
13	2,2-dimethyl propanal	MS	2420.4	3617.2	5064.1
14	unknown 1	—	2251.7	3956.1	4090.4
15	1-penten-3-ol	MS,RT	13531.5	15282.5	16930.5
16	heptane	MS,RT	9227.2	11221.1	12543.2
17	1,5-dimethyl cyclopentene	MS	43859.7	48997.8	49113.9
18	3-methyl-butanol	MS,RT	0.0	786.7	8863.1
19	2-methyl-2-butenal	MS,RT	40.8	364.9	3316.0
20	unknown 2	—	1733.2	1771.5	2022.4
21	acetic acid	MS,RT	645.9	824.2	919.5
22	toluene	MS,RT			
22	3-hexanone	MS,RT	10262.5	5582.5	2641.1
23	unknown 3	—	357.2	340.5	354.1
24	unknown 4	—	866.3	1009.9	1140.9
25	unknown 5	—	624.8	651.7	663.9
26	3-ethyl-2-methyl-pentane	MS	4606.8	5727.8	6285.1
27	ethylidene cyclohexane	MS	948.1	1751.2	2185.6
28	unknown 6	—	710.3	1156.8	1466.0
29	unknown 7	—	426.5	500.6	628.8
30	nonane	MS,RT	2026.1	2308.4	2786.4
31	unknown 8	—	1767.9	2365.7	2801.7
32	benzaldehyde	MS,RT	614.7	811.5	970.2
33	7-octen-4-ol	MS	1045.0	1308.2	1270.6
34	unknown 9	—	137.8	162.3	229.7
35	unknown 10	—	430.4	435.9	467.2
36	unknown 11	—	372.7	408.4	433.4
37	unknown 12	—	488.4	579.7	658.8
38	2-pentyl furan	MS	864.8	1090.1	1232.4
39	4-ethyl benzene methanol	MS	5440.2	6769.6	6932.0
40	3-ethyl-1,4-hexadiene	MS	563.8	575.7	585.7
41	nonanal	MS,RT	1291.4	1688.5	2114.9
42	unknown 13	—	370.4	399.4	441.5
43	unknown 14	—	647.3	768.3	892.1
44	unknown 15	—	1028.8	892.3	1386.6

[a] Ms, tentatively identified by mass spectrometry; RT, retention time consistent with that of authentic compounds.

[b] Average area counts were standardized with that of 3-hexanol as described in Materials & Methods.

Data handling and statistical analysis

A computer program was written in QuickBasic 4.5 (Microsoft Corporation) to preprocess chromatographic data. Raw data were first extracted from the stored datafiles of the integrator reports. The chromatographic area of selected peaks which were consistently detected were compiled. Standardization was done by dividing each chromatographic area with the peak area of the 3-hexanol standard and multiplying × 10000.

The Statistical Analysis System (SAS Institute, Inc., 1989) was used for multivariate analysis. Common factor analysis (CFA) was performed with the FACTOR procedure using the maximum-likelihood method and the prior communality estimates set to squared multiple correlations. Principal component analysis (PCA) was also carried out with the FACTOR procedure. Computations of linear, quadratic, and non-parametric Epanechnikov kernel discriminant analyses were performed with the DISCRIM procedure.

SYSTAT (Wilkinson, 1990) was also used for data analysis. The original spreadsheets of standardized data were first converted to compatible workfiles with the IMPORT command of the DATA module. The NPAR module was used to compute the Kolmogorov-Smirnov (KS) statistic with the Lilliefors option for testing normality. Bartlett's test for homogeneity of variance was computed using the STATS module.

RESULTS & DISCUSSION

IDENTITIES AND AVERAGE AREA COUNTS of 44 peaks were analyzed in all canned pink salmon (Table 2). Most coefficients of variation varied between 9 and 25%. Three typical chromatograms (Fig. 1A, B C) from canned pink salmon represent grades A, B, and reject. Qualitative and quantitative differences were noticed among samples of different grades during refrigerated storage. Given this complex matrix of data, CFA and PCA were initially used to assist interpretation.

CFA with varimax rotation was first performed on the 44 volatile compounds analyzed throughout the refrigerated storage study. Nine factors (Table 3) were allocated eigenvalues ≥ 1.0, explaining nearly 88% of the common variance. Factors 1 and 2 encompassed variations of a general nature including year to year variation similar to that reported in previous studies on species of Pacific salmon (Girard and Nakai, 1993) and sexual maturity of chum salmon (Girard and Nakai, 1994). However, factors 3 and 4 related to compounds having evident relationships in patterns throughout refrigerated storage. Peaks 3 (ethanol), 18 (3-methyl-1-butanol), and 19 (2-methyl-2-butenal) received loadings above 0.9 for factor 3. The standardized peak area of these three volatiles were plotted (Fig. 2) for both years of storage. Constant values over a period of time, followed by an exponential increase was common to all three peaks. For factor 4, three volatile compounds, peak 7 (2-methyl propanal), peak 11 (benzene), and peak 13 (2,2-dimethyl propanal), were allocated high loadings. As expected of variables that correlated to each other, peaks 7, 11, and 13 had similar trends (Fig. 3). They increased in concentration near the beginning of storage, and levelled off towards the end. In addition, the spread in peak area increased with time for all 6 volatiles (Fig. 2 and 3).

PCA was also carried out with a varimax rotation on the same data matrix gathered from canned salmon of the refrig-

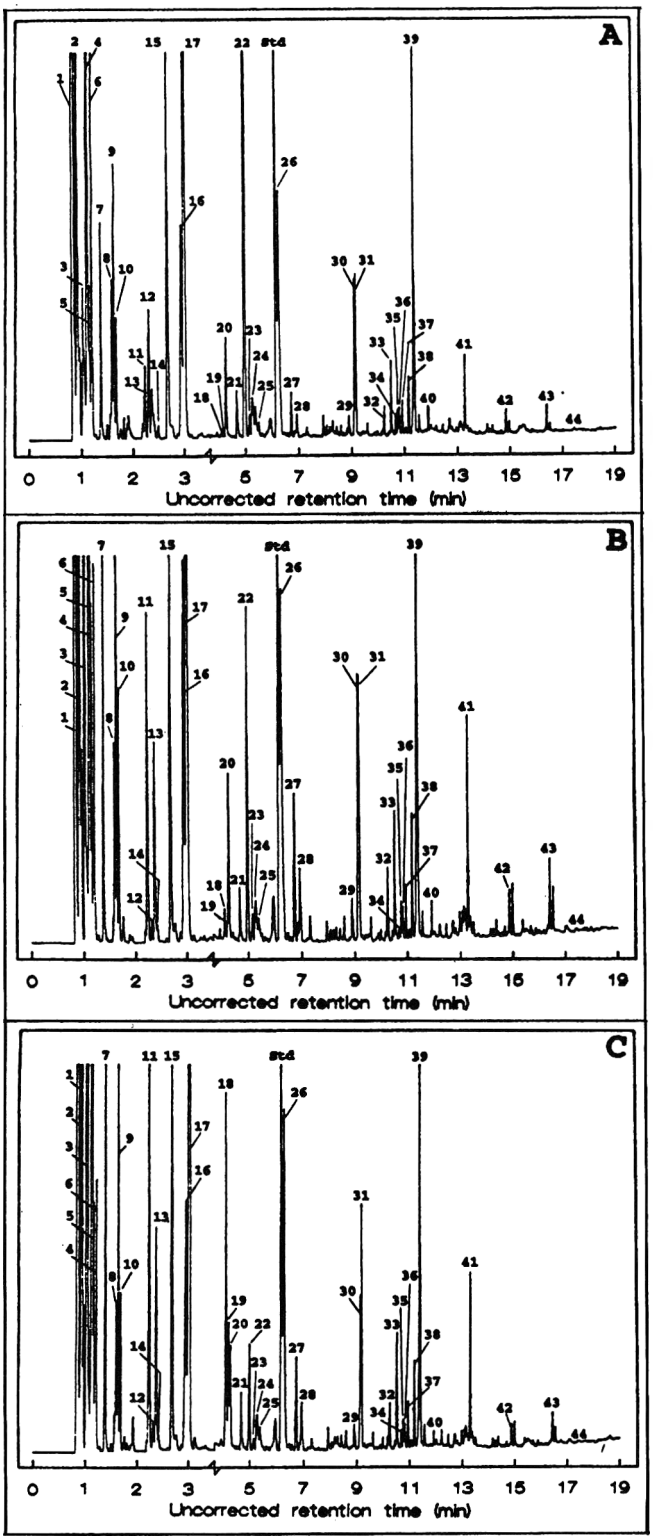

Fig. 1—Chromtograms of volatiles from canned pink salmon during refrigerated storage of year 1. (A, day 0/grade A; B, day 8/ grade B; C, day 13/grade reject).

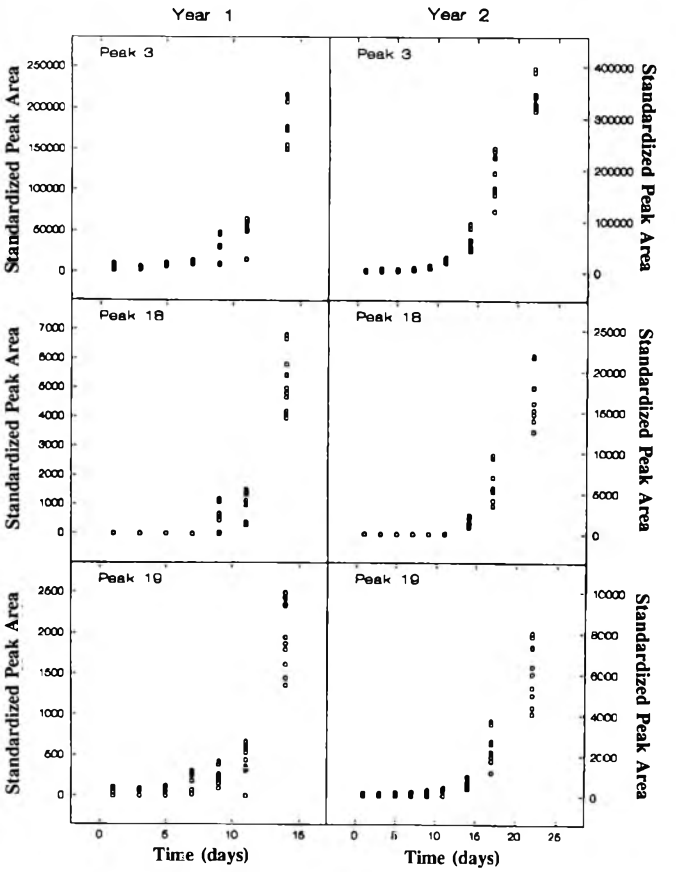

Fig. 2—Volatiles from canned pink salmon with high loadings for factor 3.

erated storage study. Our examination primarily focussed on the six volatile components discussed above. The 44 loadings were developed as vectors in the subspace (Fig. 4) spanned by PC4 and PC5. Loadings with absolute values > 0.2 have been labelled. Peaks 3, 18, and 19, and peaks 7, 11, and 13 were in the vicinities of each other and each respective group of peaks dominated PC4 and PC5. PC4 and PC5 respectively explained 3.851% and 3.750% of the total variation. Figures 5 and 6 are plots of scores of PC4 and PC5 during refrigerated storage for years 1 and 2. The trends in these figures were clearly comparable to those in Figs. 2 and 3. PC4 and PC5 essentially contained similar information to factors 3 and 4. The results of CFA and PCA were alike in terms of peak grouping and confirmed intercorrelations among several of the variables in the original dataset.

When PC4 and PC5 were plotted, segregation of the 3 quality levels became apparent (Fig. 7). PC4 separated grade reject (right side), from grade A and B (left). Score values of PC4 remained relatively constant (Fig. 5) for some time but began to increase toward the end of storage. These late changes were correlated with increases in concentration of volatiles that received especially high loadings on PC4 (peaks 3, 18, and 19) and coincided with a shift toward quality grade reject. Similarly, PC5 mainly served to vertically separate grades A and B. The increasing scores of PC5 from the start of storage to a maximum (Fig. 6) provided valuable information to discern grade A from grade B (Fig. 7).

The scores of rotated principal components of canned pink salmon from the storage study were submitted to linear discriminant analysis (LDA). The univariate tests on the 10 PC's with eigenvalues > 1.0 showed that the first five were significant at either the 0.0001 or 0.05 levels (Table 4). The two largest F ratios were assigned to PC4 and PC5, and confirmed their substantial involvement in grade categorization. Wilk's lambda, Pillai's trace, Hotelling-Lawley's trace, and Roy's greatest root are multivariate tests to determine whether the means of each quality grade grouping were equal (Morrison, 1976). These tests showed that the joint contribution of the PC's proved to be highly significant ($P<0.0001$) in discriminating the 3 quality grades. Table 4 also indicated the high statistical significance ($P<0.0001$) of both canonical variates (CV) generated from linear functions of principal components. CV1 and CV2 accounted for 89.1% and 10.9% of the variance,

Table 3—Loadings of the first nine varimax rotated factors on volatiles from canned pink salmon

	Factors								
Peak	1	2	3	4	5	6	7	8	9
1	0.372	0.674	0.080	0.096	0.019	0.182	0.105	−0.143	−0.131
2	0.232	0.032	−0.368	0.134	0.242	−0.072	−0.195	0.581	0.184
3	0.145	0.175	0.904	0.255	0.086	−0.027	0.057	−0.135	0.005
4	0.530	0.466	−0.041	0.248	0.430	0.180	0.013	0.097	0.022
5	0.297	0.826	0.243	0.258	−0.039	0.111	0.181	−0.065	−0.016
6	0.141	0.122	−0.066	−0.097	−0.191	−0.087	0.123	−0.015	0.003
7	0.285	0.270	0.156	0.868	0.069	0.007	−0.035	0.080	0.085
8	−0.053	0.115	−0.050	0.024	0.157	0.898	0.029	−0.007	−0.031
9	0.582	0.387	−0.022	0.387	0.301	−0.399	−0.079	0.219	0.029
10	0.585	0.222	0.264	0.346	0.306	−0.059	−0.030	0.220	0.029
11	0.168	0.173	0.306	0.876	0.187	−0.015	0.013	−0.110	0.048
12	0.013	0.057	−0.002	−0.052	0.163	−0.045	0.044	0.192	0.132
13	0.176	0.155	0.345	0.862	0.137	−0.029	0.004	0.007	0.039
14	0.039	0.144	0.205	0.152	−0.285	−0.071	0.546	−0.074	−0.198
15	0.360	0.374	0.217	0.040	0.542	0.166	0.501	−0.153	0.026
16	0.558	0.522	−0.008	0.143	0.277	0.083	−0.071	0.111	−0.018
17	0.798	0.084	−0.092	0.143	0.359	0.043	0.028	0.244	−0.013
18	0.059	0.111	0.963	0.171	0.010	−0.002	0.110	−0.071	−0.047
19	0.031	0.108	0.956	0.197	−0.016	−0.001	0.105	−0.065	−0.059
20	0.549	0.109	0.182	0.077	0.030	0.123	0.209	−0.012	−0.115
21	0.049	−0.003	−0.022	0.209	0.822	0.024	0.045	0.184	0.226
22	−0.059	−0.128	−0.148	−0.081	0.122	−0.124	−0.247	0.700	0.081
23	0.566	−0.163	−0.038	−0.103	0.078	−0.002	−0.041	0.111	−0.057
24	0.488	0.217	0.196	0.128	0.650	0.086	0.250	0.000	0.084
25	0.154	0.060	0.121	−0.126	0.172	0.146	0.783	−0.196	0.050
26	0.472	0.020	0.019	0.207	0.636	0.011	−0.163	0.280	0.080
27	0.421	0.761	0.256	0.297	0.076	−0.014	0.097	−0.044	0.063
28	0.549	0.704	0.268	0.277	0.074	0.002	0.086	−0.023	0.040
29	0.775	0.271	0.091	0.125	0.073	0.195	−0.118	−0.120	−0.113
30	0.570	0.467	0.004	0.006	−0.068	0.227	0.116	−0.076	0.003
31	0.614	0.061	0.213	0.233	0.477	−0.260	−0.023	−0.004	−0.013
32	0.858	0.288	0.184	0.182	0.129	0.003	0.046	−0.047	0.051
33	0.645	0.077	−0.028	0.435	0.266	−0.021	−0.149	0.280	0.230
34	0.074	−0.016	−0.069	0.112	0.143	−0.036	−0.013	0.134	0.726
35	0.358	0.063	0.031	−0.060	−0.164	0.794	0.106	−0.118	−0.023
36	0.657	0.238	0.085	−0.010	−0.221	0.072	0.462	−0.152	0.067
37	0.797	0.231	0.136	0.104	0.122	0.071	0.273	−0.168	0.241
38	0.936	0.168	0.070	0.166	0.026	−0.065	0.100	−0.030	0.130
39	0.856	0.064	−0.041	0.169	0.297	0.003	−0.059	0.206	0.019
40	0.181	0.140	0.021	−0.074	−0.472	0.353	0.257	−0.205	0.180
41	0.775	0.288	0.205	0.180	0.111	0.210	0.147	−0.098	−0.148
42	0.880	0.171	−0.041	−0.010	−0.112	0.093	0.166	−0.085	−0.008
43	0.858	0.180	0.005	0.032	−0.159	0.047	0.123	−0.113	−0.006
44	0.191	−0.017	0.123	−0.042	−0.273	0.015	0.046	−0.333	−0.211
Latent root	11.543	4.083	3.707	3.624	3.602	2.148	2.015	1.718	0.999
Variance (%)	30.371	10.743	9.755	9.535	9.476	5.652	5.303	4.520	2.630

respectively, and produced a cross-validated error rate of 6.9% in classifying samples of various quality grades.

To investigate the potential usefulness of relying directly on volatiles for classification, stepwise discriminant analysis was performed on selected peak variables (Table 5). Functions based only on peak 3 (ethanol) provided a cross-validated total error rate of 19.6%, the majority of the error on classifying grade B to grade A samples. When peak 18 was combined with peak 3 for analysis, the error rate for grade B was reduced from 75% to about 42%. Peak 7, with the highest correlation with PC5, helped achieve a further decrease in error rate to ≈17% for grade B whether or not peak 19 was used in the model. Peak 19 was redundant due to inter-correlations with peaks 3 and 18. Peaks 11 (benzene), 5 (3-methyl-1-butene), 21 (acetic acid), and 38 (2-pentyl furan), all given high loadings onto PC5, PC2, PC3, and PC1, respectively, did not improve the classification rate of 93% established with peaks 3, 18, and 7.

Bartlett's and Kolmogorov-Smirnov tests were respectively used to assess assumptions of homogeneity of variance and normality on principal components and peak variables. Important discriminating variables such as PC4, PC5, peaks 3 and 18 had significant Bartlett's Chi-square statistics at $P<0.05$, therefore indicating non-homogeneous cases of variance. Kolomogrov-Smirnov normality tests also revealed instances where assumption of normality was not respected in grade A, B, and reject. While peaks 3 and 18 showed non-normal behavior in all grades, distributions of PC4 and PC5 were statistically different from normal for only grades reject and grade B, respectively.

In view of departures from statistical assumptions, quadratic (QDA) and nonparametric (NPDA) discriminant analyses were performed for classification of canned salmon. QDA do not pool individual variances of each group (quality grades), and therefore alleviated the problem of homogeneity of variance (Dillon and Goldstein, 1984). NDPA such as kernel analysis can be used when group distributions are different from multivariate normal (Hand, 1982). Cross-validated error counts estimates were computed for the three types of discriminant functions (Table 6). Among the three quality levels, the linear functions were least reliable with grade B (12.5% and 16.7%). The quadratic and nonparametric Epanechnikov kernel methods were equally effective in lowering the error count of all grades; but grade B samples were clarified most from their implementation. Rates of correct classification were heightened to 96% to 98%. However, nonparametric discriminant functions should be regarded as more stable due to considerations encompassing the statistically nonnormal and nonhomogenous behavior of some variables. Furthermore, use of non-parametric Epanechnikov kernal DA based on the 3 peak variables had the advantage of computing the functions directly on the peak areas themselves. The number of variables handled was considerably reduced, thereby lessening the computation.

The peak areas of the 3 volatile compounds ethanol, 3-methyl-1-butanol and 2-methyl-1-propanal were translated into

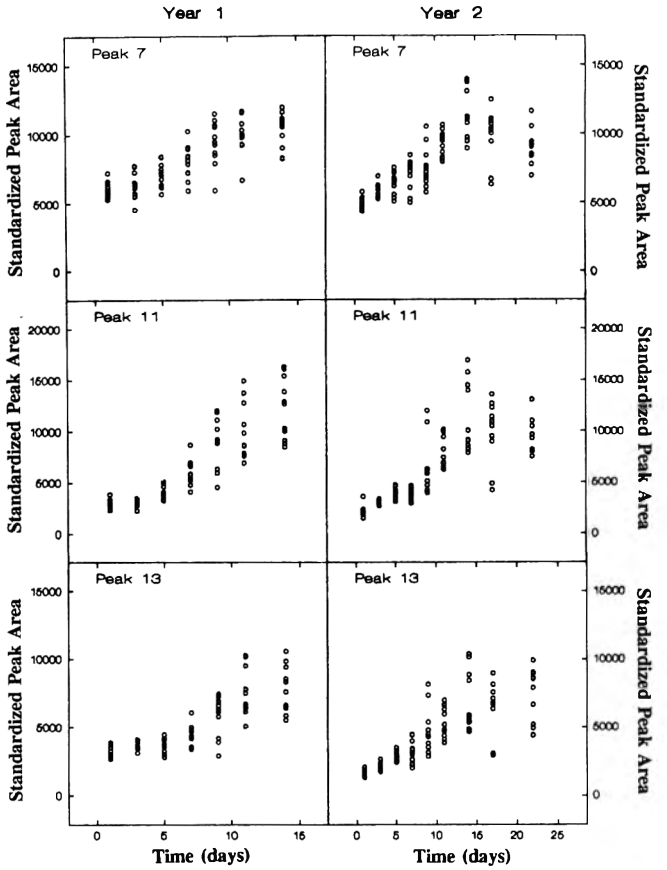

Fig. 3—Volatiles from canned pink salmon with high loadings for factor 4.

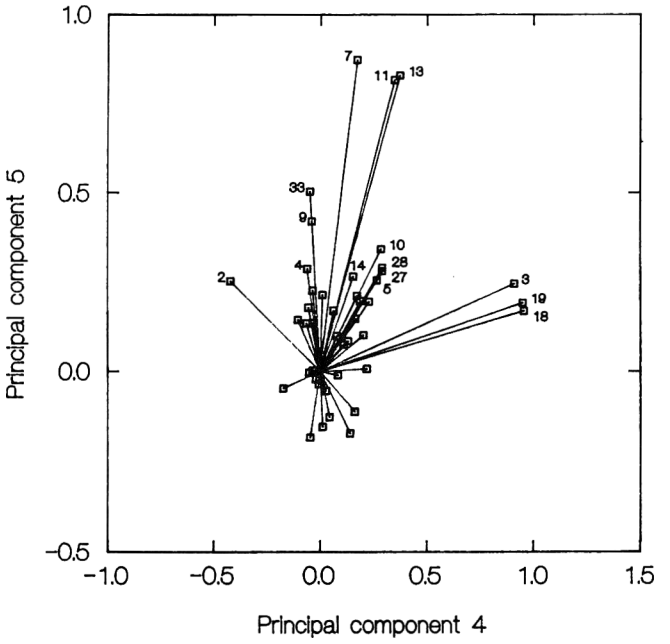

Fig. 4—Projection of gas chromatographic peak variable loadings on principal components 4 and 5 for canned pink salmon of the refrigerated storage study.

actual concentrations in the headspace samples analyzed. The ranges of concentrations within which all measurements fell, were developed for each grade level (Table 7). Ethanol and 3-methyl-1-butanol are known microbial catabolic products. Their concentration increases are derived from metabolic degradation of carbohydrates and amino acids (Brock, 1979). Eth-

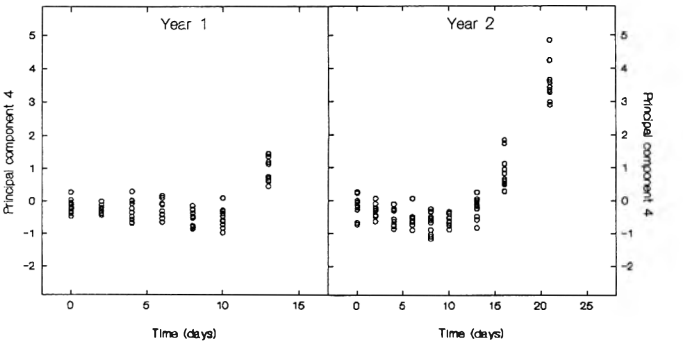

Fig. 5—Scores of principal component 4 related to refrigerated storage time for canned pink salmon.

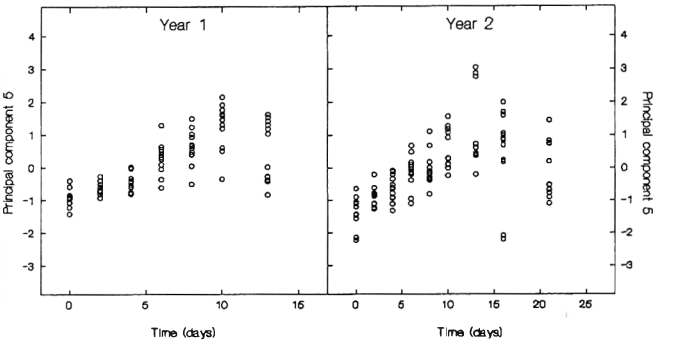

Fig. 6—Scores of principal component 5 related to refrigerated storage time for canned pink salmon.

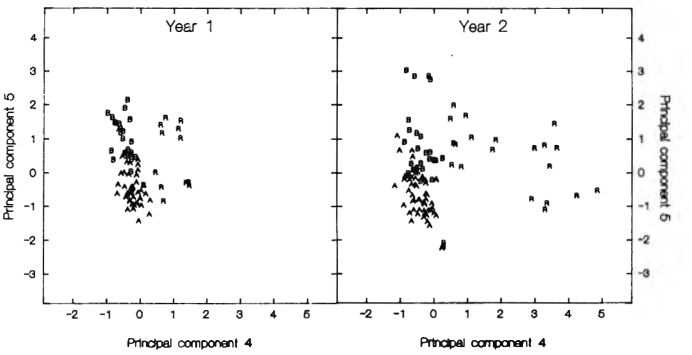

Fig. 7—Scores of principal component 5 and 4 for canned pink salmon of different quality grades.

anol has been suggested as a potential index of spoilage in canned fish (Holaday, 1939; Lerke and Huck, 1977; Crosgrove, 1978; Khayat, 1979; Iida et al., 1982; Tokunaga et al., 1982; Hollingworth et al., 1986). Regarding comparison of values for ethanol with those of Hollingworth et al. (1986) for canned salmon, a 1000-fold factor in scale was observed. The main reason was due to differences in sample preparation and analysis. Results of our study were obtained using an automated method sampling headspace volatiles above heated salmon flakes. Hollingworth et al. (1986) manually injected headspaces of liquid samples equilibrated at room temperature. Explanations for concentration increases of 2-methyl-1-propanal as well as 2-methyl-2-butenal, benzene, and 2,2-dimethyl propanal during refrigerated decomposition for canned salmon require further research.

CONCLUSION

CFA AND PCA were useful for simplifying GC data during refrigerated storage studies, and identifying primary bidimen-

Table 4—Univariate and multivariate test statistics of linear discriminant analysis on the first ten varimax rotated principal components from canned pink salmon during the refrigerated storage study

Variable	Num df,den df	F
Univariate		
PC1	2,186	3.8697 *
PC2	2,186	9.9110 ***
PC3	2,186	4.1739 *
PC4	2,186	154.0639 ***
PC5	2,186	61.4829 ***
PC6	2,186	2.5676
PC7	2,186	0.1830
PC8	2,186	2.5589
PC9	2,186	2.6270
PC10	2,186	1.2413
Multivariate		
Wilk's lambda = 0.07785	20,354	45.7376 ***
Pillai trace = 1.29703	20,356	32.8421 ***
Hotelling-Lawley trace = 7.02994	20,352	61.8635 ***
Roy's largest root = 6.26080	10,178	111.4423 ***
Canonical		
LR_1 = 0.07785	20,354	45.7376 ***
LR_2 = 0.56525	9,178	15.2118 ***

* Significant difference at 0.05 level.
** Significant difference at 0.01 level.
*** Significant difference at 0.0001 level.
LR stands for Likelihood ratio.
Num df, den df stands for numerator degrees of freedom, denominatior degrees of freedom.

Table 5—Cross-validated error count estimates of linear discriminant functions carried out on selected gas chromatographic peaks from canned pink salmon of the refrigerated storage study

	Error count estimate[a]			
Peak variable	Grade A (n=107)	Grade B (n=48)	Reject (n=34)	% Total (n=189)
3	0.000 (0)	0.750 (36)	0.029 (1)	19.6
3,18	0.000 (0)	0.417 (20)	0.029 (1)	11.1
3,18,19,7	0.037 (4)	0.167 (8)	0.029 (1)	6.9
3,18,7	0.028 (3)	0.167 (8)	0.029 (1)	6.4
3,18,7,11	0.028 (3)	0.188 (9)	0.029 (1)	6.9
3,18,7,5	0.028 (3)	0.188 (9)	0.029 (1)	6.9
3,18,7,21	0.028 (3)	0.208 (10)	0.029 (1)	7.4
3,18,7,38	0.028 (3)	0.167 (8)	0.029 (1)	6.4

[a] Numbers in parentheses are the misclassifications associated with the error count estimates.

sional structures relevant for separating quality grades. Discriminant analyses, applied to PCA scores and 3 selected volatiles, generated rates of correct classifications > 93%. Among DA methods, nonparametric Epanechnikov kernel functions provided the lowest error rates and are recommended to alleviate violations of statistical assumptions.

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Table 6—Comparison of cross-validated error count estimates for different discriminant analysis functions (DA) of selected gas chromatographic peaks and principal components from canned pink salmon of the refrigerated storage study

	Error count estimate[b]			
DA function[a]	Grade A (n=107)	Grade B (n=48)	Reject (n=34)	% Total (n=189)
Principal components				
linear	0.047 (5)	0.125 (6)	0.059 (2)	6.9
quadratic	0.028 (3)	0.083 (4)	0.000 (0)	3.7
nonparametric (r=4)	0.019 (2)	0.104 (5)	0.000 (0)	3.7
Three peaks (3, 7, 18)				
linear	0.028 (3)	0.167 (8)	0.029 (1)	6.4
quadratic	0.009 (1)	0.062 (30)	0.000 (0)	2.1
nonparametric (r=3)	0.009 (1)	0.042 (2)	0.000 (0)	1.6
Prior prob.[c]	0.566	0.254	0.180	

[a] Nonparametric method consists of the Epanechnikov kernel with smoothing parameter, r.
[b] Numbers in parentheses are the misclassifications associated with the error count estimates.
[c] Prior probability is the probability of classifying the specific quality grade in its own group by chance and is obtained from the number of samples of each grade over the total number of salmon (n=189).

Table 7—Concentration ranges of three volatile compounds from canned pink salmon of different quality grades

	Concentration (ppm)		
Grade	Ethanol	3-methyl-1-butanol	2-methyl-1-propanal
A	178 - 1668	0	812 - 1982
B	977 - 10815	0 - 218	1118 - 2633
reject	13590 - 45267	318 - 2019	1186 - 2350

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Gram Negative Bacteria Inhibition by Lactic Acid Culture and Food Preservatives on Catfish Fillets during Refrigerated Storage

CHANG R. KIM and JAMES O. HEARNSBERGER

ABSTRACT

Treating catfish fillets with sodium acetate (SA) and/or potassium sorbate (PS), with or without lactic culture (*Lactococcus lactis* spp. *cremoris* ATCC 19257 grown in 20% milk solids) was studied at 4°C. Gram negative bacterial (CVT) counts on crystal violet tetrazolium chloride agar, pH, and sensory evaluation of fillets were assessed over time. Treatment with the combination of 0.50% SA, 0.25% PS with 2.50% lactic acid culture (LC) and, also, 0.50 to 1.00% SA alone completely inhibited growth for ≥ 6 days at 4°C. After 12 days, counts with this treatment and the 1.00% SA increased ≈1.3 and 1.2 log cycles, respectively. The antimicrobial effects did not appear to be due to pH values. The combination of 0.50% SA and 0.25% PS with 2.50% LC, or 1.00% SA, alone, improved catfish odor and appearance during 13 days storage.

Key Words: catfish, bacteria, Gram negative, lactic acid, antimicrobials

INTRODUCTION

AEROBIC FOOD SPOILAGE ORGANISMS in refrigerated food can reduce shelf-life and microbiological quality (Reddy et al., 1970; Shewan, 1971; Gilliland and Specks, 1975; Post et al., 1985; Ingham, 1989; Berry et al., 1991). The combination of lactic acid bacteria and food additives could be considered as a food preservative to repress growth of such microorganisms (Reddy et al., 1970; Gilliland and Ewell, 1983; Lindgren and Dobrogosz, 1990). Metabolites produced by lactic acid bacteria have antimicrobial effect on food spoilage microorganism and food borne pathogens (Reddy et al., 1970; Gilliland and Ewell, 1983; Reddy et al., 1983; Post et al., 1985; Schaack and Marth, 1988; Berry et al., 1991; Motlagh et al., 1991; Okereke and Montville, 1991; Ray, 1992). Gilliland and Ewell (1983) reported that combinations of cells of lactobacilli and sorbate had a more inhibitory effect on psychrotrophic bacteria than either lactobacilli or sorbate alone in raw milk. They noted that addition of sorbate had no apparent effect on antimicrobial compounds produced by the lactic cultures during refrigerated storage. The use of food preservatives as antimicrobial surface treatments in fresh foods (Mendonca et al., 1989; Lindgren and Dobrogosz, 1990; El-Shenawy and Marth, 1991) has been reported. Mendonca et al. (1989) reported that surface treatment with solutions containing 10% potassium sorbate (PS) and 10% phosphates, with or without 10% sodium acetate (SA) and 5% sodium chloride, prolonged microbiol shelf-life in vacuum-packaged pork chops stored at 2–4°C.

However, food additives have received limited use in fish to improve shelf-life, quality and nutritional value. Using a lactic acid bacterial rinse may overcome the disadvantages of other food additives (Gilliland and Ewell, 1983). Research on the extension of shelf-life in refrigerated fish using a combination of food preservatives plus conditions in which lactic acid bacteria will not grow has been limited. The purpose of our study was to evaluate any extension of microbiological shelf-life or refrigerated channel catfish fillets by treating their surfaces with lactic acid culture (LC), sodium acetate (SA), and potassium sorbate (PS).

Authors Kim and Hearnsberger are with the Dept. of Food Science & Technology, Mississippi State Univ., Box 9805, Mississippi State, MS 39762. Address inquiries to Dr. C.R. Kim.

Table 1—Preservatives and concentrations studied on refrigerated (4°C) channel catfish fillets

	Preservative	Concentration (%)*
A	Sodium acetate (SA)	0.00, 0.50, 1.00, 1.50
	potassium sorbate (PS)	0.25
B	Sodium acetate (SA)	0.00, 0.25, 0.50, 0.75
	lactic acid culture (LA)	2.50
C	Sodium acetate (SA)	0.00, 0.50, 1.00, 1.50
	potassium sorbate (PS)	0.25
	lactic acid culture (LC)	2.50
D	Lactic acid culture (LC)	0.00, 1.50, 2.00, 2.50, 3.00
	potassium sorbate (PS)	0.25
	sodium acetate (SA)	0.50
E	Sodium acetate (SA)	0.50, 1.50
	potassium sorbate (PS)	0.25
	lactic acid culture (LC)	2.50
F	Potassium sorbate (PS)	0.25, 0.50
	sodium acetate (SA)	0.50, 1.00
G	Potassium sorbate (PS)	0.25, 0.50
	sodium acetate (SA)	0.50, 1.00
	lactic acid culture (LC)	2.50

* All concentrations in W/W except for lactic acid culture in W/V.

MATERIALS & METHODS

LACTOCOCCUS LACTIS SSP. *CREMORIS* ATCC 19257 was obtained from American Type Culture Collection (ATCC). The test microorganism was transferred daily for three successive days in sterile (121°C for 5 min) skim milk with 10% solids (w/v) at 30°C and then grown in resconstituted milk with 20% milk solids for 48 hr at 30°C. Fresh catfish fillets were obtained from a commercial source, stored at 7°C in a cooler, and used within 2 hr. Catfish fillets (2 kg, average weight 100g/fillet) were placed in a tumbler (Polymaid Largo, FL, Model SS 350) at room temperature (≈23°C). Food preservatives and/or lactic acid cultures (LC) were added to the catfish fillets and tumbled for 20 min at 18 rpm at room temperature. Samples were treated by a combination of preservatives (Table 1). Lactic acid culture (LC) used in preliminary treatments ranged from 9.8×10^8 to 3.2×10^9 CFU/mL of *L. lactis* ATCC 19257. After tumbling, fillets were placed in "Ziploc®" (DowBrands L.P., Indianapolis, IN) bags and stored at 4°C.

For each surface treatment, microbiological, pH, odor, appearance and flavor evaluations were performed beginning with day of treatment and at 3 day intervals thereafter. Gram-negative bacterial (CVT) counts were determined on crystal violet tetrazolium chloride (Olson, 1967) agar after incubation at 30°C for 48 hr. Each sample bag was weighed and 0.10% (w/v) peptone water was added to make a 1:1 dilution. Samples were blended for 2 min and then appropriate serial dilutions were made for spread plating using 0.1 mL of each dilution. The number of bacteria was expressed as $\log_{10}$CFU/g. Mean values were reported of duplicate platings of each sample. Sensory evaluations were performed by an eight member trained taste panel. The cooked flavor was evaluated on days 0, 3 and 6 only. Catfish fillets were blended to a homogeneous consistency. Sodium chloride (Fisher) was added at 0.05% by weight. The meat was rolled into balls about 2 cm in diameter and cooked until lightly brown in a microwave oven for 3min. Uncooked odor and appearance were evaluated on days 0, 3, 6, 9, 13, and 15. Treated samples were compared to a fresh control which was assigned a score of 5. The samples liked less than the control were scored 1 to 4, where 1 = disliked most. The samples liked more than the control were scored 6 to 9, where 9 = liked most. Untreated fillets

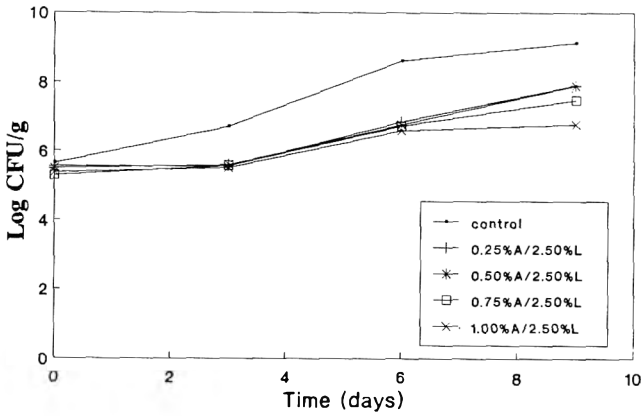

Fig. 1.—Changes in CVT counts on refrigerated catfish fillets treated by different levels of sodium acetate and 2.50% lactic acid culture. Key: control is no treatment; (A) sodium acetate; (L) lactic culture.

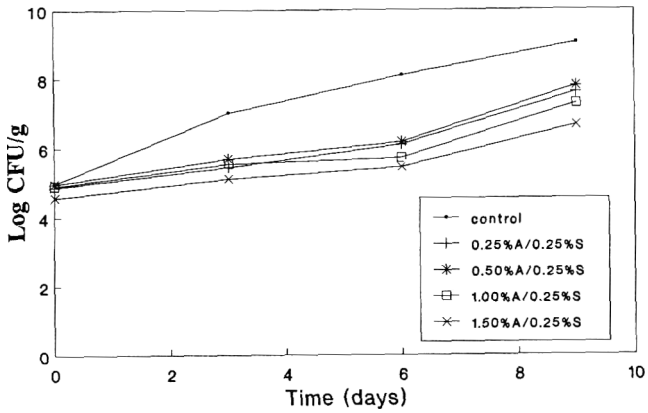

Fig. 2.—Changes in CVT counts on refrigerated catfish fillets treated by different levels of sodium acetate and 0.25% potassium sorbate. Key: control is no treatment; (A) sodium acetate; (S) potassium sorbate.

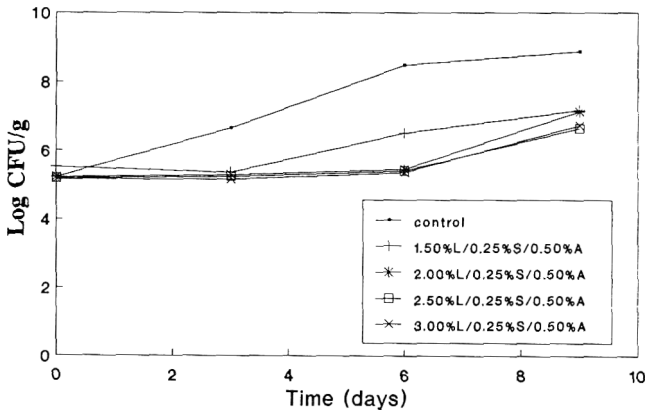

Fig. 3.—Changes in CVT counts on refrigerated catfish fillets treated by different levels of lactic acid culture, 0.25% potassium sorbate, and 0.50%, sodium acetate. Key: control is no treatment; (A) sodium acetate; (S) potassium sorbate; (L) lactic acid culture.

were also stored at 4°C for comparison against untreated fresh control fillets and treated fillets on days 3 and 6 and evaluated. A Completely Randomized Design was used and data analyzed by the Statistical Analysis System (SAS Institute, Inc., 1987).

RESULTS & DISCUSSION

GRAM-NEGATIVE (CVT) COUNTS in refrigerated (4°C) catfish were directly affected by SA and LC (Fig. 1). Treatment con-

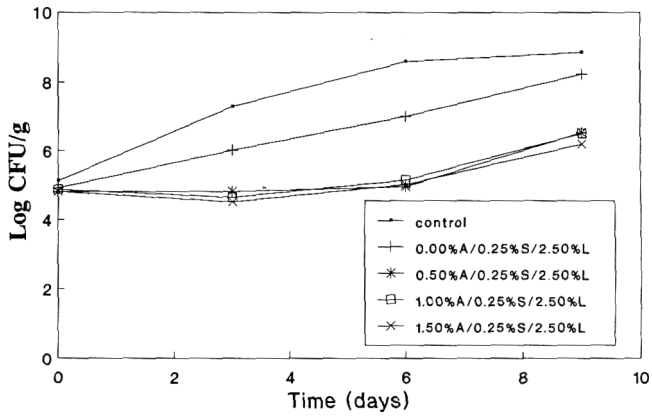

Fig. 4.—Changes in CVT counts on refrigerated catfish fillets treated by different levels of sodium acetate, 0.25% potassium sorbate, and 2.50% lactic acid culture. Key: control is no treatment; (A) sodium acetate; (S) potassium sorbate; (L) lactic culture.

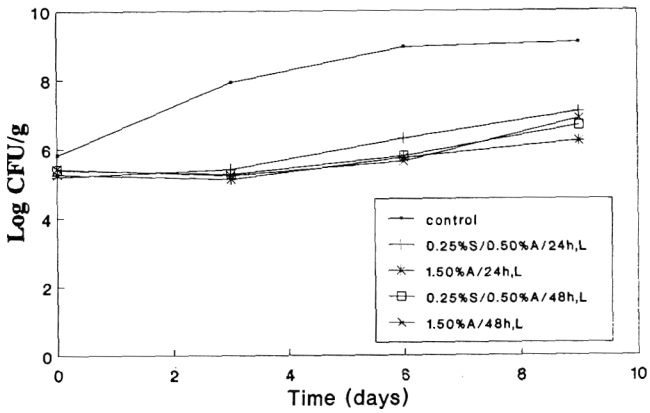

Fig. 5.—Changes in CVT counts on refrigerated catfish fillets treated by 2.50% lactic culture incubated for 24 or 48 hr (30°C) with 1.5% sodium acetate or 0.50% sodium acetate and 0.25% potassium sorbate. Key: control is no treatment; (A) sodium acetate; (S) potassium sorbate; (L) lactic acid culture.

taining 1.00% SA had slightly lower CVT counts throughout the experimental period during storage at 4°C. CVT counts in untreated fish rapidly increased for 9 days; whereas, CVT counts from a treatment with 1.00% SA and 2.50% LC increased only to 1.1 log cycles in the same period. All treatments containing SA and LC inhibited bacterial growth.

The combination of 1.50% SA and 0.25% PS initially had lower CVT counts as compared to controls (Fig. 2). In the presence of 0.25% PS, antimicrobial effects became greater as levels of SA increased up to 1.50%. CVT counts of treatments increased to the apparent lag phase at 6 days. El-Shenawy and Marth (1991) reported on the effects of PS and acetic acid. They noted that PS in the presence of acetic acid had a higher antimicrobial activity and the efficiency was greater at lower temperatures. Antimicrobial activity of SA and PS may have been enhanced by the presence of organic acids such as acetic, tartaric, lactic, and citric acids (El-Shenawy and Marth, 1991).

Treatments of 2.00, 2.50 or 3.00% LC with the combination of 0.25% PS and 0.50% SA caused complete inhibition with partial inactivation of Gram-negative bacteria at pH 6.7 for 6 days. However, undesirable microorganisms grew visibly after 9 days (Fig. 3). This suggested that the growth of LC was very slightly promoted, thereby having a pronounced inhibitory effect on gram-negative and Gram-positive bacteria (Reddy et al., 1983; Schaack and Marth, 1988; Harris et al., 1991; Okereke and Montville, 1991). The inhibitory effect was enhanced as inoculum levels of LC increased up to 2.50–3.00%. Ray

Table 2—Mean sensory scores of refrigerated (4°C) catfish fillets treated with combinations of food preservatives and/or lactic acid culture

Treatment (Storage day)	Flavor score			Odor score						Appearance score					
	0	3	6	0	3	6	9	13	15	0	3	6	9	13	15
Control	5.00^{ab}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}	5.00^{a}
1.00% SA[a]	4.60^{b}	4.85^{a}	4.00^{b}	5.05^{a}	5.70^{a}	4.90^{b}	5.00^{a}	6.05^{b}	3.90^{b}	4.85^{a}	4.20^{a}	5.60^{b}	4.00^{b}	4.90^{a}	3.85^{b}
0.50% PS[f]	5.50^{a}	5.50^{a}	5.30^{c}	4.55^{a}	5.45^{a}	5.00^{a}	5.55^{a}	6.15^{b}	4.55^{ac}	5.00^{a}	5.30^{a}	5.60^{b}	4.80^{a}	4.75^{a}	3.70^{b}
0.25% PS/0.50% SA/2.50% LC[g]	5.20^{ab}	5.85^{a}	5.30^{c}	4.65^{a}	5.05^{a}	4.70^{c}	5.55^{a}	5.90^{a}	4.15^{bc}	4.85^{a}	4.55^{a}	6.90^{c}	4.90^{a}	5.60^{a}	4.10^{b}
Stored untreated	5.00^{ab}	5.25^{a}	5.40^{d}	5.00^{a}	5.85^{a}	5.30^{d}	—	—	—	5.00^{a}	4.90^{a}	5.70^{c}	—	—	—

^{a,b,c,d} Mean values with different superscripts in the same column are significantly different ($P < 0.05$).
[a] SA = sodium acetate.
[f] PS = potassium sorbate.
[g] LC = lactic acid culture.

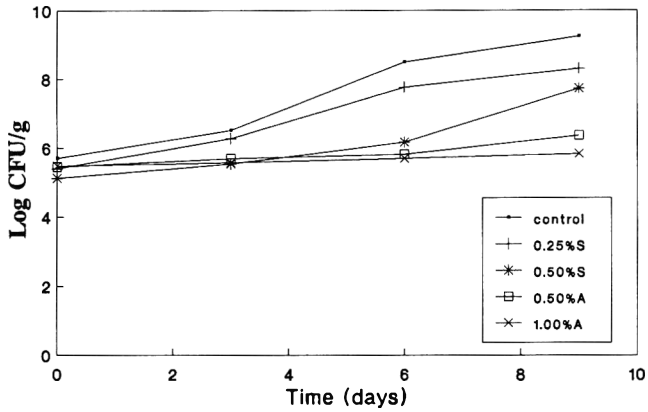

Fig. 6.—Changes in CVT counts on refrigerated catfish fillets treated by different levels of sodium acetate and potassium sorbate. Key: control is no treatment; (A) sodium acetate; (S) potassium sorbate.

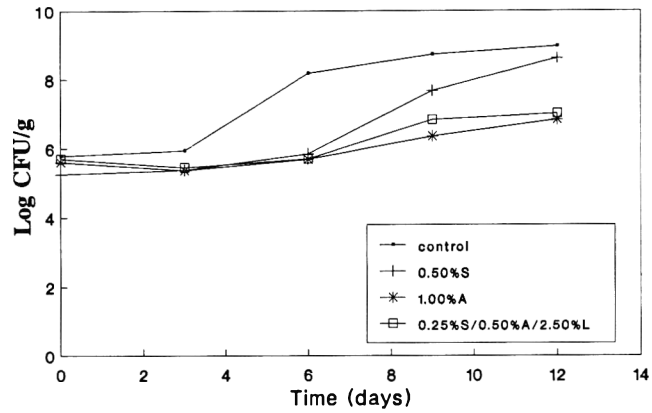

Fig. 7.—Changes in CVT counts on refrigerated catfish fillets treated by the combination of 0.50% sodium acetate and 0.25% potassium sorbate with 2.50% lactic acid culture and either 1.00% sodium acetate or 0.50% potassium sorbate alone. Key: control is no treatment; (A) sodium acetate; (S) potassium sorbate; (L) lactic acid culture.

(1992) suggested that the cells of lactic acid bacteria did not grow, yet inhibited growth and reduced viability of spoilage and pathogenic bacteria. As lactic acid and other acids are not produced by nongrowing lactic acid bacteria, live cells probably either acted as carriers of or produced other antimicrobial compounds (Ray, 1992).

The presence of 0.50–1.50% SA with 0.25% PS and 2.50% LC completely inhibited growth of CVT counts for 6 days. CVT counts in the treatment of PS plus LC without SA were increased by about 2 log cycles (Fig. 4). The pH values for all treatments dropped to 6.40 in 9 days at 4°C and were not involved in inhibition of CVT counts (data not shown). Results indicate that the combination of SA and PS with LC had a higher antimicrobial activity on undesirable microorganisms. Gilliland and Speck (1972) reported that lactic streptococci used to produce cultured dairy products inhibited growth of pathogens such as *Staphylococcus aureus* or *Salmonella typhimurium* by 86% to 99% within 6 hr at 32°C. Such antagonistic effects were slightly reduced when the organisms were grown at pH 6.50. They suggested that the antimicrobial property of starter cultures was due to lactic acid, acetic acid, H_2O_2, and possibly unknown compounds.

LC incubated for 24 or 48 hr with 1.50% SA or the combination of 0.25% PS and 0.50% SA showed antimicrobial effects (Fig. 5). When the cultivation was prolonged for 48 hr (pH 4.6), the growth and antibiotic production of LC was greater than when cultivation was 24 hr. Reddy et al. (1983) reported the same observance in skim milk containing *Lactobacillus bulgaricus* DDS 14. They also reported that extending the incubation period for 48 to 72 hr had no significant effect on antibiotic production.

Use of 0.50% PS, and 0.50 to 1.00% SA, alone, completely inhibited CVT counts for 6 days at 4°C, but after 9 days, counts rose about 1.5, 1.0 and 0.3 log cycle, respectively (Fig. 6). Use of SA in refrigerated catfish fillets had greater inhibitory effect than use of PS.

Treatment of SA and PS with LC or SA alone had higher inhibitory effect than PS alone during 12 days storage (Fig. 7). Treatments of this combination and SA alone completely inhibited CVT counts for 6 days, and counts rose 1.3 and 1.2 log cycles after 12 days, respectively. The main antimicrobial effect in refrigerated (4°C) catfish fillets was attributable to SA. Treatment of PS alone did not continue to inhibit CVT counts beyond 6 to 9 days. The antimicrobial effect could be enhanced by the combination of SA and PS with lactic acid bacteria producing natural organic acids such as lactic and acetic acid (El-Shenway and Marth, 1991).

Sensory scores indicated that treated samples were quite close in flavor, odor and appearance to fresh controls through the storage time (Table 2). Combination of 0.50% SA and 0.25% PS with 2.50% LC or 0.5% PS alone had higher ($P < 0.05$) flavor scores than fresh control fillets during 6 days storage. By day 9 untreated fillets had typical off odors associated with spoiling fish, but treated samples were liked more than untreated samples. Significant differences ($P < 0.05$) occurred in odor and appearance between fresh control fillets and treated fillets over 15 days storage. All treated samples had higher ($P < 0.05$) odor and appearance scores than untreated samples.

CONCLUSIONS

THE COMBINATION of SA and PS with LC can provide the inhibition required for extended storage with respect to growth of aerobic Gram-negative bacteria in refrigerated (4°C) catfish fillets. All treatments containing SA effectively inhibited Gram-negative bacteria. Use of specific LC in combination with SA and other food preservation techniques in refrigerated fish should prevent growth of undesirable microorganisms.

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Copper Chelation Assay For Histamine in Tuna

R.C. BATEMAN, JR., D.B. ELDRIGE, S. WADE, J. McCOY-MESSER, E.L.E. JESTER, and D.E. MOWDY

ABSTRACT

A reported copper chelation method for histamine determination was modified for use in decomposing yellowfin tuna steaks. The assay consisted of histamine extraction with hot methanol, purification by rapid cation exchange chromatography, and addition of copper and a dye to the purified sample to form an easily visualized red complex with the histamine. This method detected histamine in the low mg% range in yellowfin tuna steaks with an accuracy comparable to the standard fluorometric histamine assay. It is an alternative to the standard assay when a visual test is desirable or a fluorometer is not available.

Key Words: copper, tuna, chelation, histamine, analysis

INTRODUCTION

HISTAMINE is a biogenic amine produced during microbial decomposition of scombroid fish such as tuna and mackerel (Pan and James, 1985). Histamine has been associated with scombroid poisoning, which resembles an allergic reaction (Hughes and Merson, 1976). Since histamine is neither volatile nor destroyed by cooking, a convenient method of detecting it in seafood samples is needed, particularly where decomposition is suspected. The method of the Association of Official Analytical Chemists (AOAC) involves extraction of histamine from tuna with hot methanol, ion exchange chromatography, derivatization by o-phthalaldehyde and fluorometric quantitation (AOAC, 1990). This method, while sensitive, yields a colorless product and requires a fluorometer for product detection.

A variety of alternative assays exist for histamine in fish products. Most involve chromatography of histamine derivatives using costly instrumentation such as HPLC or GC (Staruszkiewicz and Bond, 1981; Henion et al., 1981; Pan et al., 1982; Wada et al., 1982; Walters, 1984). One simple chemical assay for histamine reported by Sakai et al. (1984) avoided covalent derivatization of histamine. It utilized the strong copper chelating properties of histamine to devise a solvent extraction assay. By adding a dye to form an ion associate with the chelate and make it extractable into the organic phase, they developed a blue/red color assay for histamine in rat tissues. Our objective was to adapt that simple copper-based histamine assay to the detection of histamine in tuna samples.

MATERIALS & METHODS

CHEMICALS were from Sigma Chemical Company (St. Louis, MO) and solvents were from Fisher Scientific (Pittsburg, PA). Accell CM Classic SepPak cartridge columns were from Waters/Millipore (Milford, MA).

Tuna samples

A yellowfin tuna loin was purchased as fresh as possible from a Boston seafood dealer. The fish had been harvested ≈7 days before receipt and had been kept packed in ice before and after dressing. The loin was sliced into cross sections, sealed in plastic bags and stored at 10°C to allow controlled spoilage. The sections were examined daily and individual sections at different stages of decomposition, determined by sensory analysis of aroma, appearance and texture by two experienced scientists trained to detect spoilage according to Food & Drug Administration (FDA) procedures, were taken as they occurred. The tuna steaks were prepared for analysis by grinding in a food processor. A portion of each sample was immediately analyzed for various diamines, including histamine quantitation by the standard method (AOAC, 1990), at FDA Laboratories at Dauphin Island. The remainder of each sample was stored frozen at −20°C without thawing for ≈2 yr. At that time they were shipped on dry ice to the Univ. of Southern Mississippi where all tests (Table 1) were performed.

Authors Bateman, Eldridge, Wade, and McCoy-Messer are with the Dept. of Chemistry & Biochemistry, Univ. of Southern Mississippi, Hattiesburg, MS 39406-5043. Authors Jester and Mowdy are with the FDA Gulf Coast Seafood Laboratories, Dauphin Island, AL 36528-0158.

Table 1—Comparison of histamine levels determined from decomposing tuna by different methods

Samples	Spoilage state*	AOAC method (mg%)	Modified AOAC method (mg%)	Copper chelation method (mg%)
1	S	12.2 ± 0.3	11.7 ± 0.5	10.9 ± 0.4
2	EP	25.8 ± 0.7	26.9 ± 1.1	25.5 ± 1.0
3	AD	45.8 ± 1.3	44.7 ± 0.9	51.5 ± 1.5
4	P	293.6 ± 8.3	273.5 ± 4.5	301.4 ± 4.9

* Spoilage state is sensory analysis using the following key: S=stale, ED=early decomposition, AD=advanced decomposition, P=putrid. Reported errors are standard errors of the mean (n = 5-20).

Table 2—Recovery of histamine from repetitive Accell CM column chromatography

Frequency of application & elution	Percent recovery after applying histamine	
	2.5 mg%	25 mg%
Initial	97.5 ± 3.4	91.0 ± 6.9
Second	94.8 ± 3.3	98.6 ± 4.5
Third	94.6 ± 3.3	89.0 ± 7.7
Fourth	95.4 ± 3.3	94.3 ± 5.0
Fifth	94.6 ± 3.3	91.0 ± 4.3

* Methanol extracts of fresh tuna containing no measurable histamine were augmented with histamine corresponding to 2.5 and 25.0 mg%. Samples were chromatographed repetitively over the same column and histamine quantitated by the copper chelation assay. Reported errors are standard errors of the mean (n = 3).

Modified AOAC histamine assay

Histamine was removed from the fish samples by extraction of 10-g portions with methanol and filtration as described in the standard AOAC (1990) procedure. Filtrates were subjected to chromatography using either a Dowex 1-X8 column (standard AOAC procedure) or an Accell CM cartridge column by the procedure described below, followed by derivatization with o-phthalaldehyde and fluorometric quantitation. The only difference between the standard and modified AOAC methods was in the chromatographic purification of histamine from the methanolic tuna extract.

In the modified AOAC method, the methanolic filtrate was diluted ten-fold with sodium phosphate buffer, 0.05M, pH 6.2. Of this diluted filtrate 5 mL were injected with a disposable syringe through a Waters Accell CM cation exchange cartridge (0.5 mL column volume) which had been preequilibrated with the same buffer. The cartridge was then washed with 5 mL of the same buffer and histamine was eluted by a further 5 mL wash with buffer containing 0.5 M NaCl (column elution buffer). This sample was then subjected to the copper chelation assay.

Recovery of histamine from the Accell CM cartridges, (Table 2) was measured using samples of fresh tuna extracts augmented with a known amount of histamine. The amount of histamine recovered in the elution was compared with the amount added to the sample. Samples were chromatographed five times over the same columns with a

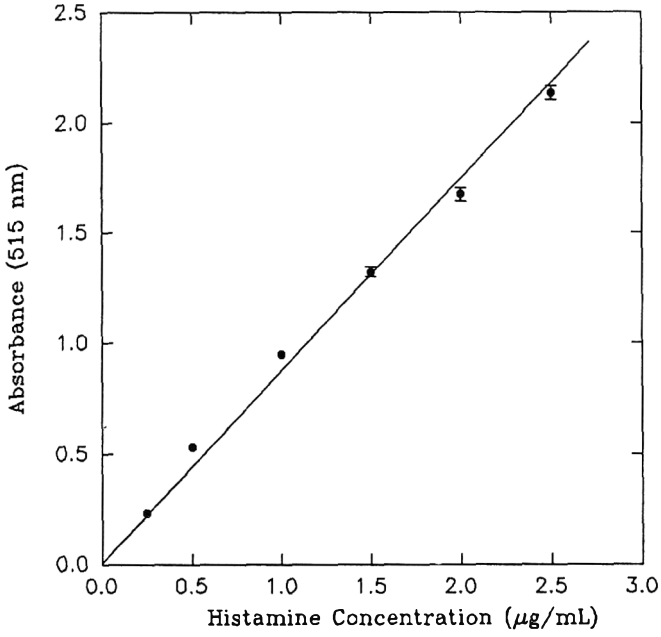

Fig. 1—Standard curve for histamine assay by copper chelation assay. A histamine concentration of 0.1 μg/mL column eluate is equal to 1 mg% of histamine in a tuna steak using this method. Error bars represent standard error of the mean (n = 5).

single re-equilibration step (20 mL wash with equilibration buffer) before each sample addition.

Copper chelation histamine assay

A smaller scaled and modified version of the method of Sakai et al. (1984) was used. Briefly, the assay consisted of the following components, in order of addition: 5 mL sample, diluted if necessary with dilution buffer; 0.21 mL 1.0M NaOH; 1.5 mL assay buffer (0.3M sodium phosphate/0.1 M sodium tetraborate, pH 9.5); 0.1 mL 0.005M copper (II) chloride solution; 0.4 mL 0.008M tetrabromophenylphthalein ethyl ester solution in ethanol or methanol; and 2 mL methylene chloride.

The assay mixture was mixed thoroughly by vortexing. The phases were allowed to separate and the lower (organic) phase clarified by brief centrifugation if necessary, followed by absorbance measurement of the organic phase at 515 nm in a Hitachi U-2000 spectrophotometer using glass cuvettes. A standard curve was generated using 5 mL of histamine standards (0.5–3 μg/mL) in dilution buffer, which consisted of column equilibration buffer containing 0.35M NaCl. This concentration of salt approximated the salt content of the column eluate. The assay mixture was stable for ≥ 1 hr, but an advance mix could not be used because of instability of the dye at basic conditions. This procedure differs from that of Sakai et al. (1984) in that twice as much tetrabromophenylphthalein ethyl ester was used to enhance sensitivity and methylene chloride replaced the highly toxic 1,2-dichloroethane. Chloroform gave comparable results, but hydrocarbon solvents were not suitable.

Statistical analysis of the results (Table 1, data set = 150 determinations) was performed using the SAS General Linear Model procedure, subjecting data to both weighted least squares and log transformations. The SAS Variance Component Model procedure was used in attributing variability to either differences among methods or measurement error (SAS Institute, Inc., 1989).

RESULTS & DISCUSSION

THE SPECTROPHOTOMETRIC ASSAY was linear (Fig. 1) with increasing histamine concentrations and sensitive enough to detect histamine in the upper nanogram/mL range (corresponding to low mg% range) in initial tuna samples. FDA guidelines establish a histamine level in tuna of 20 mg% as indicating mishandling at some stage and 50 mg% represents a serious health hazard (Pan and James, 1985). Thus the method sensitivity means that histamine could be reliably detected at and below levels indicating spoilage or toxicity. Regression analysis of the linear portion of the standard curve (Fig. 1) gave a slope of 0.835 absorbance units/μg/mL with a standard error of 0.012. From this slope a molar absorptivity of 9.3×10^4 M^{-1} cm^{-1} was calculated, showing quantitatively the high sensitivity of this assay. The standard curve became nonlinear above 3 μg/mL histamine indicating samples containing more than 30 mg% histamine must be diluted to be measured accurately.

Recovery of 0.25 μg/mL histamine in eluates from the Accell CM columns averaged 93% (Table 2) while recovery of tenfold higher histamine concentrations averaged 95%. These concentrations correspond to 2.5 and 25 mg% histamine in fish samples. This shows that columns could be reused at least five times with histamine-containing samples with no loss of recovery.

The method was tested on decomposed tuna samples (Table 1). The first column represents the standard AOAC test performed at USM after prolonged storage at −20°C. Except for the first sample, which was freezer-burned, the values were within 10% of those obtained on the same samples tested at FDA laboratories immediately after controlled spoilage of fresh tuna. This indicated the stability of histamine in the fish samples after storage in the frozen state. The second and third columns represent the same tuna samples tested by both the modified AOAC method and the proposed copper chelation method.

Two conclusions can be drawn from the results (Table 1). First, a statistical comparison of the first two columns showed that the modified AOAC method using the Accell CM cartridge column procedure was as effective as the standard AOAC ion exchange column method for recovering histamine from the tuna samples, i.e., there was no difference between assays. This equivalence in histamine recovery was especially important in the copper chelation assay because interfering compounds occur in the tuna extracts (from both tuna steaks and canned tuna) which are not removed with the standard ion exchange method and are not retarded by the column. The identity of such interfering compounds is unknown, but they were not removed by heat or acid precipitation. In addition, although cadaverine was present in high concentrations (50–105 μg/g) in all of the decomposed tuna samples tested, there was no evidence of cadaverine interference at even the lowest histamine concentrations. Control experiments showed that putrescine and cadaverine began to produce color in the copper chelation assay only at levels which would approach 1000 μg/g fish. Even then the colors varied from the burgundy red produced by histamine, with putrescine producing a brownish red (maximum 505 nm) and cadaverine a distinct green color (maximum 600 nm). The amino acids histidine and lysine produced no reaction even at concentrations up to 5000 μg/g fish. Only spermidine produced a substantial color change when substituted for histamine at equimolar concentrations. Freedom from interference by other biological amines was reported in the original copper chelation assay (Sakai et al., 1984).

The second conclusion from data of Table 1 is that the copper chelation assay compared favorably to the standard AOAC method in both senstivity and accuracy. At low and medium levels of histamine the assay had a precision comparable to the standard assay with a coefficient of variation (CV) of 3% or lower. At very high concentrations of histamine (Table 1, sample 4) the precision of the assay decreased slightly but the accuracy of the assay was maintained. This light fluxuation could be due, at least in part, to an increase in variation in recovery from the Accell columns as histamine concentration increased, as also evident in Table 2. In separate control experiments, only histamine concentrations equivalent to <2 mg% showed significant scatter in the standard curve and variation in this region is not important in terms of dangerous levels of histamine. In a statistical comparison of the standard AOAC and copper chelation methods using individual repli-

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Oxygen-Sensor-Based Simple Assay of Histamine in Fish Using Purified Amine Oxidase

MINORU OHASHI, FUMIKO NOMURA, MIEKO SUZUKI, MEGUMI OTSUKA, OSAO ADACHI, and NOBUHIKO ARAKAWA

ABSTRACT

Oxygen consumption was measured by an oxygen sensor after addition of purified fungal amine oxidase to fish extracts. The oxidation of histamine to imidazoleacetaldehyde proceeded stoichiometrically. Based on the equimolar relationship between histamine and oxygen consumption, histamine was determined selectively by the oxygen sensor. Neither sample pretreatment removing interfering materials nor daily calibration by histamine standard was required. Histamine contents in scombroid fish were determined rapidly with good accuracy. AOAC and oxygen sensor methods showed a very high correlation ($r=0.999$, $n=6$).

Key Words: fish, histamine, oxygen sensor, amine oxidase

INTRODUCTION

HISTAMINE (Hm) is a putrefactive amine which causes scombroid fish poisoning. Many studies on its determination have been conducted. Taylor and Sumner (1986) reviewed determination of Hm, putrescine and cadaverine in fish. Kawabata et al. (1960) proposed colorimetry of Hm using diazonium reagent with anion-exchange resin treatment to remove interfering materials. A fluorometric method using o-phthalaldehyde to yield a fluorophore is the most widely used for Hm determination in fish because of its high sensitivity. Even with that advanced method, a pretreatment is necessary to obtain accurate results. Staruszkiewicz (1977) and Staruszkiewicz et al. (1977) proposed anion-exchange resin treatment prior to fluorometric analysis. This method is very well known (AOAC, 1990). It requires preparation of a standard solution and a calibration procedure because of the instability of Hm in solution. Lerke and Bell (1976) proposed an alternative pretreatment method using cation-exchange resin and Taylor et al. (1978) proposed a sequential solvent extraction method. However, as these pretreatments are time- and labor-consuming, further work needs to be carried out to develop a more efficient procedure.

The main objective of our study was to develop a simpler and more rapid and selective method for determination of Hm in fish using an oxygen sensor and a novel enzyme reagent.

MATERIALS & METHODS

Apparatus

An oxygen sensor (Freshness Meter KV-101, Oriental Electric Co., Ltd. Niiza, Saitama, Japan) developed by Ohashi et al. (1987) for rapid assay of K-value (freshness index) was used (Fig. 1). For pH control of fish sample extracts, a miniature pH meter (Twin pH Meter 112, Horiba Works, Kyoto, Japan) was used.

Author Ohashi is with Moritex Co., 3-1-14, Jingumae, Shibuya-ku, Tokyo 150 Japan. Authors Nomura, Suzuki, Otsuka, and Arakawa are with the Dept. of Nutrition & Food Science, Ochanomizu Univ., 2-1-1, Otsuka, Bunkyo-ku, Tokyo 112 Japan. Author Adachi is with the Faculty of Agriculture, Dept. of Biological Chemistry, Yamaguchi Univ., 1677, Yoshida, Yamaguchi City, 753 Japan.

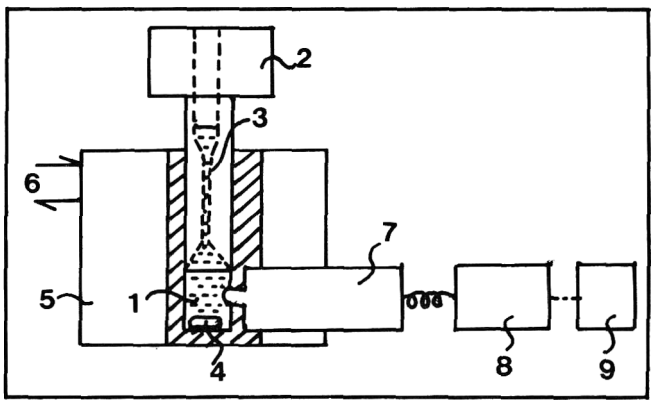

Fig. 1—Schematic of oxygen sensor, from U.S. Patent 4,650,752. (1) Reaction cell (1,150 μL); (2) Cap of the reaction cell; (3) Capillary of cap; (4) Magnetic stirrer; (5) Jacket for temperature control; (6) Exterior isothermal water; (7) Membrane-covered oxygen electrode (Clark electrode); (8) Amplifier for Clark electrode; (9) Millivolt recorder DC 10 mV/100 mm full span (SEKONIC, SS-100F, TOKYO).

Enzyme

Fungal amine oxidase, Cu-containing enzyme (E.C. 1.4.3.6), reviewed by Isobe and Yamada (1982) was prepared from mycelium extract of *Aspergillus niger* AKU-3302 strain according to Yamada et al. (1965), because commercially available amine oxidase from bovine plasma is not specific for Hm oxidation. Purified enzyme was suspended in 2M ammonium sulfate solution and stored at 5°C. Specific activity of amine oxidase suspension was measured by the Tabor et al. (1959) method using benzylamine as substrate, 1.1×10^4 U/mg protein (1.34×10^5 U/mL of amine oxidase suspension). This enzyme reagent remained stable for more than a year under the described storage conditions.

Reagents

Histamine dihydrochloride was obtained from Aldrich Chemical Co., Inc., Milwaukee, WI (purity, 98%) and Wako Pure Chemical Ind., Ltd., Osaka, Japan (purity, 97%). Cadaverine dihydrochloride, putrescine dihydrochloride, each with 98% purity, and sodium azide (NaN_3) with 97% purity were obtained from Nakarai Tesque Inc., Kyoto, Japan. Other reagents, such as trichloroacetic acid (TCA), used as extracting solvent, KOH as neutralizer and KH_2PO_4 and Na_2HPO_4 as 0.1 M phosphate buffered solution (PBS, pH8.0) were obtained from Wako Pure Chemical Ind., Ltd., Osaka, Japan. Standard solution of Hm and other putrefactive amines were prepared according to the AOAC (1990) method. These standard solutions, stored at 5°C, were stable for 1 month.

Fish samples

Canned tuna samples containing low, medium and high concentrations of Hm (about 1, 10 and 50 mg%) were obtained from Starkist Seafood Co., Long Beach, CA. Other raw fish samples were purchased from a local fish market in Japan. Commercial canned tuna samples were purchased in Taiwan.

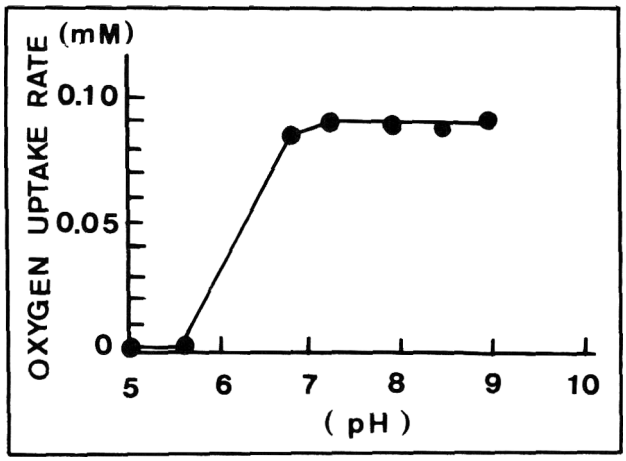

Fig. 2—Effect of pH on amine oxidase activity. Substrate: 0.18 mM histamine dihydrochloride solution, 100 μL/cell. Reaction buffer: air-saturated 0.1M phosphate buffer (PBS, pH 5-8) and 0.1M glycine-NaOH buffer (> pH 8).

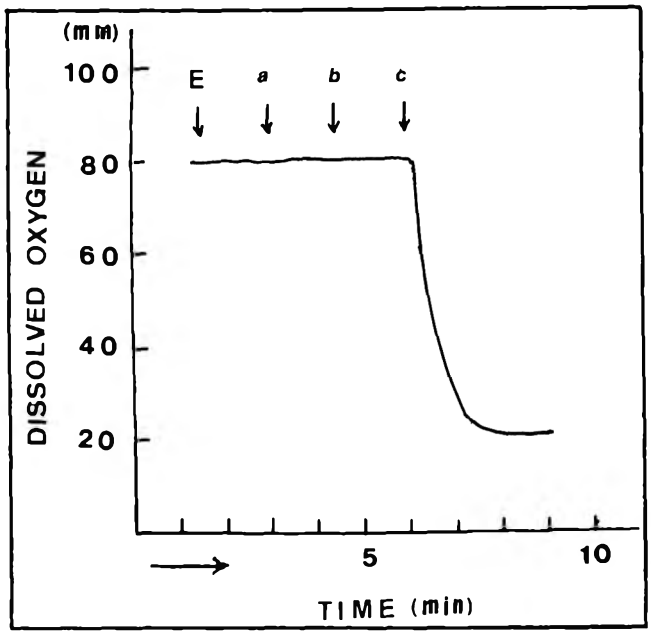

Fig. 3—Specificity of amine oxidase for putrefactive amines. (E) enzyme 20 μL; (a) 9 mM cadaverine 20 μL; (b) 9 mM putrescine 20 μL; (c) 9 mM histamine 20 μL. Reaction buffer: air-saturated 0.1M PBS (pH 8.0, 37°C).

Preparation of fish extract for oxygen sensor analysis

Fresh or processed fish meat (10g) was homogenized in 15 mL of 10% TCA solution in a small mortar or semimicro container (about 50 mL) of a high-speed blender. The homogenate was transferred to a 50-mL beaker using a miniature pH meter as agitating rod. The samples were neutralized by dropwise addition of 10N KOH until the pH reached 7.0, and then by dropwise addition of 1N KOH to obtain a final pH 8.0. An alternative method for neutralization was carried out by using 0.1% thymol blue solution (yellow to light blue at pH 8.0). The homogenate was then transferred to a 50-mL volumetric flask. The homogenate that remained was transferred to the flask with 0.1M PBS (pH 8.0) containing 2 mM NaN_3. The flask was filled to 50 mL with 0.1 M PBS containing 2 mM NaN_3 and stoppered. NaN_3 was used to prevent microbial and enzymatic contamination, which affect dissolved oxygen (DO) stability. The homogenate was mixed to a homogeneous suspension, transferred to a tube and centrifuged at 4,000 rpm for 10 min. The resulting supernatant was stored at −20°C until analyzed.

Principle of histamine determination

In the closed reaction cell of the oxygen sensor, enzymatic oxidation of Hm was determined by:

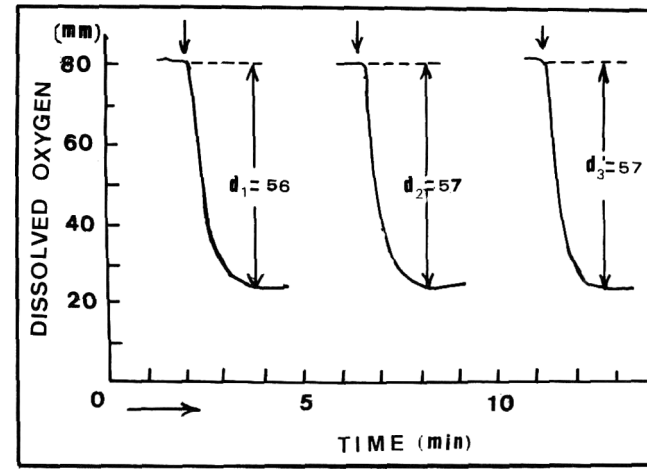

Fig. 4—Typical dissolved oxygen response curve in histamine assay. Model sample solution: 20 μL of 9 mM histamine dihydrochloride solution. Reaction buffer: air-saturated PBS (pH 8.0); reaction temperature: 37°C; ↓: injecting point of 15 μL enzyme. Three consecutive injections were made.

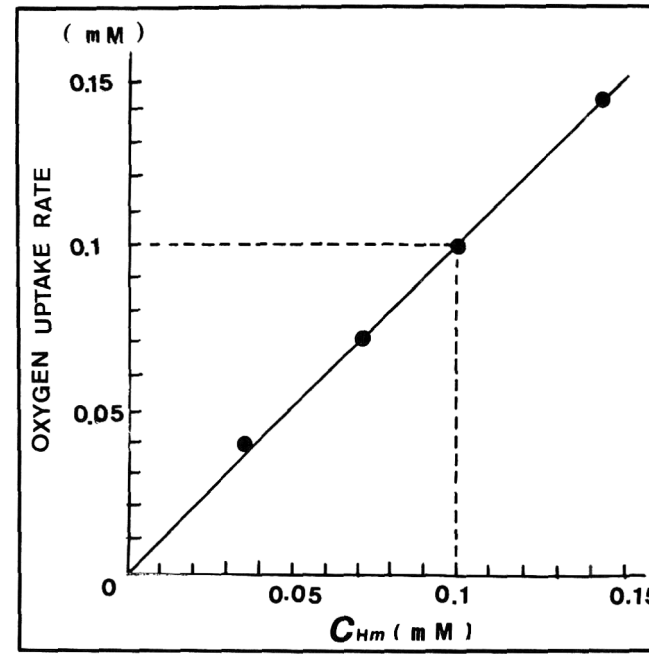

Fig. 5—Relationship between histamine concentration and oxygen uptake rate. Standard sample solution volume: 5, 10, 15, and 20 μL of 9 mM Hm solution. Reaction buffer: air-saturated PBS (pH 8.0); reaction temperature: 37°C.

$$\text{Hm} + O_2 + H_2O \xrightarrow{\text{amine oxidase}} \text{imidazoleacetaldehyde} + NH_3 + H_2O_2 \quad (1)$$

In the reaction cell, one mole DO was consumed upon oxidation of one mole of Hm. Based on this equimolar stoichiometric relation, Hm concentration (C_{Hm}) in the reaction cell could be calculated from the following equations.

$$C_{Hm}\ (\text{mM}) = C_o \times d/d_o \quad (2)$$

or

$$C_{Hm}\ (\text{mg Hm/L}) = C_o \times M \times d/d_o \quad (2')$$

where C_o is oxygen solubility of water at reaction temperature under atmospheric pressure, d is the amount of DO decrease by Hm oxidation (mm), and d_o is the amount of DO for air-saturated water (mm). M is the molecular weight of Hm (111 g/mole).

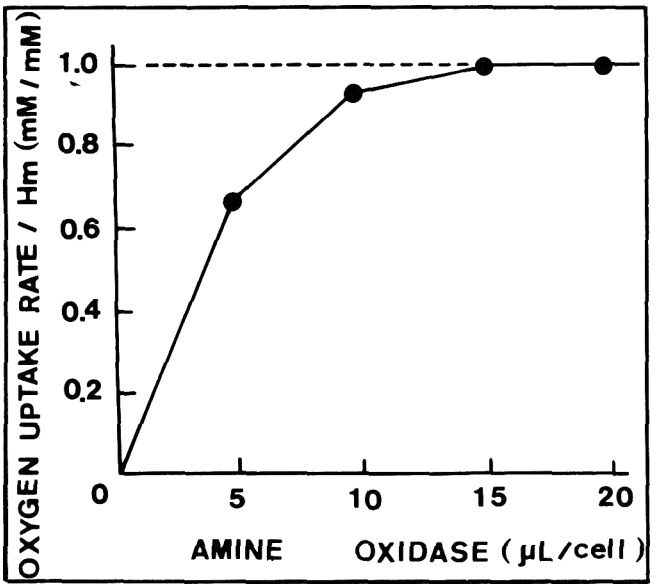

Fig. 6—Effect of amine oxidase concentration on oxygen uptake rate of histamine. Substrate: 20 μL of 9 mM histamine dihydrochloride solution; enzyme: AMO ammonium sulfate suspension (1.3×10^5 OD unit/mL); reaction buffer: air-saturated PBS (pH 8.0); temperture: 37°C.

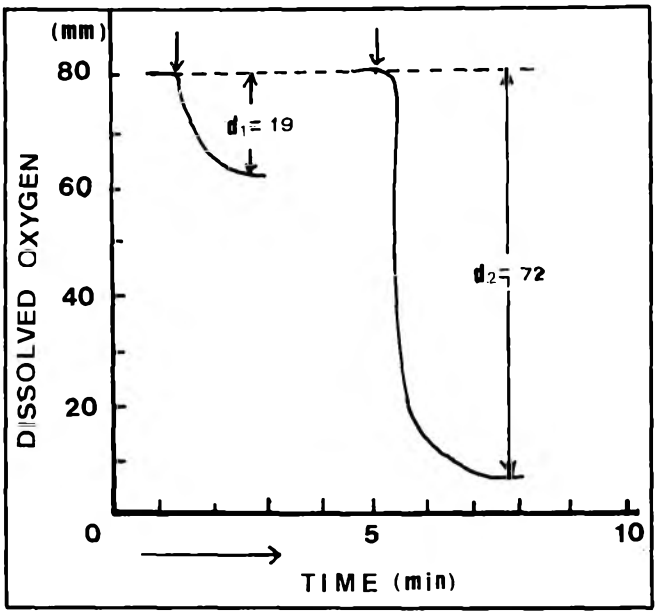

Fig. 7—Dissolved oxygen response curve in histamine assay of mackerel. d_1: sample stored 1 day at 30°C, assayed by Method I: d_2: sample stored 2 days at 30°C, assayed by Method II (V_s=100 μL).

Table 1—Recovery of histamine

Injection volume (μL)	Hm added[a] (μM)	Hm found[b] (μM)	Recovery[c] (%)
0	0	10.7	–
5	36.1	45.7	97
10	72.1	83.7	101
15	108.0	122	102
20	144.0	158	102

[a] Hm=histamine; value of the 9.2 mM Hm standard solution injected to the reaction cell.
[b] Concentration found in the reaction cell after injection of various concentrations of Hm standard solution.
[c] Recovery (%) = [(Hm found −10.7)/Hm added] × 100.

Table 2—histamine in tuna by oxygen sensor and AOAC methods

Samples	AOAC[a]	Oxygen sensor[b]	AOAC/Oxygen (%)
	mg% of Hm		
Low Hm	0.56	0.69	81.1
Medium Hm	9.88	10.2	96.5
High Hm	46.2	46.1	100

[a] Tuna samples were extracted with methanol.
[b] Tuna samples were extracted with 10% trichloroacetic acid.
Correlation coefficient value between oxygen-sensor and AOAC method was r=0.999 (n=6).

Assay procedure

Low level method. Ten mL of sample extracts containing Hm <0.2 mM was placed into a 100-mL BOD bottle and aerated using a small air pump in a water bath for about 10 min at 37°C. For foaming samples, a small amount of silicone antifoamer (Toshiba silicone #737) was added before aeration. The reaction cell, controlled at 37°C (cell volume, 1150 μL), was filled with about 1200 μL of aerated sample solution and capped. After the reading point of d_o was adjusted to 80 mm by a span controller, 15 μL of enzyme was injected into the cell through a capillary tube on the cap (Fig. 1) and output current of DO was recorded within 3–5 min.

High level method. The reaction cell was first filled with air-saturated PBS before injecting 10-100 μL of the sample extracts containing Hm >0.2 mM. The rest of the procedure was carried out as above.

Calculation of histamine in fish

Hm content in fish was calculated by the following equation derived from Eq. (2'):

Table 3—Histamine assay of raw and processed fish by oxygen sensor

Raw fish, histamine changes during storage							
		Storage condition[a] (Days)					
		0	1	2	4	6	8
Samples	Temp (°C)	mg% Hm					
Mackerel (raw)	5	0.15	—	—	—	14.5	37.8
	30	0.15	58.0	160	—	—	—
Tuna (raw)	5	N.D.	—	—	—	0.3	8.5
	30	N.D.	92.2	276	—	—	—
Tuna (frozen)	20	0.15	—	—	7.69	—	—

Processed fish[b]		
Samples		mg% of Hm
Saurel	(semi-dried)	0.33
Tuna	(canned)	0.12
Tuna	(canned)	1.51

[a] —not assayed
[b] Measured just after purchase

$$\text{Hm (mg/100g fish)} = C_o \times d/d_o \times V/V_s \times M \times V_H/1000 \times 100/W \quad (3)$$

where d is the amount of DO decrease on addition of enzyme (mm), d_o is the amount of DO for air-saturated PBS at 37°C (adjusted to 80 mm), V is volume of reaction cell (μL), V_s is volume of sample extracts injected into the cell (μL), V_H is volume of homogenate (mL), and W is weight of fish meat (g).

Changes of histamine during storage

Raw fresh mackerel, fresh and frozen tuna and semidried saurel (horse mackerel) from a local fish market in Japan and canned tuna produced in Taiwan were used. Samples were stored at 5°C, 30°C and room temperature (about 20°C), for 4–8 days.

Preparation of fish extracts for histamine assay: Fish extracts were prepared as described above. Frozen fish was thawed at room temperature before preparation. Extracts were stored at −20°C prior to analysis. Stored samples under high temperature were analyzed by method 2, and the others were done by method 1.

RESULTS & DISCUSSION

Effect of pH and selective amine oxidase (AMO) activity

The optimum pH region for the Hm oxidation (Fig. 2) was 7 to 9. Based on this range, the pH value for assay was selected

to be pH 8. When 15 μL of AMO suspension was injected into the cell filled with air-saturated PBS under stirring (Fig. 3), no change in DO was observed (point E). By successive injection of 20 μL of cadaverine and putrescine (Fig. 3 a and b, respectively), no change in DO was observed. Furthermore, injection of 20 μL of Hm standard (point c) caused a rapid decrease of DO and this reaction was completed within 90 sec. The data clearly showed that AMO had a strong oxidase activity for Hm, but not for cadaverine and putrescine. Thus the selective activity of AMO for Hm was confirmed.

Stoichiometric relation between histamine and oxygen uptake

The stoichiometric relationship between Hm oxidation and oxygen uptake (Eq. 1), was confirmed by the experimental data of the high level method using Hm standard solution prepared from the reagent with purity of 97%. Twenty μL of 9.0 mM standard Hm solution was injected into the reaction cell containing air-saturated PBS, then 15 μL of enzyme was injected and the DO decrease was recorded. The experiments were carried out at 37°C and 760 mm Hg in three replicates (Fig. 4) to examine reproducibility and accuracy of the method. Standard deviation of d given from the three experiments (Fig. 4) was 0.57 and coefficient of variance (CV) was 0.98%, showing very good reproducibility. Oxygen uptake rate (OUR) of Hm was determined as follows:

$$\text{OUR (mM)} = C_o \times d/d_o \qquad (4)$$

where C_o is oxygen solubility, (e.g., 0.214 mM at 37°C/760 mm Hg), d is the amount of DO decrease on addition of enzyme (mm), and d_o is the amount of DO for air-saturated PBS at 37°C (80 mm). When the mean value, 56.7 mm of d_1, d_2 and d_3 (56, 57 and 57 mm, respectively) was used for d, we obtained the following results by substitution of the experimental data in Eq. (4): OUR (mM) = 0.214 × 56.7/80 = 0.15 (mM).

Hm concentration in the reaction cell was calculated from the dilution rate of the original Hm solution as follows:

$$C_{Hm}\,(\text{mM}) = C \times 0.97^* \times Vs/V \qquad (5)$$

where C is concentration of original histamine solution (9.0 mM), V is volume of reaction cell (1,150 μL), Vs is volume of Hm solution injected (20 μL), and the number with asterisk is purity of histamine dihydrochloride used. Experimental data were substituted in Eq. (5): C_{Hm} (mM) = 9.0 × 0.97 × 20/1150 = 0.15 (mM).

Results showed that for 0.15 mM Hm, equimolar 0.15 mM oxygen was consumed. Excellent linear and equimolar relationships were observed (Fig. 5) between Hm concentration (C_{Hm}) and oxygen uptake rate. This correlation curve was obtained from experiments using varying volumes of Hm standard solution. Results showed that 15 μL of AMO suspension was the optimum (Fig. 6) required in the reaction cell of about 1 mL.

Histamine assay of fish

Hm recovery added to canned tuna samples (Table 1) showed reasonable recoveries (≈100%). The comparative assays with officially recognized AOAC and oxygen sensor methods using authenticated canned tuna samples (Table 2) showed high correlations. DO response curves of Hm in raw mackerel stored under different temperatures and time (Fig. 7) confirmed that, the enzyme reactions proceeded as smoothly in raw fish as in the pure Hm solution. Reaction time was ≈ 90–120 sec. Measurement of changes of Hm in raw mackerel and tuna stored various times, as well as commercial canned tuna and dried saurel (Table 3) showed Hm in all samples increased with storage. The increase was more rapid at higher temperature storage. These changes of Hm in scombroid fish were easily and rapidly detected using the oxygen sensor method. In this method, a minimum of 0.15 mg% to a maximum of 276 mg% of Hm for raw fish extracts (Table 3A) and 0.12 mg% to a maximum of 1.51 mg% Hm for the canned and dried samples (Table 3B) could be easily and rapidly assayed. The oxygen sensor method was carried out on canned tuna and raw mackerel and tuna. However, we believe that this method could be used in Hm determinations on other fish species and food products.

CONCLUSIONS

THE OXYGEN SENSOR METHOD described for determination of Hm in fish is a sensitive, simple and selective method where no specific pretreatment and calibration procedure are necessary. Analysis can be performed rapidly and semiautomatically by a compact freshness meter with a fungal amine oxidase reagent after only a simple sample preparation. The method correlated well with that of the AOAC (1990).

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Gelation of Surimi by High Hydrostatic Pressure

Y.C. CHUNG, A. GEBREHIWOT, D.F. FARKAS, and M.T. MORRISSEY

ABSTRACT

The effects of high hydrostatic pressure (HHP) on gel strength of Pacific whiting and Alaska pollock surimi were determined by torsion. Pacific whiting gels were made with and without 1% beef plasma protein (BPP) as protease inhibitor. HHP treated whiting (1% BPP added) and pollock gels showed greatly increased strain values at all pressure/temperature combinations compared with heat-set controls. Stress values for the same samples were variable depending on treatment and species. A three-fold increase in strain and stress was found for HHP treated whiting gels made without inhibitor.

Key Words: hydrostatic pressure, surimi, pollock, whiting, gel strength

INTRODUCTION

INTEREST in use of high hydrostatic pressure (HHP) in the seafood industry is increasing. HHP has been shown to decrease microorganisms, affect enzymatic activity, and cause rheological changes in several foods (Farr, 1990; Okamoto et al., 1990; Hoover, 1993). Researchers have shown that HHP could be used to induce gelation of surimi paste from several fish species. Pressure treated gels from frozen Alaska pollock (*Theragra chalcogramma*) have been produced at 0°C with treatment as low as 2.0 Kbar (Shoji et al., 1990). Gelation of pollock surimi by HHP was attributed to increased cross-linkage of the myosin heavy chain. Okazaki and Nakamura (1992) have shown that gelation of sarcoplasmic protein from different fish was related to fish species, pH, protein concentration and pressure treatment.

Surimi analogs are traditionally made from heat-set gels at temperatures approaching 90°C (Lee, 1984). Several fish species may undergo a weakening of gel structure during normal heating regimes because of endogenous proteases in the muscle tissue (Niwa, 1992). As temperature increases during cooking, leading to gelation, the product is subjected to a temperature range (50–60°C) where such proteases are most active. This potential weakening of the gel is circumvented by use of protease inhibitors (Matsumoto and Noguchi, 1992). Good quality surimi gels from Pacific whiting (*Merluccius productus*) have been produced with addition of food grade protease inhibitors such as beef plasma protein and egg white (Morrissey et al., 1993). Our objective was to determine the effects of HHP treatments on gelation of Pacific whiting surimi (with and without protease inhibitors) and Alaska pollock.

MATERIALS & METHODS

Sample preparation

Commercially frozen Pacific whiting surimi and Alaska pollock surimi were obtained from American Seafoods Co. (Seattle, WA). Both types contained 4% sorbitol, 4% sucrose, 0.3% tripolyphosphate, and 0.12% mono- and diglycerides. The commercial Pacific whiting surimi was made without protease inhibitors. Surimi blocks were stored at −20°C at the Oregon State University Seafood Laboratory (Astoria, OR). Three types of surimi paste were prepared for testing: Pacific whiting surimi, Pacific whiting surimi with 1% beef plasma protein (BPP) (American Meat Corp., Ames, IA) as protease inhibitor, and Alaska pollock surimi. Fish paste was prepared as described in the surimi testing manual (NFI, 1991). All samples were standardized at 2% NaCl and 78% moisture. Ingredients were blended in a Stephan vacuum mixer (Model UM5, Stephan Machinery Corporation, Columbus, OH) for ≈ 4 min. The mixed paste was transferred to a sausage stuffer (2.3 kg capacity, The Sausage Maker, Buffalo, NY) and extruded into stainless steel cooking tubes (17.8 × 2.2 cm i.d.) sprayed with the lecithin based release agent PAM (Boyle-Midway Household Products, New York, NY). Both ends of the tube were sealed and control samples were cooked in a water bath at 90°C (0.001 Kbar) for 15 min. After cooking, the gels were transferred to an iced water bath for 15 min. The gels were removed from the tubes, place in plastic bags and stored at 4°C for testing the following day.

Authors Chung, Gebrehiwot, and Farkas are with the Dept. of Food Science & Technology, Oregon State Univ., Corvallis, OR 97331. Author Morrissey is with the Oregon State Univ. Seafood Laboratory, 250 36th St., Astoria, OR 97103. Address inquiries to Dr. M.T. Morrissey.

Table 1—Torsion results for Pacific whiting surimi as related to pressure treatment

Treatment (Kbar/°C)	Shear stress (KPa)	Shear strain
Control	12.4[b]	1.00[b]
0.001/90		
1/28	34.1[d]	3.03[a]
1.7/28	29.3[d]	2.87[a]
2.4/28	44.0[a]	2.65[d]
1/35	34.4[d]	2.92[a]
1.7/35	28.8[c]	2.56[cd]
2.4/35	41.2[a]	2.40[c]
1/50	0.00[e]	0.00[e]
1.7/50	0.00[e]	0.00[e]
2.4/50	0.00[e]	0.00[e]

[a-e] Means within a column not followed by the same letter are significantly different at the 95% confidence level.

Pressure treatments

Experimental samples were extruded into stainless steel tubes in the same manner as described. The tubes were placed in an isostatic press (Model 1P-2-22-60, Autoclave Engineers Inc., Erie, PA) and subjected to treatments for 1 hr. The pressure/temperature settings used were: (1) 1.0 Kbar/28°C, (2) 1.7 Kbar/28°C, (3) 2.4 Kbar/28°C, (4) 1.0 Kbar/35°C, (5) 1.7 Kbar/35°C, (6) 2.4 Kbar/35°C, (7) 1.0 Kbar/50°C, (8) 1.7 Kbar/50°C, and (9) 2.4 Kbar/50°C. The temperature was kept constant with a modular temperature controller (Autoclave Engineers Inc, Erie, PA). The pressure treated gels were removed from the tubes, stored in plastic bags at 4°C and analyzed the following day.

Torsion measurement

Gel texture properties were determined by torsion. Gels were placed at 25°C for 2 hr and cut into 2.8 cm lengths and formed into an hourglass shape with a 1 cm diam on a lathe-type apparatus (Gel Consultants Inc., Raleigh, NC). Samples were subjected to torsional stress in a modified Brookfield viscometer (Gel Consultants Inc., Raleigh, NC) connected to a strip chart recorder as described by Kim et al. (1986). Shear stress and shear strain, at failure, were calculated using equations developed by Hamann (1983).

Statistical analysis

A sub-set of six samples from each treatment was used for each analysis. Statistical analysis of data was carried out using one way analysis of variance. Differences among mean values were established using the Least Significant Difference (LSD) multiple range test (Steel and Torrie, 1980). Values were considered significant when $p<0.05$.

Table 2—Torsion results for Pacific whiting surimi with 1% beef plasma protein (BPP) as related to pressure treatment

Treatment (Kbar/°C)	Shear stress (KPa)	Shear strain
Control	45.7[fg]	2.14[e]
0.001/90		
1/28	33.0[ab]	2.97[e]
1.7/28	34.0[abc]	2.88[e]
2.4/28	44.2[ef]	2.64[d]
1/35	37.8[cd]	2.81[e]
1.7/35	39.1[de]	2.64[d]
2.4/35	51.5[g]	2.42[bc]
1/50	36.7[bcd]	2.62[d]
1.7/50	28.9[a]	2.30[b]
2.4/50	35.7[bcd]	2.54[cd]

[a-g] Means within a column not followed by the same letter are significantly different at the 95% confidence level.

Table 3—Torsion results for Alaska pollock surimi as related to pressure treatment

Treatment (Kbar/°C)	Shear stress (KPa)	Shear strain
Control	31.4[b]	2.09[a]
0.001/90		
1/28	48.6[cd]	2.74[c]
1.7/28	56.7[e]	2.77[c]
2.4/28	65.7[f]	2.44[b]
1/35	48.6[cd]	2.57[b]
1.7/35	52.6[cde]	2.76[c]
2.4/35	54.6[de]	2.45[b]
1/50	34.8[b]	2.42[b]
1.7/50	22.7[a]	2.02[a]
2.4/50	47.7[c]	2.39[b]

[a-f] Means within a column not followed by the same letter are significantly different at the 95% confidence level.

RESULTS & DISCUSSION

THE SHEAR STRESS and shear strain values of Pacific whiting surimi made without BPP increased significantly for all pressure treatments compared with heat-set control gels (Table 1). Shear stress is indicative of gel hardness while strain measures cohesiveness or elasticity of surimi gels (Hamann and Lanier, 1987). The strain for Pacific whiting surimi cooked at 90°C for 15 min was 1.0 and the shear stress value was 12.4 KPa. Strain and stress tripled in value under pressure treatment of 1.0 Kbar/28°C. There was a slight decrease in strain and stress when pressure was increased to 1.7 Kbar at the same temperature. Strain continued to decrease while stress increased to 44 KPa at the highest pressures at 28°C. Similar results were found for pressure treatments at 35°C. Strain decreased with an increase in pressure while stress increased slightly at 1.7 Kbar and then increased to 44 KPa at 2.5 Kbar. When pressure treatment was undertaken at 50°C, those gels made without BPP were too weak to measure. The protease enzyme in Pacific whiting surimi has a temperature optimum at 55°C (An et al., 1994). These results indicate that the enzyme was still very active during pressure treatments at that temperature.

The control sample of Pacific whiting surimi made with 1% BPP and heat-set at 90°C for 15 min showed a strain value of 2.14 and a stress of 45.7 KPa (Table 2). Strain values for all pressure treatments were significantly higher than the control. Stress values were lower than the control except for the 2.4 Kbar treatment at both 28 and 35°C. Within the same temperature range, strain decreased with increased pressure. The highest strain value occurred at 1.0 Kbar/28°C. Stress measurements showed a direct relationshp with pressure, increasing in value with increasing pressure. The only deviation from this pattern was the pressure treatments at 50°C. The Pacific whiting gels made with 1% BPP at 50°C were high quality gels but were significantly lower in stress and strain than several of the pressure treated gels made at lower temperatures. Whether this decrease in gel strength was due to residual protease activity or pressure effects at that temperature, needs to be determined.

Pollock surimi showed significant increases in both strain and stress values for all treatments except those at 50°C (Table 3). The strain and stress values of 2.09 and 32.4 KPa, respectively, for the heat-set pollock gel indicated that the samples were made from a medium grade surimi. Strain increased to 2.74 with pressure treatment at 1 Kbar/28°C and stress increased in a similar manner to 48.6 KPa for the same treatment. No significant differences occurred between strain values for 1.0 and 1.7 Kbar at 28°C. Stress values increased with increased pressure at both 28 and 35°C. Only the 2.4 Kbar treatment showed higher stress than the control at 50°C test temperature.

HHP was very effective in forming good quality gels for Pacific whiting and Alaska pollock surimi. The pressure-treated gels had a translucent appearance compared to heat-set gels which were more opaque. Similar results were shown by Shoji et al. (1990) for Alaska pollock. The effect of HHP on gel strength was also significant for Pacific whiting surimi. The stress and strain values were among the highest we have recorded for Pacific whiting surimi both with and without BPP. A direct relationship was found between pressure and stress values. Strain measurements were, for the most part, inversely related to pressure. However, the pollock and whiting samples treated at 1.7 Kbar/50°C were an exception. Probably an increase in protease activity occurred at that pressure/temperature treatment. The main protease in Pacific whiting surimi has been identified as cathepsin L, a lysosomal protease (Seymour et al., 1993). The pressures between 1 and 2 Kbars may have induced the rupture of lysosomal membranes, releasing enzymes and increasing proteolytic activity, even in the presence of BPP. Similar results have been shown in pressure treated beef (Ohmori et al., 1991). Pollock surimi has substantially less protease but would still be affected by the destruction of lysosomal membranes and release of endogenous proteases. The heat-activated proteases, once released, would react with some of the myofibrillar protein and result in weaker gels at that temperature.

The gelation of surimi paste at room temperature (≈23%) by HHP appeared to circumvent the gel-weakening effects of endogenous proteases in Pacific whiting. Surimi paste at atmospheric pressure also forms gels at room temperature through the "suwari" or setting phenomenon (Matsumoto and Noguchi, 1992). However, Pacific whiting and pollock suwari gels, set at room temperature without heating, are usually much weaker than traditional heat-set gels (Park, 1993). HHP represents a potential processing technology for high quality surimi based seafoods. Additional research is needed to determine effects of pressure on enzyme activity and microorganisms and for optimization of processing parameters.

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Functional Protein Additives in Surimi Gels

JAE W. PARK

ABSTRACT

Medium-grade Alaska pollock surimi was used to investigate the effects of functional protein additives on texture and colors. Torsion failure and differential scanning calorimetry tests were applied to measure gel strength, gel deformability, and calorimetric properties. CIE Lab color values were also measured. Shear stress values of gels and peak temperature of DSC thermograms were all increased. Shear strain, a good indicator of protein-protein interaction, was increased only by addition of egg white and beef plasma protein. Yellowness (b*) values were affected by all additives except frozen egg white. Whiteness index (L*-3b*) was a more effective indicator to differentiate additives.

Key Words: surimi, protein additives, functionality, color index.

INTRODUCTION

THE GEL-FORMING CAPACITY of surimi enables it to assume almost any desired texture. The success of surimi-based crab products is mainly due to low cost and good taste relative to crab meat. It also is convenient to handle as a ready-to-eat food. With respect to functional properties of protein additives, interactions of protein-water, protein-protein, and protein-lipid-water are very important for the formation of a stable gel network structure (Regenstein, 1984). To maintain high-quality texture, food-grade protein additives have been used in surimi seafood formulations. Functionality studies of a few individual protein additives have been described in different comminuted muscle food systems. Gnanasambandam and Zayas (1992) evaluated wheat germ protein, corn germ, and soy flours as additives in comminuted meat products such as frankfurters. Lemmers (1991) studied milk protein additives with the objective to improve the quality of processed poultry products. Budig and Klima (1990) surveyed reasons for protein additives in meat products and compared vegetable and animal proteins. The textural and nutritional effects of protein additives such as soy protein isolate, whey protein concentrate, dried egg white, soy flour, and texturized soy protein on different forms of fish have been reported (Rockower et al., 1982; Iso et al., 1985; Chang-Lee et al., 1990; Yoo and Lee, 1993).

There has been no extensive study comparing the functionality of protein additives in a single muscle food system. The objective of this research was to investigate and compare functionalities of several types of protein sources in an Alaska pollock surimi gel.

MATERIALS & METHODS

Sample preparation

Protein sources tested were: soy protein isolate (SPI), wheat gluten (WG), dried egg white (DEW), frozen egg white (FEW), whey protein concentrate (WPC), whey protein isolate (WPI), and hydrolyzed beef plasma protein (BPP).

Three blocks (10 kg each) of frozen medium-grade Alaska pollock (*Theragra chalcogramma*) were broken into small pieces and thoroughly mixed in an attempt to provide a homogeneous mixture of surimi for all treatments. Surimi, ≈6 mo old, was obtained from Arctic Storm, Inc. (Seattle, WA). The surimi was stored at −25°C during the study. The moisture content for the composite surimi was 75 ± 0.5% (AOAC, 1984). The control group contained 80% surimi, 18% ice/water, and 2% salt. Six of seven test treatments contained 79% surimi, 18% ice/water, 2% salt, and 1% dried protein additive. The seventh treatment, frozen egg white, contained 79% surimi, 11.5% ice/water, 2% salt, and 7.5% frozen egg white (the same amount of solids as 1% dried egg white). All ingredients were commercially available.

Author Park is with Oregon State Univ., Seafood Laboratory, 250 36th St., Astoria, OR 97103.

The frozen surimi was tempered at room temperature for 2 hr and then chopped in a Stephan UM5 universal food processor (Stephan Machinery Corp., Columbus, OH) at low speed for 1.5 min. Salt and water were added and the surimi paste was chopped for 1 min. The protein additive was added and chopping was continued for 1.5 min. The ice was added and the surimi paste was chopped at high speed under vacuum (50 mm Hg) for 2 min. The chopping temperature was maintained below 8°C using a NesLab constant-temperature bath (Portsmouth, NH) containing a solution of ethylene glycol and water (50:50). A sausage stuffer (The Sausage Maker, Buffalo, NY) was used to stuff raw paste into stainless steel tubes (i.d. = 1.86 cm, length = 17.5 cm) with stainless steel screw caps. The interior wall of the tubes was coated with a film of PAM® no stick cooking spray (Boyle-Midway, Inc., New York, NY). The tubes were submerged in a 95°C water bath, and the water temperature was adjusted immediately to 90°C using ice. Total cooking time was 15 min. According to Yongsawatdigul et al. (1994), 8 min were required to reach the internal gel temperature of 80°C. The cooked gels were cooled quickly by immersion in ice water. The gels were kept in a refrigerator for texture and color analysis.

Textural properties

The cooled gels (5°C) were held 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 geometry (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 failure were measured (Hamann, 1983; NFI, 1991). Texture characteristics of gels was categorized based upon the relationship between shear stress and shear strain: *mushy*: low strain, low stress; *brittle*: low strain, high stress; *rubbery*: high strain, low stress; *tough*: high strain, high stress.

Color analysis

Five samples from each treatment were removed for color analysis using a Minolta CR300 colorimeter (Osaka, Japan). CIE L*(black [0] to light [100], a*(red [60] to green [−60]), b*(yellow [60] to blue [−60]) values were measured to evaluate the coloring effects of protein additives. Two whiteness indices (I&II) were used to compare effectiveness. One method was based on a procedure recommended by NFI (1991):

$$\text{Whiteness I} = 100 - [(100-L^*)^2 + a^{*2} + b^{*2}]^{1/2}$$

The other method was a conventional method used in Japan, defined by the following:

$$\text{Whiteness II} = L^* - 3b^*$$

Differential scanning calorimetry (DSC)

The effects of protein additives on thermal denaturation of surimi proteins were studied in a DuPont 910 DSC (DuPont Instruments, Wilmington, DE). The samples were prepared on a small scale (100 gm: 97% pollock surimi, 2% salt, 1% dried ingredients) using a mortar and pestle in a cold room. For frozen egg white treatment, 92% surimi, 2% salt, and 7.5% FEW were used. Samples were weighed to ≈10 mg and sealed in aluminum capsules. As a reference, an empty pan was used. The scanning temperature was 15-100°C at a heating rate of 10°C/min. Each experiment was repeated.

Statistical analysis

Ten replicates were tested for torsional measurement and 5 for color measurement. Statistical analysis (t-test) was applied on the data for significant differences ($P<0.05$). Two replicates were used for DSC so that a simple mean value was obtained without further statistical analysis.

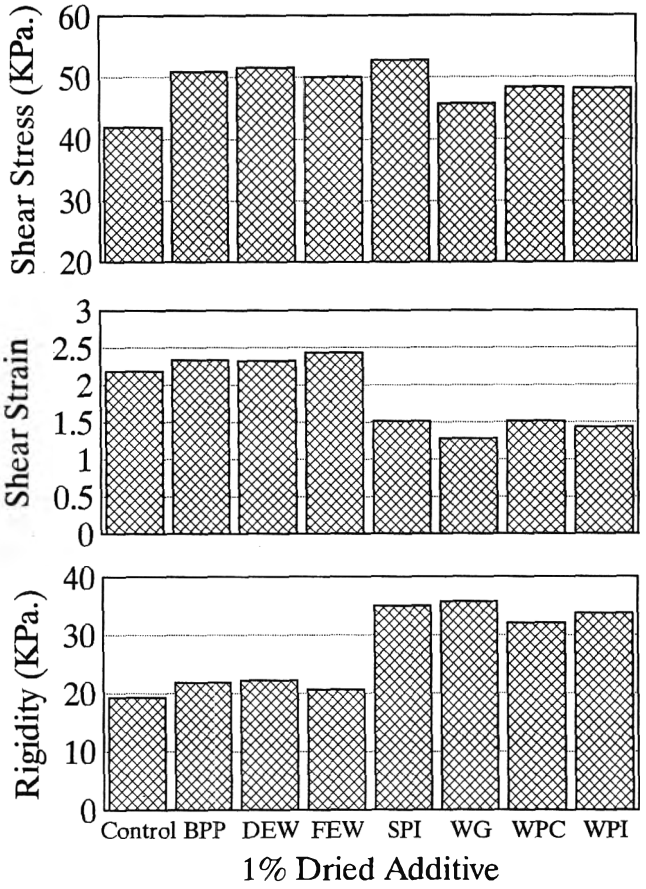

Fig. 1—Effects of protein additives on shear stress (hardness), shear strain (deformability), and rigidity.

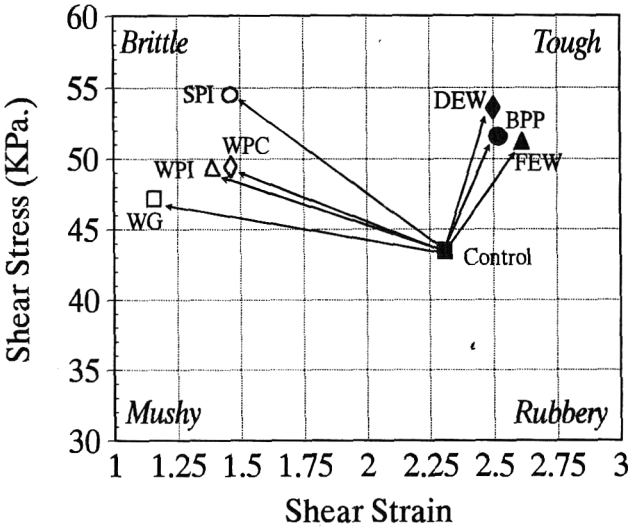

Fig. 2—Texture modification as affected by protein additives.

RESULTS & DISCUSSION

Effects of textural properties

The shear strain-at-failure measurement indicates the cohesive nature of a gel, while the stress-at-failure measurement relates to the strength of the material (Park et al., 1988). Shear stress values were all ($P<0.05$) improved by protein additives in a descending order of SPI, DEW, BPP, FEW, WPC, WPI, and WG (Fig. 1). The results observed for Alaska pollock were different from those reported by Chang-Lee et al. (1990). They reported that the hardness of Pacific whiting gel was not changed at a 1% level of DEW and WPC, while SPI reduced gel hardness by 10% at the same level. However, the presence of 3% DEW resulted in 30% improvement of gel hardness. Hamann and MacDonald (1992) also confirmed that the failure stress of surimi gels was influenced by filler ingredients.

Shear strain values at failure were measured as affected by addition of 1% protein additives. BPP, DEW, and FEW improved gel strain ($P<0.05$), while SPI, WG, WPC, and WPI decreased strain ($P<0.05$) (Fig. 1). BPP, DEW, and FEW appeared to improve the binding functionality (shear strain) of surimi proteins through protein-protein interactions. Hamann (1988) also reported that strain, as an indicator of protein interactions, was strongly affected by protein functionality.

The effects of protein additives on rigidity was also compared (Fig. 1). Shear rigidity is shear modulus calculated by the simple division of shear stress by shear strain (Hamann and MacDonald, 1992). BPP, DEW, and FEW did not show strong effects of rigidity, while SPI, WG, WPC, and WPI ($P<0.05$) increased rigidity. Thus although BPP, DEW, and FEW increased the strength (stress) and the deformability (strain) of gels, they did not increase rigidity (brittleness) of the gel (Fig. 1) due to rubberiness of the gel. Burgarella et al. (1985) showed that the negative interaction, with respect to gel rigidity was more pronounced when croaker surimi was mixed with egg white than with whey protein concentrate. The texture modification by protein additives was also illustrated as a function of stress and strain (Fig. 2). Based on four typical characteristics (mushy, rubbery, brittle, and tough) of the gels, BPP, DEW, and FEW made the gels tougher, while the other test materials made the gels more brittle.

Effects of color/appearance

The CIE L*, a*, and b* value of gels (Fig. 3) showed protein additives reduced lightness values ($P<0.05$) except WG. The greenness of gels was not affected by addition of protein additives. This confirmed results of Park (1990). Regardless of surimi grades, a* values were consistent, with small differences in the range of -3.0 to -4.5. Yellowness of the gels was affected ($P<0.05$) by the addition of protein additives except for FEW. BPP, DEW, SPI, WG, and WPC increased yellowness by 35–50%.

The surimi industry has used different whiteness indices to determine functionality of color. Two widely used methods were compared (Fig. 4) to evaluate the effects of protein additives on whiteness indices. Whiteness I, based on NFI (1991) recommendations, considered all components of the coloring fraction, L*, a*, and b*. Whiteness II was based on the conventional equation used in Japan, which does not consider the function of a*. Whiteness II showed that protein additives affected the whiteness index (Fig. 4). Whiteness I showed only slight differences when compared to the control. Visual differences between gels were observed and no difference occurred in a* value between protein additives. Thus, the conventional equation from Japan (whiteness II = L^*-3b^*) appeared more effective. This also confirmed reports of Park (1994). The a* value of pollock and whiting gels was very consistent regardless of cooking/setting conditions, moisture contents, sample size, or frozen storage.

Effects on DSC properties

Heat-induced calorimetric properties of surimi proteins, affected by addition of protein additives, were compared by differential scanning calorimetry (Table 1). The myosin peak temperature was shifted to higher values with addition of protein additives: SPI, WG, WPC, and WPI produced a greater shift than BPP, DEW, and FEW. These protein additives appeared to delay denaturation (unfolding) of fish proteins. The temperature shifting was also observed when non-protein additives (sugar) were mixed with surimi (Park and Lanier,

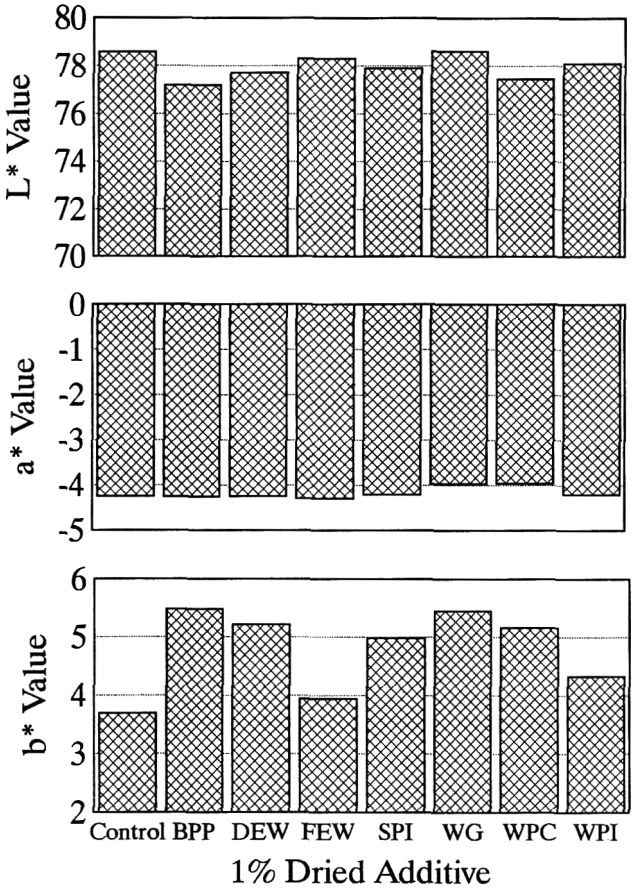

Fig. 3—Effects of protein additives on L* (dark to light), a* (−: green; +:red), and b*(−:blue; +:yellow).

1990). Two continuous mechanisms affect thermal gelation; denaturation and aggregation. Protein additives reduced the enthalpy of endothermic peaks (Table 1). Reduced enthalpy may be explained by the increased exothermic contribution. Denaturation is associated with intensive heat absorption, while aggregation is exothermic (Biliaderis, 1983). The endothermic peak is the net enthalpic change which represents the sum of positive (endothermic, e.g., denaturation) and negative (exothermic, e.g., aggregation) contributions (Park and Lanier, 1990). The reduction in enthalpy was possibly due to increased protein aggregation enhanced by protein additives. The results agreed with increased shear stress (Fig. 1).

CONCLUSIONS

THE TWO EGG WHITES used and hydrolyzed beef plasma proteins acted as very functional binders in surimi gels and the other protein additives were functional fillers. The whiteness II equation for surimi gels, L*-3b*, was more effective than the whiteness I equation to differentiate effects of protein additives.

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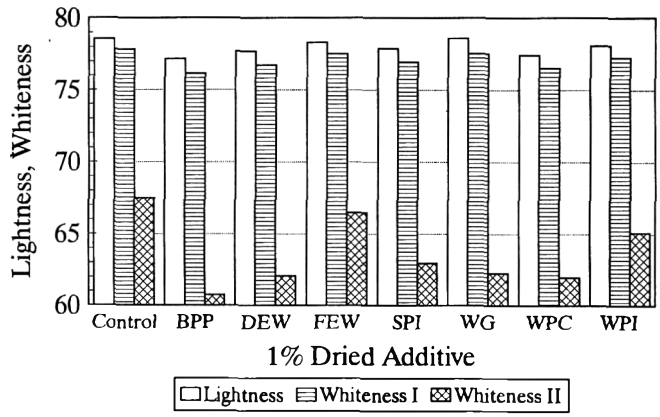

Fig. 4—Two whiteness indices as compared with lightness values. Whiteness I is based on $100-[100 - L^*)^2 + a^{*2} + b^{*2}]^{1/2}$, while Whiteness II is basedon L*-3b*.

Table 1—DSC properties of myosin peak by protein additives

Additives[a]	Peak temp (°C)	ΔH (cal/g)
Control	36.7	1.62
BPP	45	0.56
DEW	40.7	0.73
FEW	38.1	0.71
SPI	48.1	0.71
WG	49.6	1.04
WPC	49.2	0.48
WPI	47.4	0.31

[a] BPP = beef plasma protein; DEW = dried egg white; FEW = frozen egg white; SPI = soy protein isolate; WG = wheat gluten; WPC = whey protein concentrate; WPI = whey protein isolate.

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Reverse-Phase HPLC Analysis of Cheese Samples Aged by a Fast-Ripening Process

V. FURTULA, S. NAKAI, G.F. AMANTEA, and L. LALEYE

ABSTRACT

Cheese ripening accelerated by adding enzyme extracts derived from lactic bacteria promoted proteolysis and produced an acceptable novel cheese based on sensory tests. However, HPLC profiles for water-soluble compounds were considerably different from those for control Cheddar cheese without the added enzyme extracts, probably indicating a different mechanism of proteolysis. The samples did not follow the usual aging pathway on a similarlity scattergram, when HPLC data were processed by principal component similarity (PCS) analysis. Such analysis was useful for evaluating effects of accelerated cheese ripening.

Key Words: cheese ripening, cheddar cheese, sensory, hydrolysis

INTRODUCTION

ACCORDING TO LAW (1990), the research trend in shortening ripening time is turning from enzyme addition methods and focusing on starter culture technology. This appears logical because of the complexity of cheese flavor profiles and the use of mixtures of enzymes. The balance of hydrolysis products derived mainly from casein and also, probably to a lesser extent, from milk fat, comprises the traditional flavor profile of Cheddar cheeses (McGugan et al., 1979). The complexity of cheese flavor is exemplified in differences in HPLC patterns of different varieties of cheeses (Mohler-Smith and Nakai, 1990) and GC patterns (Aishima and Nakai, 1987). Therefore, in principle, if cheese ripening is to be successfully accelerated, the flavor profile by HPLC for water-soluble peptides, as well as GC for volatiles should be similar to those of traditional cheese. The only differences should be in the speed of ripening.

Based on this principle, analysis of the principal component similarity (PCS) of chromatography data was applied (Furtula et al., 1994). Reference samples of Cheddar cheese prepared by the traditional process were analyzed by HPLC. Similarity parameters computed using principal component (PC) scores could discriminate a cheese sample abnormally aged from one normally aged by comparing pathways of ripening on the plots of PCS analysis. Our objective was to compare patterns of cheese ripening on the PCS plot of HPLC data, when ripening was accelerated by adding enzymes from lactic acid bacteria.

MATERIALS & METHODS

Cheese samples

Commercial Cheddar cheeses of different ages were used for reference. These included mild, medium, sharp and extra-sharp cheeses aged for 3, 6, 9 and 12 mo, respectively (Furtula et al., 1994). The two regularly aged batches (with and without symbol "j") were used as references. One reference batch (without symbol) was the control of the fast-ripening experiment. Cheese batches (A through E) experienced fast-ripening under slightly different conditions. These were used as test batches to investigate similarity of HPLC profiles with those of the control. Altogether six batches were prepared from the same milk. The industrial storage conditions were the same as conditions for commercial Cheddar cheese.

Authors Furtula and Nakai are with the Dept. of Food Science, The Univ. of British Columbia, 6650 N.W. Marine Drive, Vancouver, BC, Canada V6T 1Z4. Authors Amantea and Laleye are with Ault Foods Ltd., Research & Development, 75 Bathurst St., London, ON, Canada N6B 1N8.

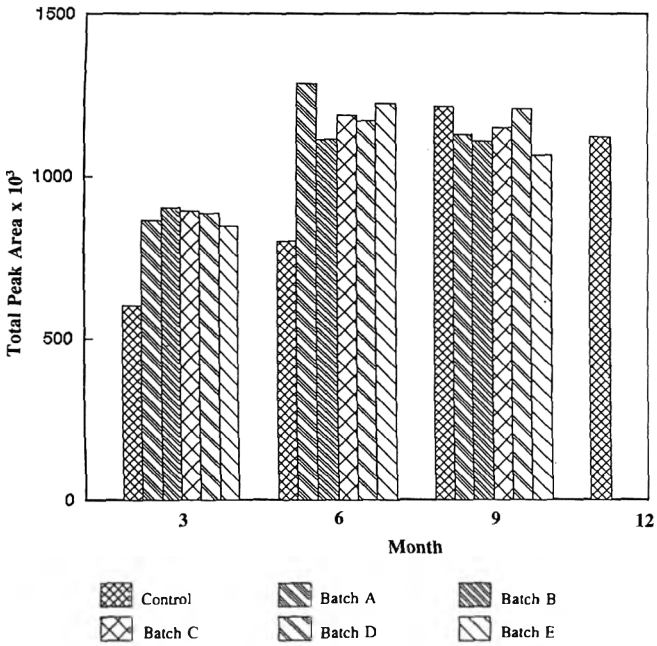

Fig. 1—Changes in total HPLC peak areas during aging of fast-ripened cheese samples (A to E). Total peak area is proportional to concentration of water-soluble compounds.

HPLC and statistics

These analyses were carried out as described (Furtula et al., 1994).

Sensory analysis

Sensory analysis was by two official graders (Agriculture Canada) and three professional cheesemakers, under industrial conditions. The procedure of grading was according to the grading manual (Agriculture Canada, 1983). The 18.2 kg cheese blocks were graded after 3, 6 and 9 mo aging. The cheese gradings were based primarily on overall quality, not on flavor quality alone. The quality can be judged to have a maximum compiled score of 100. A minimum of 92/100 was considered grade 1. Graders were asked to comment on intensity of flavor as well as flavor defects. The estimated age was reported whenever possible.

RESULTS & DISCUSSION

HPLC analysis

The total peak area as a function of aging (Fig. 1) showed no appreciable differences in the total peak area among accelerated samples A–E within the same age groups. Based on degree of hydrolysis without considering HPLC profiles, the total peak area of fast-ripened samples at 3 and 6 mo were about the same as those of reference samples at 6 and 9 mo. This indicated an acceleration of ripening of about 3 mo.

Table 1—Component loadings of PC scores 1 to 5

Peak	Retention time (min)	Std Dev	PC_1	PC_2	PC_3	PC_4	PC_5
Var40	52.06	0.23	0.9549	0.0540	0.2190	0.0017	0.0501
Var18	28.07	0.22	0.9485	0.0315	−0.1371	−0.1420	0.0324
Var37	48.48	0.23	0.9367	−0.0780	0.2598	0.0467	−0.0423
Var2	4.41	0.10	0.9344	−0.1060	0.0129	−0.2589	0.1402
Var39	49.82	0.29	0.9269	0.0152	0.1252	−0.1593	−0.1243
Var45	57.70	0.22	0.9247	−0.2831	0.0015	−0.1388	0.0661
Var21	30.87	0.24	0.9218	0.1573	0.0541	0.0130	0.0679
Var11	23.93	0.29	0.9156	0.2728	0.1895	−0.0083	0.0347
Var5	11.45	0.32	0.8962	0.2952	−0.0003	−0.1121	0.0823
Var46	61.10	0.21	0.8938	0.0944	−0.1805	0.1525	−0.0399
Var20	30.32	0.33	0.8920	0.2993	−0.1713	0.0796	0.0775
Var10	23.79	0.22	0.8887	0.0818	0.3147	−0.1215	0.0272
Var8	21.67	0.23	0.8822	0.1524	0.3428	−0.0215	0.1049
Var33	42.18	0.26	0.8788	−0.2814	0.2370	−0.0210	−0.1924
Var16	26.81	0.20	0.8767	−0.3714	0.0161	0.1676	0.1158
Var29	30.72	0.20	0.8757	−0.1007	0.3319	0.0948	−0.1310
Var13	25.29	0.25	0.8753	−0.1038	−0.2100	0.2877	0.0227
Var17	27.55	0.21	0.8672	−0.3496	0.2258	−0.0741	0.0790
Var42	54.67	0.25	0.8664	0.1306	−0.1115	0.2592	0.0339
Var50	72.92	0.31	0.8629	0.1657	−0.3760	−0.0931	−0.0078
Var48	66.71	0.27	0.8575	−0.1995	−0.1424	0.0608	0.0499
Var49	72.06	0.24	0.8553	−0.3988	−0.0354	−0.0808	0.1329
Var15	26.07	0.28	0.8507	0.3656	−0.2432	−0.0249	0.0278
Var4	11.11	0.36	0.8503	−0.1175	−0.1157	−0.2279	0.0714
Var31	39.61	0.28	0.8488	0.2415	−0.1036	0.3116	−0.1032
Var30	37.88	0.22	0.8477	0.0394	0.4120	0.0816	−0.0684
Var41	53.42	0.23	0.8304	−0.0307	0.4290	0.0139	0.1358
Var28	35.73	0.21	0.8133	0.5098*	0.1984	0.0076	0.0477
Var9	22.96	0.29	0.8071	0.3288	−0.1188	−0.0730	−0.0894
Var22	31.31	0.18	0.8012	0.3641	−0.3848	−0.0472	0.0282
Var25	33.35	0.25	0.7977	−0.3937	0.2026	0.0380	−0.1785
Var32	40.80	0.23	0.7968	−0.4082	0.0761	0.0607	−0.2886
Var43	55.32	0.36	0.7899	−0.2562	0.2047	0.2718	−0.2334
Var27	34.56	0.23	0.7441	−0.5145*	−0.2758	0.1098	−0.0916
Var26	33.83	0.30	0.6943	0.5520*	−0.3958	0.0769	0.0990
Var24	32.59	0.32	0.6972	0.1874	−0.1367	−0.0417	−0.1832
Var23	32.02	0.21	−0.5856	−0.1368	0.1007	0.7510*	0.0449
Var36	47.26	0.14	0.5732	−0.7278*	0.2076	−0.0281	0.0140
Var19	29.18	0.20	0.5213	−0.7064*	−0.0212	−0.3096	0.2060
Var3	6.21	0.16	0.1695	0.9166*	−0.0649	−0.1282	−0.2488
Var6	12.41	0.29	0.4575	0.7750*	−0.2830	0.2289	−0.0830
Var7	15.59	0.34	0.1803	0.7713*	−0.3187	0.4724*	−0.0618
Var12	24.13	0.22	0.1169	−0.6323*	−0.6038*	0.3138	0.1837
Var34	73.37	0.23	−0.0934	−0.5790*	0.1913	0.6724*	0.2222
Var14	25.59	0.29	0.3740	0.5523*	0.5595*	0.0191	−0.0065
Var47	64.72	0.33	0.3840	−0.5388*	−0.5132*	−0.2161	−0.3260
Var1	3.63	0.12	−0.3882	0.5259*	0.6371*	0.1810	−0.2189
Var38	48.93	0.28	0.2941	−0.2931	−0.7127*	0.1241	−0.4530*
Var35	44.54	0.16	0.3466	−0.0648	0.0592	0.8143*	0.1355
Var44	55.89	0.21	−0.3528	−0.4166	0.3383	0.1199	−0.6886*

* Loadings >0.45 for PC_2–PC_5.

Table 2—Summary of deviations of corrected PC-scores from extra-sharp cheese used as a standard*

Sample		PC_1	PC_2	PC_3	PC_4	PC_5
Control						
	Mild	−		+		−
	Medium	−		+	+	
	Sharp			+		
A	3	−	−	+	−	−
	6		−	+		−
	9		−	+		
B	3	−	−	+	−	+
	6		−	+		−
	9		−	+		
C	3	−	−	+	−	+
	6		−	+		−
	9		−	+		−
D	3	−	−	+	−	+
	6		−	+		−
	9		−	+	+	−
E	3	−	−	+	−	+
	6		−	+		−
	9		−	+		−

* +,− indicate that PC score of sample is larger and smaller, respectively, than that of reference sample (extra-sharp sample of control batch).

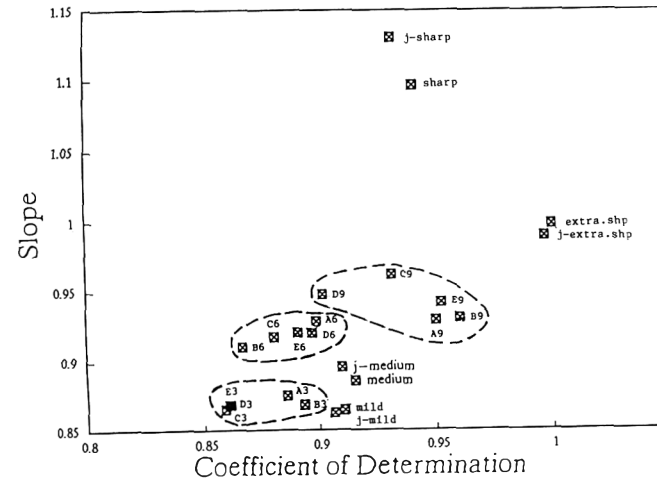

Fig. 2—PCS scattergram of fast-ripened and control cheeses. Extra-sharp control Cheddar cheese as reference (r^2 = 1.0, slope = 1.0).

Principal component analysis of fast-ripened samples provided five principal components with eigenvalues >1.0, which accounted for 89.7% of total variation. Component loadings of these principal components (Table 1) indicated variables involved were not much different from those of traditional cheese (See Table 2, Furtula et al., 1994) in terms of order of

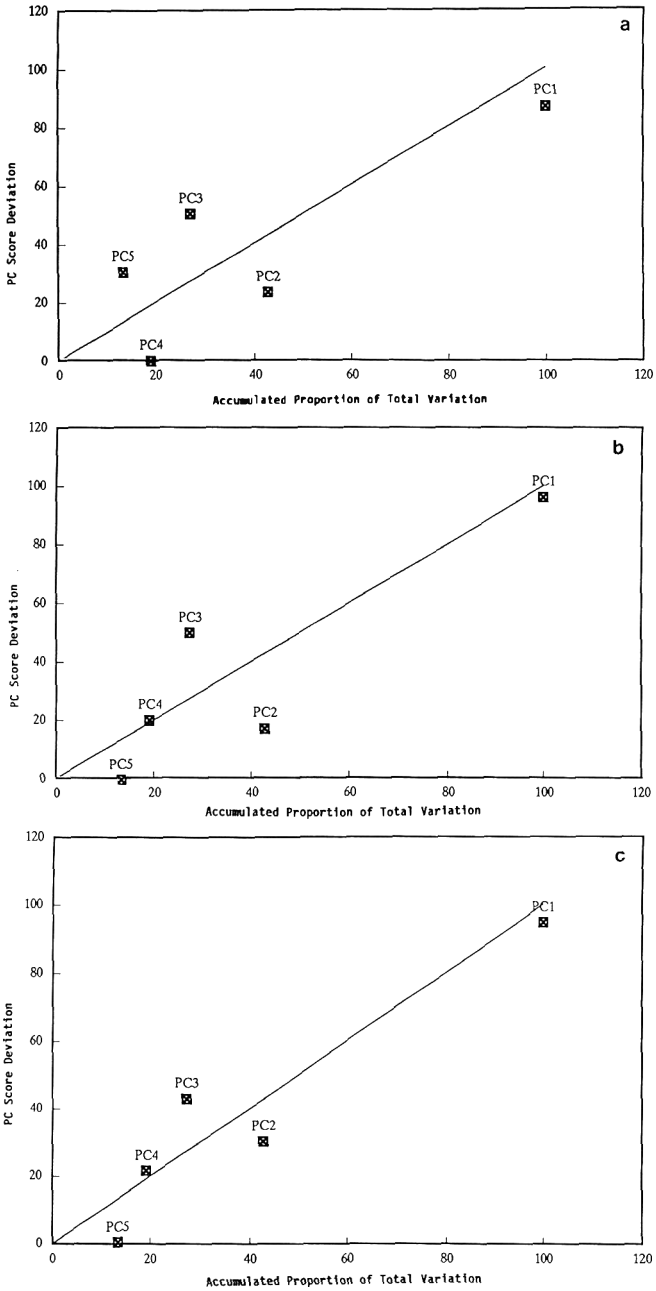

Fig. 3—PC score deviation (scores for batch E of different ages from that of extra-sharp Cheddar reference) as related to accumulated proportion of total variation that each PC score can account for. (a) mild E3 cheese; (b) medium E6 cheese; and (c) sharp E9 cheese. (———) Extra sharp cheese (PCS reference).

variables and those with high loadings. This may indicate that compounds important for cheese aging are not greatly different between traditional and fast-ripened cheeses. The PCS plots of all cheese samples (Fig. 2) showed at 3 mo aging, fast-ripened batches A and B were between mild and medium of reference samples, thus certain acceleration effects were observed. However, batches C, D and E were separated away from the regular aging pathway from mild to extra-sharp and after 6 and 9 mo aging, no accelerated samples were near "sharp" and "extra-sharp" (reference samples). Batches A and B were relatively closer to the regular ripening pathway.

PC plots of batch E demonstrated the approach of sample PC1 to that of PCS reference samples as cheese aged (Fig. 3). This is the change similar to traditional Cheddar cheese (Fig. 6, Furtula et al., 1994). Since PC1 accounted for the major part of total variation, the aging changes between traditional and fast ripenings may not be much different.

This change is also shown in Table 2 (similar to Table 3, Furtula et al., 1994). Minus, minus changed to none for control cheeses, and minus to none and none in the case of accelerated cheeses, as the cheeses aged from mild to medium and then to sharp. Since the minus sign indicates HPLC peaks represented by principal components were smaller than corresponding peaks of controls (extra-sharp Cheddar cheese), these peaks increased during aging and approached those of controls, unless the sign of the loading was negative. The behavior of PC3 was not much different between control and accelerated batches of cheese samples (Table 2). Patterns of changes in PC4 and PC5 were difficult to summarize as signs were mixed compared to plus and minus for PC4 of medium and PC5 of mild of the control batch. They represent only minor peak variabilities. The major difference of accelerated cheeses from controls, therefore, appears in the behavior of PC2 (Table 2) as minus throughout aging vs none. Major contributions to this principal component were variables 3, 6, 7, 36, 19 and 12 (Table 1). This appears as differences (Fig. 4) in peak areas between controls and accelerated HPLC profiles, e.g., peaks 6 (12.4 min) and 7 (15.6 min) with plus loadings smaller in the accelerated cheese and peaks 19 (29.2 min) and 47 (64.7 min) with negative loadings, which were larger. These differences could be why the accelerated group did not follow the regular aging pattern. Note that trends of abnormally aged cheese samples (Furtula et al., 1994) showed a similar behavior from plus for mild to none for medium and sharp compared to none to minus, here.

At 9 mo aging when accelerated samples were expected to be similar to "extra-sharp" as judged from total peak area (Fig. 1) and the deviation of accelerated samples from extra-sharp (PCS reference) should be minimum. However, in all accelerated samples, PC2 and PC3 showed appreciable deviations (Table 2). PC3, which should not deviate from PC3 of extra-sharp if acceleration of 3 mo were effective, contained variables 38, 1, 12 and 14 as major components (Table 1).

These results may not necessarily mean a total failure of ripening accelerated by this method, because the deviation of HPLC patterns from those of PCS reference did not occur in PC1 which contained major components of all HPLC peaks (Table 2), accounting for almost 60% of total variation. A combination of PC2 and PC3 accounted for about 20% of total variation, which may need more improvement in acceleration technology for promoting "typical Cheddar cheese ripening" while maintaining high pattern similarity. The acceleration process we used accelerated the protein hydrolysis (Fig. 1). However, HPLC patterns were considerably different from normally aged Cheddar cheese samples, especially after 6 mo aging (Fig. 2). Importance of pattern similarity in judging effects of fast-ripening should, therefore, be emphasized.

Sensory analysis

That both the control and experimental enzyme-treated cheese were classified grade 1 (Table 3) except for the 3-mo control. However, the score for RD enzyme-treated cheese (Batches C to E) was 1 point higher. The commercial-enzyme cheese (Batch B) produced a good-flavored cheese, the only defect being mealy texture. The cheese broke down and crumbled when pressed in the palm of the hand. This defect could be explained by proteinase content of the commercial enzyme (Choi, 1992). Furthermore, no increase of apparent age was evident in these samples.

All RD enzyme-treated cheese (Batches C and E) had good texture, good flavor and showed significant accelerated aging. At the 6-mo mark, the age of the RD enzyme-treated cheese was accelerated by 1 or 2 mo, for a total of 7 to 8 mo of true aging. At the 9-mo mark, the accelerated age was almost equivalent to 12 mos. In addition to the advantage of acceleration in aging, favorable comments were made regarding

Table 3—Grading of fast-ripened cheese by sensory analysis

Age (months)/Sample	3		6		9	
	Score	Comments	Score	Comments	Score	Comments
Set A (control)[a]	91.2	Slightly bitter	92.2	Bitter, metallic flavor	92.4	Bitter, metallic flavor
Set B (Commercial enzyme)[b]	91.6	Slightly mealy texture	91.7	Mealy texture Good flavor 6-7 months old	92.2	Mealy texture Good flavor 8-9 months old
Set C (RD-1 Enzyme)[c]	92.5	Good texture Good flavor	92.6	Good texture Good flavor 7-9 months old	92.8	Good flavor Taste like 12 months old
Set D (RD-2 Enzyme)[c]	92.5	Slight acid	92.5	Good flavor Slightly weak texture, 7 months	92.3	Good flavor Pasty texture 12 months old
Set E (RD-3 Enzyme)[c]	92.8	Good flavor Good texture	93.4	Good flavor Good texture 7-9 months old	93.3	Good flavor Good texture 12 months old

[a] Under the processing conditions same as other sets but without enzymes.
[b] *Accelerase* (Imperial Biotechnology, London, UK).
[c] Cell-free extracts of *Lactobacillus casei* (RD) (Ault Foods, London, Ontario).

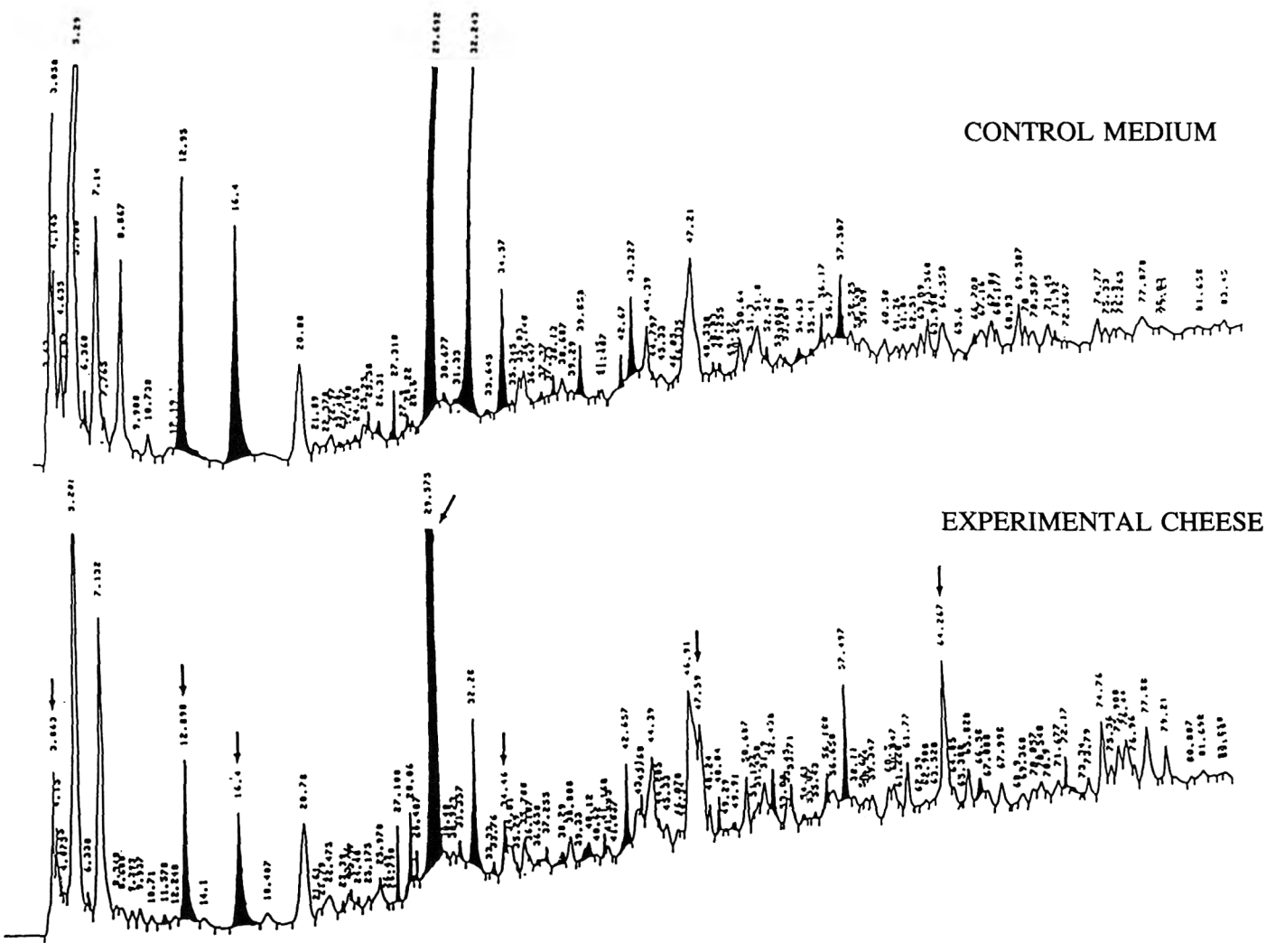

Fig. 4—HPLC profiles of control Cheddar cheese and fast-ripened cheese B3. Shaded peaks have principal component loadings >0.8. Arrows indicate peaks highly influencing principal component 2 with high loadings.

flavor. It was unique, not necessarily comparable to the usual flavor of naturally aged Cheddar.

Analytical results

To determine whether accelerated samples belonged to the Cheddar cheese category, further analysis of HPLC data were required using supervised classification methods (Mohler-Smith and Nakai, 1990). Note similarity in the behavior of fast-ripened cheese to that of abnormal traditional cheese (Furtula et al., 1994). This suggests a similarity in protease activity between the two groups of cheese, probably promotion of endo-enzyme action to a greater extent than exo-enzyme action. Hydrolysis deep inside casein molecules probably occurred rather than splitting of small peptides from the end of casein molecules. Deviation of accelerated cheese from traditional cheese (Fig. 2) with lower r^2 may mean deviations of HPLC profiles from those of traditional cheeses.

Multivariate analysis of accelerated cheese ripening

Pattern similarity is frequently used for comparing chromatograms or spectra. Since successful accelerated cheese ripening shortens the aging time of cheese without changing similarity to traditional cheese, use of pattern similarity to compare chromatograms may be applicable. The pattern similarity is the cosine of vectors of 2 patterns (Aishima et al., 1987), which is a measure of the extent of similarity. Unlike pattern similarity of Aishima et al. (1987), the similarity parameters in PCS are eventually statistical parameters, i.e., slope of regression line (s) and coefficient of determination (r^2) or correlation coefficient (r), derived from linear regression analysis of principal component scores instead of original pattern data. Therefore, two statistical parameters, s and r^2 (or r), could be used to illustrate the similarity on a 2-D scattergraph. This is an advantage in terms of classification capacity as the 2-D PCS scattergraph can be used instead of single dimensional comparison of size of similarity constants. This characteristic property of PCS would enhance classification capacity. Furthermore, reasons for nonsimilarity are readily apparent, as shown here. Since PCS is capable of tracing the pathway of cheese aging with simultaneous assessment of similarity, it can be a powerful tool for evaluating effects of accelerated cheese ripening.

Comparison of similarity measurement

Three expressions describe similarity (Massart and Kaufman, 1983), namely, distance, correlation and information content. However, the information-type measure is useful only when information aspects are specifically included and it does not appear advantageous over correlation coefficient, popular for analysis of quantitative data. Occasionally, two different approaches, i.e., distance and correlation, yield different results, mainly because distance detects differences best and correlations find similarities. The Euclidian distance led to distorted conclusions when variables were correlated (Massart and Kaufman, 1983). Depending on the mathematical definition the distance computed may not be in agreement with a lack of similarity in mathematical terms.

The similarity based on correlation generally uses cos α, (the angle between vectors of two patterns), as a measure of similarity (Aishima et al., 1987). The pattern similarity takes a similar form as correlation coefficient in linear regression analysis, with a slight difference. Similarity uses raw values, while correlation uses values compared to the mean. Similarity employed in PCS is not the same as cos α, as it is a result of linear regression of plot of differences of PC scores used to reduce the dimension of raw data and also to decrease multicolinearity effects. This may be useful, especially when size of the data matrix is large. When the number of data are small, the advantage of PCS may diminish.

Comparison of multivariate analysis techniques

Multivariate analysis can be classified into two categories in terms of objectives, namely, computations for relationships or for classification. Computation for relationships includes multiple regression analysis, principal component regression (PCR), partial least squares (PLS) regression, and artificial neural networks (ANN). Computation for classification is carried out using stepwise discriminant analysis, principal component similarity (PCS) analysis, and ANN. ANN is, in principle, a technique for pattern recognition; however, it can also be used to study relationships, because it can predict the property of unknown samples, (Arteaga and Nakai, 1993), once a training set is established. Cheese ripening is a continuum therefore, the relationship study is more appropriate. However, classification of cheese samples based on flavor and age, i.e., mild, medium, sharp and extra-sharp is frequently imposed in commercial practice. Therefore, classification of continuum cases, such as cheese by age which should be processed by relation study, would adversely affect the accuracy of classification.

Banks et al. (1993) applied PLS to GC data of Cheddar cheese and reported excellent correlation with sensory flavor scores. For a model including both volatiles and measures of proteolysis, up to 98% of total variation in maturity scores was explained using PLS, while PCR did not provide reliable predictions. However, there may be limitation when PLS is applied to fast-ripened cheese samples. As long as pattern similarity vs control Cheddar cheese is high, it may be useful, otherwise prediction accuracy may quickly diminish.

ANN is rapidly gaining applications because of its superior capacity for classification as well as relationship studies. It outperforms most other multivariate analysis techniques. For example, in QSAR (quantitative structure activity relationships) study of antiarrhythmic activity of phenylpyridines, the predictability of ANN was 93% compared to 78% of PLS and PCR (Hasegawa et al., 1992). Similarly, ANN was also better than PLS in estimating composition of mixtures computed from fluorescence spectra (McAvoy et al., 1992). No application of ANN to cheese ripening has been reported. However, application to accelerated cheese ripening may be restricted by the same reason as application of PLS, discussed above.

The usefulness of PCS in the study of accelerated cheese ripening is premature to judge, because of limited experience of application. However, because of its favorable nature, i.e., similarity based on correlation, unsupervised learning methods (such as PCS) without need of establishing learning sets, may be exploited for new product development, e.g., fast-ripening when available data matrix is rather limited. This is also true in the case of emergency, e.g., the abused cheese batch reported previously (Furtula and Nakai, 1994). There, it is assumed that the cause of the abnormality is unknown beforehand, meaning an unsupervised case where ANN could not be directly applied. More powerful classification of PCS than PCA should be useful here. PCS should be a useful tool for routine quality control, as it can be readily employed as a subroutine program in specific data processing systems. The programming of PCS is simple and computer programs for both linear regression and PCA are available in relatively simple, short forms (Yokoh, 1985).

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Reverse-Phase HPLC Analysis of Reference Cheddar Cheese Samples for Assessing Accelerated Cheese Ripening

V. FURTULA, S. NAKAI, G.F. AMANTEA, and L. LALEYE

ABSTRACT

Applying water extracts from Cheddar cheese to an octadecyl vinyl alcohol copolymer column using a reliable auto-sampling system provided highly repeatable HPLC patterns. Two batches of standard samples of mild, medium, sharp, and extra-sharp and one batch of abused samples (rapidly aged under abnormal conditions) were analyzed. Principal component similarity (PCS) analysis indicated similar shifts of plots due to aging of the standard batches, while the plot for the abused batch deviated from the pathway of normal aging. PCS may be useful for analysis of accelerated ripening effects as well as finding unusual samples during quality control.

Key Words: Cheddar cheese, HPLC, principal component, similarity, cheese ripening

INTRODUCTION

DUE TO ECONOMIC ADVANTAGES, development of methods to shorten natural maturation in cheeses has been extensively studied and well documented (Law, 1990). Sensory evaluation of texture and flavor is conventionally used to assess the age of cheese. However, more reliable, instrumental analyses are desirable when judging ripening acceleration effects. As an objective method for analysis of Cheddar cheese age, Pham and Nakai (1984) proposed reverse-phase (RP) HPLC analysis of a water-soluble fraction extracted from cheese samples based on the method of McGugan et al. (1979). To circumvent the disadvantage of the relatively short life-time of silica-based HPLC columns, a vinyl alcohol copolymer column was introduced for analysis of cheese water extracts (Li-Chan et al., 1992) which almost tripled column life.

Cliffe and Law (1991) applied RP-HPLC to fast-ripened Cheddar cheese and reported a good correlation of the area of one peak with Cheddar flavor intensity. Although a mixture of Neutrase and starter peptidase resulted in a significant shortening of ripening time, HPLC profiles were considerably different from those of control cheese. In principle, accelerated ripening is intended to promote ripening of Cheddar cheese, i.e., reproduction of the hydrolysis profiles of control cheeses in a shorter ripening time.

Pham and Nakai (1984) applied linear discriminant analysis to RP-HPLC data to judge age of Cheddar cheese samples using age discrimination based on label declarations of commercial products. The age of cheese is usually defined differently by manufacturers, or even for different production batches. Moreover, cheese aging is a continuum, making it difficult to discriminate precise stages. Banks et al. (1992) applied partial least squares (PLS) regression to GC data of Cheddar cheese and reported excellent correlation with sensory flavor scores of samples of different ages. However, the reliability of the PLS analysis is totally dependent on reliability of the objective function for classification (sensory flavor scores). This is a characteristic property of supervised learning methods of multivariate analysis (Aishima and Nakai, 1991). The PLS has excellent regression capacity which was claimed to be superior to that of principal component regression (Martens and Martens, 1986). However, PLS would have the inherent deficiency in finding relationships with the original analytical data because of hidden functions (Martens and Martens, 1986). This is sometimes critical for studying the chemical mechanisms of reactions, especially for new or unknown mechanisms.

To utilize the information from chemical analysis without relying on information known *a priori* on the sample grouping (unsupervised learning cases), principal component similarity (PCS) analysis was proposed by Vodovotz et al. (1993). This method may be useful in analyzing samples prepared by a new process, e.g., fast-ripening, in comparison to samples prepared by conventional Cheddar cheese-making.

The objective of our work was to apply PCS to HPLC data of typical Cheddar cheese samples and use PCS in classifying samples based on age. Special emphasis was placed on an autosampling system in conjunction with a non-silica column (Li-Chan et al., 1992) to provide good repeatability for HPLC profiles with a large number of cheese samples.

Authors Furtula and Nakai are with the Dept. of Food Science, The Univ. of British Columbia, 6650 N.W. Marine Drive, Vancouver, BC, Canada V6T 1Z4. Authors Amantea and Laleye are with Ault Foods Ltd., Research & Development, 75 Bathurst St., London, ON, Canada N6B 1N8.

MATERIALS & METHODS

Cheese samples

Commercial Cheddar cheese samples of different ages (mild, medium, sharp and extra-sharp aged for 3, 6, 9, and 12 mo, respectively) were supplied by Ault Foods Ltd. (Winchester, ON). Three batches of cheese samples per each age of the 4 age types were selected from many batches analyzed. The third batch was delayed almost a month in delivery from London, ON, to Vancouver, BC, due to a disturbance in the mailing system. That batch was exposed to unknown ambient temperatures so long that ripening was accelerated in comparison to the other two regularly aged batches.

Sample preparation

The water-soluble fraction was extracted from cheese samples according to the method of Li-Chan et al. (1992) with slight modifications. The MeOH/H_2O layer after initial extraction was concentrated by rotary evaporation, diluted with deionized water, filtered through a 0.45-μm Millipore Millex-GV_{13} disposable filter, and then freeze-dried. Prior to HPLC, samples were freshly made from the lyophilized water extract by dissolving 0.01g of powder in 2 mL of mobile phase containing 25 μg of glycyltyrosine (Sigma Chemical Co., St. Louis, MO) as an internal standard.

HPLC

The HPLC system consisted of a Hewlett Packard 1050 series pump and autosampler, a Shimadzu SPD-EAV UV detector (220 nm) C-R3A integrator, RS-232C transmitter interface, and a PC computer with Shimadzu software. A C-18 RP Asahipak ODP 50 column (150 × 6 mm) and its guard column (Asahi Chemical Industry, Tokyo) were purchased from Keystone Science Inc., Bellefonte, PA. Samples (5 μL) were automatically injected onto the HPLC column and eluted using water-acetonitrile (AcN) gradient 0–30.6% with 22.7 mM phosphoric acid added as an ion-pair maker. The column was kept at 30°C and chromatograms were recorded for 83 min. Elution conditions were as reported by Li-Chan et al. (1992). Each sample was analyzed three times and average values were used for statistical computation.

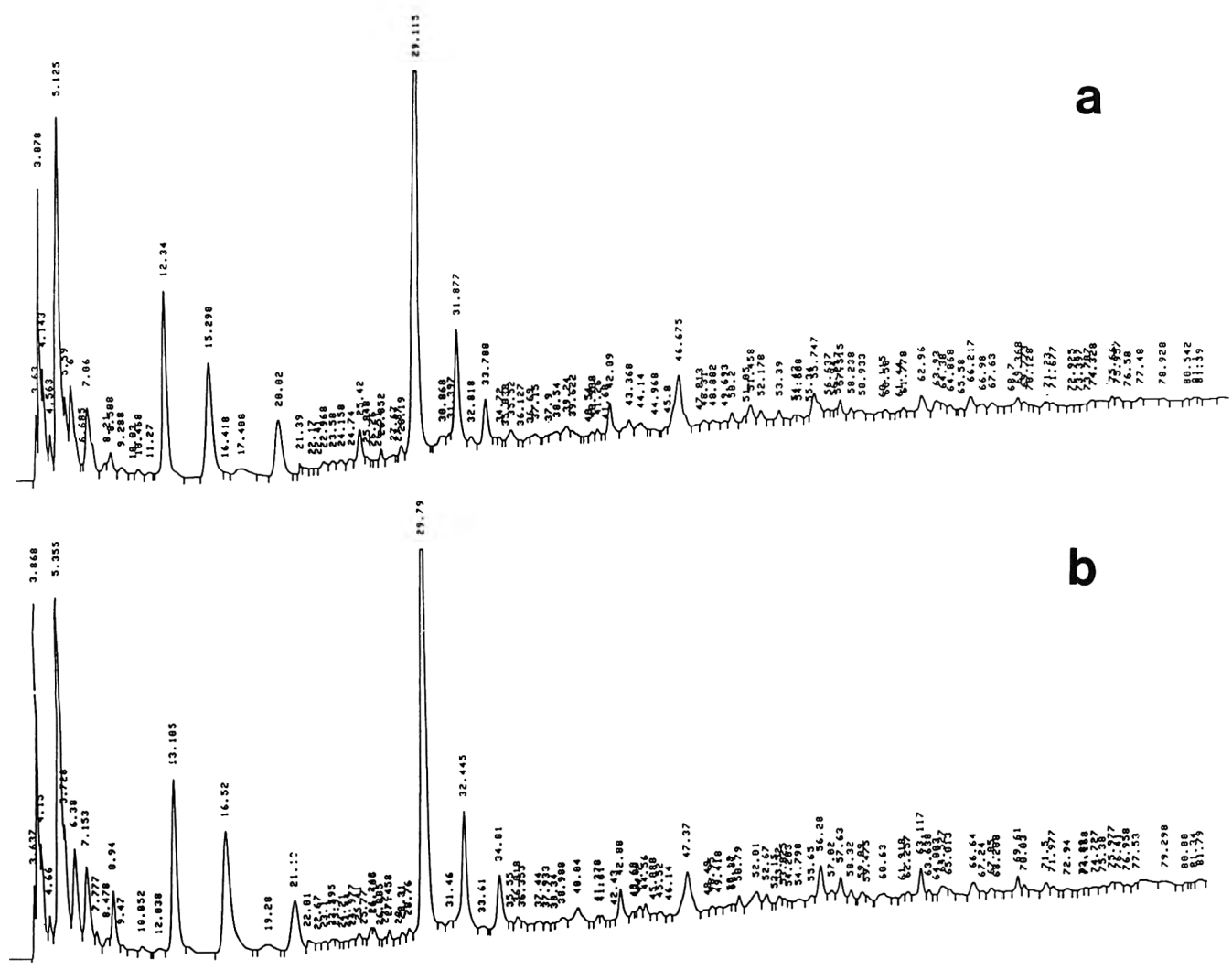

Fig. 1—HPLC chromatograms of water-soluble fraction from two batches of mild Cheddar cheese separated with different ODP-50 columns: (a) Injection #100; (b) Injection #16. Digit above each peak is the elution time in min.

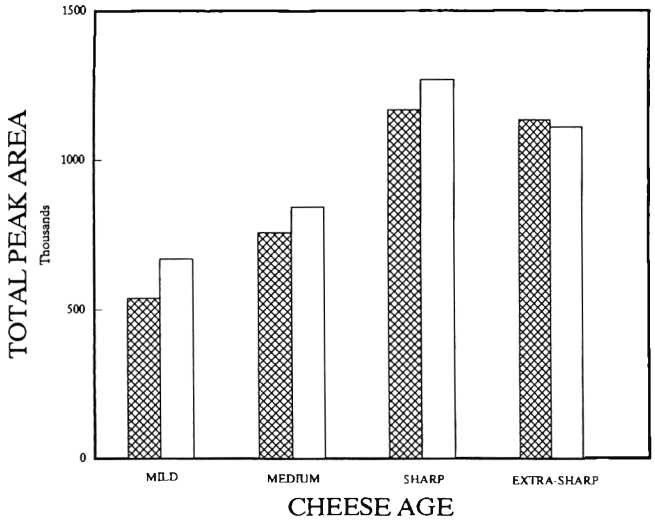

Fig. 2—Changes in the total peak area, proportional to total water-soluble components extracted from first (shaded) and second (open) batches of cheese samples.

Statistical analysis

The procedure of PCS computation was reported (Vodovotz et al., 1993). Matching of chromatography peaks was by visual comparison. A program was written to apply principal component analysis using SAS Institute, Inc. (1985) to HPLC data. Each peak area was considered a "variable" for a given cheese sample which was expressed as "case." Variables with component loading > 0.8, chosen arbitrarily, were selected for further computation, thereby decreasing the total number of variables from 137 to 50. Subsequently, the pattern similarity computation was made on a PC computer using SYSTAT (Wilkinson, 1988).

RESULTS & DISCUSSION

HPLC data

The combination of octadecyl polymeric (ODP) column and the autosampling system chosen in this study provided excellent repeatability (Fig. 1). HPLC profiles of two different batches of mild cheese samples analyzed using different ODP-50 columns on different dates were quite similar. More than 100 runs could be performed per column with similar repeatability. Note that Fig. 1a and 1b represent later and earlier runs, respectively, within the life of different ODP columns. The coefficient of variation for peak areas rarely exceeded 15%. As cheese aged, the total peak area increased, but it slightly decreased from sharp to extra-sharp (Fig. 2). This has been uniformly observed in almost all other batches analyzed (Furtula et al., 1994). A possible explanation is that proteases were denatured during aging, thereby discontinuing proteolysis. Simultaneous insolubilization of peptides by aggregation probably occurred during freeze-drying, or by decrease in polarity due to changes in amino acid composition of peptides. This resulted in less available water-extractable compounds. Fifty

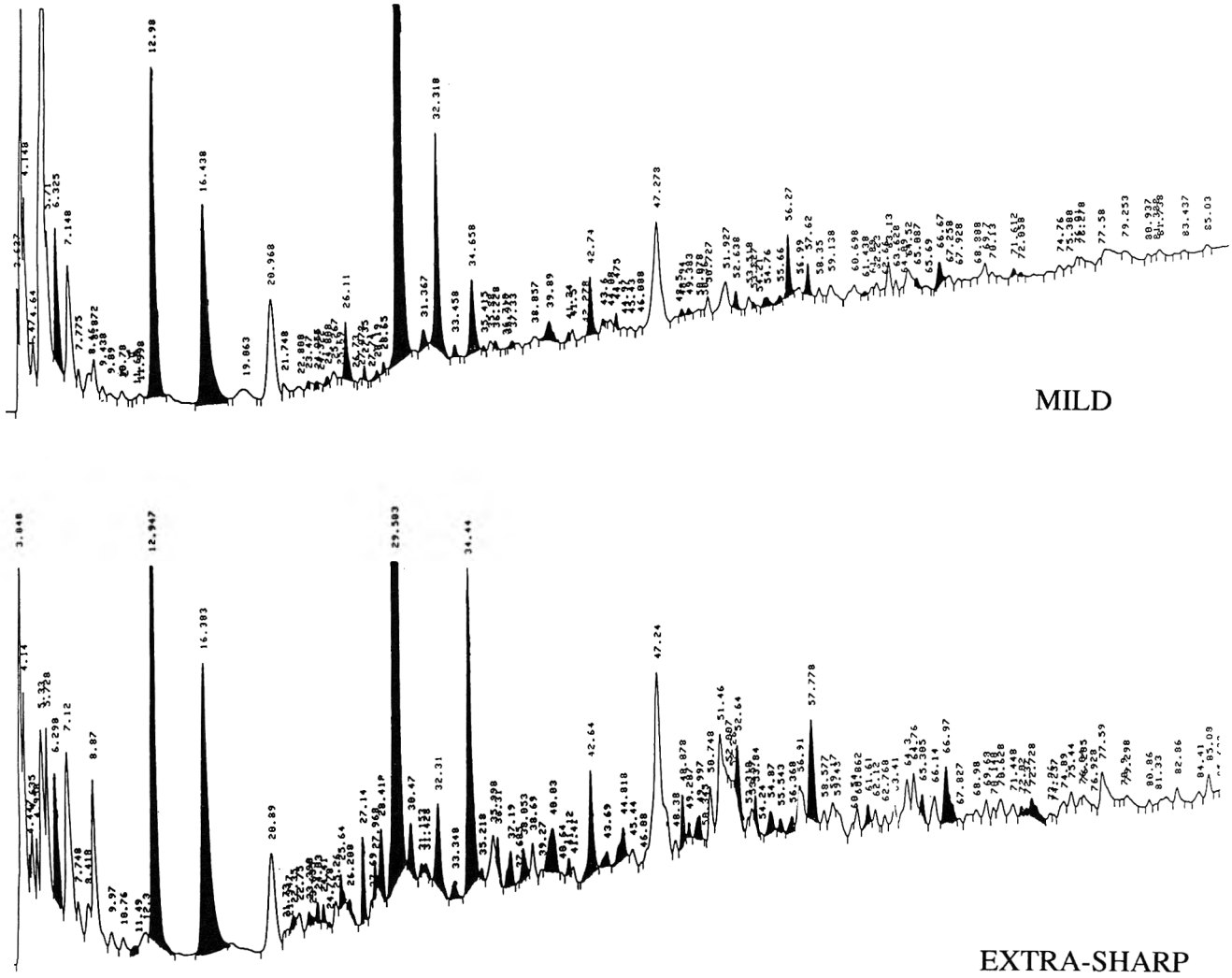

Fig. 3—The most important peaks (shaded) believed involved in Cheddar cheese ripening, with principal component loadings >0.8.

peaks were selected (Fig. 3) and are representative of changes during aging which should be useful for classifying samples based on ripening effects.

Statistical analysis

To assess the aging effect, the area percentage of the 50 selected peaks was calculated compared with corresponding peaks of extra-sharp cheese in the first batch (Table 1). Blanks in the table represent no detectable peak. The majority of peaks showed increases in area during aging (peaks 6, 7, 19, 26, 27, 46, 48 and 50) or increases during the first 9 mos and then decreases (peaks 8, 14, 16, 17, 25, 29, 30, 31, 32, 35, 36, 37, 40, 41, 43, 45 and 59). Other peak areas did not change much or even decreased (peak 44), suggesting degradation of compounds. In the abused sample (third batch), peak areas showed a trend of faster increase (peaks 14, 23, 26, 31 and 32) or of producing new peaks earlier (peaks 21, 22 and 25) especially in mild and medium cheeses. Sometimes, no peak or only small peaks appeared in batch 3 when there were definite peaks in batches 1 and 2 (peaks 4, 24, 36 and 37). In sharp and extra-sharp cheeses, decreases in many peaks were observed in the peak area of abused cheese, suggesting protein hydrolysis considerably different from normal aging.

Principal component analysis (PCA) is customarily used for dimension reduction when the dimension of original data (X_p) is extremely large by replacing with principal components (Z_p) that are mutually uncorrelated based on the relation $\mathbf{Z} = \mathbf{Xe}$, where $\mathbf{e}$ is the matrix of eigenvectors. By using first several Z_p, customarily with eigenvalues > 1.0, important information (variation) in the original data can be retained (Manly, 1986).

PCA of HPLC data from the first cheese batch yielded 11 principal component (PC) scores which accounted for 96.5% of total variation, (first three PC scores illustrated in Fig. 4). Factors 1–3 were used as axes to represent Z_1 to Z_3. When 137 variables of five batches of normal cheese samples (without including the abnormal third batch) were used, the shift from A through D due to aging was smooth. However, when 50 variables, selected as described, were used, D fell between B and C.

Six principal components had eigenvalues (λ) >1.0 (component loadings (L) shown in Table 2). Loading is a coefficient in a relation $\mathbf{L} = e\Lambda^{1/2}$, where Λ is the matrix of eigenvalues. PC1 represents the majority of variables in the table, while PC's from PC2 to PC5 represent characteristic peaks (marked with *) in contribution to PC scores, and PC6 was less characteristic, in representing any specific HPLC peaks.

The result of PCS analysis (Fig. 5) was a plot of slope (s) vs coefficient of determination (r^2) derived from linear regression of data in Fig. 6. Therefore, as the sample approached the reference standard (PCS reference: Ext.Shp-1), the deviation decreased thus approaching the diagonal line. Then both the slope and r^2 became close to 1.0. Figure 5 demonstrates the aging changes from mild to medium, then sharp and finally extra-sharp. Similar aging patterns were observed for the two regularly aged batches (first and second batches). Changes due to aging were observed from the data points for mild ($s = 0.86$, $r^2 = 0.87$) to medium with a slight increase in both s

Table 1—Area percentage for peaks of cheese samples calculated vs corresponding peaks of the extra-sharp Cheddar cheese in the first batch

			Mild			Medium			Sharp			Extra-sharp		
Peak	R_t	SD	1	2	3[a]	1	2	3[a]	1	2	3[a]	1	2	3[a]
1	3.62	0.12												
2	4.41	0.10						80	116	118	74	100	92	88
3	6.21	0.16	100[b]	100	28	45	40	102	94	102	91	100	104	93
4	11.11	0.36						53	88	94		100	85	
5	11.45	0.32						101	121	128	147	100	98	156
6	12.41	0.29	65	68	68	58	74	115	82	93	90	100	92	88
7	15.59	0.34	69	85	88	83	98	100	89	93	127	100	106	132
8	21.67	0.23	10	15	50	28	19	53	213	182	38	100	73	36
9	22.96	0.29	30	47	36	19	32	100	98	90	145	100	85	112
10	23.49	0.22	30	40	30	32	27	66	185	182	105	100	98	35
11	23.93	0.29						54	161	167	90	100	88	46
12	24.13	0.22			172	83	89	106				100	81	96
13	25.29	0.25			19	46	55	53	97	95	38	100	91	42
14	25.59	0.29	175	99	75	102	81	233	227	286	169	100	123	94
15	26.07	0.28							82	82	44	100	82	
16	26.84	0.20	20	26	130	57	69	59	123	127	112	100	103	100
17	27.55	0.21	9	12	89	32	33	90	154	208	72	100	110	95
18	28.07	0.22	11	11	24	10	11	39	105	83	28	100	83	42
19	29.18	0.20	57	67	118	69	75	85	93	102	76	100	99	77
20	30.32	0.33					25	62	91	93	63	100	81	81
21	30.87	0.24			7		20	88	120	137	81	100	112	44
22	31.31	0.18						66	53	80	69	100	92	112
23	32.02	0.21	164	204	294	303	370	208	125	139	119	100	89	79
24	32.59	0.32							97	101		100	156	
25	33.35	0.25			130			192	192	200	200	100	122	200
26	33.83	0.30	19	21	31	27	35	68	69	81	81	100	103	102
27	34.56	0.23	28	14	65	49	37	40	76	68		100	86	31
28	35.73	0.21	12	13	28			59	167	196	123	100	99	161
29	36.72	0.20	45	23	76	27	37	111	212	256	119	100	118	127
30	37.88	0.22						68	270	357	76	100	100	175
31	39.61	0.28	36	36	40	53	62	90	104	133	102	100	118	127
32	40.80	0.23	25	33	52	30	38	86	99	200	103	100	125	108
33	42.18	0.26	52	47	90	42	39	86	133	185	91	100	106	79
34	43.37	0.23	111	102	357	416	526	104	204	286	115	100	101	76
35	44.54	0.16	33	39	55	57	93	61	145	189	57	100	127	127
36	47.26	0.24				49	82		212	222		100	102	39
37	48.48	0.23	25	16	60	31	27	63	145	192	85	100	91	63
38	48.93	0.28	31	32	60	39	32	94			61	100	127	34
39	49.82	0.29	33	20	100			119	127	147	44	100	92	42
40	52.06	0.23	10	11	32	18	17	39	147	143	24	100	70	21
41	53.42	0.23	25	30	34	44	47	48	222	175	24	100	70	57
42	54.67	0.25			35	30	53	98	96	135	55	100	74	101
43	55.32	0.36	13	35	49	42	57	84	122	147	59	100	56	110
44	55.89	0.21	455	588	625	384	417	370	270	270	326	100	156	147
45	57.40	0.22	14	21	73	22	31	64	112	122	36	100	104	54
46	61.10	0.21			25	25	20	84	106	85		100	85	26
47	64.72	0.33	21	14	18			33			26	100	46	47
48	66.41	0.27	33	26	44	51	38	63	85	79	57	100	66	72
49	72.06	0.24	10	15	43	49	34	43	111	100	40	100	88	35
50	72.92	0.31						43	67	82	37	100	115	72

[a] Sample batches.

[b] Mean of triplicate injections. Coefficients of variation of all peaks were less than 15%.

and r^2, then to sharp with large increases in s to 1.1–1.15 and r^2 to 0.94. Finally the slope decreased to 1.0 along with shift of r^2 towards 1.0 for extra-sharp.

The abused batch (third batch) did not follow the regular route and formed a smaller circle near the medium zone of normal cheeses with s of 0.88 to 0.96 and r^2 of 0.82 to 0.91. Probably the age difference was minimized by a different mechanism of degradation of proteins due to unusual storage conditions at temperatures higher than normal for cheese curing. Compared to smooth shifts from mild towards extra-sharp (Fig. 5), PCA plots (Fig. 4b) when the same number of peaks were used showed a twisted shift from sharp (C) to extra-sharp (D). Similar results showing PCS was superior to PCA were reported for mango samples from 2 cultivars ripened under different conditions (Vodovotz et al., 1993).

In PC score plots of sample vs PCS reference ("extra-sharp") (Fig. 6), the diagonal line is the line of equal PC scores. Therefore, vertical deviations of sample PC plots from the diagonal line indicated the departure of sample PC scores from reference PC scores. Compared to "extra-sharp", "mild" (Fig. 6a) had departures of −PC1 and +PC3; "medium" (Fig. 6b) had −PC1, +PC2 and −PC5; and "sharp" (Fig. 6c) had +PC2 and +PC3. They were deviations characteristic of each sample from the PCS reference. In the case of regular aging (batches 1 and 2), mild and medium did not change the slope (Table 3) which increased considerably in sharp cheeses. Changes were from negative to zero for PC1 and zero to positive for PC2, while not much change was observed between sharp and extra-sharp.

Contrary to similar deviations between the two regularly aged batches 1 and 2, the abused samples (batch 3) were quite different in deviations of PC scores. This could be traced to the effective HPLC peaks by referring to component loadings (Table 2). The third batch demonstrated a characteristic change in PC2 in mild, similar to medium of batches 1 and 2. However, in medium, sharp and extra-sharp no detectable changes in PC1 and PC2, (most influential to the slope) were observed. This would explain why abused cases did not follow the pattern to move toward sharp cases of regular aging. That is, at higher temperatures, aging was promoted during the first 3 mos, thereafter not many major changes occurred, probably due to denaturation of enzymes.

This difference of the abused batch suggests an effective use of PCS for the detection of unusual samples among normal samples, which may be useful, in routine quality control. By changing the PCS reference, a different detection efficiency could be expected. When cheese samples are prepared to make Cheddar cheese using fast-ripening processes, the data points

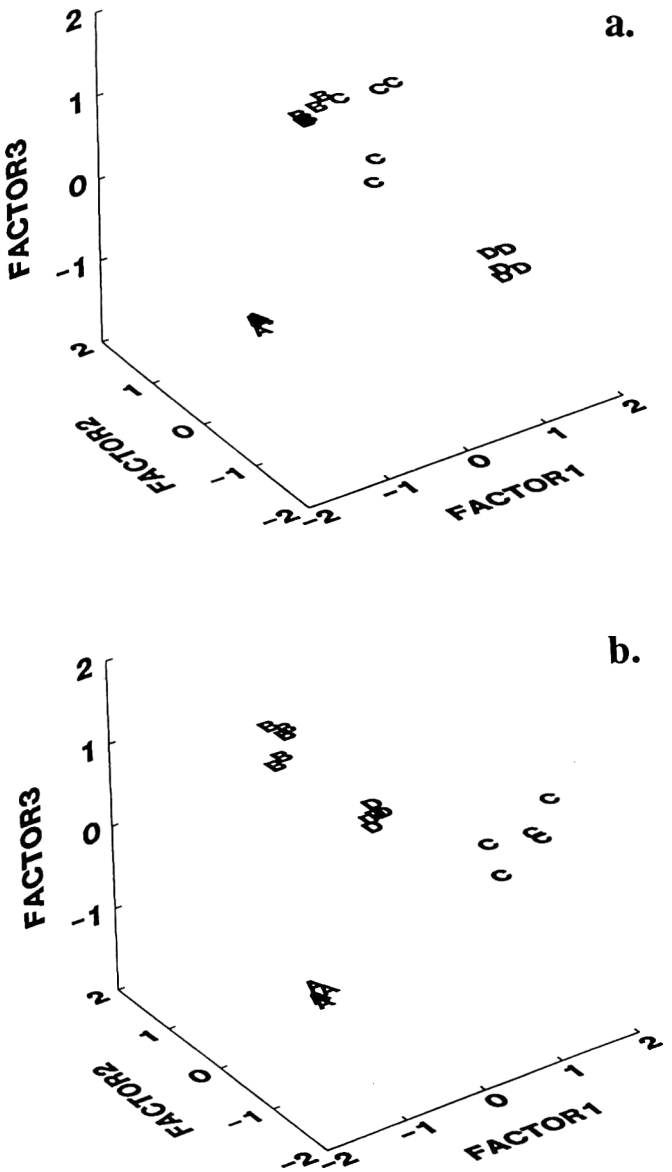

Fig. 4—PC (factor) scores for mild (A), medium (B), sharp (C) and extra-sharp (D) cheeses, when different numbers of variables were used for PCA: (a) All 137 variables; (b) 50 selected variables.

representing accelerated samples at different ages should follow the pathway of standard Cheddar cheese much faster. Deviation from the regular pathway should be interpreted as the protein degradation occurring in samples being different from that of typical Cheddar cheese. Therefore categorizing the sample cheeses into mild, medium, sharp or extra-sharp may be difficult.

Comparison of PCS with other multivariate analysis techniques

PCS, an unsupervised classification method, should be useful for investigating new processes for which limited data of unusual cases or limited information of the product is available. For classification especially for supervised cases, PLS and artificial neural networks may be the most advanced, accurate methods as reported by Banks et al. (1992) and Arteaga and Nakai (1993), respectively. However, classification for supervised cases requires reliable sample grouping known *a priori*. If the sample grouping relies on non-instrumental methods, it immediately affects the accuracy of classification.

Table 2—Component loadings, result of PCA for all three cheese batches

	PC_1	PC_2	PC_3	PC_4	PC_5	PC_6
VAR2	0.9685	−0.1864	0.1249	−0.0118	−0.0312	0.0642
VAR11	0.9554	0.1329	0.1804	−0.0862	−0.0004	−0.0725
VAR20	0.9466	−0.2429	0.0269	0.0306	−0.1418	0.0785
VAR18	0.9385	0.0496	−0.2185	−0.1945	−0.0050	−0.0393
VAR21	0.9367	0.0067	0.1275	−0.0439	0.0674	0.0272
VAR37	0.9314	0.2789	0.1237	0.0571	0.0928	−0.0897
VAR17	0.9254	0.2336	0.0482	0.2058	0.1566	−0.0219
VAR45	0.9170	0.1791	−0.2677	0.0191	0.1947	−0.0430
VAR29	0.9090	0.2219	0.2919	0.1169	0.1090	−0.0367
VAR32	0.9076	−0.0344	0.1735	0.1459	0.0225	−0.0759
VAR48	0.9038	−0.1112	−0.1896	0.0498	−0.1392	0.1407
VAR50	0.8998	−0.3075	−0.1875	−0.1248	−0.1291	−0.0628
VAR28	0.8998	−0.0724	0.3342	0.1535	−0.0589	−0.1582
VAR33	0.8996	0.2523	0.1295	0.0570	0.2452	−0.1046
VAR31	0.8980	−0.2438	0.1460	0.1891	−0.2235	0.0026
VAR42	0.8932	−0.1000	−0.0990	0.2208	−0.0938	0.3102
VAR49	0.8925	0.2580	−0.3035	−0.0926	−0.0561	−0.0660
VAR49	0.8860	0.4116	−0.2651	−0.1417	0.0496	0.0051
VAR4	0.8792	0.1245	0.2213	−0.3249	0.0615	0.1490
VAR10	0.8774	0.3085	−0.3112	−0.1566	0.0590	−0.1160
VAR13	0.8753	0.1394	0.1654	−0.0011	−0.2561	0.1178
VAR43	0.8698	0.1425	0.3322	0.2805	−0.0823	0.2184
VAR30	0.8637	0.2397	−0.2071	0.0998	−0.0772	0.0016
VAR15	0.8601	0.0637	0.0506	−0.2897	−0.0731	−0.2372
VAR8	0.8536	0.4753	−0.3370	−0.0691	0.0669	−0.0111
VAR46	0.8393	0.1343	−0.0739	−0.1616	0.0741	0.3076
VAR26	0.8329	−0.5049*	0.3215	0.1265	−0.1530	−0.0519
VAR5	0.8271	−0.3997	0.0267	0.1769	−0.0757	−0.0228
VAR22	0.8187	−0.5368*	−0.0729	0.0861	−0.1326	0.0291
VAR39	0.8117	0.1496	0.0724	−0.0257	0.5344*	0.1284
VAR41	0.7982	0.5175*	0.0724	−0.1002	−0.0825	0.0373
VAR36	0.7692	0.5515*	−0.0322	−0.0692	−0.2748	−0.0264
VAR25	0.7628	−0.2344	0.2813	−0.3899	0.3003	−0.0128
VAR44	−0.7480	0.3723	0.1280	0.0595	0.5058*	−0.0548
VAR16	0.7209	0.0975	−0.1574	0.5297*	0.1497	−0.3515
VAR9	0.7066	−0.5376*	0.3395	0.1364	0.0615	−0.0577
VAR6	0.6988	−0.4788	0.0360	0.0459	0.2052	0.4161
VAR23	−0.6813	0.4824	−0.2475	0.3378	0.0254	0.2498
VAR24	0.6684	−0.0065	−0.4154	−0.3585	−0.0867	−0.2932
VAR27	0.6111	0.2518	−0.7164*	−0.1350	0.0290	0.0371
VAR19	0.5994	0.1501	−0.5049*	0.3236	0.4690	−0.1616
VAR3	0.5232	−0.3977	0.3963	−0.6112*	0.0351	0.0885
VAR14	0.5102	0.3046	0.5515*	−0.2632	0.2157	0.2928
VAR47	0.3009	−0.7164*	−0.4459	−0.1854	0.0130	0.0718
VAR34	−0.3034	0.6915*	−0.2754	−0.4794	−0.2217	0.0714
VAR7	0.3596	−0.6742*	0.1656	0.5329*	−0.2000	−0.1541
VAR38	0.0768	−0.6655*	−0.6063*	−0.1282	0.2025	0.0526
VAR35	0.2718	0.5170*	−0.1425	0.1903	−0.7011*	0.1136
VAR1	−0.2312	0.1465	0.9142*	0.1206	0.0446	0.1879
VAR12	−0.1452	−0.2064	−0.7215*	0.5377*	0.1875	0.2246

*Loadings >0.5 for PC_2–PC_6.

Table 3—Summary of PCS analysis. Corrected PC scores responsible for differences from PCS reference (extra-sharp Cheddar cheese of batch 1)

Sample*	PC_1	PC_2	PC_3	PC_4	PC_5	PC_6
Mild-1	−		+			
Mild-2	−		+			
Mild-3	−	+		+	+	−
Medium-1	−	+				
Medium-2	−	+		+		
Medium-3			+		+	−
Sharp-1		+	+			
Sharp-2		+	+			
Sharp-3			+	+		−
Extra-sharp-2						−
Extra-sharp-3			+	+		

* Digits attached to the age of cheeses are batch numbers.
[b] +, − indicate that PC score of sample is larger and smaller, respectively, than that of reference sample (extra-sharp Cheddar cheese of batch 1).

For unsupervised classification purposes, PCA and cluster analysis (CA) may be the most useful. PCA can be considered a method for dimension reduction, with a limited classification capability compared to PCS. However, PCS is an extended version of PCA, representing a larger number of PC scores than PCA for graphic illustration of computed outputs. Therefore, the advantage of PCS diminishes as the number of PC scores for computation decreases.

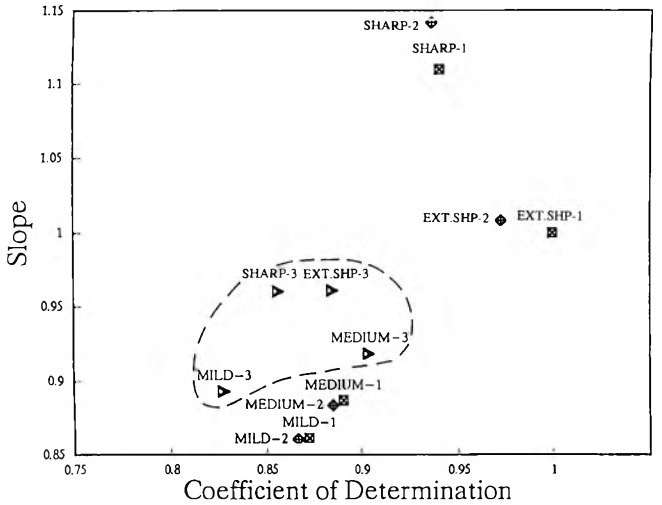

Fig. 5—PCS scattergram of three batches of Cheddar cheese samples.

Cluster analysis, in principle, has inadequate classification capability as it is qualitative rather than quantitative. PCS, however, may be useful for classification of unsupervised cases by utilizing the original data to a greater extent. PCS may also be useful for preliminary data processing before conducting more detailed analyses using discriminant analysis or artificial neural networks. These require a large body of analytical data for many different cases in order to obtain the highest classification accuracy.

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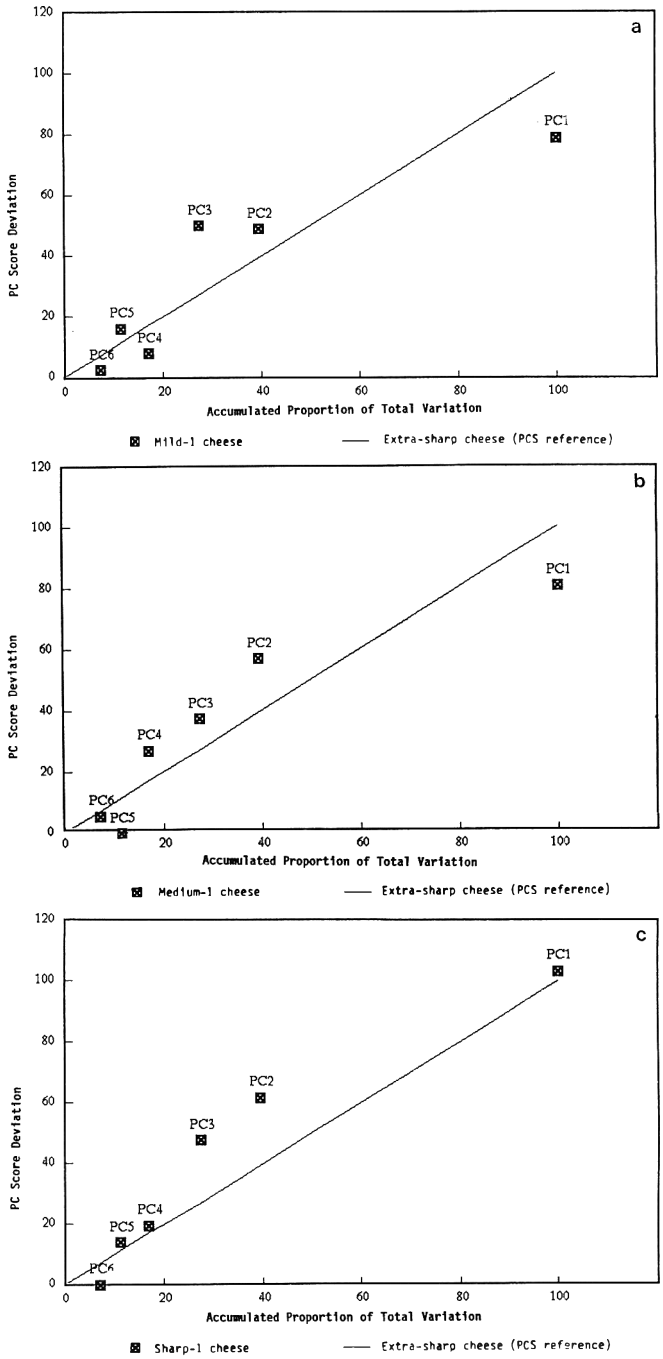

Fig. 6—PC score deviation (samples of different ages from that of extra-sharp) as related to accumulated proportion of total variation that each PC score can account for. (a) mild; (b) medium; and (c) sharp.

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Analysis of Textural Changes in Hard Cheese during Mastication by Progressive Profiling

FRANCES R. JACK, JOHN R. PIGGOTT, and ALISTAIR PATERSON

ABSTRACT

Progressive profiling allowed study of changes in textural attributes of hard cheese during mastication, each chew stroke having an effect. Perceptions of attributes were scored individually at each chew stroke from the start of chewing to swallowing, producing a changing profile of texture. Differences among cheeses in terms of coarseness/crumbliness vs smoothness/creaminess and in rubberiness vs graininess were determined. The profile characteristics changed in different ways and at different rates, depending on the sample. Limited correlations were found between progressive profiling, descriptive analysis and instrumental force-deformation data.

Key Words: force deformation, Instron, sensory, Cheddar cheese, progressive profiling

INTRODUCTION

TEXTURE IS HIGHLY IMPORTANT in Cheddar cheese. Untrained consumers can readily discriminate between Cheddars of different styles and maturities, solely in terms of textural characteristics (Jack et al., 1993b). Texture in immature cheeses also is important in prediction of mature Cheddar character by expert graders (Bodyfelt, 1988). A good understanding of the sensory textural properties of such cheese is necessary.

In the Texture Profile Method of Brandt et al. (1963), rating scales were used to score major textural attributes of products, standard samples being provided for each point on the scale. Although that technique is used widely in texture research (Skinner, 1988), vocabulary development and extensive panel training make it costly. Descriptive analysis (Stone et al., 1974), another popular sensory approach, involves scoring samples in terms of a fixed vocabulary of descriptors, also using a trained panel. Piggott and Mowat (1991), using descriptive analysis to study sensory properties of Cheddar cheese, concluded that texture and mouthfeel were important discriminatory factors.

Free Choice Profiling (FCP) (Williams and Langron, 1984), where subjects generate their own unique vocabularies, has also been used successfully for study of cheese texture (Jack et al., 1993b; McEwan et al., 1989). Since FCP requires no training it is relatively low cost and can be used to obtain data from untrained consumers. Time-intensity methodology studies changes in sensory characteristics throughout a time scale. Developments in computer technology have assisted collection and analysis of such data (Guinard et al., 1985). Time-intensity can give additional information on individual attributes not available through the other approaches (Lundahl, 1992).

Szczesniak (1963) defined texture as "the composite of structural elements of food and the manner in which they register with physiological senses". Mastication deforms and breaks down a foodstuff, and sensory feed-back from the actions of mastication supply essential information for oral texture perception (Christensen, 1984). Mastication is of obvious importance in relation to texture. In simple masticatory studies (Harrington and Pearson, 1962; Gacula et al., 1971), chew number has been related to tendernessin meat. Other studies have involved the use of electromyography (EMG) to examine muscle activity during chewing (Pierson and Le Magnen, 1970; Boyer and Kilcast, 1986; Eves and Kilcast, 1988; Eves et al., 1988). Jack et al. (1993c) reported use of this technique to study Cheddar cheese texture, different EMG traces being related to sensory and instrumental measures of texture.

Cheddar cheese changes markedly during mastication. Our objective was to develop appropriate sensory methodology to account for mastication allowing examination of textural transformations. Relationships with descriptive analysis and force-deformation properties were also considered.

Authors Jack, Piggott, and Paterson are affiliated with the Centre for Food Quality, Dept. of Bioscience & Biotechnology, Univ. of Strathclyde, 131 Albion St., Glasgow, G1 1SD, Scotland, U.K.

MATERIALS & METHODS

Cheese samples

Cheddar cheeses of a range of maturities, styles and qualities, obtained from supermarkets and specialist commercial outlets, were selected to represent a wide range of textural characteristics; half fat Cheddar style cheese (**HAF**), mild Scottish Cheddar (**MDS**), vintage Canadian Cheddar (**VIN**), mature Scottish Cheddar (**MTS**), Tobermory Cheddar (**TOB**), vegetarian Cheddar (**VEG**), mild English Cheddar (**MDE**), mature English Cheddar (**MTE**) and vegetarian farmhouse Cheddar (**VFM**). The vegetarian Cheddars were produced using a microbial generated coagulant, rather than rennet. Cheeses were stored at 4°C, wrapped in cheese-cloth moistened with a 5% solution of food-grade sodium chloride to minimize moisture loss.

Progressive profiling

Computerized time-intensity, using the PSA-System v1.64 (OP&P Inc., P.O. Box 14167, 3508 SG Utrecht, The Netherlands), was initially considered to study the effect of mastication on cheese texture. Since the most important changes in texture occur on each chew stroke, it was important to record their occurrence. The time-intensity module of the PSA-system provides an event marker facility which, as well as recording the continuously varying attribute magnitude could be used for this purpose. However in preliminary trials, assessors were unable to carry out both procedures simultaneously. Progressive profiling was developed to allow easy profiling of textural characteristics at defined stages during mastication.

Progressive profiling employed a fixed vocabulary of textural descriptors, developed from a Free Choice Profiling study of Cheddar cheese texture (Jack et al., 1993b); dry (**Dr**), grainy (**Gr**), crumbly (**Cru**), hard (**Ha**), coarse (**Co**), creamy (**Cre**), smooth (Sm), sticky (**St**), rubbery (**Ru**) and pasty (**Pa**). The term "pasty" was later removed, assessors expressing difficulty in its definition. Attribute intensity was scored on category scales of 0-5, 0 being defined as "not present" and 5 as "extremely." One cheese was assessed per session, scoring for each textural attribute individually. Printed sheets were provided with forty sets of category scales, assessors working thorough this series of scales recording one score on each chew stroke, from the onset of chewing through to swallowing. The assessors chewed the samples in as natural a manner as possible, and recorded the time from the beginning of mastication up to swallowing using a stopwatch. The number of chews, determined from the number of scales used on the score sheets, plus chew times were used to calculate chew rate.

Samples of uniform dimension, 1 cm cubes, were used, as sample size could affect both textural perception (Cardello and Segars, 1989) and rate of breakdown. Samples were cut on the day of analysis, at least 3 cm from the surface of the cheese to overcome surface drying, and stored in polythene bags at 4°C. Prior to assessment, the cheese was allowed 20 min to equilibrate at room temperature, (≈ 20°C).

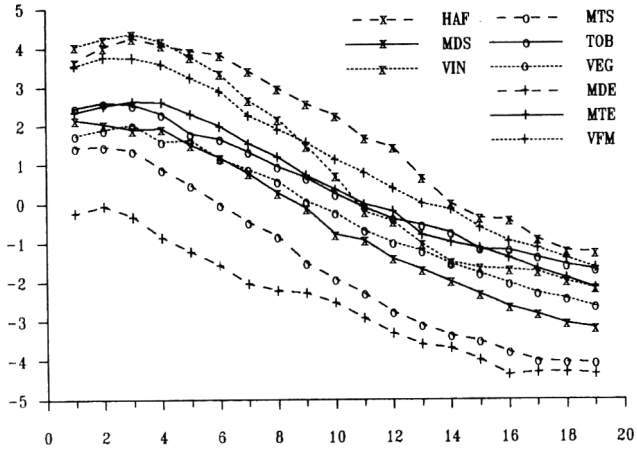

Fig. 1—Sample scores on Factor 1 (horizontal) and 2 (vertical) after PCA of panel means for nine Cheddar cheeses (half fat Cheddar style cheese **HAF**, mild Scottish Cheddar **MDS**, vintage Canadian Cheddar **VIN**, mature Scottish Cheddar **MTS**, Tobermory Cheddar **TOB**, vegetarian Cheddar **VEG**, mild English Cheddar **MDE**, mature English Cheddar **MTE**, vegetarian farmhouse Cheddar **VFM**).

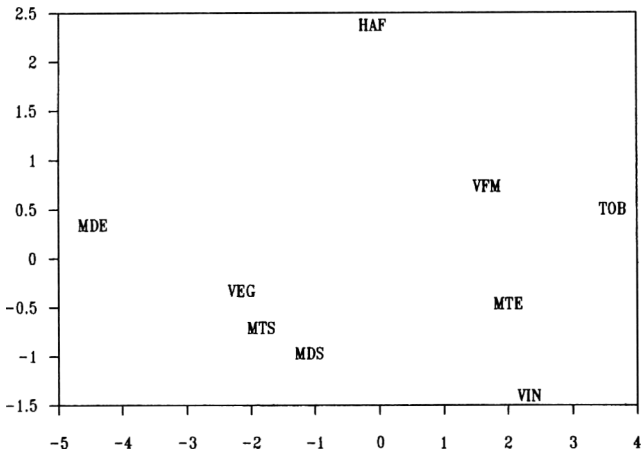

Fig. 2—Sample scores on Factor 1 after PCA of panel means for the nineteen stages of chewing in progressive profiling of nine Cheddar cheeses (half fat Cheddar style cheese **HAF**, mild Scottish Cheddar **MDS**, vintage Canadian Cheddar **VIN**, mature Scottish Cheddar **MTS**, Tobermory Cheddar **TOB**, vegetarian Cheddar **VEG**, mild English Cheddar **MDE**, mature English Cheddar **MTE**, vegetarian farmhouse Cheddar **VFM**).

Table 1—Mean force-deformation parameters for nine Cheddar cheeses

Sample	Peak height	Break point	Between curves	A1	A2	A2/A1
HAF	35.3	16.3	20.7	646	93	0.15
MDS	36.7	15.3	21.7	789	95	0.12
VIN	56.0	7.3	22.7	1152	82	0.07
MTS	36.7	15.7	23.3	820	101	0.12
TOB	37.0	10.7	20.3	751	75	0.10
VEG	38.7	12.3	23.7	789	92	0.12
MDE	23.3	20.7	20.7	432	78	0.18
MTE	41.0	13.0	23.7	742	84	0.11
VFM	31.7	11.3	24.0	581	71	0.12
s.d.[a]	4.8	1.0	1.0	64	13	0.01
p value[b]	<0.001	<0.001	<0.001	<0.001	<0.141	<0.001

[a] s.d. – pooled standard deviation.
[b] p value – probability of samples being the same.

Samples were presented in 150mL capacity clear glass tumblers covered by watch-glasses and assessed in individual booths under white fluorescent light, with water and dry crackers provided to cleanse the palate.

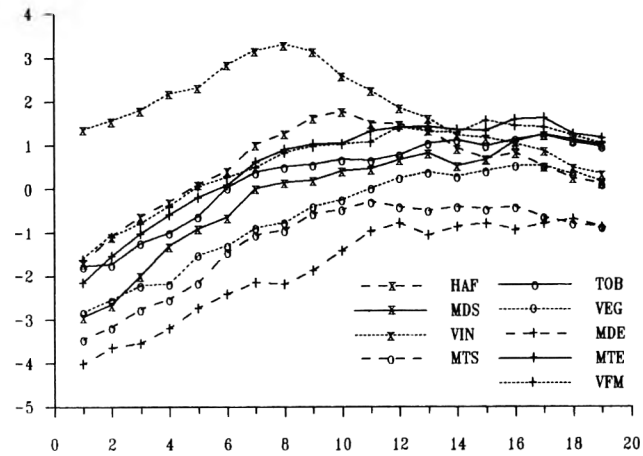

Fig. 3—Sample scores on Factor 2 after PCA of panel means for the nineteen stages of chewing in progressive profiling of nine Cheddar cheeses (half fat Cheddar style cheese **HAF**, mild Scottish Cheddar **MDS**, vintage Canadian Cheddar **VIN**, mature Scottish Cheddar **MTS**, Tobermory Cheddar **TOB**, vegetarian Cheddar **VEG**, mild English Cheddar **MDE**, mature English Cheddar **MTE**, vegetarian farmhouse Cheddar **VFM**).

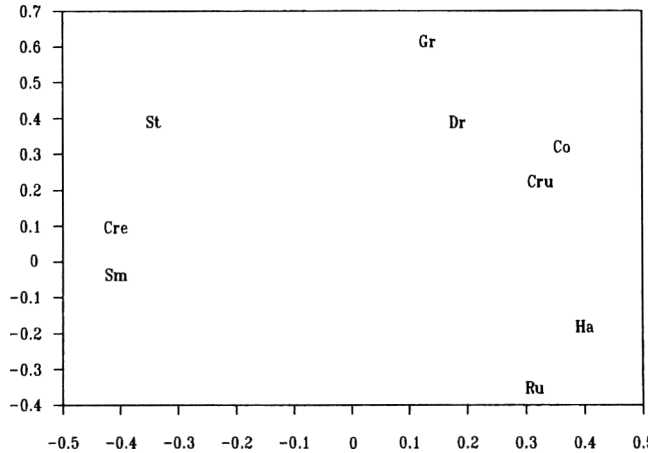

Fig. 4—Descriptor loadings on Factor 1 (horizontal) and 2 (vertical) after PCA of panel means for the nineteen stages of chewing in progressive profiling of nine Cheddar cheeses (dry **Dr**, grainy **Gr**, crumbly **Cru**, hard **Ha**, coarse **Co**, creamy **Cre**, smooth **Sm**, sticky **St**, rubbery **Ru**).

Samples were assessed in duplicate, presentation order being balanced betweens essions (MacFie et al., 1989). Sessions were carried out on sequentiald ays. Seventeen assessors, all with previous sensory experience of Cheddar cheese texture assessment, took part in the progressive profiling, at least twelve attending each session. The first of three training sessions refamilarised assessors with vocabulary and introduced the progressive profiling method, while the second and third sessions allowed practice in scoring and time recording.

Descriptive analysis

Descriptive analysis (Stone et al., 1974) was carried out, using the vocabulary, scale, sample size and preparation used for progressive profiling. All nine samples were presented in a single session. Replicates were presented in a second session using a balanced order of presentation. Data were recorded and stored in the PSA-System.

Instrumental analysis of force-deformation characteristic

Force-deformation curves were measured, using the Instron Universal Testing Machine (Instron Corporation, Canton, MA 02021). Triplicate samples of all 9 cheeses were prepared (Jack et al., 1993a) and subjected to a double 60% compression at 20mm min^{-1}, using a cycling mode. A flat 45mm diameter probe, lubricated with vegetable oil

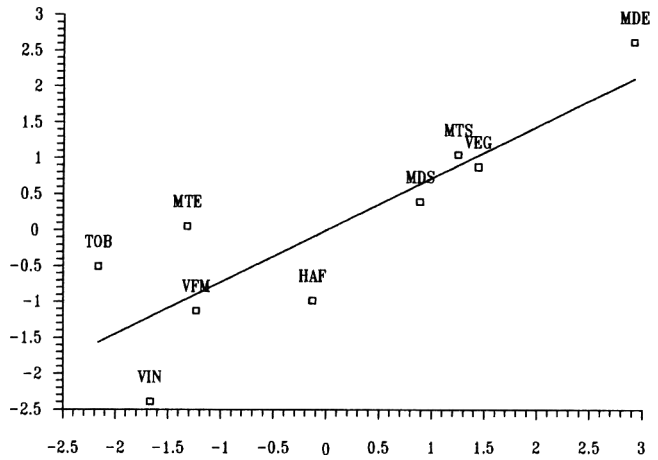

Fig. 5—Prediction of Factor 1 after PCA of Descriptive data (Y-axis) from panel means of chew stage 1 of progressive profiling of nine Cheddar cheeses against actual scores on this factor (X-axis) (half fat Cheddar style cheese **HAF**, mild Scottish Cheddar **MDS**, vintage Canadian Cheddar **VIN**, mature Scottish Cheddar **MTS**, Tobermory Cheddar **TOB**, vegetarian Cheddar **VEG**, mild English Cheddar **MDE**, mature English Cheddar **MTE**, vegetarian farmhouse Cheddar **VFM**).

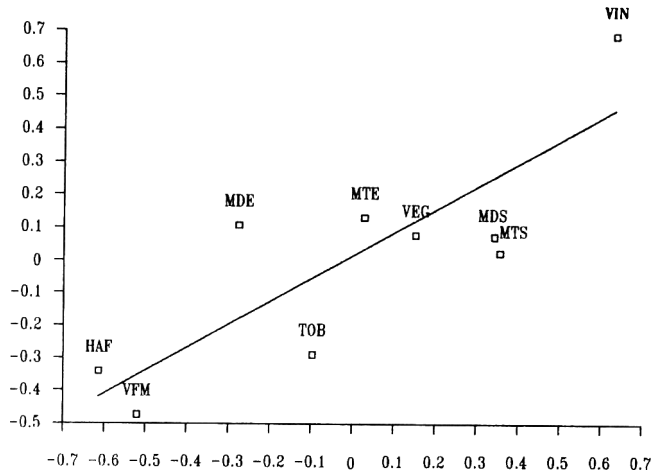

Fig. 6—Prediction of Factor 2 after PCA of Descriptive data (Y-axis) from panel means of chew stage 1 of progressive profiling of nine Cheddar cheeses against actual scores on this factor (X-axis) (half fat Cheddar style cheese **HAF**, mild Scottish Cheddar **MDS**, vintage Canadian Cheddar **VIN**, mature Scottish Cheddar **MTS**, Tobermory Cheddar **TOB**, vegetarian Cheddar **VEG**, mild English Cheddar **MDE**, mature English Cheddar **MTE**, vegetarian farmhouse Cheddar **VFM**).

Table 2—PLS correlation coefficients: Prediction of Factors 1 and 2 after PCA of Descriptive data from each of the nineteen chew stages in progressive profiling (one factor optimal for prediction in each case)

Chew stage	Prediction of factor 1	Prediction of factor 2
1	0.85	0.82
2	0.84	0.82
3	0.84	0.81
4	0.84	0.76
5	0.82	<0.70
6	0.83	<0.70
7	0.82	<0.70
8	0.82	<0.70
9	0.79	<0.70
10	0.76	<0.70
11	0.76	<0.70
12	0.75	<0.70
13	0.78	<0.70
14	0.80	<0.70
15	0.80	<0.70
16	0.83	<0.70
17	0.83	<0.70
18	0.84	<0.70
19	0.83	<0.70

Table 3—PLS correlation coefficients: Prediction of Factors 1 and 2 of each of the nineteen chew stages of progressive profiling using Instron force-deformation data (one factor optimal for prediction in each case)

Chew stage	Prediction of factor 1	Prediction of factor 2
1	<0.70	0.85
2	<0.70	0.80
3	<0.70	0.82
4	<0.70	0.79
5	<0.70	0.79
6	<0.70	0.82
7	<0.70	0.81
8	<0.70	0.80
9	<0.70	0.77
10	<0.70	0.72
11	<0.70	0.72
12	<0.70	<0.70
13	<0.70	<0.70
14	<0.70	<0.70
15	<0.70	<0.70
16	<0.70	<0.70
17	<0.70	<0.70
18	<0.70	<0.70
19	<0.70	<0.70

to minimize frictional effects, was used to effect the compression at a full scale load of 5 kg. Parameters related to a range of textural characteristics (Jack et al., 1993a) were measured from the resulting force-deformation curves; height of the first peak, distance to break point, distance between curves, areas under first (A1) and second (A2) curves and ratio of areas under curves (A2/A1).

Data analysis

Progressive profiling and descriptive data were analyzed by Principal Component Analysis (PCA) (Piggott and Sharman, 1986) using the Unscrambler II version 3.1 (CAMO A/S, Jarleveien 4, N-7041 Trondheim, Norway). PCA calculated linear combinations of variables (components) describing as much in the variance of the original data as possible. This allowed the original multi-dimensional matrix to be simplified without substantial loss of information, and so eased interpretation of complex data matrices. The results of PCA were graphically displayed as two sets of plots. In the first the correlations of variables with successive components were plotted, to aid interpretation of components; in the second the sample scores were plotted to show relationships between samples.

Partial Least Squares Regression, using the Unscrambler II version 3.1 (CAMO A/S, Jarleveien 4, N-7041 Trondheim, Norway), was used to examine relationships between progressive profiling data, descriptive analysis and force-deformation characteristics of the cheeses. In this technique a few orthogonal latent variables (factors) were sought to describe inter-relationships between two blocks of variables, with primarily those facors relevant to the prediction of the regressed matrix (Y) extracted from the regressor matrix (X). Thus PLS obtained pairs of directions, one from each set, which showed strong covariance with each other. Cross-validation was used to minimize problems with overfitting of data (Martens & Martens, 1986).

RESULTS & DISCUSSION

Descriptive analysis

Principal Component Analysis of panel means from the descriptive analysis gave two meaningful factors. The first, accounting for 72% of variance, related to smooth/creamy (negative), opposed to coarse/crumbly (positive). The second factors, relating to rubberiness (positive), accounted for 15% of the variance (Fig. 1).

From instrumental analysis of force-deformation characteristics, the measured parameters were recorded and compared (Table 1).

Progressive profiling

No differences occurred among samples in terms of rates at which they were masticated, $p<0.001$. The mean chew rate was 85 ± 19 chews per minute. Although attribute scoring probably slowed chewing, it still proceeded at a relatively natural rate. Chew number (mean = 19 chews) was assessor dependent ($p<0.001$). Two explanations can be advanced. The first is that samples were swallowed at different stages in their breakdown, a sample chewed only six times was swallowed when only partially broken down but a sample chewed 35 times, was more fully broken down. The alternative is that the assessors masticated the samples to "swallowable" consistency, and at that loosely defined point swallowed them. The question arose—Are samples swallowed at different stages of breakdown by different assessors or are all samples chewed to a predetermined texture then swallowed? To explore this, the panel means of scores at the first chew, at the sixth chew, which was the first point where any assessors swallowed, and at the last chew, in each individual data set, were examined using Principal Component Analysis. Two factors were produced, accounting for 50% and 24% of the variance, respectively. Factor 1 separated smooth, creamy, sticky samples (negative) from hard, coarse samples (positive), while Factor 2 separated rubbery samples (negative) from grainy, sticky, dry ones (positive). At the first chew differences occurred among the nine samples in term of both Factors 1 and 2 ($p<0.02$ and $p<0.04$) and this sample diversity was again observed at the sixth chew ($p<0.02$ and $p<0.04$). At the final chew clustering, no differences among samples ($p>0.07$ and $p>0.95$), were observed. Results supported the hypothesis that samples were being masticated until they reached a defined texture before swallowing.

Since samples were apparantly masticated to a textural endpoint, scaling of data sets to a uniform length could be justified. Data were scaled to the equivalent of 19 stages, the mean chew number, allowing study of textural changes through mastication. Panel means were taken at each of the nineteen chew stages, and subjected to PCA. Although PCA allowed examination of data from an unbalanced data set, a full data set may have yielded additional information on assessor differences.

PCA of the panel means at each chew stage produced two interpretable factors, accounting for 51 and 24% of variance. Samples scores on the two factors (Fig. 2 and 3) and descriptor loadings (Fig. 4) indicated Factor 1 separated smooth, creamy, sticky samples (negative) from coarse, hard samples (positive). Factor 2 separated rubbery samples (negative), from grainy samples (positive). Analysis of variance scores for Factor 1, showed differences among samples ($p<0.001$), and among chew stages ($p<0.001$), and also interaction between sample and chew stage ($p<0.05$). Similar observations were made for Factor 2 (all p values <0.001).

Early in the chew sequence, samples were well separated on Factor 1 (Fig. 2), possessing relatively coarse/crumbly characteristics in comparison to later stages. The mild English Cheddar was an outlier at this stage, with relatively smooth/creamy characteristics. As chewing progressed, all samples decreased in coarseness and crumbliness and became increasingly smooth and creamy. A slight increase in coarseness/crumbliness was observed after the first one/two stages of chewing, which may indicate textural characteristics such as coarseness and crumbliness required a certain amount of sample manipulation and breakdown in the mouth before assessors could make rational judgements. Changes in textural character progressed at similar rates for all cheeses, with exception of vintage Canadian Cheddar. In the early stages of chewing that sample was perceived relatively high in coarse/crumbly characteristics but it rapidly became smoother during chewing. Factor 2 showed that sample was an outlier, significantly less rubbery than the other samples. Such characteristics have

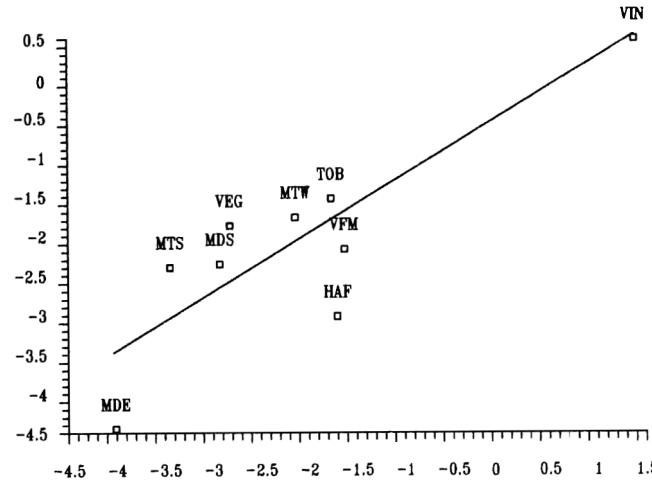

Fig. 7—Prediction of Factor 2 after PCA of panel means of chew stage 1 of progressive profiling (Y-axis) from force-deformation data for nine Cheddar cheese against actual scores on this factor (X-axis) (half fat Cheddar style cheese **HAF**, mild Scottish Cheddar **MDS**, vintage Canadian Cheddar **VIN**, mature Scottish Cheddar **MTS**, Tobermory Cheddar **TOB**, vegetarian Cheddar **VEG**, mild English Cheddar **MDE**, mature English Cheddar **MTE**, vegetarian farmhouse Cheddar **VFM**).

beenr elated to textural maturity (Jack et al., 1993b). A mature texture, with low cohesiveness, may explain the rapid breakdown.

Factor 2 (Fig. 3), showed that in the early stages of chewing the cheeses were perceived as having diverse textural characteristics of graininess/rubberiness, corresponding to the range of maturities. Over the first half of the chew cycle, rubberiness decreased and graininess increased. The vintage Canadian Cheddar and half fat Cheddar style cheese showed a shift back towards the "rubberiness" end of the factor, in the second half of the chew cycle. Granular structures could have become apparant as the sample broke down, these in turn being broken down further as chewing progressed. In the final stages of chewing all samples were of similar texture, showing no extremes in rubberiness or graininess.

PLS revealed that Factor 1 sample scores from the descriptive analysis could be predicted using the panel means from the first chew stage of progressive profiling (correlation coefficient 0.85, Fig. 5). Predictions were observed (correlation coefficients >0.7) through each of the 19 chewing stages (Table 2) indicating that when making an overall judgement for smooth/creamy, hard/coarse perceptions throughout the complete chew cycle were accounted for.

Factor 2 of the descriptive analysis data could be predicted, by PLS, using certain stages of progressive profiling data (Table 2). Predictions (correlation coefficients >0.7), were found from the first stage of chewing (correlation coefficient 0.82, Fig. 6), through to the fourth chew stage, indicating that the overall perception of rubberiness was gathered in the early stages of mastication.

The Instron data could not be used to predict sample scores on Factor 1 of the progressive profiling at any chew stages (Table 3). Although previous studies have related force-deformation characteristics to hardness (Lee et al., 1978), the lack of prediction in our study may have beend ue to secondary textural characteristics, such as smoothness and creaminess. The Instron data could however be used to predict Factor 2 of the progressive profiling data at the first stage of chewing (correlation coefficient 0.85, Fig. 7). Significant predictions, >0.7, were found through the eleenth chew stages, but no predictions were found later (Table 3). Sample scores on Factor 2 (Fig. 3) showed that at the eleventh stage samples began to group.

Changes in textural characteristics occurred in different ways and at different rates among cheeses. Additional instrumental approaches would be required for textural properties throughout the chew sequence to be adequately accounted for in routine analyses.

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cates (Table 1) and either a weighted least squares or log transformation analysis, the two assays were strongly correlated (correlation coefficient >0.99). A variance component analysis showed that the small statistical difference between the two assays was almost completely due to measurement error rather than differences among methods.

CONCLUSIONS

THE COPPER CHELATION METHOD of histamine determination is a simple and rapid alternative to the standard AOAC fluorometric histamine assay. It is sensitive enough to accurately determine levels of histamine which may be cause for regulatory action. It is also more convenient since it is quantitated with a simple spectrophotometer rather than a fluorometer or could be observed visually without instrumentation.

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Phosphopeptides from Comté Cheese: Nature and Origin

F. ROUDOT-ALGARON, D. LE BARS, L. KERHOAS, J. EINHORN AND J.C. GRIPON

ABSTRACT

Thirteen low-molecular-weight phosphopeptides were isolated from the water-soluble fraction of Comté cheese. The sample was fractionated and purified by gel permeation chromatography and reverse-phase HPLC. The peptide sequences were identified by Edman degradation and primary molecular structure was confirmed by mass spectrometry. The different peptides purified correspond to fragments of the sequence Val13-Lys 28 of β-casein and of the sequence Glu 5-Lys 21 of α_{s2}-casein. These fragments probably originated from an initial proteolysis of the two caseins by plasmin, followed by further endopeptidase aminopeptidase and, possibly, carboxypeptidase digestions. Partial dephosphorylation of some β-casein fragments was observed. These peptides probably influence the flavor profile of comté cheese.

Key Words: cheese, flavors, phosphopeptides, proteolysis

INTRODUCTION

PROTEOLYSIS DURING CHEESE MATURATION is important in development of flavor of most cheese varieties. Free amino acids are flavor precursors since their catabolism results in several compounds, including amines, aldehydes, alcohols, phenols, and indole which contribute to odor and taste. Bitterness in cheese is associated with the presence of hydrophobic bitter peptides. However, bitterness is not the only taste from peptides since brothy and sweet-tasting peptides have been described (Noguchi et al., 1975; Nishimura and Kato, 1988). In cheese, McGugan et al. (1979) reported that the nonvolatile water-soluble substances and, hence, proteolysis products were very important for cheese flavor development. Aston and Creamer (1986) ascribed the taste intensity of a Cheddar cheese with bitter and brothy notes, to the water-soluble peptide-containing fraction. In Swiss cheese, intermediate-size peptides may contribute to brothy flavor (Biede, 1977) and correlation has been reported between perception of the flavor and concentration of small peptides and free amino acids (Biede and Hammond, 1979).

Several methods have been used to study proteolysis in cheese: solubility of peptides, various forms of chromatography or electrophoresis. Published reports were reviewed by Grappin et al. (1985), Rank et al. (1985), Fox (1989), and Ardö and Gripon (1992). The specific action of some enzymes on caseins has also been studied. Le Bars and Gripon (1989) identified some peptides occurring from the action of plasmin on α_{s2}-casein. The degradation of β-casein by rennet was studied by Pélissier et al. (1974) and Guillou et al. (1991). Mc Sweeney (1993) studied the proteolytic activity of plasmin, chymosin and some cell wall proteinases of bacteria from the genus *Lactococcus* on α_{s1}-casein, α_{s2}-casein and β-casein, respectively.

Except for the work of Mojarro de Guerra et al. (1991) on Vacherin Mont d'Or cheese, Addeo et al. (1992) on Parmigiano Regiano, and Roudot-Algaron et al. (1993, 1994) on Gruyère de Comté, no detailed studies have been reported on low-molecular-weight peptides in cheeses. Our objective was to purify and identify low-molecular-weight phosphopeptides from Comté cheese. Reverse-phase high-performance liquid chromatography (HPLC) and Edman degradation were used to purify and characterize these compounds. Mass spectrometry was used to confirm primary structures. We also considered the enzymatic mechanisms involved in generation of observed peptides.

Authors Roudot-Algaron, Le Bars, and Gripon are with Station de Recherches Laitières, INRA, Domaine de Vilvert, 78352 Jouy en Josas, France. Authors Kerhoas and Einhorn are with Station de Phytopharmacie, INRA, Route de St Cyr, 78026 Versailles, France.

MATERIALS & METHODS

Sample preparation

Cheese of the type "Gruyère de Comté" (12 mo old) was obtained from a local manufacturer and stored at −20°C. Cheese (600g) was ground and then extracted by liquid carbon dioxide in a Nova Swiss extraction cell (Nova Werke AG, Effretikon, Switzerland). The experimental conditions were: T° extractor = 16°C, P = 250 bar; T° separator = 26°C, P = 50 bar; (flow of CO_2 was 7–10 kg.hr^{-1}). After 24 hr extraction, 10g of the defatted residue, remaining in the extractor, was finely ground and dissolved in 40 mL of a 0.5M citrate buffer, pH 7. After stirring for 1 hr at 37°C, another 40 mL of citrate buffer was added and the mixture centrifuged (30,000 × *g*, 30 min). The supernatant was filtered through a GFC filter (Whatman, Maidstone, England). Caseins and large peptides were then precipitated at pH 4.6 and the suspension was centrifuged again (30,000 × *g*, 30 min). The supernatant was filtered through a Millipore (Milford, MA) 0.45 μm filter. The solution was immediately further purified.

Gel permeation chromatography

Ten mL of the solution was placed onto a Sephadex G10 column (58 × 1.5 cm) which was eluted with 30% acetic acid. Flow rate was 14 mL.hr^{-1}, and detection was at 280 nm in order to observe aromatic amino acids. The volume of each collected fraction was 3.5 mL.

Reverse-phase HPLC

The HPLC equipment consisted of an SP 8750 pump, an 8700 gradient controller (Spectra Physics, San Jose, CA) and a 990 diode array detector assisted by a data module for recording and treatment of results (Waters, Milford, MA). Fractions 3 to 7 from gel permeation were purified by reverse-phase HPLC in three steps. A first separation was made on a preparative C-18 Nucleosil column (SFCC-Shandon, Gagny, France), 250 × 10 mm, with a linear gradient from solvent A [0.15% trifluoroacetic acid (TFA)] to 70% B [0.1 TFA, 60% acetonitrile (ACN)] in 30 min. The flow rate was 2.5 mL.min^{-1}. The second step was carried out on a C-18 Nucleosil semi-preparative column, 250 × 6.2 mm, under the same gradient conditions with solvent A' (10 mM phosphate buffer, pH 7) and solvent B' (40% A', 60% ACN). The flow rate was 1.8 mL.min^{-1}. To desalt the sample, a third step, similar to the first step (solvents, gradient conditions), was carried out on a C-18 Nucleosil analytical column, 250 × 4.6 mm, at a flow rate of 1 mL.min^{-1}.

Amino acid analysis and sequencing

Samples were dried under vacuum in a Speed Vac concentrator (Savant, Hicksville, NY) and hydrolyzed in glass tubes with 6N HCl, under vacuum, at 110°C during 24 hr. Amino acid analysis was then performed on a LC 5000 amino acid analyzer (Biotronik, Maintal, Germany). The N-terminal sequence of the peptides was determined after derivatization with phenylisothiocyanate (PITC) and Edman degradation. These steps were carried out automatically on a 477A protein sequencer (Applied Biosystem, Foster City, CA). Identification was by on-line HPLC on a C-18 Brownlee narrowbore column (Applied Biosystem) using the manufacturer's procedure. Phenylthiohydantoin

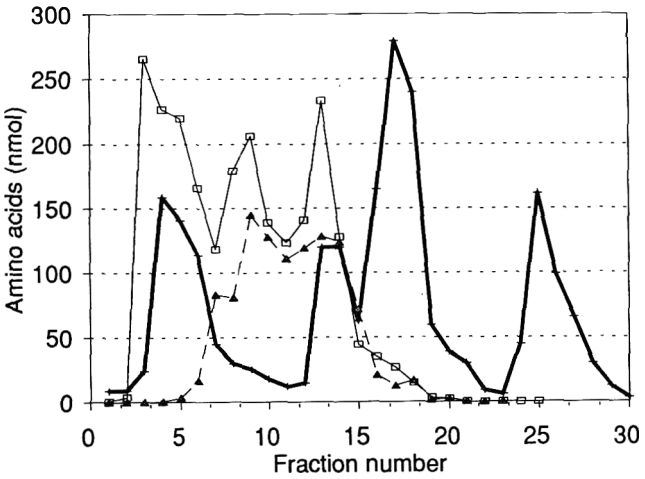

Fig. 1—Gel permeation chromatogram, total and free amino acid analysis for each fraction collected. —|— Gel permeation profile (280 nm); ▲—▲ amino acids before hydrolysis; □—□ amino acids after hydrolysis.

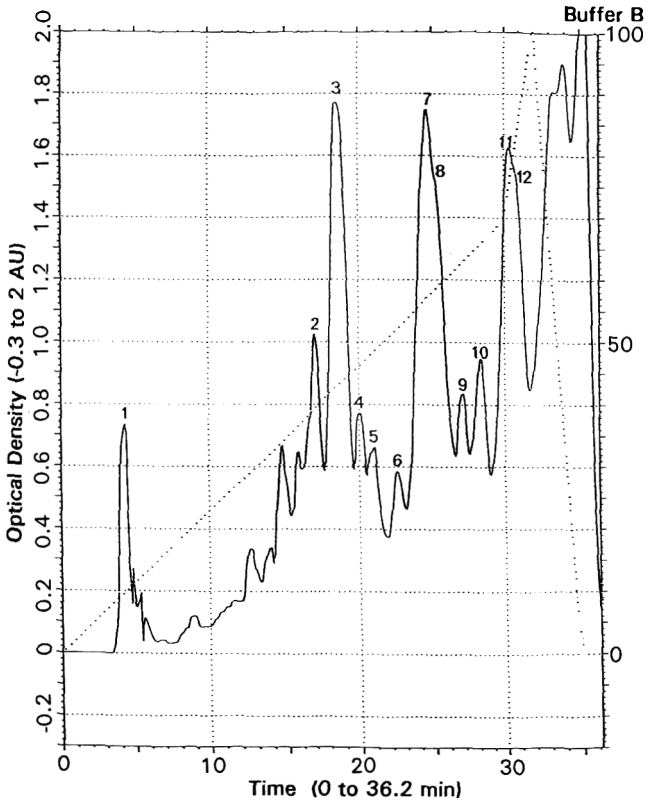

Fig. 2—RP-HPLC chromatogram of gel permeation fraction 5.

(PTH) amino acids were detected at 270 nm. Detection limit of the method was 10–20 pmol.

Mass spectrometry

Fast atom bombardment (FAB) mass spectra (positive mode) were obtained on a Nermag R30-10 triplicate quadrupole instrument equipped with an M-Scan Ltd atom gun. Xenon atoms with energies of 8 keV and a beam flux of 0.3 mA were used as ionizing beam. Samples were dissolved in MeOH-H_2O (1/1) and mixed with a thioglycerol matrix deposited on a stainless steel target. Spectra were recorded in the 1000–2000 mass range with a 3-sec scan time. Electrospray (ES) mass spectra (positive mode) were recorded on a VG Trio 2000 instrument equipped with an electrospray ion source and a quadrupole mass analyzer of m/z 3000 Da upper mass limit. Ten-µL sample solutions (solvent: acetonitrile/water/formic acid, 50/49/1) were introduced into the source at a 2 µL/min.

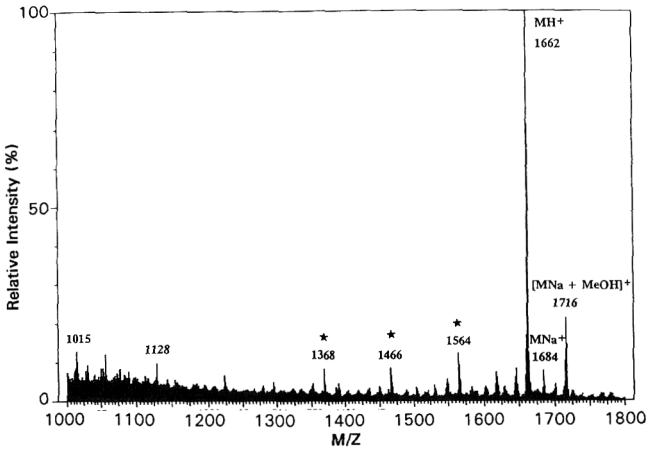

Fig. 3—FAB mass spectrum of the peptide from fraction **6-2**. [*Fragment ion resulting from MH^+ by loss(es) of H_3PO_4 unit(s).]

RESULTS

Purification

Four peaks were detected at 280 nm after gel permeation chromatography of the sample (Fig. 1). The third and fourth peaks were identified as tyrosine and trytophan, respectively. The comparison of the amino acid content of the fractions before and after acid hydrolysis indicated that most of the peptides were in the first peak corresponding to fractions 3 to 7. Fractions 8 to 15 contained mainly free amino acids and a few peptides (Roudot-Algaron et al., 1994). Reverse-phase HPLC of fractions 3 to 7 gave similar profiles. The chromatogram of fraction 5 is shown as an example (Fig. 2). It shows the complexity of the cheese extract. In order to avoid the isolation of bitter peptides, which are fairly rich in hydrophobic amino acids and would exhibit long retention times on a reverse-phase column, further purification was performed on a lower-retention-time fraction, peak 3 (Fig. 2). This fraction should contain more hydrophilic and shorter peptides which could have flavor properties. Rechromatography of peak 3 with phosphate buffer gave 9 fractions. The reverse-phase HPLC desalting step resulted in purification of 15 compounds.

Identification

The isolated peptides, presumed to result from casein degradation, were identified by amino acid analysis and N-terminal sequencing. Except for those from fractions **3-1** and **8-1**, the primary structures, some showing partial dephosphorylation, were confirmed after determination of molecular weights by FAB-MS or ES-MS. The spectra obtained under FAB-MS generally exhibited MH^+ (base peak in some cases), MNa^+ and (MNa^+, MeOH) ions as molecular species (for instance, at m/z 1662, 1684, and 1716, respectively, for fraction **6-2**, see Fig. 3). Fragment-ions which corresponded to the loss (or successive loss) of 98 u (e.g., H_3PO_4 molecule) from the MH^+ ion may appear, indicating a minimum number of phosphoryl groups in the molecules. Although most peptides could be examined by FAB-MS, two whose molecular weights exceeded the mass range of our instrument were analyzed by ES-MS. Amino acid analysis and N-terminal sequences (Table 1) and molecular masses determined by MS (Table 2) and identified sequences (Fig 4) were recorded. In spite of imprecision in determination of Ser and Glx, 13 peptides were identified, 10 originating from the N-terminal part of β-casein and 3 from the N-terminal part of α_{s2}-casein. Most had a C-terminal lysine (Lys21 for α_{s2}-casein peptides and Lys28 for β-casein peptides), except 4 peptides whose C-terminus was Asn27 of β-casein. The N-terminal residues found for α_{s2}-casein peptides

Table 1—Amino acid analysis of the purified compounds (a) and comparison with calculated values (b) according to identifications

Compound	Asx	Thr	Ser	Glx	Val	Ile	Leu	Tyr	His	Lys	Arg	N-terminal sequence
1-1 (a)	1	1	8.8	4.9	0.2	2	0.9	0.1	0.1	0.1	0.9	Glu-SerP-Leu-
(b)	1	1	5	3	0	2	1	0	0	0	1	
1-2 (a)	0	1	4.3	4.4	0.9	1.1	0	0.8	0.1	1.1	0	Val-SerP-SerP-
(b)	0	1	5	4	1	2	0	1	0	1	0	
2-1 (a)	0.8	1	8.5	9.2	0.5	1.5	0.4	0.65	0.2	1.2	0.3	blend
(b)	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	nd	
2-2 (a)	0.1	1	4.4	6	1.2	1.1	0	0.7	1.2	1.1	0	Glu-His-Val-
(b)	0	1	5	5	1	2	0	1	1	1	0	
3-1 (a)	1	0.8	6.3	3.8	0.1	3.1	0.9	0	0	0.1	0.9	SerP-Leu-SerP-
(b)	1	1	5	2	0	2	1	0	0	0	1	
3-2 (a)	0.3	1	4.8	4.6	0.9	1.2	0	0.8	0.9	1.1	0	His-Val-SerP-
(b)	0	1	5	4	1	2	0	1	1	1	0	
4-1 (a)	1.1	1	4.3	4	0.9	1.7	0.8	0.1	0.2	0.6	0.7	Val-Glu-SerP-
(b)	1	1	5	3	1	2	1	0	0	1	1	
5-1 (a)	1	0.9	6.2	2	0	1.8	0.9	0	0	0.1	0.9	Leu-SerP-SerP-
(b)	1	1	4	2	0	2	1	0	0	0	1	
5-2 (a)	1	0.9	7.7	3.2	0.1	1.9	0.9	0	0	1	0.9	Glu-SerP-Leu-
(b)	1	1	5	3	0	2	1	0	0	1	1	
6-1 (a)	1	0.9	6.6	1.9	0	1.8	0.9	0	0	1	0.9	SerP-Leu-SerP-
(b)	1	1	5	2	0	2	1	0	0	1	1	
6-2 (a)	1.3	0.8	5.8	4.5	1	2.2	0.7	0.1	0.1	0.4	0.7	Ser-Leu-SerP-
(b)	1	1	5	2	1	2	1	0	0	0	1	
7-1 (a)	1	1	4.4	2.4	0.8	2.1	1	0	0.1	0.5	0.9	Val-Glu-Ser-Leu-
(b)	1	1	5	3	1	2	1	0	0	0	1	and Ser-Leu-SerP-
8-1 (a)	1	0.9	6	2	0.1	1.9	0.9	0	0	0	0	Leu-SerP-SerP-
(b)	1	1	4	2	0	2	1	0	0	0	0	
8-2 (a)	1.2	1	4.6	3.3	0.2	2	1	0	0.1	1.1	1	Glu-Ser-Leu-
(b)	1	1	5	3	0	2	1	0	0	1	1	
9-1 (a)	1	0.9	6.8	2	0.1	1.9	0.9	0	0.1	1	0	Ser-Leu-SerP-
(b)	1	1	5	2	0	2	1	0	0	1	1	

Table 2—Molecular weights determined by FAB or ES-MS and final identification

Compound	M(FAB-MS)	M(ES-MS)	Identification	Phosphorylation[a]
1-1	1870		14-27 β-casein	Ser15-P
1-2			7-21 α_{s2}-casein	
2-1	1739		Blend	
2-2			5-21 α_{s2}-casein	
3-1[b]			15-27 β-casein	Ser15-P
3-2		2144.5	6-21 α_{s2}-casein	Ser16-P
4-1	1969		13-28 β-casein	Ser15-P
5-1	1574		16-27 β-casein	
5-2		2000	14-28 β-casein	Ser15-P
6-1	1869		15-28 β-casein	Ser15-P
6-2	1661		15-27 β-casein	Ser15
7-1a			13-28 β-casein	Ser15
7-1b	1661		15-27 β-casein	Ser15
8-1[b]			16-27 β-casein	
8-2	1918		14-28 β-casein	Ser15
9-1	1789		15-28 β-casein	Ser15

[a] Ser15-P means that the seryl residue at position 15 is phosphorylated. Ser15 means that this residue is not phosphorylated.
[b] Compounds not studied by mass spectrometry.

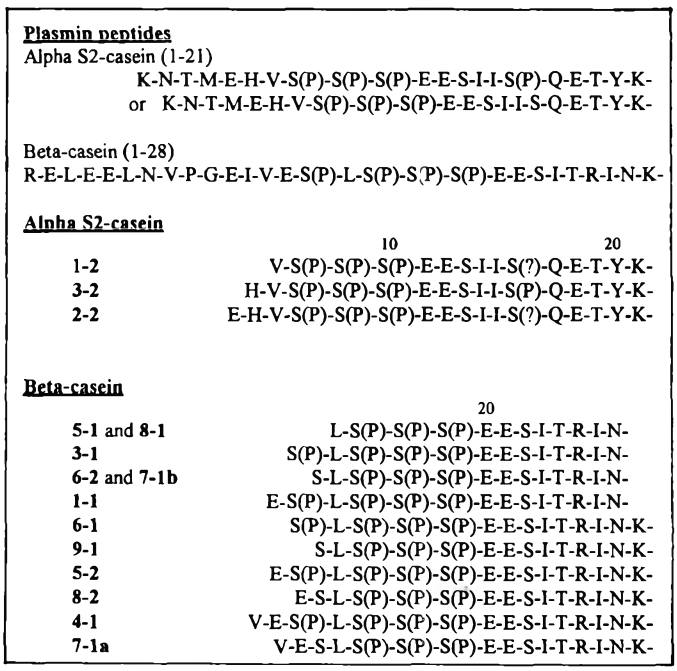

Fig. 4—Amino acid sequence of identified compounds. (P)=phosphate group.

were Glu5, His6, Val7 (for peptides **2-2, 3-2, 1-2**,, respectively) suggesting sequential degradation of peptide Glu5-Lys21. The same observation was made with the β-casein peptides since their N-terminus corresponded to residues 13, 14, 15, and 16 of β-casein. In the case of peptides **6-2, 8-2** and **9-1** arising from β-casein, Ser was detected by Edman degradation in position 15 where phosphoserine was present in β-casein. The dephosphorylation of the residue was confirmed by FAB-MS for the three peptides. The structures of peptides **1-2** and **2-2** were not confirmed by mass spectrometry because of insufficient amounts. Nevertheless, the amino acid compositions and N-terminal sequences were non-ambiguous and support the identifications. Fraction **7-1** appeared to be a mixture of two peptides, one being peptide 13-28 of β-casein, the other (peptide 15-27) being a contamination from the HPLC fraction **6-2** which eluted just before.

DISCUSSION

THIRTEEN PEPTIDES were identified, all of them phosphopeptides from α_{s2}- or β-casein (Fig. 4). Their sequences indicate

the action of several proteolytic enzymes on caseins in Comté cheese. Most of the peptides had lysine as the C-terminal end. This indicated that they were released by plasmin (the alkaline milk proteinase). This enzyme has a trypsin-like specificity and cleaves Lys-X and Arg-X peptide bonds. Lys28-Lys29 of β-casein and Lys21-Gln22 of α_{s2}-casein are plasmin-sensitive bonds and phosphopeptides 1-21 from α_{s2}-casein (Le Bars and Gripon, 1989) and 1-28 from β-casein are rapidly produced. These two peptides were further hydrolyzed, probably by an endopeptidase followed by aminopeptidases. The aminopeptidase activity was clearly observed since several peptides differed by only one N-terminal amino acid: peptides 5-21, 6-21, 7-21 from α_{s2}-casein and 13-28, 14-28, 15-28 and 14-27, 15-27, 16-27 from β-casein were detected. Aminopeptidases from lactic acid bacteria have been widely studied. A metallo-aminopeptidase (PepN) and a cysteine aminopeptidase (PepC) with broad specificities, as well as an aminopeptidase specific for glutamyl and aspartyl residues (PepA), were characterized in *Lactococcus lactis* (Monnet et al., 1993). Less information is available on the thermophilic species used in Comté cheese manufacture. A metallo-aminopeptidase with properties similar to PepN, hydrolyzing peptides with aliphatic or negatively charged N-terminal residues, has been isolated from *Lactobacillus helveticus* (Khalid and Marth, 1990; Miyakawa et al., 1992), *Lactobacillus delbruecekii* subsp. *lactis* (Eggiman and Bachmann, 1980) and *Streptococcus thermophilus* (Tsakalidou and Kalantzopoulos, 1992). Other aminopeptidases are probably in the thermophilic lactobacilli since 4 different aminopeptidases have been reported by Atlan et al. (1990) in *Lactobacillus delbrueckii* subsp. *bulgaricus*. Before being digested by the aminopeptidases, the plasmin peptides 1-21 from α_{s2}-casein and 1-28 from β-casein were probably cleaved by a proteinase. This action could be attributed to the cell wall-associated proteinase, observed in thermophilic lactobacilli (Ezzat et al., 1985; Laloi et al., 1991).

Two different forms of the phosphopeptides 13-28, 14-28, 15-27 and 15-28 were obtained, differing by the state of phosphorylation of Ser15 in β-casein. Thus, peptide **4-1** had the sequence Val-Glu-SerP-Leu-..., while peptide **7-1a** had the following: Val-Glu-Ser-Leu-.... The same differences could be observed in peptides **5-2** and **8-2, 3-1** and **6-2** or **6-1** and **9-1**. Dephosphorylation of an α_{s1}-peptide in Parmigiano Reggiano was reported by Addeo et al. (1992): peptide 41-75 was partly dephosphorylated, containing only 3 of the 7 native phosphate groups. These observations suggested that phosphatases were active in those cheeses. Two indigenous phosphatases are present in milk: acid and alkaline phosphatases. Acid phosphatase is present at a much lower level than alkaline phosphatase, but its heat stability, and optimum pH (~ 4) make it technologically important (Fox and Morissey, 1981). It is active during cheese manufacture and this enzyme has been suggested as rate-limiting in protein degradation because normal proteinases cleave peptide chains near phosphorylated residues (Dulley and Kitchen, 1973). An acid phospatase activity was detected in dairy microorganisms such as *Lactococcus lactis, Lactococcus cremoris, Streptococcus thermophilus,* and *Penicillum roqueforti,* the last one was most efficient (Larsen and Parada, 1988). The rate of dephosphorylation depends on the initial state of phosphorylation of the phosphoprotein or peptide. For example, for a peptide derived from casein which contained 4 closely located phosphoserine residues, the first phosphate group was more easily lost than the second and so on (Weller, 1979). The properties of protein phosphatases differ markedly, but they generally have an optimum pH between 5.5 and 6 and are heat-resistant. These properties allow them to act, when present, in cheese.

The structure of only one α_{s2}-casein peptide was confirmed by mass spectrometry. The molecular mass for peptide **3-2** made it possible to determine the phosphorylation state of the seryl residue in position 16. Since α_{s2}-casein has different degrees of phosphorylation, from 10 to 13 (Brignon et al., 1977), the seryl residue at position 16 may be phosphorylated (or not). Because lack of product did not allow MS determination for peptides **2-2** and **1-2,** the state of phosphorylation of Ser 16 in these peptides could not be determined.

At the C-terminal end of the identified peptides, residue Lys28 was missing for peptides **1-1, 3-1, 5-1, 6-2, 7-1b, 8-1.** For all of them, except peptides **5-1** and **8-1,** the corresponding peptide, including Lys28, was found. This could be explained by the action of a carboxypeptidase. Except for the intracellular enzyme of *Lactobacillus casei* (El Soda et al., 1978), no carboxypeptidase has been identified in lactic acid bacteria. However, a lysine carboxypeptidase is present in the plasma of almost all mammals. In the same way that plasmin in milk originates from blood, probably the same mechanism of exchange occurs for lysine carboxypeptidase present in plasma. We could also explain the presence of these peptides by the action of an endopeptidase on the bond Asn27-Lys28 before action of the plasmin.

CONCLUSION

THIRTEEN PHOSPHOPEPTIDES were identified from Comté cheese. They differed from each other by one or two residues. They all originated from two short regions of β- and α_{s2}-caseins. The original cleavage of these caseins was no doubt due to plasmin, probably followed by the action of another endopeptidase and of aminopeptidases, as well as a lysyl carboxypeptidase. Some of the peptides appeared to be partly dephosphorylated. The original molecules containing both phosphoserines and high glutamate may be of importance in flavor development.

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Caseinate at Low Temperatures: Calcium Use in β-Casein Extraction by Microfiltration

M.H. FAMELART and O. SUREL

ABSTRACT

Calcium-induced modifications of 2% sodium caseinate at 4°C and β-casein extraction by microfiltration on membranes were studied. Pore diameters and tangential flow rates were investigated. Calcium addition up to 3 $g.L^{-1}$ was followed by a pH decrease, particle size increase and a shift of casein towards colloidal state. The higher purity of β-casein in the soluble fraction was obtained between 1 and 1.5 $g.L^{-1}$ of added calcium. Increasing calcium to 1 $g.L^{-1}$ induced hydraulic resistance increase, and higher retention of β-casein. Particle size increases were related to physicochemical modifications induced by the calcium addition.

Key Words: milk, caseinate, β-casein, microfiltration, calcium

INTRODUCTION

STORAGE of milk at 4°C induces a dissociation of caseins from micelles (Pierre and Brulé, 1981; Dalgleish and Law, 1988a). This increase of serum casein is first due to displacement of colloidal calcium phosphate towards the aqueous phase (Brulé, 1981) because of ionization regression of soluble citrate, phosphate, and because of lowered calcium phosphate saturation (Pierre and Brulé, 1981). Also, it is due to the lowering of intermolecular hydrophobic interactions at low temperatures. β-casein constitutes more than 60% of serum casein, because of its high hydrophobicity. In the presence of calcium, α_s- and β-caseins in pure solutions associated at room temperature and aggregation occurred, while at < 10°C, β-casein was soluble (Farrell et al., 1988). Methods for fractionating β-casein from milk at low temperature by membrane processing have been optimized by ultrafiltration (Renner-Nantz et al., 1990) or microfiltration (Love, 1990), from caseinate in the presence of calcium (Terré et al., 1986, Surel and Famelart, 1991) or without calcium addition (Murphy and Fox, 1991; Le Berre and Daufin, 1994).

Microfiltration of sodium caseinate at low temperature in the presence of calcium between 0.5 and 2 $g.L^{-1}$, (Terré et al. 1986) gave low retention of β-casein and high fluxes. They reported the effects of calcium addition up to 3 $g.L^{-1}$ to sodium caseinate at 4°C on physicochemical characteristics, such as size of particles, casein solubility and partition of calcium and sodium between serum and colloidal phases. Our objective was to investigate modifications induced by added calcium in the caseinate and to optimize microfiltration. Addition of calcium was then tested during microfiltration and process performance was investigated.

MATERIALS & METHODS

Substrates

Microfiltration. Sodium caseinate at 2% (w/v) was reconstituted with commercially produced sodium caseinate (Armor Proteines, St Brice en Cogles, France) in 5-μm-filtered tap water using a Triblender (Guérin, Mauzé-sur-le-Mignon, France). The caseinate powder contained total dry matter 950, calcium 0.07, and sodium 17. Calcium was added by 0.5-L solution of $CaCl_2$ during microfiltration to increase final concentrations from 0 to 1, 2 and 3 $g.L^{-1}$ Ca. The pH of the caseinates was not readjusted to its value without $CaCl_2$ addition (6.7). Microfiltrations with constant concentration of calcium were also carried out to study the effect of filtration time on fluxes and retention.

Physicochemical experiments. Sodium caseinate (2%, w/v) was prepared in MilliQ water with 0.02% (w/v) sodium azide using a paddle laboratory mixer. Solutions were cooled in a water-bath at 4°C. Calcium chloride powder was added to caseinate solutions at 4°C to reach final Ca concentrations of 0.5, 1, 1.5, 2, and 3 $g.L^{-1}$. The pH of the caseinates was not readjusted to 6.7, except in one experiment on mineral distribution.

Caseinate ultrafiltrates used for mineral partition were prepared on a Centriflo CF25 (Amicon, Paris, France) ultrafiltration membrane (mol. wt. cut-off 25,000) at 500g. The first 2 mL of ultrafiltrate and the concentrated caseinate were discarded and 7 mL of caseinate was then filtered. Ultrafiltrates used to resuspend ultracentrifuged pellets were prepared on an Amicon HIP 1043 membrane (mol. wt. cut-off, 10,000). The first 100 mL of ultrafiltrate from 1 L of caseinate was discarded.

Microfiltration

Module. The temperature of caseinate in the feed vat was maintained by circulating through a plate heat exchanger supplied with 0°C water, while the 0.2 m^2 module (Fig. 1) was equipped with a tubular heat exchanger supplied with water-ethylene-glycol at −10°C with a cryostat (model HS40; Huber, Offenburg-Elgersweier, Germany). The module was connected to a hydraulic backflush defouling device (SCT, Bazet, France) in which a piston pushed 100 mL of the filtrate towards the retentate side at an appropriate frequency (1 stroke/min). This removed the fouling layer at the retentate side of the membrane. Membralox membranes (SCT, Bazet, France) of sizes 0.1 μm (zirconia-alumina), 0.2 and 0.5 μm (alumina) were tested. The pH values of zero point charge, estimated by flow potential measurements, were 5.7 for zirconia-alumina membrane and 8-8.2 for alumina (Soria, 1993).

Experimental runs. Before each run, membranes were conditioned with Ultrasyl 11 at 0.1%. Caseinate was prechilled at 2°C with the plate heat exchanger. The defouling device was not started until the filtrate compartment was filled, after 3 min. At each calcium concentration (0, 1, 2 and 3 $g.L^{-1}$), 3 tangential flow rates (4, 6 and 7.3 $m.s^{-1}$) were tested. After 15 min, volumic flow rates were measured and filtrate samples were frozen until analyzed. Volumes of samples were used to calculate the concentration factor.

Calculations. Normalized fouling hydraulic resistance (R_f/R_m) was calculated by Darcy's law as follows:

$$R_f/R_m = \frac{J_0}{J_t} - 1$$

where R_f is the fouling hydraulic resistance with time, R_m is the hydraulic resistance of the clean membrane, J_0 and J_t the fluxes at 4°C with water and caseinate. Retention, TR (%), was calculated as:

where C_p and C_r are permeate and retentate casein concentrations.

Casein solubility and analysis

Soluble caseins were separated from aggregates by centrifugation (model C8-55, Beckman Instrument France, Gagny) at 75000 × *g* for 2 hr at 4°C, immediately after calcium addition, and 3 hr after storage

Author Famelart is with INRA, Laboratoire de Recherches de Technologie Laitiére, 65, rue de St Brieuc, 35042 Rennes Cedex, France. Author Surel is with Société des Céramiques Techniques, 65460 Bazet, France.

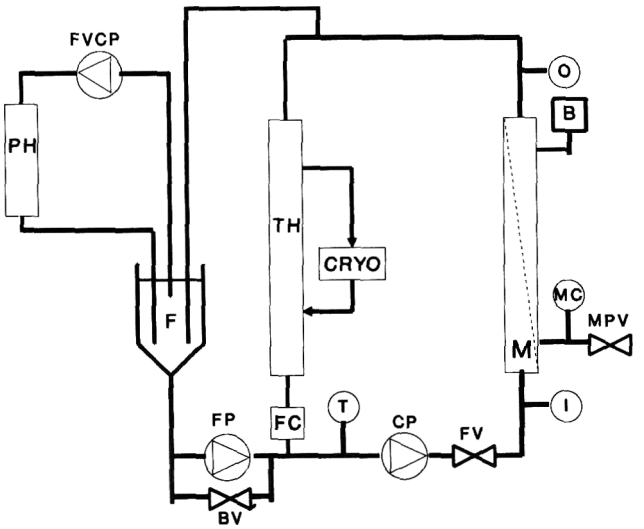

Fig. 1—Schematic of 0.2m² microfiltration plant. FP = feed pump, CP = circulation pump, FVCP = feed vat circulation pump, F = feed vat, M = microfiltration module, TH = tubular heat exchanger, PH = plate heat exchanger, B = backflush device, BV = feed pump by-pass valve when backflush is running, FC = flow controller, FV = flow control valve, I = input pressure sensor, O = output pressure sensor, MC = microfiltrate pressure controller, MPV = microfiltrate pressure valve, T = temperature controller, CRYO = cryostat.

Table 1—Average particle size of unimodal population (d_{unimod}) and particle size of the major particles (i.e., those with greatest weights) (d_{maj}) obtained with Coulter N4MD granulometer[a]

Calcium (g.L⁻¹)	pH	I.S.	d_{unimod} (nm)	d_{maj} (nm)	%
0	6.68	0.004	333	11	80
0.5	6.19	0.030	142	25	97
1.0	6.02	0.065	131	75	88
1.5	5.92	0.100	136	81	87
2.0	5.86	0.130	151	86	81
3.0	5.79	0.200	193	213	100

[a] Ionic strength (I.S.) and pH of sodium caseinate samples with calcium addition. % = weight percentage by N4MD for the major population.

in the presence of calcium at 4°C. Supernatants were frozen until analyzed. Casein contents in supernatants and microfiltrates were determined by FPLC (Pharmacia LKB Biotechnologie, Les Ulis, France) in an anion-exchange MonoQ column (Guillou et al., 1987). β-casein purity was calculated as follows:

$$\frac{\beta\text{-casein}}{\beta\text{-casein} + \alpha_s\text{-casein}} \times 100$$

Protein contents of supernatants were estimated from N determinations (Kjeldahl) with 6.38 conversion factor.

Particle size analysis

Particle sizes were obtained by light scattering and photon correlation spectroscopy on a Coulter granulometer N4MD (Coultronics, Margency, France). Samples diluted in water at 4°C were thermostated 10 min in the Coulter cell before measurement of light intensity scattered at 90° during 300 sec. The size measurement of caseinate particles diluted in water gave constant diameter within at least 2 hr. Index of refraction and viscosity are those of water at 4°C. In the simplest case, the N4MD model provides the average particle size of a unimodal population obtained from the diffusion coefficient. Size distribution information can be obtained from the deconvolution of autocorrelation function.

For each calcium concentration, caseinate was fractionated by successive centrifugation steps as was done on milk by Dalgleish et al. (1981) or Davies and Law (1983). The supernatant of a centrifugation step was subjected to further centrifugation. Conditions were 12500 × *g*/15 min, 55000 × *g*/15 min, 55000 × *g*/1 hr, 75000 × *g*/2 hr. The pellets obtained successively (C1, C2, C3 and C4) were resuspended

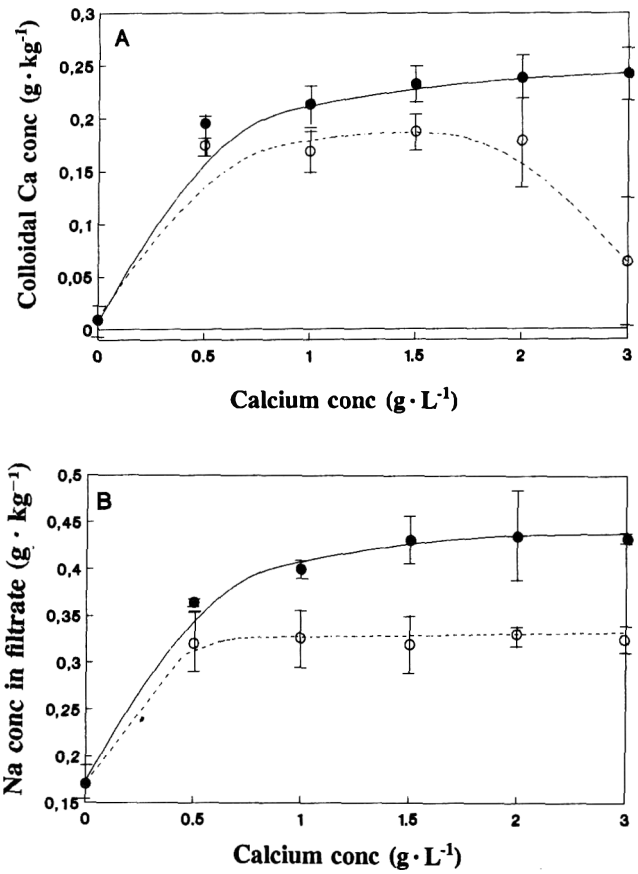

Fig. 2—Ca and Na partitions in calcium caseinates, at 4°C: mean of two repetitions and 95% confidence intervals. --o--, non-pH-maintained; ——●——, non-pH-maintained at 6.7 with 1N NaOH.

in 15 mL of corresponding ultrafiltrate at 4°C, maintained at 4°C overnight with magnetic stirring, and subjected to homogenization in a Polytron 10-35 (Kinematica, Littau-Luzern, Switzerland) at 15000 rpm for 1 min. The dry matter (total solids) of each fraction was used to calculate the dry matter distribution of particles.

Calcium and sodium partition

Calcium and sodium determinations on caseinates and Centriflo ultrafiltrates were made using an atomic absorption spectrophotometer (model AA300, Varian France, Les Ulis) (Brulé et al., 1974). Mineral partition between aqueous and colloidal phases was deduced. In one experiment, mineral partition was also determined on caseinates with calcium addition and pH maintained at the value of the caseinate without calcium, i.e., 6.7, with 1N NaOH.

Binding equilibrium constants

The thermodynamic linkage approach of Farrell et al. (1988) was used, assuming that:

$$p + nx \overset{k_1}{\Leftrightarrow} PX_n + mx \overset{k_2}{\Leftrightarrow} PX_nX_m$$

where p is the unbound protein, x the free calcium and n and m the number of calcium bound to protein. k_1 and k_2 are the calcium binding equilibrium constants. For each observed variable, the calcium concentration in mole.l^{-1} at the midpoint of the curve was graphically determined. At that point, $[PX_n] = p$, so that the inverse of this calcium concentration led to an approximate value of k_1 constant, in L.mole^{-1}.

RESULTS

THE pH VALUES of caseinate solutions were compared (Table 1). They showed a sharp decrease between 0 and 0.5 g.L^{-1} calcium and a slight reduction up to 3 g.l^{-1}. Calcium bound

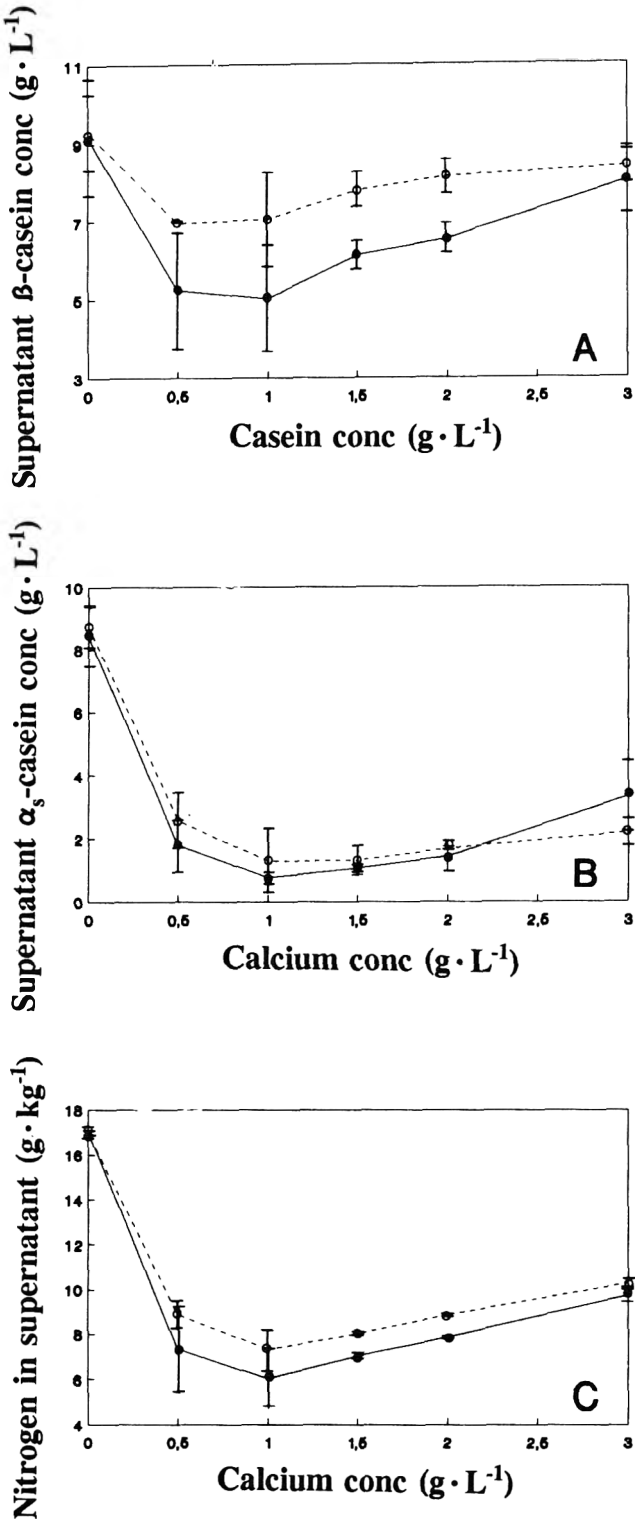

Fig. 3—Caseins in centrifuged caseinate supernatants with added calcium at 4°C immediately after calcium addition (—●—); after 3 hr storage in the presence of calcium at 4°C (--○--). Mean of two repetitions.

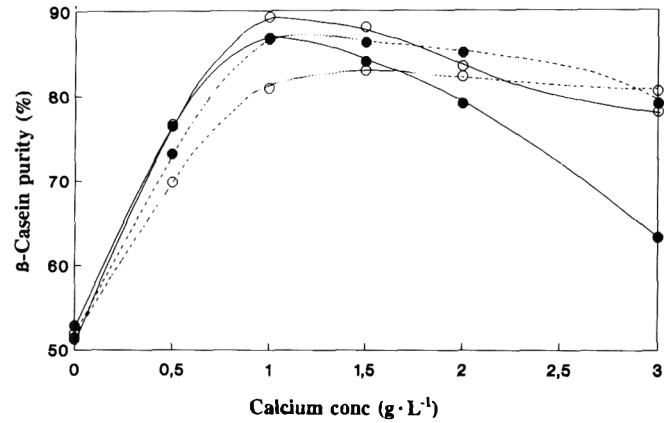

Fig. 4—β-casein purity (%) of centrifuged caseinate supernatants with calcium addition at 4°C for two repetitions (——— and - - -). (●): immediately after calcium addition; (○): after 3 hr storage in the presence of calcium at 4°C.

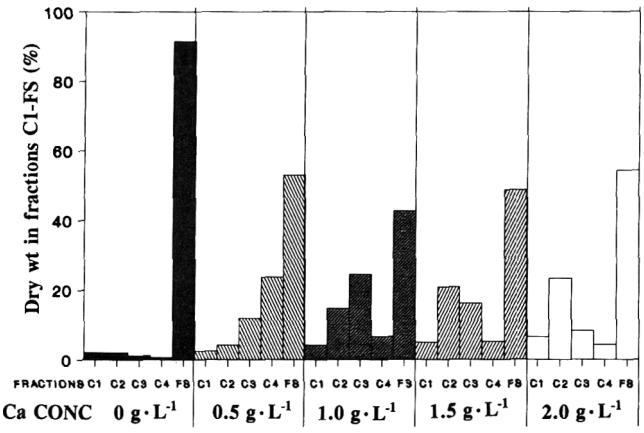

Fig. 5—Dry weight repartition (in percent total dry matter) in centrifuged pellets C1, C2, C3 and C4 and in final supernatant (FS) after successive ultracentrifugations at 4°C. Size of particles decreased from C1 to C4 fractions and from C4 to FS.

to colloidal casein (Fig. 2) increased with calcium addition up to a plateau value of 0.24 g.L^{-1} with constant pH (i.e., 12 mg calcium bound to 1 g caseinate or 6.9 moles calcium bound to 1 mole caseinate using casein atomic weight of 23160, Holt, 1982). Without pH correction, a maximum bound level of 0.2 g.L^{-1} was found for calcium addition between 0.5 and 1.5 g.L^{-1}. Above 1.5 g.L^{-1}, the colloidal calcium level decreased to 0.1 g.L^{-1}. Soluble sodium in ultrafiltrate, without calcium addition, was 163 mg for 347 mg of total sodium (47% soluble), and calcium addition at 0.5 g.L^{-1} led to total sodium solubilization (97%). NaOH additions for pH adjustment accounted for sodium discrepancies.

The effects of calcium addition at 4°C on casein and N in supernatants were compared (Fig. 3). When increasing calcium was added, part of the casein (or N) became "insoluble" in sediment. This pellet casein was maximal at 1 g.L^{-1} calcium and further calcium addition led to casein resolubilization. At 1 g.L^{-1} calcium, 90%, 50% and 65% of α_{s1}, β-casein and N were centrifuged, while calcium at 3 g.L^{-1} led to 62%, 15% and 44%. β-Casein was less sensitive to sedimentation by calcium, and showed a storage time effect with nonmicellar levels higher after 3 hr at 4°C. Solubilization of β-casein can be promoted by long storage time at 4°C. Calcium addition between 1 and 1.5 g.L^{-1} was optimal with respect to β-casein purity (Fig. 4). Five g.L^{-1} and 0.8 g.L^{-1} of β- and α_s-casein were non-micellar in 1 g.L^{-1} calcium. These values became 6–7 g.L^{-1} and 1 g.L^{-1} after 3 hr storage at 4°C.

Calcium addition was accompanied by an increase in turbidity. Without calcium, centrifuged samples showed a turbid zone at the top of the supernatant. With increased calcium, this zone was smaller and included large particles in its lower part. At > 1 g.L^{-1} calcium, supernatants were totally homogeneous. The heterogeneous materials observed at low calcium contents were considered to be insoluble and lipid matter.

Without calcium, more than 80% of light-diffusing material was composed of 11-nm particles, whose sizes increased to

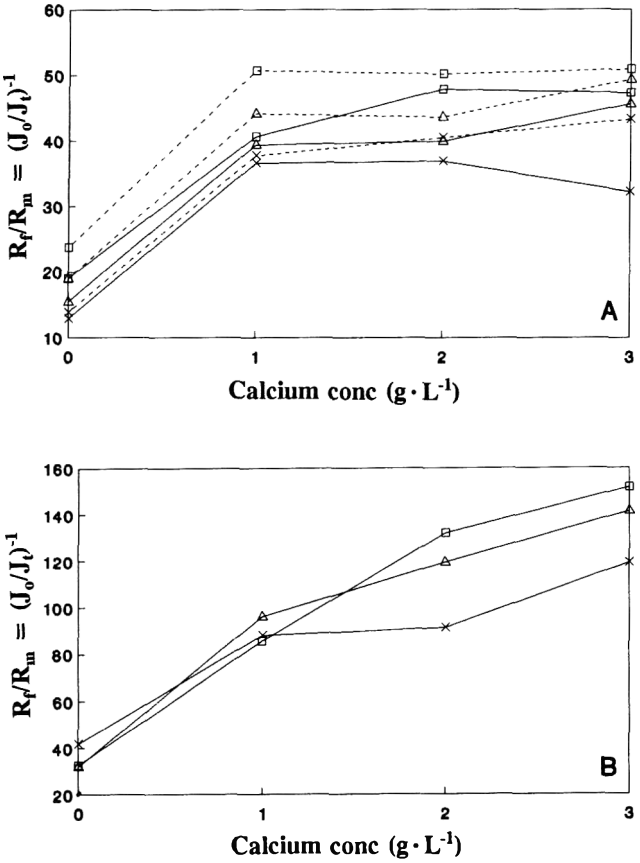

Fig. 6—Normalized fouling hydraulic resistance (R_f/R_m) evolution with calcium addition during sodium caseinate microfiltration at 4°C. Tangential flow rates: 4(X), 6(Δ) and 7.3(□) $m.s^{-1}$. A: 0.1 μm (———); 0.2 μm (- - -) membrane. B: 0.5 μm membrane.

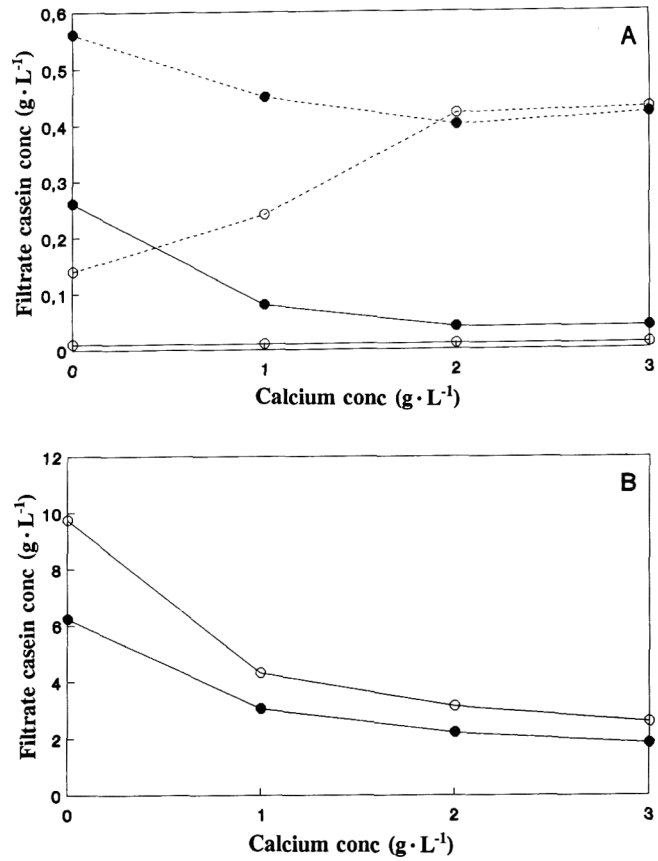

Fig. 7—Casein concentrations in microfiltrates from sodium caseinate at 4°C with calcium addition. α_s-casein concentration (○); β-casein concentration (●). A: 0.1 μm membrane (———), 0.2 μm membrane (- - -). B: 0.5 μm membrane.

210 nm in presence of 3 $g.L^{-1}$ calcium (Table 1). Dry matter distributions from successive centrifugation steps in fractions C1 to FS were summarized (Fig. 5). It was extremely difficult to resuspend pellets at 4°C in ultrafiltrates, even after overnight agitation and Polytron homogenization. Residual aggregates were found in resuspended pellets at any calcium concentration and in final supernatants at low calcium levels, resulting in overestimations of mean diameters. As shown, calcium addition resulted in a 40% reduction in dry matter of final supernatant and an increase in centrifuged material in C1, C2, C3 and C4. This indicated an increase in particle sizes.

Microfiltration results from controlled conditions of hydrodynamics and of calcium level were identical to those obtained with variable conditions. The calcium effect on retention and fluxes was identical. Controlled condition experiments showed filtration fluxes were constant over ≤ 2 hr. Thus the backflush defouling device was efficient and evolutions in filtration experiments with variable conditions were due to calcium addition or modification of flow rates. Normalized hydraulic resistance evolutions with the three membranes (0.1, 0.2 and 0.5 μm), at three tangential flow rates (4, 6 and 7.3 $m.s^{-1}$) with calcium addition were compared (Fig. 6). Evolutions were similar for different flow rates. In the case of 0.1 and 0.2 μm membranes, R_f/R_m increased up to 1 $g.L^{-1}$ calcium and remained constant, while fouling continuously increased to 3 $g.L^{-1}$ calcium level on the 0.5 μm membrane. Maximal R_f/R_m values between 40 and 50 were obtained for 0.1 and 0.2 μm membranes, with the 0.2 μm membrane always more fouled, while the 0.5 μm membrane reached 140. Maximal filtration fluxes at 7.3 $m.s^{-1}$ were 24.2, 23.6 and 30.8 $l.hr^{-1}.m^{-2}.bar^{-1}$, respectively, for 0.1, 0.2 and 0.5 μm, in the absence of calcium.

α_s-Casein and β-casein concentrations in filtrates (Fig. 7) showed without calcium, β-casein in permeates was 0.26, 0.56 and 6.23 $g.L^{-1}$ with purities of 96, 80 and 39%, respectively, with 0.1, 0.2 and 0.5 μm membranes. Casein levels increased with pore diameter. On 0.1 μm, casein concentration (almost entirely β-casein) decreased with calcium. Filtrates of 0.2 μm membranes were less enriched in β-casein with calcium addition. The 0.5 μm membrane showed no selectivity for any casein, and the casein level decreased also with calcium addition. α_s-Casein and β-casein retentions increased with calcium addition, with exception of α_s-casein on 0.2 μm (Fig. 8). k_1 and k_2 values (Table 2) showed k_1 values for the microfiltration process (Rf/Rm and filtrate concentration) were lower than those for physicochemical variables (pH, mineral partition).

DISCUSSION

Evolution of fluxes with calcium addition

Permeation fluxes without calcium were similar to those reported with sodium caseinate (Le Berre and Daufin 1994) on ultrafiltration and microfiltration. Fluxes on Membralox in presence of calcium were lower than those of Terré et al. (1986) and maximum fluxes and β-casein concentrations were obtained in the absence of calcium. However, this condition was not tested by them or described in their patent.

Calcium addition led to fouling conditions on the three membranes. These conditions appeared mainly with 1 $g.l^{-1}$ calcium on 0.1 and 0.2 μm membranes. On 0.5 μm membrane, increase of calcium to 3 $g.L^{-1}$ led to fouling. This fouling could be explained, first, by particle size or shape modifications that led to a pore blocking. They could also be due to physicochemical modifications that led to new interactions between solutes, particles and chemical groups at the surface or inside membrane layers.

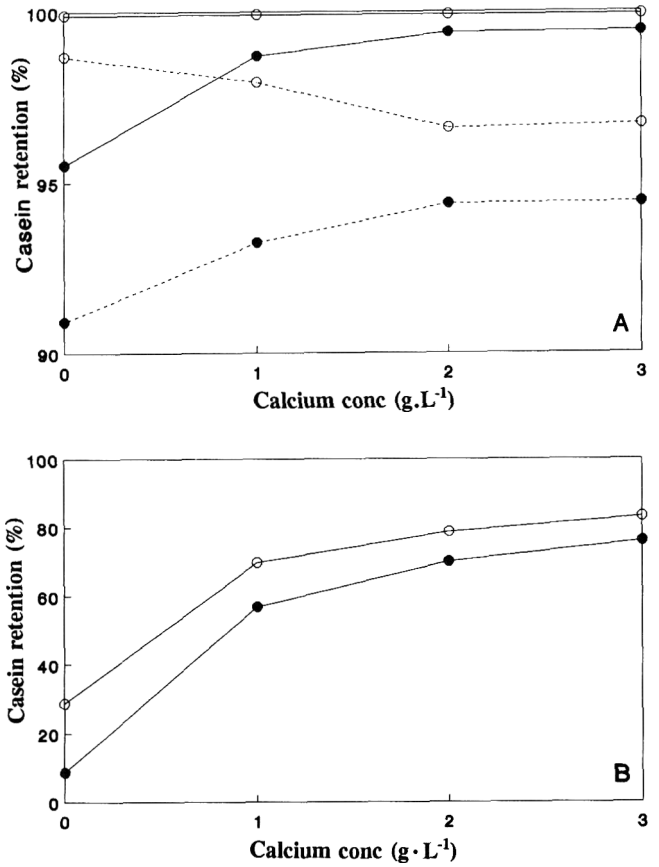

Fig. 8—Casein retention as (1-(Cp/Cr))×100 in percent in sodium caseinate microfiltration at 4°C with calcium addition. α_s-casein (○); β-casein (●). A: 0.1 μm membrane (———), 0.2 μm membrane (- - -). B: 0.5 μm membrane.

Table 2—k_1 and k_2 calculations according to the Farrell et al. (1988) approach[a]

Measured factor		k_1 (L. mole^{-1})	k_2 (L. mole^{-1})
pH		100	—
Colloidal calcium (pH-maintained)		90	—
Colloidal calcium (non-pH-maintained)		101	15
Soluble sodium (pH-maintained)		95	—
Soluble sodium (non-pH-maintained)		124	—
Soluble β-casein		147 159	21 27
Soluble α_s-casein		136 127	17 19
Soluble nitrogen		136 136	20 21
β-casein purity		110 102	17 19
% weight in low-size fraction (FS)		145	26
R_f/R_m 0.1 μm	4 m.s^{-1}	82	15
	6 m.s^{-1}	65	—
	7.3 m.s^{-1}	60	—
R_f/R_m 0.2 μm	4 m.s^{-1}	65	—
	6 m.s^{-1}	67	—
	7.3 m.s^{-1}	79	—
α_s-casein conc	(0.1 μm)	—	—
	(0.2 μm)	33	—
	(0.5 μm)	61	—
β-casein conc	(0.1 μm)	64	—
	(0.2 μm)	55	—
	(0.5 μm)	58	—

[a] From Fig. 2 to 8, and from Table 1, the Ca concentration (in Mole L.$^{-1}$) at the midpoint of the curves was graphically determined. The inverse of this concentration led to an approximate value of k_1 and k_2 in L.mole^{-1}. Soluble caseins and nitrogen (analyzed from supernatants of 75000 × *g* 2-hr/centrifugtion) after 3 hr storage in the presence of calcium at 4°C are underlined.

Particle size or shape modifications. R_f/R_m evolution curves (Fig. 6) with calcium addition could be explained by the particle size increases (Table 1, Fig. 5). This size increase was expected since calcium addition resulted in an increase in turbidity, (Dalgeish and Law 1988b). Baumy and Brulé (1986) pointed out that calcium binding to caseinate led to solubility decrease and aggregation. A 0.5 g.L^{-1} calcium addition led to a total solubilization of colloidal sodium and a binding of about 0.2 g.L^{-1} calcium at pH near 6. Baumy and Brulé (1986) observed a binding of 0.26 g.L^{-1} of calcium on casein at pH 7 in a 21 g.L^{-1} caseinate at the onset of precipitation. According to Adachi (1963), as cited by Baumy (1987), calcium addition led to progressive reconstitution of casein micelles and to their precipitation. Particle size in caseinate and particle size evolution with calcium addition have received very little attention. Dalgleish and Law (1988b) obtained a 110 to 206 nm size range for sodium caseinate at 0.6 g.L^{-1} calcium. Our results pointed out a substantial size increase for major particles with calcium addition (Table 1), and a shift towards larger size (Fig. 5). This particle size increase was more obvious between 0 and 0.5 g.L^{-1} calcium addition.

Mean size measurements were unexpected. Calcium addition from 0 to 0.5 g.L^{-1} led to an unexpected mean size reduction (Table 1) from 333 to 142 nm. According to Le Berre and Daufin (1994), caseinate at 4°C is composed of submicellar particles with sizes from 30 to 40 nm and of monomeric β-casein, measuring a few nm. Light scattering of such 40-nm-sized particles cannot result in turbid appearance. Without calcium, turbidity was caused by lipid particles > 100 nm (Dalgleish and Law, 1988b). As calcium was added, caseins formed particles. Such particles were smaller than the initial lipid particles. They contributed to almost all light scattering, because they are numerous, which explains the size reduction with calcium addtion between 0 to 0.5 g.L^{-1}.

Physicochemical environment modifications. Calcium addition led to strong mutations of membrane and particle environments. Ionic strength increased from 0.004 to 0.200 with 3 g.L^{-1} calcium addition, due to Ca^{2+} mainly in a soluble and ionic form >1.2 g.L^{-1} (90% soluble, Fig. 2) and due to Cl^-. This ionic strength increase caused the dissociation of ionizable groups, phosphoseryl and carboxyl, and this apparent pK decrease consequently led to proton release and pH reduction from 6.7 to 5.8 (Table 1). All these modifications resulted in overall negative charge reduction of casein and calcium solubilization from 0.24 to 0.06 g.L^{-1}, (Fig. 2) because of the great ionization reduction. Salt increase also led to the shielding of protein negative charges by small ions in solution, and so to repulsion reduction and aggregation. Studies cited by Olmos-Rogissart (1991) reported high flux decreases after NaCl addition. This salt effect was due to modifications of protein conformation that lead to a higher precipiation susceptibility, to easier binding between membrane and solutes, or to a membrane fouling layer of higher density. Furthermore, lowering the pH towards pH_i led to interparticular repulsion reduction, and to particle adsorption on membrane sites, and flux decrease.

Also, calcium addition resulted in its binding, about 12 mg/g of caseinate, and in a release of sodium ions (Fig. 2). Calcium binding was 0.18 g.L^{-1} (4.5 mM or 9 mEq of Ca), vs 0.18 g.L^{-1} of sodium released (7.8 mEq). Binding was greater than release probably because Na exists in a more ionized form than Ca (Pierre and Brulé, 1981).

Reduction of negative charge of casein led to unstable particles because of low interparticular repulsion-stabilizing forces. During concentration polarization, calcium-aggregated particles of low diffusion coefficient gather in the membrane vicinity, thus forming deposits on the membrane. The cake filtration model of Le Berre and Daufin (1994) proved particularly relevant.

Evolution of retention with calcium addition

The 0.1 and 0.2 μm membrane permeate compositions in the absence of calcium were similar to those reported by Murphy and Fox (1991) by ultrafiltration of 1% caseinate, by Love

(1990) by milk microfiltration or by Nau (1991) by acid casein microfiltration. Process relevance comparisons are not reliable because of different casein analytical methods employed. Terré et al. (1986) obtained β-casein concentrations of 0.6 $g.L^{-1}$ with 95% purity with a highly critical gel permeation analysis. Love (1990) did not take into account the presence of contaminants in β-casein, such as whey proteins.

The 0.5 μm membrane had no selectivity to β-casein. The relative casein composition of the 0.5 μm permeate (Fig. 7) was quite similar to that of initial caseinate, while calcium addition induced an obvious reduction in casein. This 0.5 μm membrane had a broad pore diameter, and was porous to every form of casein.

A steric retention mechanism might be expected, since β-casein content showed sharp increases with pore diameter. Solutions of individual casein presented a high tendency to form various-size aggregates, depending on concentrations, temperature, calcium contents and ionic strength. Sodium caseinate at 20°C may contain casein molecules held together by hydrophobic interactions in 30–40 nm submicellar type aggregates. After our investigations on centrifugal fractionation of casein material, we still could not determine the size distribution of casein particles in a sodium caseinate held at 4°C. Aggregates together with monomeric soluble casein (namely β-casein) were to be expected, first, because hydrophobic interactions were weakened at the low temperature and, second, because of β-casein behavior in pure solutions (Payens and van Markwijk, 1963; Schmidt, 1982). Major particles in caseinate at 4°C were observed at 11 nm. Monomeric β-casein has been found at 10 nm (Buchheim and Schmidt, 1979) or at 16 nm by Whitney (1988) according to a radius of gyration of 4.6 nm obtained by Andrews et al. (1979) cited by Whitney (1988).

Studies have been published on milk casein solubilization at low temperatures. Soluble casein is commonly determined using centrifugal techniques, pellets and supernatants representing colloidal and soluble casein. Hence, β-casein contributed to half the total solubilized casein, either in monomeric or polymeric state (Pearce 1975) and Pierre and Brulé (1981), or in submicelles (Dalgleish and Law, 1988a). Caseins other than β-casein are solubilized, which raises the possibility of a submicellar model of casein solubilization. Our study showed that in the absence of calcium, 97–98% N was soluble, no micelles being present.

β-Casein is not sensitive to calcium at 4°C, while α_s-caseins are precipitated at 0.2 $g.L^{-1}$ (Parker and Dalgleish, 1981). While a pure solution of β-casein is soluble at 4°C in the presence of calcium, Ali et al. (1980) and Pierre and Brulé (1981) showed that calcium addition to milk at 4°C resulted in a reduction of soluble β-casein. Calcium at 1 $g.L^{-1}$ led to a 50% decrease of soluble β-casein (Fig. 3) which was in agreement with results of Pierre and Brulé (1981) obtained with 0.7 $g.L^{-1}$ calcium added to milk. Calcium binds to casein, and calcium linkage leads to precipitation. Beyond 1 $g.L^{-1}$ calcium, casein, namely α_s-casein (Fig. 4), and calcium concentrations in the aqueous phase, increased. This solubilization was partly due to salting-in effects reported by Farrell et al. (1988) for α_s-casein solutions at low temperature with increasing calcium, but was mainly due to pH effect. According to Ali et al. (1980), Roefs et al. (1985) and Dalgleish and Law (1988a), casein solubility increases at acidic pH, with maximum solubility at pH 5.4. Lowering pH from 6.6 to 5.4 at 4°C results in greater solubilization of α_s-casein (Dalgleish and Law, 1988a). However α_s-casein association is by electrostatic interaction, while β-casein interacts more by hydrophobic interaction.

Hence, the content of β-casein in microfiltrates was considerably lower than that in supernatants. Less than 2 and 10% of soluble β-casein were, respectively, recovered in 0.1 and 0.2 μm filtrates. However, with a β-casein monomeric size of about 11 nm, retention levels were unexpected, based on sieving steric retention mechanisms. A more complex model of retention is required: a cake build-up as proposed by Le Berre and Daufin (1994) that imposed its own selectivity on the system.

The better recovery of α_s-casein on the 0.2 μm membrane with calcium addition was unexpected. The 0.2 μm membrane presented a high positive charge, unlike the 0.1 μm membrane which was weakly negative without calcium addition and almost neutral with calcium addition. pH values of zero point charge were estimated under rather different physicochemical conditions. However, they are reliable because ionic strength modifications have no effect on pH of zero point charge (Soria, 1993). β-Casein in a monomeric state would be weakly negatively charged with calcium addition, being the less ionized casein (pH_i around 5.2). The residual aggregates would hold a net negative charge, for example with pH_i of α_s-casein from 4.9 to 5.0. A bigger attraction between negative aggregates and positively charged 0.2 μm membrane could be expected, enhancing the protein transmission. Another explanation which, in our opinion, is more probable, comes from the physicochemical properties of caseins: β-casein has amphipathic properties, which means that hydrophilic residues such as phosphoseryl and carboxyl are located at the external surface, whereas these ionic sites are uniformly distributed for α_s-casein. Ca-binding would consequently lead to intraparticle interactions for α_s-casein-rich particles, while more interparticle bondings would be observed in β-casein-rich particles, with particle size increases. Ca addition would lead to formation of rather dense α_s-casein-rich particles of high microfiltration transmission, and to high-molecular-weight polymers of β-casein-rich particles.

k_1 and k_2 values were comparable to those of Farrell et al. (1988). Curves were either monophasic (pH, R_f/R_m) or biphasic (casein solubility) in the two groups, physicochemical or microfiltration variables. Microfiltration efficiency was less sensitive to calcium addition than the physicochemistry of suspensions. The microfiltration process was much more complicated than calcium binding or solubility decreases.

Maximum fluxes were obtained without calcium addition. A better fractionation process between soluble β-casein and aggregates lies in higher soluble-casein contents, and greater size differences between soluble and colloidal particles. Calcium addition resulted in size increases, but prevented high fluxes. Acidification of caseinate could be another way to promote casein aggregation and increase casein solubilities without lowering fluxes. Optimization of β-casein-soluble contents has been reported by Famelart et al. (1989), while a microfiltration step on a 1.4 μm membrane has been tested by Nau (1991). Optimization of solubilization, together with the microfiltration stage, is a much needed study for continuous extraction of β-casein from caseinate.

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Proteose Peptones and Physical Factors Affect Foaming Properties of Whey Protein Isolate

HAIMING ZHU and SRINIVASAN DAMODARAN

ABSTRACT

Effects of peptides and nonprotein components of whey on whey protein isolate (WPI) were studied using a differential pressure method. Decay of WPI foam followed biphasic first-order kinetics, but was affected by solution conditions. WPI foam stability exhibited two pH optima (5.0 and 8.5). Addition of 0.02–0.15M NaCl progressively decreased foaming capacity and foam stability. Addition of 0.01–0.2% proteose-peptones caused a sharp decrease in foam stability, but did not affect WPI foaming capacity. Foam stability was increased by addition of up to 20% lactose. Removal of proteose-peptones should greatly improve foaming properties of whey proteins.

Key Words: protein, peptides, foaming properties, whey protein isolate

INTRODUCTION

WHEY PROTEIN CONCENTRATES (WPC) prepared by various methods differ in functional properties. In a detailed study, de Wit et al. (1986) reported that the components of WPC varied depending on method of preparation. The WPC prepared with membrane processes had whey protein compositions similar to that of sweet whey. Those prepared with ion exchange methods showed altered ratios of major and minor whey proteins. The fat content of membrane-processed WPC was higher than that prepared by ion exchange. Several studies have shown that variability in composition of WPC caused variations in foaming properties (Richert et al., 1974; de Vilbiss et al., 1974; Richert, 1979; Morr, 1985). Liao and Mangino (1987) observed that the free sulfhydryl content and protein solubility of several WPC preparations correlated positively, whereas lipid and ash contents of the same WPC samples correlated negatively with foaming properties. Studies on effects of calcium on foaming properties of whey proteins have been very ambiguous. Some researchers reported that addition of $CaCl_2$ decreased overrun and firmness of whey protein foams (Cooney, 1974; Richert et al., 1974), whereas others showed that those properties were increased (Hansen and Black, 1972; McDonough et al., 1974).

Because of wide variations in composition of commercial WPC, results from studies on functional properties of WPC are not reliable for understanding potential uses of whey proteins in food products. Whey proteins have potential as foaming agents in foam-type food products, such as ice cream, whipped toppings, and angel food cakes. Systematic studies based on sound methodologies are needed to understand the influence of various components, such as lactose, lipids, proteose-peptones, salts, and environmental conditions on their foaming properties. Our objective in this was to conduct systematic studies on the effects of whey components on foaming properties of whey protein isolates.

MATERIALS & METHODS

COMMERCIAL WHEY PROTEIN ISOLATE (BiPRO), prepared by ion exchange process was obtained from Le Sueur Isolates Co., Le Sueur, MN. It contained 95% protein, 3.6% moisture, < 1% lactose, and < 1% ash. The WPI was 100% soluble in the concentration range studied. The protein composition of WPI was analyzed by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE) according to the method of Laemmli (1970) using 5–20% linear gradient slab gels (Fig. 1). Densitograms of SDS-PAGE of the WPI sample indicated that it contained 64% β-lactoglobulin, 27% α-lactalbumin, and 4% bovine serum albumin. Comparison of high-performance liquid chromatography (HPLC) profiles of raw Cheddar cheese whey proteins and the WPI from Le Sueur Isolates indicated that the Le Sueur WPI did not contain a significant amount of low-molecular-weight proteose-peptones (Fig. 2).

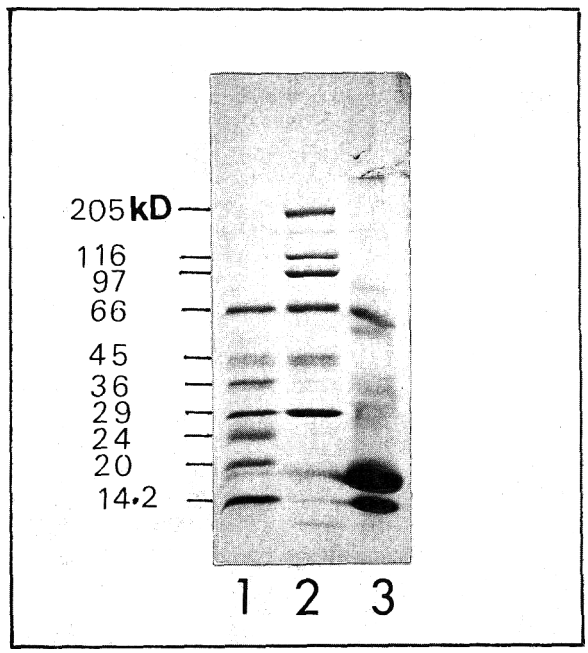

Fig. 1—SDS-PAGE of Le Sueur WPI. Electrophoresis was run under reducing conditions. Columns 1 and 2 were molecular weight markers, and column 3 was WPI dissolved in sample buffer containing 0.5M β-mercaptoethanol. Samples were heated in boiling water for 5 min prior to electrophoresis.

A proteose-peptone fraction was prepared from skim milk according to the method of Andrews and Alichanidis (1983). Protein solutions were prepared in ultra-pure water by using a Milli-Q Plus water purification system, and adjusted to pH 7.0 by addition of 0.1N HCl or NaOH. Protein concentration was measured by the biuret method. All foaming studies were performed with 5% (w/v) protein solutions.

Viscosity and surface tension

Viscosities of WPI solutions were determined using a Cannon-Fenske-type capillary viscometer immersed in a constant-temperature water bath at 25 °C. Specific viscosities of protein solutions were calculated from the relation (Bradbury, 1970):

$$\eta_{sp} = (t_s - t_o)/t_o \quad [1]$$

where t_s and t_o are flow times for protein solution and water, respectively. Surface tensions of the protein solutions were determined by the Whilhelmy plate method using an electrobalance (Cahn Instruments Co., Cerritos, CA, Xu and Damodaran, 1992).

Authors Zhu and Damodaran are affiliated with the Dept. of Food Science, Univ. of Wisconsin-Madison, 1605 Linden Drive, Madison, WI 53706.

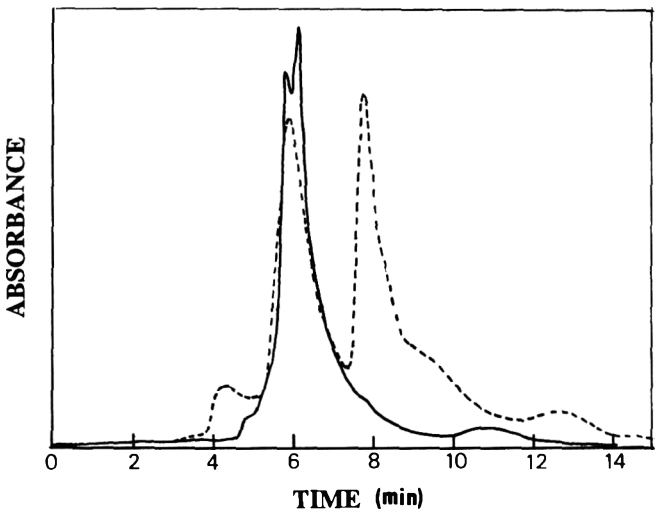

Fig. 2—HPLC profiles of a Le Sueur WPI (solid line) and microfiltered (0.2 μm) Cheddar cheese whey (broken line). Twenty μL sample solution (≈0.6% protein) was injected into a Bio-gel SEC 30XL (300 × 7.8 cm) gel permeation column (Bio-Rad, Hercules, CA) and eluted at 1 mL/min and 20 mM phosphate buffer, pH 6.8. Absorbance was monitored at 270 nm.

Kinetics of foam decay

We have developed a simple and highly reproducible method to study foaming properties of proteins (Yu and Damodaran, 1991a,b). The method is based on physical principles that govern the equation for state of spherical foam bubbles. According to the Laplace equations, that equation is:

$$P_i - P_e = 4\gamma/r \quad [2]$$

where P_i and P_e are the pressures inside and outside of the foam bubble, r is the bubble radius, and γ is the surface tension. If the foam is contained in a closed foam column, breakage of the foam would result in an increase in pressure inside the vessel. That is, the rate of breakage of a foam inside a vessel could be monitored by measuring the rate of pressure change inside the vessel. The pressure change inside the foam column can be transformed to changes in the interfacial area of the foam according to the relation (Nishioka and Ross, 1981):

$$A_t = (3V/2\gamma)(\Delta P_\infty - \Delta P_t) \quad [3]$$

where A_t is the interfacial area of the foam at time t, V is the total volume of the foam apparatus, and ΔP_t and ΔP_∞ are net pressure changes at time t and when the foam would completely collapse at infinite time, respectively.

Based on these principles, an apparatus containing a single foam column with a differential pressure transducer attached at the top was constructed. The details of the apparatus and procedures for measuring pressure change in the column as a function of time have been described (Yu and Damodaran, 1991a). The apparatus was housed in an incubator to precisely control temperature. In a typical experiment, 20 mL of 5% WPI solution was placed in the solution chamber of the apparatus and pre-equilibrated at 25 ± 0.1 °C. Pre-purified nitrogen was bubbled through the solution at 40 mL/min until the foam rose to a pre-marked point near the top of the column. The time to raise the foam was recorded. All valves of the apparatus were closed, and the change in pressure of the head space in the column was continuously recorded. When the rate of pressure change approached a plateau, an antifoaming agent stored in a bent side tube of the apparatus was dropped into the foam column to completely collapse the foam. The final pressure change (ΔP_∞) was recorded. Each experiment was done at least in triplicate.

A dimensionless fractional interfacial area at any given time during foam decay was calculated by using the relation (Yu and Damodaran, 1991a):

$$A_t/A_o = (\Delta P_\infty - \Delta P_t)/\Delta P_\infty \quad [4]$$

where A_o is the initial interfacial area of the foam, which is given by (Yu and Damodaran, 1991a):

$$A_o = 3V\Delta P_\infty/2\gamma \quad [5]$$

The decay of WPI foams was analyzed according to the biphasic first-order equation (Yu and Damodaran, 1991a):

$$A_t/A_o = Q_g \exp(-k_g t) + Q_d \exp(-k_d t) \quad [6]$$

where k_g and k_d are first-order rate constants for decay due to gravitational liquid drainage and inter-bubble gas diffusion, respectively, and Q_g and Q_d are amplitude parameters of the two kinetic phases.

Since A_o is related to the foamability of the protein, the foaming activity index (FAI) of the protein, defined as the interfacial area per unit volume of foam, was developed from the relation:

$$FAI = A_o/\upsilon \quad [7]$$

where υ is the total volume of foam at t=0.

RESULTS & DISCUSSION

Effect of pH

β-Lactoglobulin, the major component of WPI, undergoes pH-dependent association-dissociation reactions (Swaisgood, 1986). In the pH range 3.5–5.2, β-lactoglobulin exists predominantly as an octomer; at pH 5.2 ≤ 7.0 it predominantly exists as a dimer; and > pH 7.0 the dimer dissociates to the monomer. To determine whether such complex equilibria of β-lactoglobulin affect foaming properties of WPI, the foaming capacity and foam stability were studied in the pH range 3 to 9.

The kinetics of WPI foam decay at various pHs were compared (Fig. 3). Effects of pH on gravitational rate constant k_g and gas diffusional rate constant k_d were also compared (Fig. 4). The WPI was very unstable at pH 3.0, and the foam collapsed rapidly within 10 min. As pH was increased the stability of WPI foam sharply increased and reached maximum at pH 5.0. As pH was increased further, foam stability decreased in the pH range 5.0 to 7.0, and then increased again between pH 7.0 and 8.5. The foam stability at pH 8.5 was comparable to that at pH 5.0.

The stability of WPI foam exhibited two pH optima. One was at pH 4.5- –5.0, near the isoelectric pH of major whey proteins, and the other was at pH 8.5. Several studies of other globular proteins have shown that the stabilities of protein-stabilized foams were usually maximum at or near the isoelectric point when there was no loss of solubility (Buckingham, 1970; Mita et al., 1978; Yu and Damodaran, 1991a). At the isoelectric pH, absence of electrostatic repulsion promotes cohesive interactions which result in improvement of viscoelastic and mechanical properties of the protein film (Yu and Damodaran, 1991a). Furthermore, the lack of electrostatic repulsion between adsorbed protein molecules and those approaching the interface also enhances surface coverage and formation of multilayers. This increases viscosity and improves other rheological properties (Damodaran, 1990). The high viscosity of the film retards drainage of lamella fluid (Mita et al., 1978). Apart from the influence of electrostatic forces on film stability, the dimer⇄octomer equilibrium of β-lactoglobulin at pH 5.0 also may influence viscoeleastic properties.

The stability of WPI foam at alkaline pH 8.5 must come predominantly from conformational changes in β-lactoglobulin at that pH. The β-lactoglobulin dimer dissociates to the monomer at pH > 7.0. This dissociation is preceded by reversible conformational changes and ionization of an abnormal carboxyl group at pH 7.5 (Tanford and Nozaki, 1959; Timasheff et al., 1966; Basch and Timasheff, 1967). In addition, exposure and ionization of the single thiol group in β- lactoglobulin increases its reactivity. This results in rapid denaturation and polymerization at pH 8.3, presumably via intermolecular di sulfide-sulfhydryl interchange reactions. The observed improvement in stability of WPI foam at pH 8.5 might be attributable to conformational transition in β-lactoglobulin and subsequent disulfide-sulfhydryl interchange reactions at the interface.

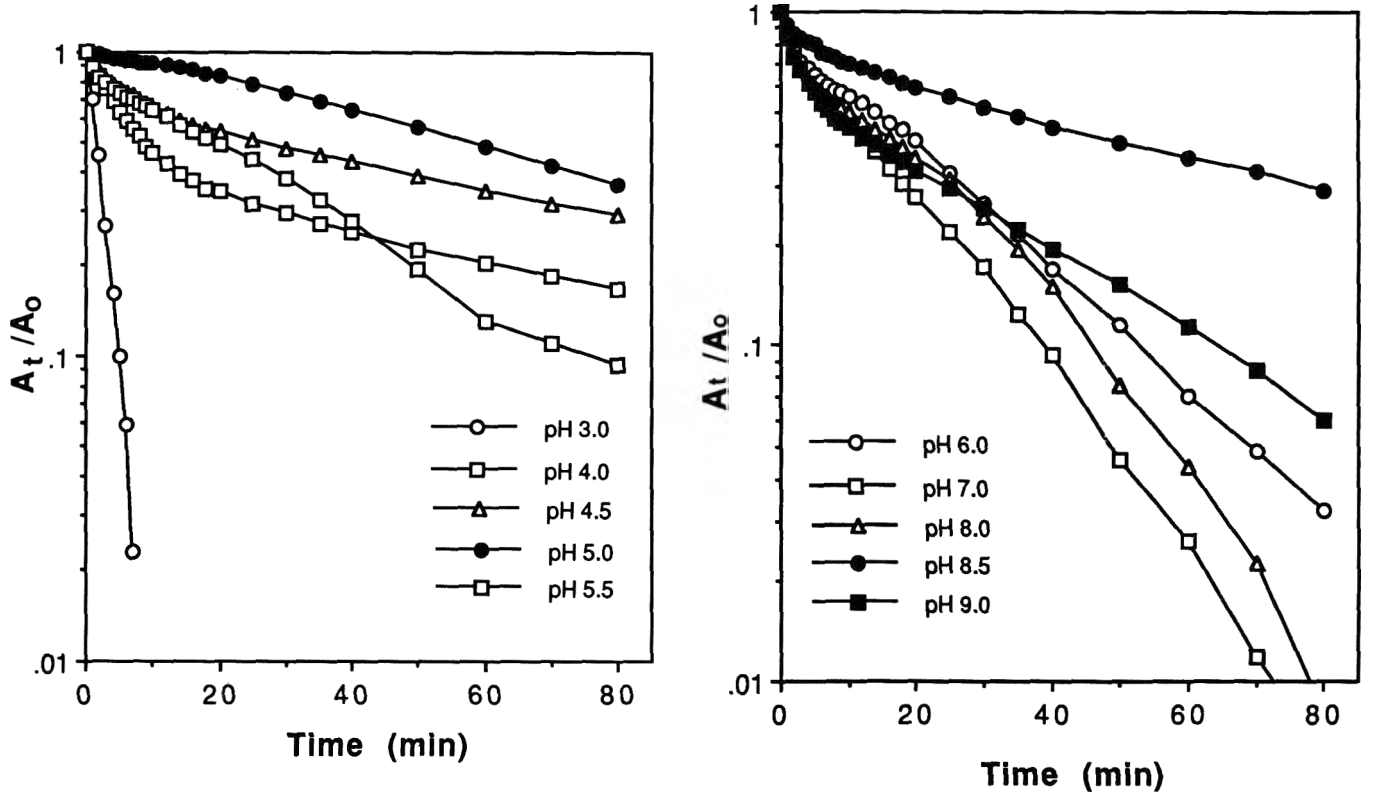

Fig. 3—Effect of pH on interfacial area decay of WPI foams at 25 °C. Continuous decay curves. Data points do not represent individual measurements as a function of time. Symbols provided to distinguish curves (averages of triplicates). Average standard error of A_t/A_o for all curves was 1.73×10^{-3}. Protein concentration was 5% (w/v).

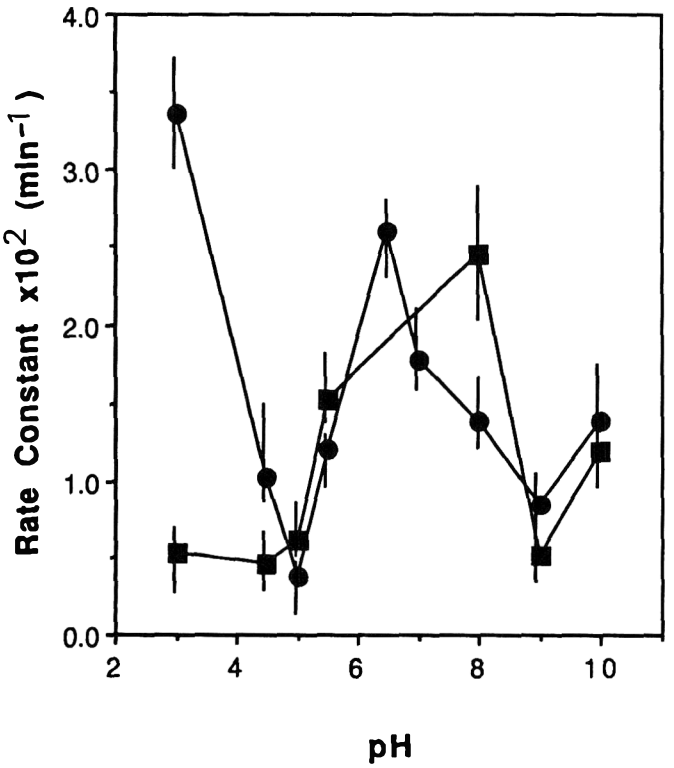

Fig. 4—Effect of pH on the rate constants of foam decay due to gravitational liquid drainage, k_g, (●—●), and interbubble gas diffusion, k_d (■—■). Error bars represent standard error.

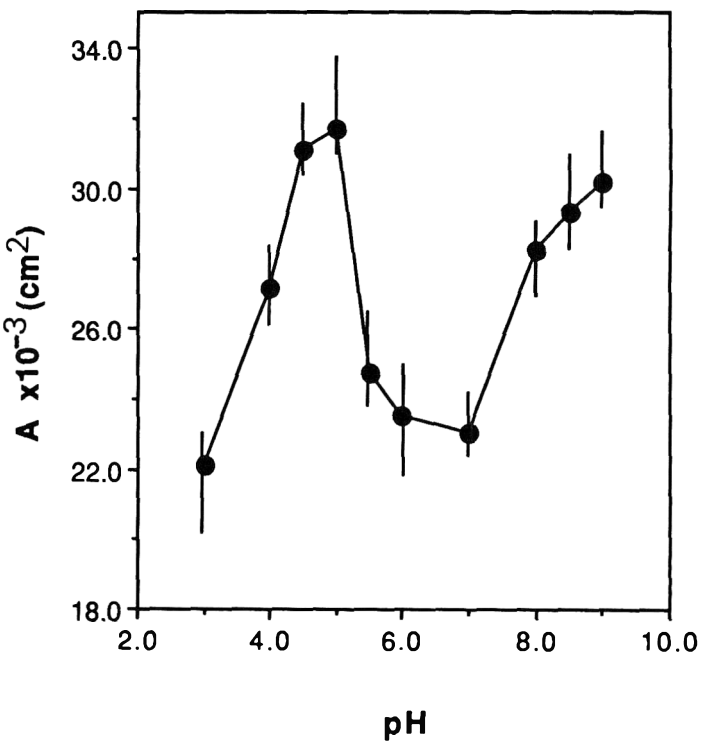

Fig. 5—Effect of pH on initial interfacial area, A_o, of WPI foam. Total volume of foam generated was 67 mL at 25 °C. Bars represent standard error.

The effects of pH on initial interfacial area (A_o) of WPI foams (67 mL) (Fig. 5) and on the time to form 67 mL of foam at a gas flow rate of 40 mL/min (Table 1) were compared. The A_o increased as pH increased from 3.0, and reached a maximum at pH 5.0; whereas the foaming time decreased to a minimum at pH 5.0. At pH 5.0, the A_o decreased rapidly and reached a minimum at pH 7.0. Foaming time increased and

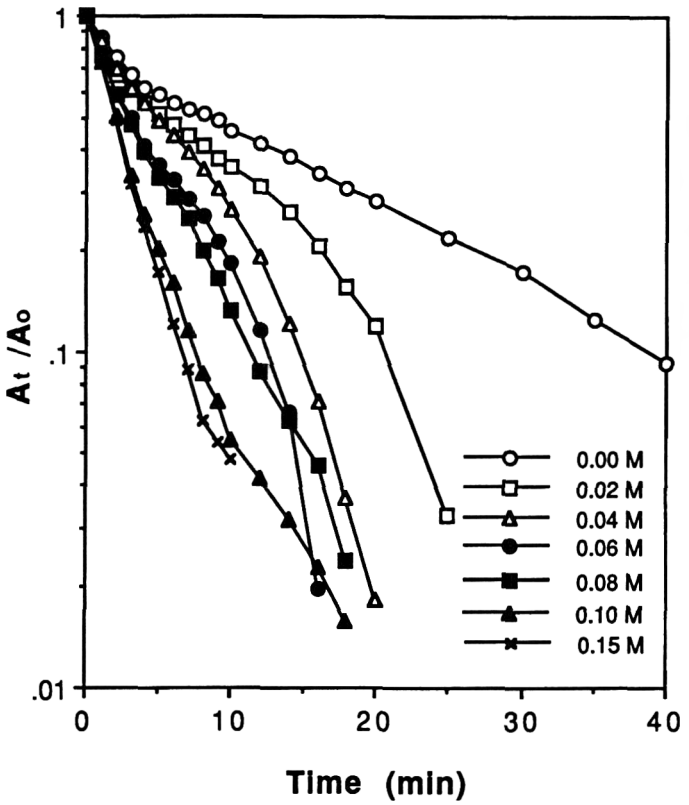

Fig. 6—Effect of NaCl concentration on interfacial area decay of WPI foams at 25 °C, pH 7.0. (Averages of triplicates.) Average standard error of A_t/A_o for all curves was 1.61×10^{-2}.

Table 1—Effect of pH on time required to form 67 mL of WPI foam

pH	Foaming time (sec)
3.0	199.2 ± 2.8*
4.0	110.7 ± 1.4
4.5	85.0 ± 2.1
5.0	77.4 ± 3.0
5.5	87.0 ± 2.4
6.0	90.5 ± 3.3
7.0	108.7 ± 1.8
8.0	93.0 ± 2.0
8.5	82.9 ± 4.3
9.0	89.8 ± 1.2

* Standard error based on triplicate measurements.

reached a maximum at pH 7.0. At pH > 7.0, increasing the pH resulted in increases in A_o up to pH 9.0, whereas foaming time decreased up to pH 8.5 and exhibited a slight increase at pH 9.0. These data clearly indicate that foamability was better at pH 5.0 and at 8.5 than at any other pH. The volume of foam generated was the same at all pHs. The higher A_o value at pH 5.0 and at 8.5 indicates that the average size of bubbles in the foam was smaller at those pH values than at any other pH. The short foaming times at pH 5.0 and 8.5 to create foams with smaller bubble sizes and larger interfacial areas indicate that rates of adsorption, molecular rearrangement, and film formation were faster at pH 5.0 and 8.5.

The changes in A_o, foaming time, k_g, and k_d with changes in pH were congruous indicating that both foam stability as well as foamability of WPI were maximum at pH 5.0 and at pH 8.5. The mechanisms responsible for improvement in foaming properties at pH 5 and at pH 8.5, however, might be quite different. At pH 5.0, the lack of electrostatic repulsion between protein molecules and between adsorbed molecules and those approaching the film might facilitate rapid adsorption and formation of a stable viscous film. In addition, the octomeric form of β-lactoglobulin at pH 5.0 might also contribute to high viscoelasticity of the film. However, at pH 8.5,

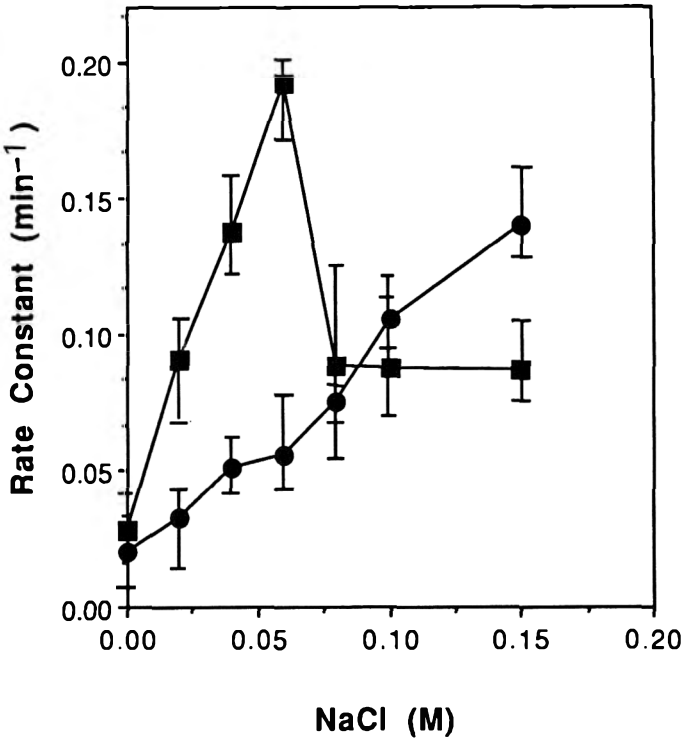

Fig. 7—Effect of NaCl concentration on rate constants of foam decay due to gravitational liquid drainage, k_g, (●●), and inter-bubble gas diffusion, k_d (■■). Bars represent standard error.

dissociation, conformational change, and polymerization of β-lactoglobulin via disulfide-sulfhydryl interchange are likely responsible for formation of stable films.

Effect of ionic strength

The effect of NaCl concentration on kinetics of decay of WPI foam was studied in (Fig. 6). Foam stability decreased with increasing NaCl concentration up to 0.1M. This indicated that, even at very low ionic strength, (0.02M) partial screening of charges on whey proteins decreased stability of WPI foam. Note that up to 0.06M NaCl, decay curves were distinctly convex in shape; above 0.06M NaCl, however, the curves were concave (Fig. 6). The convex shape implied that decay due to gravitational drainage was rate-limiting, and the concave shape indicated the decay due to inter-bubble gas diffusion was rate-limiting (Yu and Damodaran, 1991a). The transformation from convex to concave-type with salt concentration clearly indicated that changes in electrostatic free energy induce fundamental changes in rheological properties. In relative terms, the type of changes that occurred in physical properties at 0 to 0.06M NaCl greatly affected gas permeability of the film. Above 0.06M NaCl, the factors promoting liquid drainage were affected to a greater extent. The relationship between ionic strength and k_g and k_d were compared (Fig. 7). k_d increased sharply as salt concentration increased up to 0.06 M, and decreased sharply at 0.08M and remained unchanged thereafter. This indicated a fundamental change in physical properties of the film occurred at 0.06–0.08M NaCl concentration. In contrast, k_g increased progressively as salt concentration increased.

The effect of NaCl concentration on A_o of WPI foam (Fig. 8) showed the foamability of WPI also decreased with increased salt concentration. However, the decrease was bimodal. That is, A_o decreased rapidly up to about 0.04M NaCl, did not change much between 0.04–0.08M NaCl, and rapidly decreased above 0.08M, up to 0.15M NaCl. Note that the transition from convex to concave-type decay in foam stability

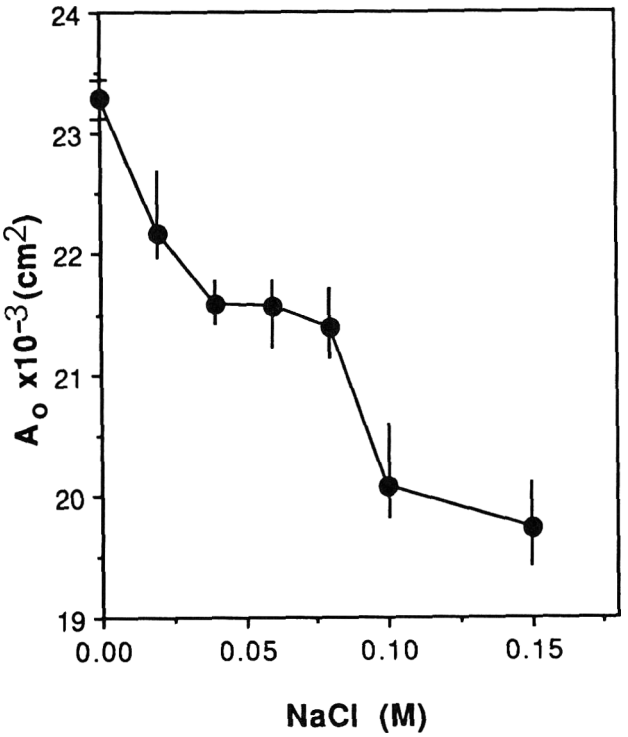

Fig. 8—Effect of NaCl concentration on initial interfacial area, A_o, of WPI foam. Total volume of foam generated was 67 mL at 25 °C. Bars represent standard errors.

(Fig. 6) at 0.06M NaCl coincided with the transition in A_o vs salt concentration (Fig. 8). This further confirmed that, at 0.06 ≤ 0.08 M NaCl concentration, the protein film has a critical change in physical properties, which further affects foamability and relative rates of decay from gravitational drainage and gas diffusion. The fundamental nature of this change, however, is difficult to ascertain.

The stability of soy protein isolate foam reportedly increased with increasing NaCl up to 0.1M (Yu and Damodaran, 1991b). Foaming studies of other globular proteins, such as bovine serum albumin, egg albumin, and gluten, also have shown that addition of NaCl improved foamability and foam stability (Halling, 1981; Poole et al., 1984). This was usually attributed to a decrease in electrostatic repulsive interactions which promotes increased protein adsorption at the interface and formation of a viscous and cohesive film via noncovalent interactions. These include van der Waals, hydrophobic, and hydrogen bonding interactions. However, the behavior of WPI foam in the presence of NaCl apparently did not follow such a simple mechanism. Cooney (1974) reported a decrease in stability of whey protein concentrate with increasing NaCl concentration and attributed it to a decrease in mutual repulsion of electrical double layers between adsorbed protein films of the lamellae, which might promote film-film interactions. This is not logical, because if true then the same situation should also be operative with other protein foams. Note that, while addition of H^+ ions to decrease electrostatic free energy (i.e., at pI) caused an increase in foaming properties of WPI (Fig. 3), addition of NaCl to lower electrostatic free energy (at pH 6.85) had the opposite effect. If electrostatics were the underlying mechanism then effects also should be similar. This contraindication suggests that adverse effects of NaCl on foaming properties may not be due to electrostatic, but more due to ionic-specific "salting-in" effects of NaCl on whey proteins. These proteins, especially β-lactoglobulin, are extremely salt-soluble, and β-lactoglobulin has a 100-fold increase in solubility when the salt concentration is increased from 0 to 0.1M (Edsall and Wyman, 1958). Even at high NaCl concentrations, β-lactoglobulin remains soluble at neutral pH. This is attributed to its unique charge distribution on the surface, which facilitates strong preferential interaction with NaCl (Arakawa and Timasheff, 1987). Since the WPI we used was extremely soluble in the salt concentration range studied, we suggest that the adverse effects of NaCl on foaming properties might have arisen from its salting-in effects especially on β-lactoglobulins. Because of increased solubility in NaCl solutions, whey proteins may not readily adsorb at the air-water interface during bubbling. This would result in formation of large foam bubbles with less interfacial area per unit volume of foam. In addition, once the foam was formed, the whey proteins at the bubble interface might be salted-in by the lamella fluid, resulting in destabilization of the film and rapid collapse of the foam.

Effect of proteose-peptones

Proteose-peptone fragments, formed by hydrolysis of β-casein by plasmin, account for about 4% of milk proteins (Swaisgood, 1986). In raw sweet whey and in whey protein concentrates, the proteose-peptone content is about 20% on protein basis (Marshall, 1986). Volpe and Zabik (1975) reported that incorporation of whey proteins in bread depressed loaf volume. This was attributed to the proteose-peptone component in the whey protein concentrate. Phillips et al. (1987) reported that addition of proteose-peptones depressed the overrun of egg-white foams. Later, however, they reported that proteose-peptones did not act as foam depressants with egg-white foams, but increased the overrun (Phillips et al., 1989a). To resolve such contradictory reports we studied the effect of proteose-peptones on foaming properties of WPI. Since the WPI we used apparently did not contain important amounts of proteose-peptones, we could systematically study the effect of proteose-peptones on foaming properties.

The effects of proteose-peptones (0.01 to 0.2%) on stability of WPI (5%) foams were compared (Fig. 9). The levels of proteose-peptones represented 0.2 to 4% (protein weight basis), much less than amounts normally found in whey protein concentrates. Foam stability progressively decreased with increasing proteose-peptones concentrations (Fig. 9). However, the A_o of the foams (67 mL) was not affected by addition of proteose-peptones (Table 2). The data indicated that proteose-peptones affected foam stability, but not foamability of WPI. The depression of loaf volume by proteose-peptones reported by Volpe and Zabik (1975) might have been a result of reduced foam stability during baking. Such reduction might be attributed to competitive adsorption of proteose-peptones with other whey proteins. Proteose-peptones, being low-molecular-weight and random-coil peptides, may adsorb faster and also may partly displace adsorbed β-lactoglobulin and α-lactalbumin from the interface. This could prevent formation of a cohesive viscous film and contribute to rapid foam decay. The time to create 67 mL of foam progressively increased with increasing proteose-peptone content (Table 2). This indicated that foams formed in the presence of proteose-peptones were relatively unstable and disintegrated during foam formation. The adverse effects of proteose-peptones on the foaming properties of WPI apparently are related to its inability to form a cohesive viscoelastic film along with other whey proteins, but not due to its inability to adsorb and reorient at the interface.

Effect of lactose

A 35% protein WPC typically contains about 55% lactose. Incorporation of WPC at 10% protein level in foam-type food products would result in addition of about 16% lactose. Several studies have reported that addition of sucrose before whipping caused a decrease in overrun of WPC and β-lactoglobulin

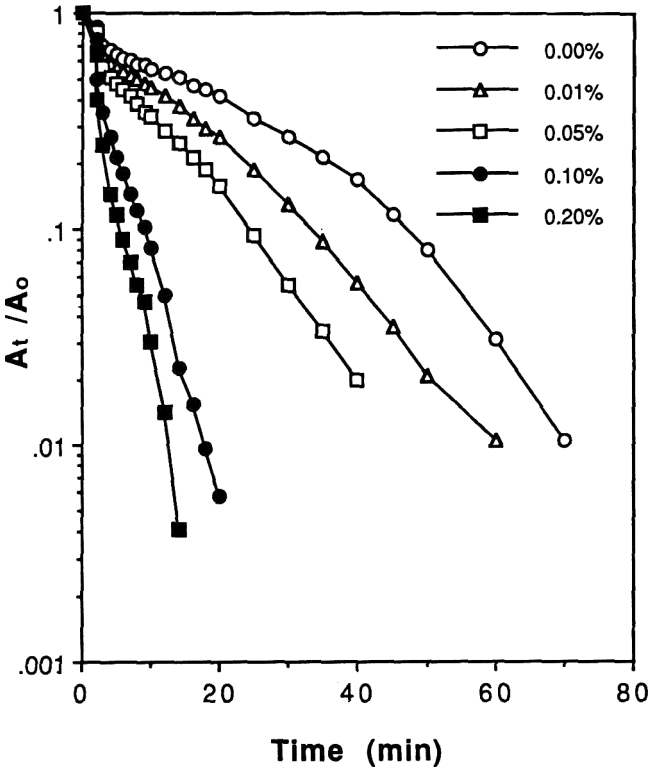

Fig. 9—Effect of proteose-peptones concentration (w/v) on interfacial area decay of WPI foams at 25 °C, pH 7.0. (WPI concentration 5%; Averages of triplicates.) Average standard error of A_t/A_o for all curves was 5.44×10^{-3}.

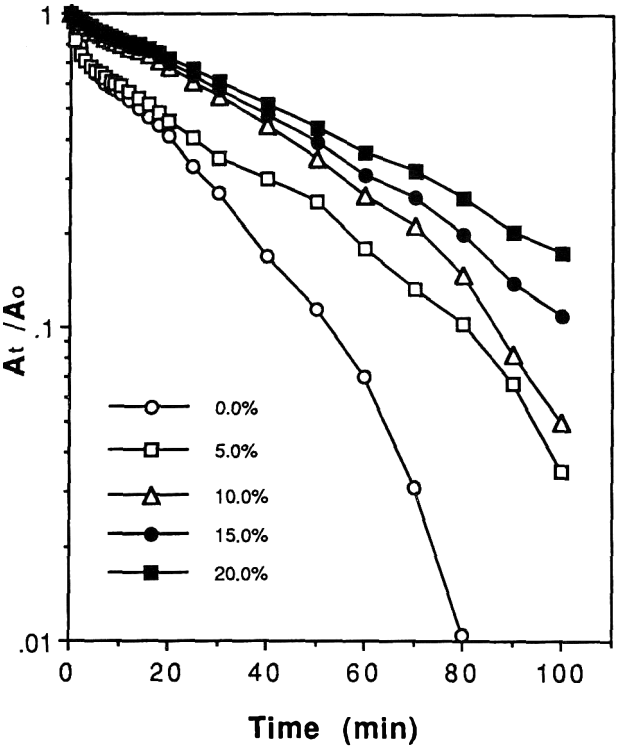

Fig. 10—Effect of lactose concentration (w/v) on interfacial area decay of WPI foams at 25 °C, pH 7.0. (Protein concentration 5%; (Averages of triplicates.) Average standard error of A_t/A_o for all curves was 4.08×10^{-3}.

Table 2—Effect of proteose-peptones concentration on initial interfacial area (A_o) and foaming time of WPI foams[a]

Proteose-peptones[b] (%)	$A_o \times 10^{-3}$ (cm^2)	Foaming time (sec)
0.0	23.78 ± 0.45[c]	113.0 ± 3.8[c]
0.01	21.729 ± 0.19	131.5 ± 4.5
0.05	22.717 ± 0.58	146.3 ± 4.8
0.10	22.792 ± 0.50	160.0 ± 3.1
0.20	22.681 ± 0.60	164.7 ± 6.2

[a] Total volume of the foam was 67 mL.
[b] On w/v basis.
[c] ± Standard error based on triplicate measurements.

foams, but sometimes resulted in an increase in foam stability (Hansen and Black, 1972; de Vilbiss et al., 1974; Haggett, 1976; Phillips et al., 1989b). At 0–20% lactose concentration, foam stability increased gradually with increased lactose (Fig. 10). As lactose concentration increased, the decay curve transformed from convex-biphasic first-order to monophasic first-order behavior. The relationships between specific viscosity of WPI solutions and the decay rate constant were compared with respect to lactose concentration (Fig. 11). Specific viscosity of the protein solution increased and decay rate constant (k_g) decreased with increasing lactose concentrations. This indicated that the improvement in stability of WPI foam was directly related to the increasing viscosity of the solution. In contrast to foam stability, the foamability of WPI was not affected by addition of ≤ 20% lactose (Table 3). The A_o of the foam slightly decreased at 5%, but values at higher lactose concentrations were similar to controls. These results were in variance with those reported for effects of sucrose on overrun of whey protein foams (Hansen and Black, 1972; de Vilbiss et al., 1974; Haggett, 1976; Phillips et al., 1989b). A probable reason for the difference is the source of whey proteins, i.e., WPC we used, instead of whey protein isolates used in the other studies; or the method of preparation of the foam, i.e., whipping vs bubbling methods.

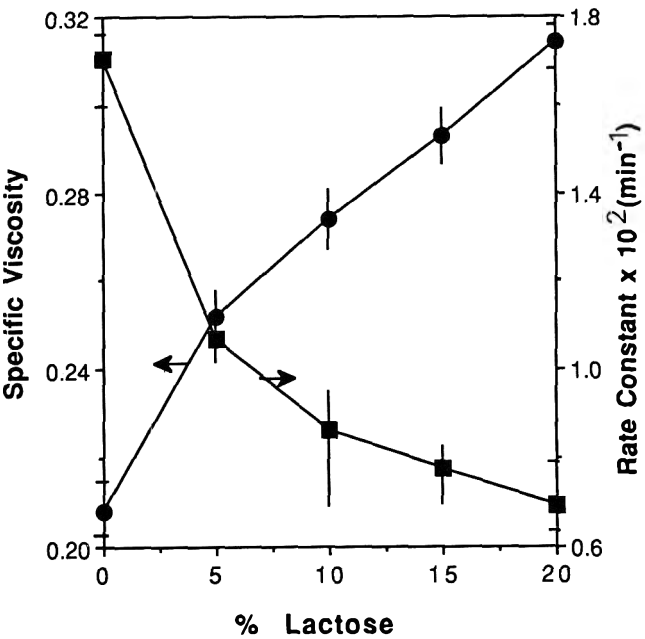

Fig. 11—Relationship between decay rate constant, k_g (■), of WPI foams, specific viscosities (●) of WPI solutions (5%) and lactose concentration. Bars represent standard error.

CONCLUSIONS

PEPTIDES AND NONPROTEIN components of whey affect foaming properties of whey protein isolate. Proteose peptones as low as 0.01–0.2% w/v (0.2–4% protein weight basis) decreased the stability of WPI foams. Processing methods that remove both milk fat globule membranes and proteose peptones would effectively improve foaming properties of whey protein concentrates and isolates.

Table 3—Effect of lactose concentration on initial interfacial area (A_o) of WPI foam[a]

Lactose (%)	$A_o \times 10^{-3}$ (cm^2)
0.0	23.2 ± 1.9[b]
5.0	20.5 ± 4.2
10.0	22.6 ± 1.4
15.0	23.2 ± 2.3
20.0	22.7 ± 5.1

[a] Total volume of foam was 67 mL.
[b] ± Standard error based on triplicate measurements.

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Oxidative Stability and Sensory Quality of Stored Eggs From Hens Fed 1.5% Menhaden Oil

A.C. MARSHALL, A.R. SAMS, and M.E. VAN ELSWYK

ABSTRACT

Effects of refrigerated storage on thiobarbituric acid (TBA) values and sensory scores of shell eggs from hens fed 1.5% menhaden oil (MO) and a no-added fat control diet were investigated. Eggs were collected weekly (0, 1, 2, 3, 4) for each of three replicates/diet and all weeks were analyzed within 24 hr of 0 week. The flavor of scrambled samples from stored shell eggs (wk 0, 2, 4) was determined by scoring difference scale administered to two consumer panels (n = 40/panel). Flavor scores for eggs from all treatments were not different. TBA values were greater ($P < 0.05$) in MO eggs at week 0 but did not increase during storage. Storage stability of shell eggs from hens fed 1.5% dietary menhaden oil is comparable to that from hens fed a no-added fat diet.

Key Words: TBA, storage, egg, menhaden oil, flavor

INTRODUCTION

EFFORTS BY THE POULTRY INDUSTRY to market health-value-added products have stimulated research concerning the fatty acid modification of poultry meat and eggs (Van Elswyk, 1993). Some such efforts have focused specifically on increasing the very long chain polyunsaturated fatty acid (VLCPUFA) content of poultry meat and eggs (Yu and Sim, 1987; Ajuyah et al., 1993). The VLCPUFA found predominantly in fish and fish oil, (n-3 fatty acids) have been associated with reduced risk for coronary heart disease (Harris, 1989; Nettleton, 1991; Kromhout, 1992). Adults have been recommended to consume at least one to two 100 g servings of fish per week to increase dietary availability of potentially healthful fatty acids (Kromhout, 1992). The addition of marine sources of VLCPUFA, such as menhaden oil, to broiler and layer rations has resulted in the incorporation of those fatty acids into poultry meat and eggs (Yu and Sim, 1987; Adams et al., 1989; Van Elswyk et al., 1992a; Ajuyah et al., 1993). The production of VLCPUFA-enriched poultry products would increase the dietary sources of such fatty acids.

The double bonds of VLCPUFA are particularly susceptible to oxidative deterioration (Frankel, 1984). Previous investigators have demonstrated that native egg yolk lipids were inherently resistent to oxidative deterioration (Pike and Peng, 1988a). However, the typical shell egg yolk contains only 1.4% VLCPUFA (Burley and Vadehra, 1989). Yolks of eggs from hens fed menhaden oil can contain up to 5% VLCPUFA as n-3 fatty acids (Hargis et al., 1991). This change in yolk VLCPUFA content may lower the stability of egg yolk lipids. Enhanced susceptibility of VLCPUFA-enriched shell eggs to oxidative breakdown may indicate a need to alter storage conditions. Due to their oxidative susceptibility, the potential for development of off-flavors during storage may exist. Therefore, the objectives of our current study were to determine the oxidative stability (as TBA) and off-flavor (as sensory score), during storage of shell eggs from hens fed menhaden oil (MO) as compared to shell eggs from hens fed a "no-added fat" layer ration.

The authors are affiliated with the Dept. of Poultry Science and Faculties of Food Science & Technology and Nutrition, Texas Agricultural Experiment Station and Texas A & M Univ., College Station, TX 77843-2472.

MATERIALS & METHODS

Animals and diets

One hundred fifty (n = 50/replication) 65-wk-old Single Comb White Leghorn hens were fed a layer ration containing 1.5% feed grade menhaden oil (Table 1) stabilized with 500 ppm ethoxyquin by the supplier (Zapata Haynie, Reedville, VA). Another 150 hens (n = 50 hens/replication) were maintained on a corn and soybean-based layer ration containing no added fat. Hen diets were formulated to meet the National Research Council requirements for nutrient and energy content for laying hens (SPN, 1984) and were isonitrogenous (Hargis et al., 1991). Diets were prepared bimonthly and stored in airtight containers at −20°C. Hens were fed *ad libitum* daily.

Egg sample collection and storage design

Egg sample collection commenced after 4 wk of feeding the dietary treatments since previous studies indicated that yolk VLCPUFA incorporation leveled off following 4 wk of feeding (Hargis et al., 1991). Egg sample collections for each of the three replications/dietary treatment were staggered 1 wk to facilitate analysis of all storage lengths from each replication within 24 hr of the baseline collection. Eggs from each replication (n = 30/replication) were collected on the same day each week for 5 wk and stored. Eggs were not commercially processed prior to storage and were held in corrugated egg flats at 4°C and 96% relative humidity until analyzed. Lengths of storage times were chosen in an effort to simulate potential duration of storage of shell eggs by consumers.

Yolk fatty acid analysis

For determination of fatty acid composition of shell eggs, pools of six yolks from each replication, dietary treatment, and storage length were utilized. Prior to breaking out yolks, whole eggs were weighed and weights recorded. Yolks used in pools were broken out and adhering albumen removed with a paper towel. Yolks were weighed, pooled, and mixed with a wire whisk. Four 1-g aliquots were taken from each pool and fat was extracted with chloroform:methanol (2:1, v/v) (Folch et al , 1957). Extracted samples were saponified, methyl esters were prepared, and fatty acids were quantitated using gas chromatography (Van Elswyk et al., 1992a). Fatty acid peaks were identified by comparison of retention times of fatty acid methyl ester standards (AOCS mixture #6, Alltech, Deerfield, IL, plus the addition of 20:5 n-3, 22:6 n-3, Nu-Chek-Prep, Elysian, MN) and areas quantified by comparison with an internal standard (C13:0, Alltech, Deerfield, IL) using the Expert Chromatography System (version 4.0, Waters, Millford, MA).

Egg yolk pH and thiobarbituric acid numbers (TBA)

Duplicate pH values were determined from each yolk pool using a Corning pH Meter 245 (Corning, Corning, NY). Determination of yolk lipid oxidation products was conducted according to the method of Tarladgis et al. (1960), as modified by Rhee (1978), for quantitation of malonaldehyde and other thiobarbituric acid reactive substances (TBARS). Four dispersions were prepared from each egg yolk pool using 20g yolk, 30 mL double distilled water, and 10 mL .5% propyl gallate/ethyleneciamine-tetraacetic acid solution (Rhee, 1978). Each sample was prepared by combining 30g dispersion with 78 mL double distilled water and 2.5 mL 4N HCl. Samples were distilled using a Kjeldahl distillation apparatus (Labconco Corp., Kansas City, MO) and

Table 1—Polyunsaturated fatty acid composition of menhaden oil[a]

Fatty acid[b]	%
Linoleic (18:2n-6)	1.15
Linolenic (18:3n-3)	1.06
Arachidonic (20:4n-6)	1.67
EPA (20:5n-3)	13.48
DPA (22:5n-3)	2.08
DHA (22:6n-3)	9.08

[a] Polyunsaturated referring to n-3 and n-6 fatty acid classes.
[b] EPA = eicosapentaenoic acid; DPA = docosapentaenoic acid; DHA = docosahexaenoic acid.

Table 2—Effect of dietary menhaden oil on yolk fatty acid composition[a]

Fatty acid	Dietary treatment	
	Control	Menhaden Oil
	(mg/yolk)	
16:0	1392 ± 52	1247 ± 28*
16:1n-9	211 ± 8	206 ± 5
18:0	470 ± 23	398 ± 16*
18:1n-9	2394 ± 154	2162 ± 71
18:2n-6	577 ± 56	540 ± 32
18:3n-3	13 ± .8	18 ± 1.5*
20:4n-6	99 ± 6	35 ± 2*
20:5n-3	<1	16 ± 2*
22:6n-3	20 ± 1	106 ± 6*

[a] Values are means ± SEM summarized throughout the experiment and differ significantly ($P < 0.05$) due to diet as indicated by asterisk.

50 mL distillate were collected from each sample. Duplicate 4 mL aliquots of each distillate were combined with 4 mL of 2-thiobarbituric acid in 90% glacial acetic acid and boiled 35 min. Duplicate 1 mL aliquots were taken from each sample of boiled solutions and optical densities determined at 538 nm on a Beckman spectrophotometer (Beckman Instruments, Fullerton, CA). Optical densities of sample solutions were compared to a 1,1,3,3,-tetra-ethoxypropane standard curve and multiplied by 7.8 to correct for incomplete recovery of malonaldehyde and other TBARS during distillation (Tarladgis et al., 1960). The correction factor was determined for the specific apparatus used as described by Tarladgis et al. (1960). Results were reported as TBA numbers representing mg TBARS/kg yolk.

Flavor evaluation

Flavor evaluation was completed using scrambled egg samples from both dietary treatments at baseline, wk 2, and wk 4 storage time. Two of the three replicates from each dietary treatment were used for the consumer sensory panel on two different dates, with a total of 40 untrained consumers participating in each panel replicate. Eggs were equilibrated from 4°C to room temperature (≈23°C) for about 3 hr prior to mixing. Three eggs/dietary treatment/storage length were pooled and thoroughly mixed using a wire whisk. All eggs were scrambled in a nonstick saute pan on a gas stove using a medium flame to a final internal temperature of 75°C. Fresh samples were prepared at 15 min intervals during evaluation. Bite-sized pieces (2×2×1 cm) were then cut from each warm scrambled egg sample for presentation to panelists. A scoring sheet was used to evaluate flavor. Panelists were asked to evaluate each coded sample individually, choosing the statement that best matched their evaluation of sample flavor. Choices included "inedible, very poor," "poor," "fair," "good," "very good," "ideal, excellent." In each sitting, participants were presented with six successive warm scrambled egg samples, each on a separate plate and independent of each other. Samples were served under red lighting to eliminate color discrepancies. Consumers were given distilled water and crackers to cleanse their palates between samples. Afterwards, consumer flavor evaluations were assigned numerical codes, 1 representing "excellent, ideal" and 6 representing "inedible, poor," for data entry and statistical analysis.

Statistics

Yolk fatty acid content, whole egg and yolk weights, yolk pH measurements, yolk lipid oxidation products, and egg flavor scores were analyzed using one-way analysis of variance (SAS Institute, Inc., 1992) with replication, storage length, and dietary treatment as main effects. Egg yolk pH measurements were transformed into hydronium ion concentration prior to analysis. Results were reported as pH. In all analyses, the interaction of replication and dietary treatment was not significant for any parameter, therefore replications within dietary treatments were combined. Significantly different treatment means were further separated using Duncan's Multiple Range test (SAS Institute, Inc., 1992). Significance was reported at $P < 0.05$.

RESULTS & DISCUSSION

NO EFFECTS WERE FOUND for storage length on shell egg weight (data not shown). Typically, shell egg weights decrease during storage due to carbon dioxide and water losses (Stadelman, 1990). In our study, eggs from hens fed MO maintained an average weight of 64.8 ± 0.4 g during storage, those from hens fed the control diet averaged 65.2 ± 0.4 g. The high relative humidity, 96%, of the refrigerated storage area most likely contributed to the maintenance of shell egg weight during storage. A dietary treatment effect was noted, however, as yolk weights of eggs from hens fed 1.5% menhaden oil were significantly lower (18.9 ± 0.2) than those of controls (19.5 ± 0.2). Previous reports have noted a 1–2g decrease in yolk size in response to feeding 3% menhaden oil, and this effect has been attributed to the influence of dietary VLCPUFA on hen liver lipid metabolism but has not been associated with a change in percentage of yolk that is fat (Van Elswyk et al., 1991, 1992b). As expected, yolk pH increased during storage. No consistent effects of diet, however, were noted. The pH of yolks from the baseline collection was 5.9, following 4 wk storage the yolk pH was 6.4. Typically, yolk pH gradually increases from 6.0 in fresh shell eggs to a range between 6.4 and 6.9 during storage (Powrie and Nakai, 1990). No evidence has suggested that increased pH values would have any impact on lipid oxidation state. Only low pH values have been associated with a significant increase in yolk lipid oxidation (Pike and Peng, 1988b).

Consistent with previous results obtained by this laboratory, dietary MO dramatically altered yolk fatty acid composition (Table 2). The VLCPUFA linolenic acid (LNA, 18:3n-3), eicosapentaenoic acid (EPA, 20:5n-3), and docosahexaenoic acid (DHA, 22:6n-3) increased. Linolenic acid increased twofold in eggs from hens fed 1.5% menhaden oil. No detectable levels of EPA were present in control eggs while those from hens fed menhaden oil contained 16 mg. A 6-fold increase in DHA was noted in eggs from hens fed menhaden oil as compared to those of control fed hens. While n-3 fatty acids increased, the amount of n-6 PUFA, arachidonic acid (20:4n-6), decreased. These findings were consistent with previous results of Hargis and co-workers (1991). Significant reductions in yolk saturated fatty acids, palmitic (16:0) and stearic (18:0), were also noted in response to dietary MO. No effects of storage on egg yolk VLCPUFA content were noted (data not shown). Stability of VLCPUFA during storage was reported by Deslypere et al. (1993) who observed that the VLCPUFA, EPA and DHA, in VLCPUFA-enriched human adipose samples were stable for several months when stored at 4°C.

While yolk VLCPUFA were not degraded in such manner to cause reduction in total concentration in yolks, potential for oxidative degradation of lipids could have been initiated. Lipid oxidative deterioration may result from very low concentrations of free radicals (Frankel, 1984). TBA numbers for yolk from stored shell eggs (Fig. 1) indicated no effects of storage and no interactions between dietary and storage treatments. However, a consistent and significant dietary treatment effect was observed. The TBA numbers for yolk from eggs of hens fed 1.5% MO were greater each week compared to values for yolk from control-fed hens (Fig. 1). The origin of such lipid oxidation products in the egg yolk remains to be determined. Some oxidative changes may have occurred during distillation. However, development of TBARS from distillation should be minimal as antioxidant and chelating agents were added prior to heating. Potentially, hens may have consumed the lipid oxidation products in the feed and deposited them in egg yolks. However, stringent attempts (e.g., frozen storage of feed, an-

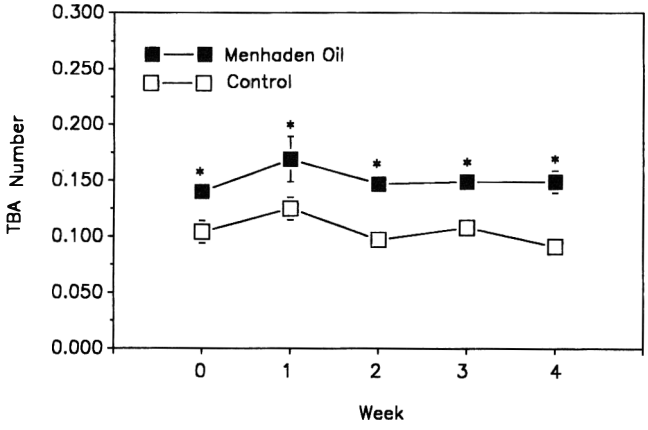

Fig. 1—Effect of dietary and storage treatments on TBA numbers of eggs from hens fed 1.5% menhaden oil. Means ± SEM differ significantly ($P < 0.05$) due to diet as indicated by asterisk.

tioxidant stabilized oil) were made to diminish the contribution of feed to oxidative potential of resulting shell eggs. TBA numbers of hen diets were not determined in our study. Alternatively, the oxidative deterioration of VLCPUFA may have been initiated in the hen's liver and oxidation products may have been directly transported from the liver along with other lipophilic materials for yolk deposition.

The persistence of TBA values during storage of eggs from both dietary treatments seemed to indicate that lipid oxidation products, regardless of origin, once deposited in the yolk were stable during storage. Egg yolk is uniquely protected from lipid oxidation by a natural chelating compound, phosvitin. Lu and Baker (1986) demonstrated the ability of this substance to chelate iron, binding several moles of the metal per mole of protein. The metal-chelating capacity of phosvitin may have prevented iron from acting as a pro-oxidant in the oxidative degradation of yolk VLCPUFA. Also, the structure of phospholipids in the yolk may have aided in preventing oxidation (Burley and Vadehra, 1989). Phospholipids and protein are interwoven in the exterior structure of low-density lipoprotein (LDL) in the yolk, and this compact surface layer can partly exclude oxygen from the lipid core of the particle (Burley and Vadehra, 1989). Thus, VLCPUFA within the LDL particles of yolks may have been partially protected from oxygen. Another possible explanation is that, potentially, the rate of formation and degradation of malonaldehyde precursors were similar. Therefore, yolk malonaldehyde concentrations would appear constant. This would depend on a similar diffusion rate of atmospheric oxygen through the egg shell during storage. Verification of such occurrence would entail measurement of volatile degradation products of malonaldehyde precursors in stored egg yolks (Pryor et al., 1976). The production of volatile products in the presence of stable malonaldehyde concentrations would be indicative of such.

The occurrence of lipid oxidation is often associated with deleterious changes in food flavors (Frankel, 1984). The formation of flavor compounds may be initiated in the lipid portion of foods during storage and heating (Grosch, 1982). Due to low threshold concentrations, off-flavors can be developed during cooking (Grosch, 1982). In our study, consumers ranked all eggs, regardless of storage length or dietary treatment, between 3 and 4, indicating an evaluation between "average" and "above average." Average scores were: for eggs from hens fed MO 3.6, and for eggs from hens fed the control diet, 3.8. Apparently the level of oxidation products, (indicated by TBA numbers), in stored and fresh eggs from hens fed 1.5% MO were below the threshold of flavor detectability by the taste panel. Although previous studies have indicated good correlations between higher TBA numbers and lowered sensory quality of powdered eggs stored at low temperatures (Kline et al., 1964), correlations between TBA numbers and sensory quality of cooked whole egg may not be as high.

CONCLUSION

THE LEVEL OF LIPID OXIDATION products in the yolk of eggs from hens fed 1.5% MO was not associated with a deleterious change in sensory quality. The lipid oxidation products in eggs from hens fed 1.5% MO did not contribute to detectable off-flavors. Therefore, the level of 1.5% MO would be acceptable in feed of hens for commercial production of VLCPUFA-enriched shell eggs. Storage stability of eggs enriched with VLCPUFA was comparable to that of typical shell eggs.

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Enzymatic Browning in Apple Pulps

J.E. LOZANO, R. DRUDIS-BISCARRI, and A. IBARZ-RIBAS

ABSTRACT

Enzymatic browning in Golden Delicious apple pulp was studied as related to degree of ripeness and temperature (3.5–31°C). Green apple pulp showed the highest rate of browning. This was attributable to differences in ascorbic acid (AA) content and polyphenoloxidase activity in young fruits. The rate of browning determined by CIE L* measurements followed complex temperature dependent kinetics, represented by a multiple linear effects with log time. Equal changes in L* parameter yielded straight lines in a log temperature vs log time plot. Inhibition with AA caused an initial slow rate of browning and a well defined break point associated with exhaustion of antioxidant properties of the AA. The greater the AA concentration the longer the initial period.

Key Words: enzymatic browning, ascorbic acid, apple pulp, polyphenoloxidase activity

INTRODUCTION

CONTROL OF ENZYMATIC BROWNING during processing of fruits is important to fruit pulp manufacturing. In clarified apple juice little browning is acceptable and a typical amber-like hue is commercially desirable. However, both apple purée and cloudy juice are expected to have the yellowish or greenish color which characterize the fresh product. Enzymatic browning in apples and their products is caused by the action of polyphenoloxidase, (PPO) which catalyses oxidation of phenolic compounds containing two o-dihydroxy groups to the corresponding o-quinone (Joslyn and Ponting, 1951). The quinone is very reactive and forms dark brown polymers, called melanins. The prevention and control of enzymatic browning has been the focus of several studies, reviewed by Labuza et al. (1992).

Enzymatic browning is the result of fast reactions. It has been claimed that refrigeration (0 to 4°C) retarded browning. However, in pulps, purées and cloudy apple juices, the cellular tissue has been practically destroyed and low temperatures are not adequate to inhibit PPO activity. Also, about 10 sec at 90°C are minimal conditions for PPO inactivation (Dimick et al; 1951) and such conditions are easily provided during heat processing of pulps. However, in practice a long delay occurs between mash formation and thermal processing. Moreover, during milling and finishing operations enzymatic browning is difficult to control even with high levels of SO_2 or ascorbic acid (AA) because of incorporation of air. Montgomery and Petropakis (1980) reported that the amount of AA required to prevent enzymatic browning in pear juice was dependent on length of time between milling and heating. In addition, production of cloudy or opalescent apple juice usually requires a maceration period, with or without adding specific enzymes, to improve characteristics and stability or suspended particles (Fukutani et al.; 1986).

To effectively inhibit or control enzymatic browning in pulverized apple products, accurate determination of the kinetics of this catalyzed-oxidative reaction is required. Sapers and Douglas (1987) developed a reflectance method for measurement of enzymatic browning and the effectiveness of AA as browning inhibitor at cut surfaces and in juice of apple and pear at 20°C. However, the degree of cellular destruction and air incorporation is greater in fruit pulp, than in juice or cut fruits. Our objective was to determine the influences of time and temperature on extent of enzymatic browning in apple pulp, and to study the browning inhibition capacity of ascorbic acid on pulps.

Authors Lozano and Ibarz-Ribas are with PLAPIQUI (UNS-CONICET), 12 De Octubre 1842, 8000 Bahia Blanca, Argentina. Author Drudis-Biscarri is with the Dept. de Tecnologia d'Aliments, E.T.S.E.A. Lleida Av. Rovira Roure 177, 25006 Lleida, ESPAÑA.

Table 1—Specifications of Golden Delicious apple pulp

	Green	Mature	Overripe
Soluble solids, °Brix (±0.2)*	13.0	15.0	17.0
Total acidity, g/L (±0.15)	3.25	2.6	2.48
pH (±0.05)	3.68	3.95	4.10
CIELAB L_o (±0.7)	64	65	66
CIELAB a_o (±0.3)	−9	−4	−1
CIELAB b_o (±0.6)	28.5	35	38

* Values in parentheses are standard errors.

MATERIALS & METHODS

PULPS WERE PREPARED from the cultivar Golden Delicious. This cultivar was chosen because it is currently grown commercially in several countries. The fruits were stored at 1°C prior to processing. No evidence of chill injury was observed. Apples were grouped in three degrees of ripeness designated green (GA); mature (MA); and overripe (OR) apples, based on the soluble solids, acidity, pH and peel color criteria. This procedure was done in duplicate at each temperature and AA level. More than 200 kg of apples were processed. Characteristics of Golden Delicious apple pulp at these three degrees of ripeness were summarized (Table 1). On day 0, all pulps had essentially the same L_o value. However, initial a_o and b_o values indicate a change in hue from yellowish green to yellow, as apples matured.

Preparation of samples

Twenty apples of the same degree of ripeness were conditioned to the assay temperature, peeled, cut in ≈ 1cc cubes, and pulverized in a laboratory crusher (Hamilton Beach Blendmaster, model 582W-220, Hamilton Beach/Proctor Silex, Inc., Washington, NC 27889), thermostated to the selected temperature. After 40 sec of mashing the pulp was poured into a thermostated glass from which 40cc samples were analyzed for color variation at selected times from 1 to 120 min. Pulp was agitated periodically (every 2 min) by gentle stirring to avoid decreases in PPO activity due to decreases in oxygen availability in the assay sample. Working temperatures were 3.5, 5, 8.5, 16.5. 21, and 31°C.

Ascorbic acid treatment and color measurements

To determine the kinetics of browning inhibition with AA, MA samples had added 150, 300, 600, 900 and 1200 ppm AA at 18°C. Reflectance spectra and CIE L*, a*, b* values were determined with a Macbeth Color-Eye 3000 (Macbeth-Kollmorgen Inst. Corp.; Newburgh, NY). All measurements were made with a light source D75, large viewing area and the observer at 2°. Color of pulp samples was measured in duplicate in black painted plastic cells (40 cm^3). Sapers and Douglas (1987) reported that decreases in CIE L* value correlated well with increases in apple browning. Labuza et al. (1992) proposed the normalized $\Delta L/L_o$ (%) values as a measure of browning when initial (L_o) values varied slightly between samples. Soluble solids were determined as °Brix with an Abbe refractometer at 20±0.1°C. Total acidity and pH were determined in accordance with methods reported

Table 2—Slopes for linear portion of CIE (L*, a*, b*) parameters vs log time curves, at 5°C for three types of apple pulp

Sample[a]	Parameter	Slope[b]	Std. error	r^2
OA	L*	−8.733	0.3138	0.991
	a*	3.624	0.2684	0.973
	b*	−5.664	0.3064	0.986
MA	L*	−7.885	0.2539	0.993
	a*	3.198	0.1079	0.992
	b*	−5.826	0.3265	0.978
GA	L*	−11.703	0.6491	0.994
	a*	7.529	0.5778	0.971
	b*	−5.519	0.4353	0.969

[a] GA = Green, MA = Mature, OA = Overripe.
[b] Slopes are the fitting of at least seven pairs of data points.

by the International Federation of Fruit Juice Producers, IFFJP (Lozano, 1991).

RESULTS & DISCUSSION

Effect of ripeness on color change

As reported by Sapers and Douglas (1987), tristimulus reflectance values were strongly nonlinear and changes in rates of browning were better followed when plotted against log time (Fig. 1). The rate of luminosity decrease could be divided into three periods. The first period was characterized as an induction or flat period probably due to the inhibitory action of natural AA (Ponting and Joslyn, 1948). During the second period the rate of browning (reduction of L*) was linear in this semilog plot. This could be attributed to the consumption of substrates of PPO (Sapers and Douglas, 1987). After that logarithmic decrease of L*, browning approached a plateau at a time which depended on the degree of apple ripeness. Induction time also was a function of degree of ripeness, the lower induction time corresponding to GA samples. This was in agreement with the results of Koch and Bretthauer (1956) who found considerable seasonal variations in the amounts of AA and dehydroascorbic acid in apples. Their results for apples at 3 stages of maturity indicated that green apples had one order of magnitude less AA than ripe apples, and that PPO activity was greater in young fruit than in fully ripe apples. Regression slopes were developed only for the linear portion (Table 2) of CIELab curves. Data were fitted with the Statgraphics program (STSC, Inc.; Statistical Graphics Corp.). Reduction in b* values and change in sign (from − to +) in a* parameter (Fig. 2) clearly indicated that browning development occured in apple pulp. Negative a* values were given by the green pigmentation of apples and it was pronounced in GA samples.

When the reflectance spectra for OR, MA and GA were compared (Fig. 3), green apple pulp showed a marked depression in the red region. OR and MA showed generally higher reflectance values across the spectrum. Extended enzymatic browning attenuated differences between spectra and reduced reflectance variations.

Effect of temperature on color change

The L* decreased in MA apple pulp with time at all temperatures studied (Fig. 4). Color development during pulping of apples includes Michaelis-Menten type reactions followed by several reactions, both reversible and irreversible, leading to dark brown pigments. The combined effect of these browning reactions results in a nonlinear behavior strongly related to temperature. Experimental data on the dependence of the rate of CIELAB L* on temperature were fitted to the equation:

$$L^* = a - k \log t$$

where a and k are fitting parameters and t = time in min. The selected range is in agreement with the second linear period in the L* vs log t plot. Slope k was assumed to be the inverse

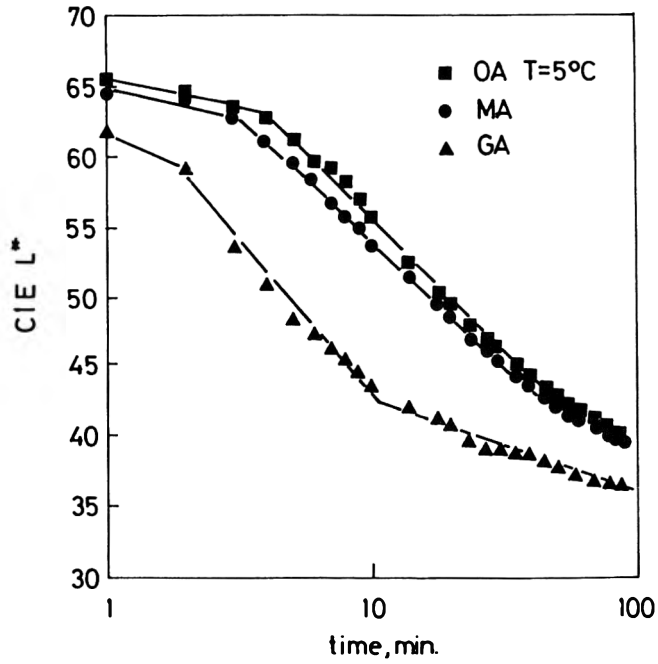

Fig. 1—Relationship of CIE L* value in Golden Delicious apple pulp to time and degree of ripeness at 5°C.

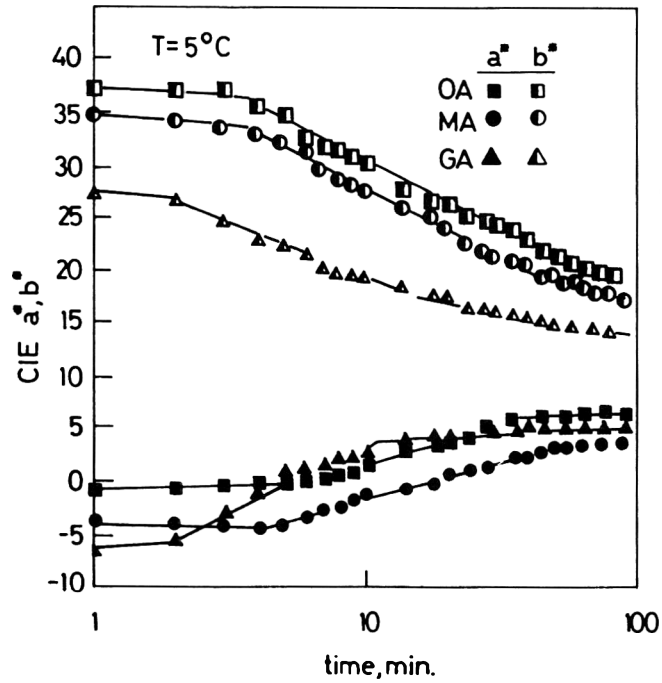

Fig. 2—Relationship of CIE a* and b* values in Golden Delicious apple pulp to time and degree of ripeness at 5°C.

of the time necessary to reduce L* value from 59 to 46 at every temperature. Calculated k values and correlation coefficients for MA samples at the different temperatures were compared (Table 3).

Kinetic measurements on complex systems, such as fruit pulp, usually give reaction constant values which may or not be dissociation constants, but which are frequently the combination of rate constants for several steps. Equal changes in L* parameter could be represented as straight lines (Fig. 5) when the logarithm of time was plotted vs. logarithm of temperature.

Effect of AA content on color change

Color changes of apple pulp treated with various AA concentrations at 18°C (Fig. 6) also showed three linear regions.

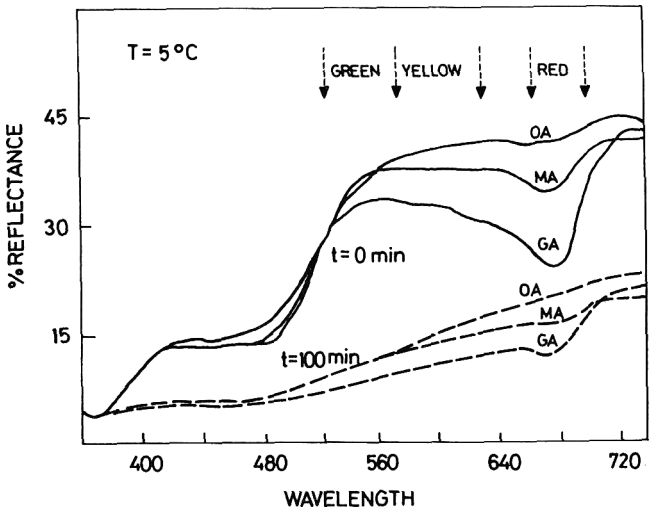

Fig. 3—Reflectance spectra for GA, MA and OA apple pulp at 5°C. Solid lines: Spectra at t=0 (fresh pulp). Dashed lines: Spectra after 100 min.

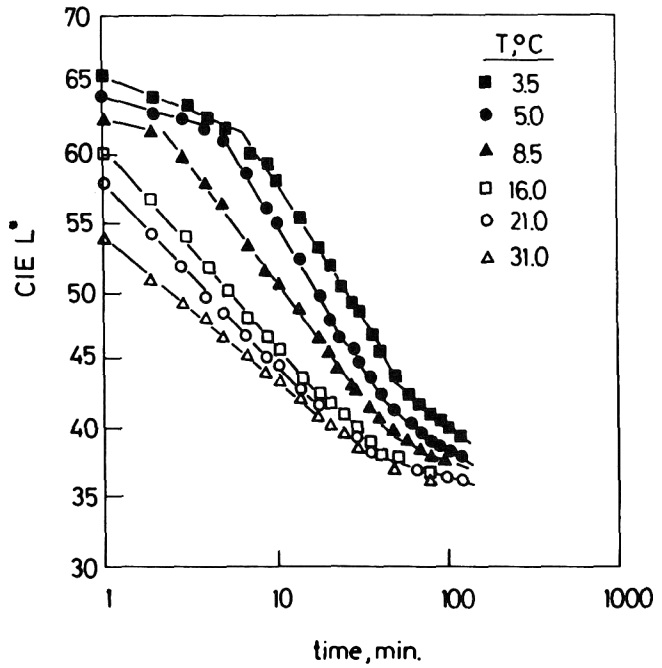

Fig. 4—Effect of temperature on CIE L* value in MA apple pulp for different times of heating.

Table 3—Slope for linear region of L* vs log(t) curve (Eq. 1) and calculated activation energy

Temp (°C)	k (slope)*	r^2	E_a (Kcal/mol)
3.5	0.47	0.996	
5.0	0.58	0.992	
8.5	0.74	0.997	9.3
16.5	1.36	0.996	
21.0	1.53	0.998	
31.0	2.16	0.994	

* Slope = 13 L* units/time; min^{-1}. (valid in the range from L* = 59 to L* = 46).

However, the initial flat period was strongly dependent on the amount of AA and a very well defined breaking point was observed at the point where the AA lost its inhibitory properties. Similar effects were reported by Matsui et al. (1957) in a contribution regarding the oxidative darkening during processing of "natural apple juices". Slopes of the initial (inhibition) and the second linear portion in the L* vs log t plot, were

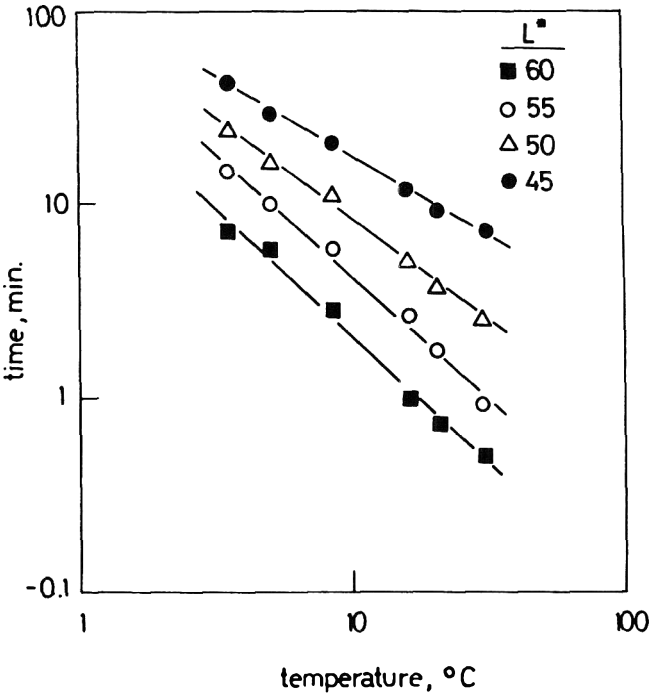

Fig. 5—Lines of identical color changes as CIE L* parameter, for MA apple pulp.

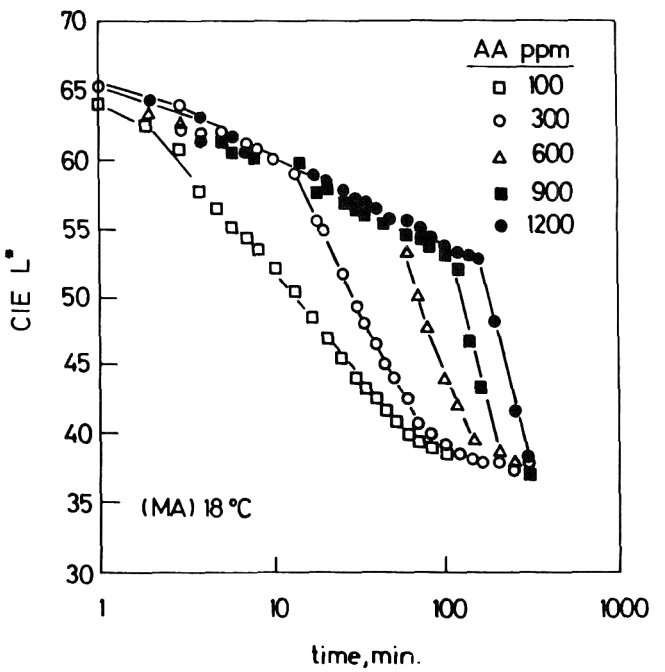

Fig. 6—Influence of ascorbic acid on enzymatic browning in MA apple pulp for different AA concentrations and times of treatment, at 18°C.

Table 4—Slopes of inhibition and second linear portion in the L* vs log t plot as a function of AA content, at 18°C

AA (ppm)	1st. portion slope	r^2	2nd. portion slope	r^2	Breaking point (min)
100	−2.688 (2)*	—	−15.259(16)	0.997	1.49
300	−5.376(14)	0.958	−24.375(11)	0.983	11.47
600	−5.431(18)	0.982	−37.820 (5)	0.989	49.69
900	−5.538(22)	0.992	−46.213 (5)	0.957	122.0
1200	−5.386(22)	0.989	−55.815 (4)	0.992	178.2

* Number in parentheses = number of pairs of data points.

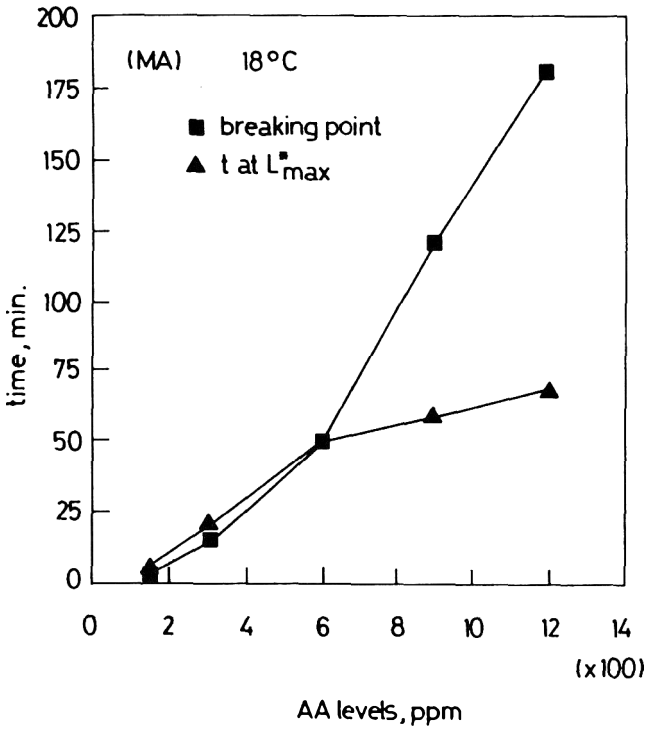

Fig. 7—Time to reach L* = 55 and breaking point (end of browning inhibition) for MA sample as related to AA concentration, at 18°C.

compared (Table 4) with the calculated intersection points (breaking points) and corresponding regression coefficients. Experimental breaking points as a function of AA levels were plotted (Fig. 7). Addition of AA at levels > 600 ppm resulted in a linear increase of the browning breaking point with a slope of 0.217 t(min)/AA(ppm) (r^2 = 0.997).

Visual observation of AA treated pulp samples by trained judges indicated that for L* values > 55 the browning would be considered unacceptable. Without intending to recommend L* = 55 as a limit, for comparison purposes the time required at 18°C to attain that level at various AA amounts was also plotted (Fig. 7). These values indicate overtreatment with AA (>600 ppm) would not proportionally increase the time to reach maximum browning.

SUMMARY

ENZYMATIC BROWNING in Golden Delicious apple juice can be monitored by measuring CIE L* values. A significant influence of degree of ripeness on rate of browning was observed. Pulp made with unripe (GA) apples browned at a faster rate. This was attributable to differences in AA content and PPO activity in young fruits. Rate of browning increased with temperature. However, the browning mechanism was strongly nonlinear and simplified kinetics equations were not applicable. For production of light-colored apple pulp, the time between milling of fruit and heat treatment must be as short as possible. Prevention of enzymatic browning of cloudy apple juice by adding AA was possible. The effect showed a very well defined breaking point after which browning proceeded at the usual rate. When retention of apple pulp in maceration tanks is required, as for cloudy juice, level of ascorbic acid necessary to inhibit enzymatic browning must be related to the maceration time and temperature.

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Edible Coating Effects on Storage Life and Quality of Tomatoes

HYUN J. PARK, MANJEET S. CHINNAN, and ROBERT L. SHEWFELT

ABSTRACT

Tomatoes at breaker and pink stage maturities were coated with corn-zein film. Color, weight and firmness changes and sensory quality were compared with noncoated tomatoes during storage at 21°C. Corn-zein film delayed color change and loss of firmness and weight during storage. Shelf life was extended by 6 days with film coatings as determined by sensory evaluation.

Key Words: tomatoes, storage life, color, texture, edible coatings

INTRODUCTION

SEVERAL TYPES OF EDIBLE FILMS have been applied successfully for preservation of fresh products. Mixtures of sucrose fatty acid esters have been used for coating fresh fruits and vegetables to extend shelf life and minimize quality changes (Banks, 1984; Chu, 1986; Santerre et al., 1989; Smith and Stow, 1984). Corn-zein coating has been used on nuts to extend their marketing period (Cosler, 1958). Modification of internal gas composition by edible coatings can increase disorders due to high CO_2 and low O_2 (Ben-Yehoshua, 1969, 1985; Smith et al., 1987). Several problems have been associated with edible coatings, such as anaerobic fermentation of apples and bananas (Banks, 1984; Smock, 1940). Tomatoes coated with a thick corn-zein film had rapid weight loss (Park et al., 1993). Elevated level of core flush for apple (Smith and Stow, 1984) and increased incidence of decay in cucumbers (Risse et al., 1987) have also been reported.

Most research on edible coatings has not studied critical factors such as gas permeation properties of the fruit skin and beneficial internal gas composition. The effectiveness of edible coatings for fruits and vegetables depends primarily on selection of appropriate films or coatings which result in beneficial internal gas composition (Kader et al., 1989; Park et al., 1993). Coatings on fruits and vegetables that exceed a critical thickness can cause detrimental effects by reducing internal O_2 and increasing CO_2 concentration leading to anaerobic fermentation.

Our specific objectives were to coat tomatoes with an appropriate thickness of corn-zein film to provide beneficial internal O_2 composition and determine the effects on color change, alcohol formation, weight loss, and sensory quality.

MATERIALS & METHODS

Fruit

Tomatoes of two developing stages (breaker, pink), *Lycopersicon esculentum* (cv. 'Mountain Pride'), were sorted for uniform size, color, and physical damage and dipped in chlorinated water. A total of 180 tomatoes (90 of each stage) were used. Lots were subdivided into two groups each (corn-zein film coated and noncoated) and held at 21°C to study changes in selected quality parameters during storage. In each group, 10 tomatoes were used for measuring color change and weight loss, 10 tomatoes were used for instrumental firmness and the remainder were used for sensory analysis.

Author Park, formerly with the Dept. of Food Science & Technology, Univ. of Georgia, is now with the Dept. of Food Engineering, Mokpo National Univ., Mokpo, Korea. Authors Chinnan and Shewfelt are with the Center for Food Safety & Quality Enhancement, Dept. of Food Science & Technology, Univ. of Georgia, Agricultural Experiment Station, Griffin, GA 30223-1797. Address inquiries to Dr. M.S. Chinnan.

Corn-zein film

A commercial corn-zein product, Regular Grade F4000, produced by INC Biomedicals, Inc. (Cleveland, OH) was used as coating material. The corn-zein solution was prepared using 54g corn-zein, 14g glycerine, and 1g citric acid dissolved in 260g ethanol (95%). Tomatoes were brushed twice with corn-zein solution, dried at room temperature (≈23°C) by blowing air with a table fan, and were held at 21°C (Park et al., 1993). The thickness of the corn-zein film coating was assured to be within the limits of 1.01 ± 0.18 mil (Park et al., 1992).

Ethanol content

Every two days, two tomatoes from each group were ground in a blender, transferred to a bottle containing 10 mL distilled water and sealed with a septum cap. The bottle was shaken vigorously and immersed in a water bath at 95°C for 30 min and then cooled and held at 50°C until sampling. After 10 min at the reduced temperature, a 0.5 mL headspace sample was injected into a gas chromatograph for alcohol analysis (Banks, 1984; Davis and Chace, 1969; Park et al., 1993).

Color measurement

Ten tomatoes from each group were monitored for surface color change using a Gardner XL-845 colorimeter. Instrumental color readings (L, a and b) were determined every 2 days and were taken as an average of 8 different points on the circumference and blossom end of the tomatoes using a standard pink calibration tile (L = 69.1, a = 23.4 and b = 9.3) (Yang and Chinnan, 1987). A correction factor to offset the effect of the film coating on instrumental color readings was obtained on a separate group of tomatoes where surface color changes were recorded for the same tomato with and without the coating (Park et al., 1993).

Firmness

An Instron Universal Testing Machine (Model 1122, Canton MA) mounted with a 25.8 cm^2 compression anvil (Assembly No. A327-19) operating at cross head speed of 2 mm/min, chart speed of 100 mm/min and 5 kg full scale load was used to measure tomato firmness by a nondestructive test adapted from Bourne (1982). Force readings were recorded at 2% constant deformation at one point on the circumference of each of 10 fruits from coated and non-coated groups.

Sensory evaluation

Eleven experienced sensory panelists scored samples on a 150 mm unstructured line scale with anchor points at 12, 75, and 138 mm. For each sensory panel session four tomatoes were selected from each group. Two tomatoes from each group were washed, diced into ≈1 cm cubes and mixed in a bowl, and samples were presented in coded cups on a white dish for flavor evaluation. Another two tomatoes were used for sensory color and firmness and were discarded after using. Each panelist rated each sample by placing a vertical mark on each scale and labeling each mark with the three digit sample code. Samples were evaluated by using scales for three flavor attributes, including sweetness, acidity, and "off-flavor," and sensory color and firmness (Resurreccion and Shewfelt, 1985; Shewfelt et al., 1987). Corn-zein

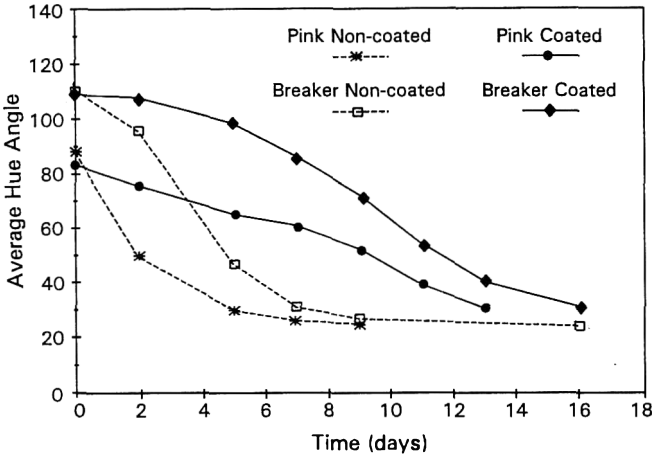

Fig. 1—Average hue angle change of coated and noncoated tomatoes during storage.

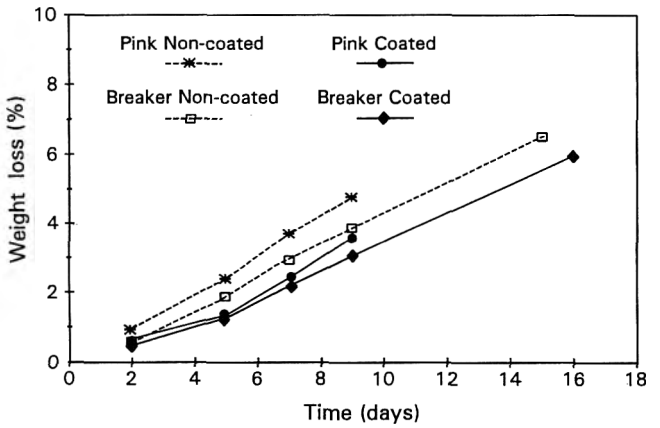

Fig. 2—Weight changes of coated and noncoated tomatoes during storage.

film on the surface of coated tomatoes was peeled away before sensory evaluation.

RESULTS & DISCUSSION

Ethanol production

Ethanol production is an indicator of degree of anaerobic fermentation taking place. No ethanol production was observed in any coated pink or breaker tomatoes. Accumulation of ethanol and alcoholic off-flavors (Smith et al., 1987) have been reported when internal atmosphere was affected by restricting gas exchange. Extremely low O_2 contents for broccoli and cauliflower resulted in off-flavor as soon as the product changed from aerobic to anaerobic metabolism (Weichmann, 1987). Tomatoes coated with corn-zein film (2.6 mil) produced ethanol during storage (Park et al., 1993). The internal O_2 concentration of green, pink and red stage tomatoes coated with corn-zein (1.01 mil) was 4.2 ± 1.5, 5.9 ± 1.9 and 7.8 ± 1.7 (%), respectively (Park et al., 1992). The lack of observable ethanol production in our study suggested that the thickness of edible coating applied was within beneficial limits that did not cause deleterious modification in internal atmospheres.

Color change

Color changes during tomato ripening are characterized by loss of chlorophyll and rapid accumulation of carotenoids, particularly lycopene, as chloroplasts are converted to chromoplasts (Khudairi, 1972). This is important in consumer acceptability (Beattie et al., 1983). The color change, as indicated by hue angle, of the coated breaker group reached the

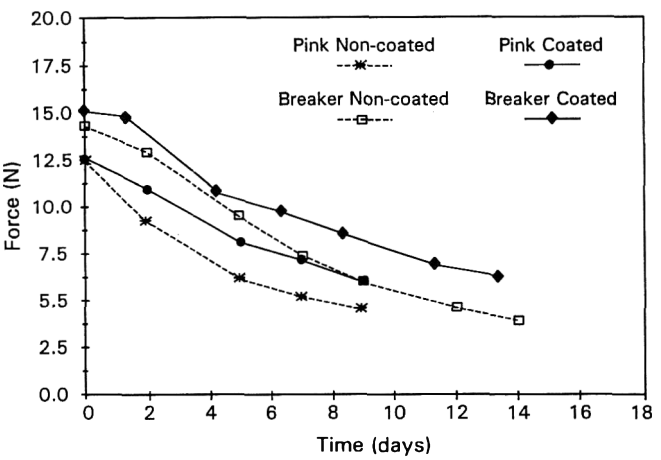

Fig. 3—Firmness changes of coated and noncoated tomatoes during storage.

red stage (hue angle = 30) after 16 days storage. In the coated pink group, after 13 days storage a red color was attained. Only 5 and 7 days storage of the noncoated pink group and the noncoated breaker group resulted in their change to red. Hue angles were not significantly different ($\alpha = 0.05$), after 5 days and 7 days for 'pink noncoated' and 'breaker noncoated' respectively. Significant differences for hue values of 'breaker-coated' were: no difference between 0, 2 and 5 days; 7 days different from 0 and 2 days; 9 days different from 5 days and less; no difference between 11 and 13 days; 16 days different from 11 days and less. Results for 'pink coated' were as follows: no differences between 0 and 2 days, 2 and 5 days, 5 and 7 days, 7 and 9 days, and 11 and 13 days; 0 different than 5 days and greater, 2 different than 7 days and greater, 5 different than 9 days and greater, 7 and 9 days different than 11 and above.

Color development of tomato was influenced by gas composition of its environment (Yang and Chinnan, 1987). In ripening of tomatoes, high CO_2 levels decrease ethylene synthesis, which can delay color changes (Buescher, 1979). Shewfelt et al. (1987) suggested that individual seal packaging might delay ripening at the pink stage. Yang et al. (1987) reported that seal-packaging delayed ripening from the breaker to the red stage by 3 days at both 12°C and 21°C; whereas, no delay in ripening was reported beyond the pink stage. Our results suggested that edible coatings delayed ripening from the pink stage to red stage tomatoes (Fig. 1) when pink stage tomatoes were used. Coating of tomatoes with corn-zein film delayed color change which was probably due to an increase in CO_2 level and a decrease in O_2 level (Buescher, 1979; Yang and Chinnan, 1987).

Weight loss

The weight losses of noncoated and coated tomatoes during storage were compared (Fig. 2). Corn-zein film coatings resulted in reduced weight loss during storage. The weight loss values within each group of samples during storage were significantly different ($\alpha = 0.05$) from each other. Edible coatings have been applied to reduce weight loss during storing and handling of fresh produce. Wax coating resulted in marked reductions in weight loss of oranges during storage (Ben-Yehoshua, 1969). Slight reductions in weight loss in bananas and apples were found with sucrose ester-based coating (Banks, 1984; Smith and Stow, 1984). Park et al. (1993) however, reported that tomatoes coated too thickly showed too-low O_2 and too-high CO_2 concentration and produced ethanol. The primary explanation for increased weight loss of thickly coated tomatoes is loss of sugars, generation of heat and production

Table 1—Changes in sensory characteristics of noncoated (NC) and coated (C) tomatoes stored at pink stage of maturity

	Day 2		Day 5		Day 9		Day 15
Characteristic	NC	C	NC	C	NC	C	C
Flavor							
Sweetness	61.89	54.78	67.78[a]	39.33[b]	56.22	58.67	82.25
Acidity	64.33	48.11	51.78	56.11	66.33	52.00	77.62
Overall	74.33	54.56	67.56	56.78	68.56	66.89	92.38
Off-flavor (if any)	0.61	2.02	0.44	1.80	0.99	1.90	1.86
Firmness	90.56	102.33	88.22	88.33	37.56[a]	85.44[b]	42.75
Color	102.22[a]	59.11[b]	128.22[a]	67.89[b]	133.67[a]	93.89[b]	130.38

[a,b] Sensory attribute values within the same day with different letter superscripts are significantly different as determined by Duncan's Multiple Range Test ($\alpha = 0.05$).

of end products from anaerobic fermentation (Weichmann, 1987).

Firmness

Firmness measures of coated and noncoated tomatoes (Fig. 3) showed coating delayed loss of firmness. Firmness of 'breaker noncoated' and 'pink noncoated' did not change after 11 days and 5 days of storage, respectively. For 'breaker-coated' fruit no significant differences were found for 0 and 2 days, 5, 7 and 9 days, 9 and 11 and 11 and 13 days storage. For 'pink coated' group no differences in firmness were found between 5 and 9 days storage. Park et al. (1992) reported that respiration and O_2 consumption of coated tomatoes were lower than those of non-coated tomatoes. Ben-Yehoshua et al. (1985) reported that seal packaging of citrus fruits had reduced respiration rate. Reduction in respiration rates of coated tomatoes could be responsible for delaying ripening which resulted in reduction of firmness loss during storage.

Sensory evaluation

Mean scores for sensory attributes during storage at 21°C were determined, and values with and without corn-zein coating were compared (Table 1). Note that the zein coating was removed from the tomatoes when preparing samples for sensory tests. We did not want panelists to be influenced by the physical presence of coating or any flavor imparted by the coating. In commercial conditions the coatings would usually remain on the fruit when consumed. The effect of the coating at the point of purchase and point of consumption needs further study.

Acidity, overall flavor and off-flavor attributes were not affected by coating. Increased perception of sweetness was observed later in the coated tomatoes than those not coated. Firmness and color are major factors in price and market value of tomatoes (Jordan et al., 1984). Softening (loss of firmness) and color development were noticeably delayed in the coated tomatoes. The noncoated tomatoes were not evaluated after 9 days storage at 21°C due to spoilage.

CONCLUSIONS

NO ETHANOL PRODUCTION was observed in coated tomatoes. The corn-zein film coating on the surface of tomatoes delayed color change, firmness loss and weight loss during storage. Sensory studies indicated an increase in sweetness and reduction in firmness in both coated and noncoated fruit. However, loss of firmness was slowed in coated tomatoes as was color development.

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Non-Invasive Determination of Freezing Effects in Blueberry Fruit Tissue by Magnetic Resonance Imaging

GARY R. GAMBLE

ABSTRACT

Water and sugar distribution in a single blueberry were mapped both before and after freeze/thaw using magnetic resonance imaging. Images were obtained using a slice selective spin echo pulse sequence which included a T_1 inversion recovery time period, allowing selection of either the water signal or the sugar signal. Freeze/thaw results in the rupture of water retaining membranes within discrete locations of the fruit tissue. This causes a change in the ratio of motion modified water (i.e., hydrogen bonded or chemically exchanged) to unmodified (i.e. mobile and not chemically exchanged) in those regions, as well as a concomitant change in sugar concentration, due to diffusion to other tissues.

Key Words: blueberries, freezing effects, sugar distribution, magnetic resonance imaging

INTRODUCTION

THE QUALITY OF FOOD MATERIALS may be characterized either by the structure imparted by constituent components (i.e., water, oil) or by some parameter correlated with food quality. An example is soluble solids, notably sugars, present in fruit tissues. Food production and marketing industries seek to nondestructively measure alterations in food quality which result from processing or maturation. Non-destructive methods (Dull, 1986) exist for evaluation of histological changes in plant materials, X-ray or optical transmission, near-infrared diffuse reflectance, and ultrasound. None of these can nondestructively determine with high resolution, the internal structure of a complex material. One method with this potential is Magnetic Resonance Imaging (MRI) (Mansfield and Morris, 1982). MRI is routinely used in medical applications but its use for investigating the histochemistry and structure of plant materials has been limited (Chen et al., 1989; Ishida et al., 1989; Pope et al., 1991; Duce et al., 1992; Wang et al., 1988, 1992).

Several combinations of pulse and gradient sequences are possible in MRI. The most commonly used is the Hahn spin-echo (Fig. 1). A nonselective 90°, or $\pi/2$, pulse places ^1H magnetization in the plane transverse to the B_0 field of the magnet. The subsequent application of a variable amplitude y gradient (G_y), (phase encoding gradient) causes dephasing of spin coherence along the y axis in the laboratory frame of reference. After dephasing, a frequency selective 180°, or π, pulse applied in combination with a z gradient (G_z), (slice selective gradient) allows isolation of a single slice perpendicular to the z axis. The resulting spin echo is produced during a constant amplitude x gradient (G_x), called the read gradient. A spin echo is produced at each increment of the phase encoding gradient, and 2D Fourier transformation of the data produces an image.

In order to observe the internal structure of a material various methods of tissue contrast may be employed. The simplest is to measure different densities of ^1H present. This information is provided by an experiment as in Fig. 1. Further contrast may be obtained if selective observation of protons with different chemical shifts is required by applying a chemical shift selective $\pi/2$ pulse in place of the hard $\pi/2$ pulse of Fig. 1. This may be difficult, however, since ^1H linewidths from compartmentalized systems (such as fruit tissues) tend to be so broad as to cause considerable overlapping of different resonances. Another method, used for our research, relies upon distinguishing regions of a tissue based upon differences in ^1H relaxation rates. Two time constants regularly used to define ^1H relaxation are T_1 (spin-lattice) and T_2 (spin-spin) relaxation (Abragam, 1961).

T_2 describes the mechanisms by which magnetization induced into the plane perpendicular to the applied field dephases, or loses coherence. ^1H nuclear spins will vary in T_2 due to different chemical or physical environments. In the case of H_2O imaging, the physical environment (i.e., the extent of motion restraint on the nucleus) will be of primary importance, though chemical exchange between H_2O and sugar will also affect T_2. T_2 contrast is achieved by varying the echo time TE (see Fig. 1) which weights the components of magnetization based upon T_2 relaxation times. TE is varied by altering the time between application of the hard 90° pulse and the selective 180° pulse.

T_1 describes the mechanisms by which the induced magnetization decays to equilibrium with the applied field, and is also affected by the environment. T_1 contrast is achieved by applying an initial RF pulse. The time τ between this pulse and the subsequent $\pi/2$ pulse (which relocates the magnetization back to the plane perpendicular to the applied field) is varied to allow weighting of different components based on T_1 relaxation times.

In this study both T_1 and T_2 relaxation of ^1H were used as contrast agents to observe the effects of freezing upon sugar and water distribution in blueberries. Cho et al. (1991), applied the inversion recovery method to selectively observe the sugar resonance while suppressing that of water. The objective was to use this method in conjunction with the conventional spin-echo imaging sequence to selectively map sugar distribution in blueberries based upon T_1 relaxation times. Differences in T_2 of water which occur as a result of freezing were also utilized.

MATERIALS & METHODS

BLUEBERRY SAMPLES (var. Burlington) were obtained at a commercial market and used immediately after purchase. These samples were analyzed utilizing gas chromatography (Chapman and Horvat, 1989) as 5.5 wt% fructose and 5.8 wt% glucose. Duplicate spectroscopic and imaging experiments were performed on 3 different blueberries. Results presented are those on a single berry with which the highest quality images were obtained.

All experiments were performed on a Bruker MSL 300 spectrometer, utilizing a 7.05T superconducting magnet tuned to 300 MHz for proton observation and equipped with an 87 mm vertical bore. The sample was placed in a 15 mm tube and inserted into a 15 mm imaging coil. Gradient rise- and fall-times were about 300μs. The sample remained in the bore for the entire sequence of experiments, including freezing and subsequent thawing, and was not reoriented. Low temperatures (≤0 C) were achieved using a variable temperature unit with liquid N_2 boiloff as cooling gas.

One dimensional NMR spectra were averaged over 8 scans and were obtained with a spectral width of 45454 Hz and a recycle delay of 8s. Spectra were transformed without window functions. Selection of the

Author Gamble is with the USDA-ARS, Richard B. Russell Agricultural Research Center, P.O. Box 5677, Athens, GA 30613.

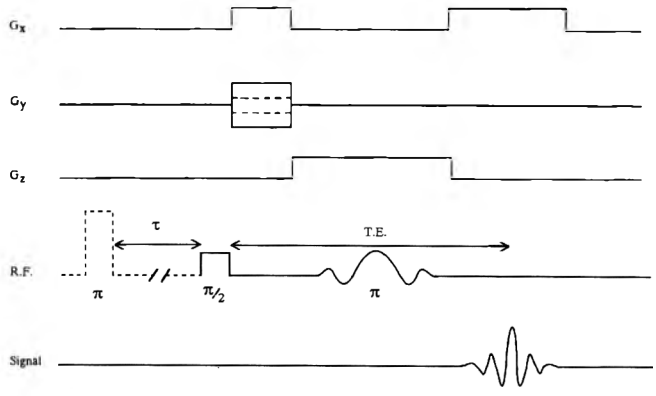

Fig. 1—Spin echo pulse sequence, modified to include initial π pulse for spin inversion.

sugar resonance was achieved by a T_1 inversion recovery sequence (Fig. 1). The sugar free induction decay (FID) was acquired at the null point of the water signal inversion recovery, where there was equal intensity along the z, -z axes. Sampling of the FID without the 180° inversion pulse results in a ^1H spectrum representative of the entire sample. T_1 of unbound water was measured at its null point in the inversion recovery sequence based on the relation

$$I = I_o (1 - 2e^{-t/T1})$$

where I is the signal intensity (positive or negative) at time t after the 180° pulse, I_0 is the signal intensity without an initial 180° pulse and inversion recovery period. At the null point, I = 0 and T_1 = t/0.693. T_1 of water varied considerably from one berry to another; measured values ranged from 750 ms to 1.75 s. T_2 was measured using the CPMG pulse sequence (Carr and Purcell, 1954; Meiboom and Gill, 1958). Data were fit assuming mono-exponential decay. An additional 180° inversion recovery pulse was utilized for sugar to isolate its signal from that of water. Error limits were reported as standard deviations.

Images were obtained using a slice selective spin echo technique (Fig. 1), with an echo delay time (TE) of 16 ms and a recycle delay (TR) of 8s. For sugar imaging, an additional hard 180° pulse was added for inversion of ^1H magnetization, with the sugar signal measured at the null point of water. Each increment of a 256 × 256 data matrix was averaged over 16 scans for H_2O and 32 for sugar. Slice thickness was 1 mm. Application of 6.75 Gauss/cm gradient strengths, with a field of view of 1.58 cm, and ^1H linewidths of 170 Hz (FWHH) for water and 560 Hz for sugar resulted in theoretical resolutions of 10 μm for water and 30 μm for sugar. The true resolution of each image, however, is determined by the size of the data matrix, each pixel being 62 μm × 62 μm. The 2D serial file was processed without window functions, and the resulting image was stored on a film using a Mitsubishi Color Video Copy Processor. Intensity integration over an entire image was achieved by summing the intensity of all pixels in the image.

RESULTS & DISCUSSION

THE ^1H SPECTRUM of a single blueberry is presented in Fig. 2. This signal was due primarily to water, with ~7.5% (based on calculation from GC) of the intensity due to dissolved sugars. T_1 (H_2O) for this berry was 1.23 s, and when the T_1 inversion recovery pulse was included, with τ = 850 ms, the water signal was nulled and the resulting spectrum (Fig. 2), had 10% of the intensity of the total spectrum. This signal was due primarily to dissolved sugars and to an additional component which was not nulled during the inversion recovery. Because GC results indicated no appreciable other soluble solids, it was concluded that this component was water which had shorter T_1 relaxation times as a result of either adhesion to cell wall surfaces or from differences in sugar concentration which may occur in different regions of the berry. Both mechanisms have decreased observed T_1 values (Ablett et al., 1991). Whatever the mechanism it is referred to as water molecules with "motion modified", while the water component with a longer

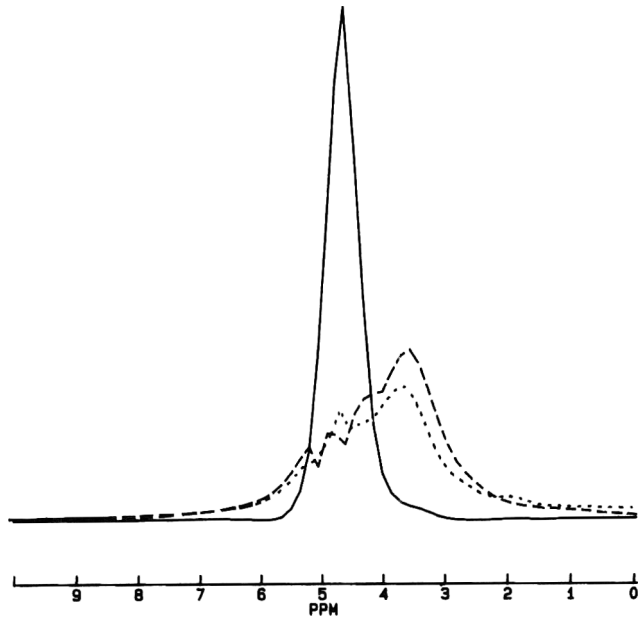

Fig. 2—Total ^1H spectrum (———) and sugar-(motion modified) water spectrum (- - -), magnified 10×, of fresh blueberry, and sugar-(motion modified) water spectrum (· · ·), magnified 10×, of freeze/thaw blueberry.

T_1 is "unmodified." T_2 (unmodified H_2O) was determined to be 14.0 ± .2 ms and T_2 (sugar, motion modified H_2O) was 6.6 ± .2 ms for the fresh berry.

When a blueberry is frozen and subsequently thawed, T_1 (unmodified H_2O) decreases to 1.08 s and the null point of the unmodified water in the inversion recovery experiment is at τ = 750 ms. The integrated intensity of the resultant sugar-(motion modified) water signal is 22% smaller than before freezing. T_2 (unmodified H_2O) concomitantly increases to 15.0 ± 0.2 ms while T_2 (sugar, motion modified H_2O) increases to 6.9 ± 0.2 ms for the freeze/thaw berry. T_1 (sugar, motion modified H_2O) could not be accurately measured due to overlap of sugar and water signals. The observed reduced echo intensity cannot be related to the increase in T_2 (sugar, motion modified H_2O), as this should cause an increase in intensity (Ablett et al., 1991). The reduced intensity may be due to a decrease in relative amount of bound water, as evidenced by the increase in T_2 (unmodified H_2O). Such increase in T_2 is due to the rupture of water retaining cell walls during freezing, which increases the amount of unmodified water relative to motion modified water. Motion modified water exhibits more solid-like transverse relaxation behavior, i.e. shorter T_2's, than unmodified water (Ablett et al., 1991).

The image of the unmodified water in an unfrozen blueberry was recorded (Fig. 3A). Comparison of the integrated image intensity with that of the frozen/thawed berry (Fig. 3B) shows that the integrated intensity for the frozen/thawed berry increased by 7% relative to that of the fresh. The total amount of water had not changed, thus the cause of this behavior is attributed to the fact that T_2 (unmodified H_2O) increased from 14.0 ms to 15.0 ms upon freezing/thawing which resulted in a more intense echo for the frozen/thawed berry when TE = 16 ms. Although intensity increased throughout, it appeared to be related to location. The image in Fig. 3C resulted from subtraction of Fig. 3A from Fig. 3B and was magnified 8× relative to Fig. 3A. It shows the regions of greatest intensity increases; primarily placental tissue and locules, where seeds are located.

The image of sugar and motion modified water in the unfrozen berry, (magnified 8× relative to 3A) was obtained by inversion recovery with τ = 850 ms (Fig. 4A). Localization of the sugars and motion modified water components to dis-

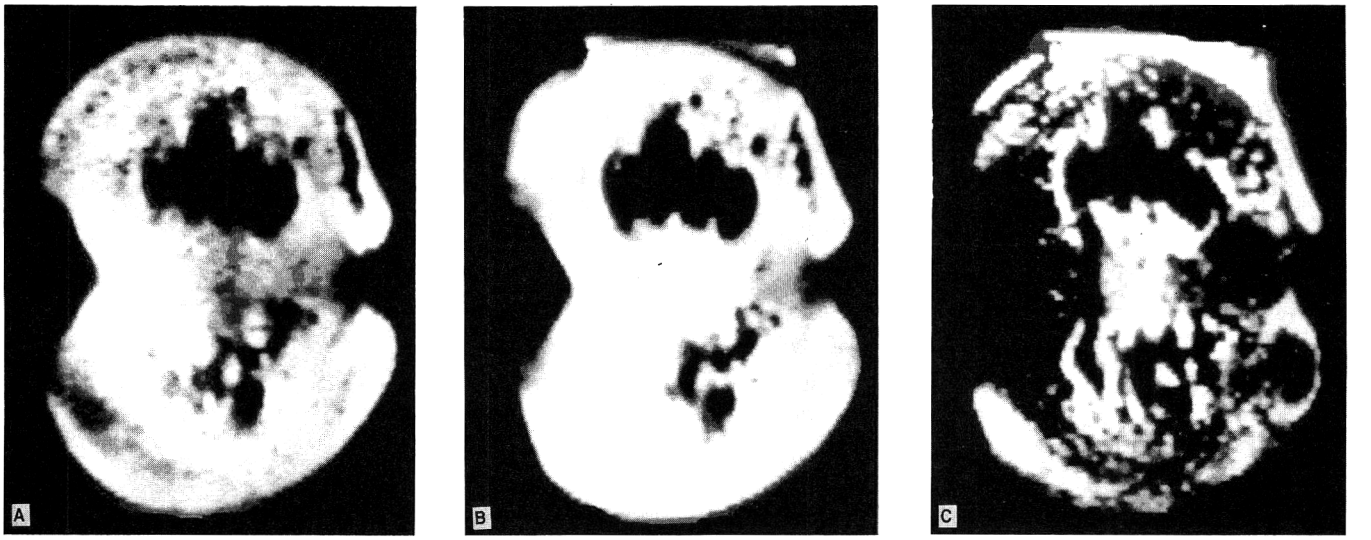

Fig. 3—(A) Unmodified water weighted image of fresh blueberry; (B) unmodified water weighted image of freeze/thawed blueberry; (C) difference image, magnified 8×.

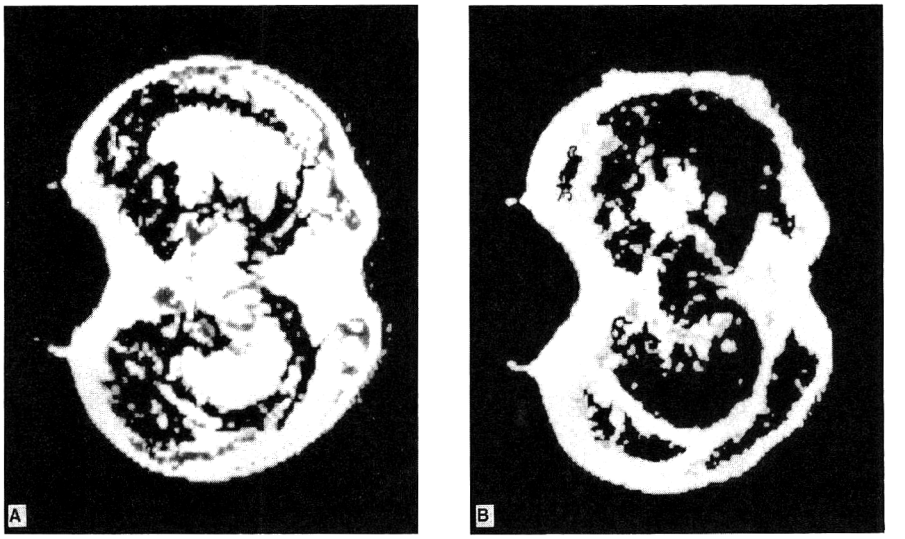

Fig. 4—(A) Sugar-(motion modified) water weighted image of fresh blueberry, magnified 8× relative to Fig. 3(A); (B) sugar-(motion modified) water weighted image of frozen/thawed blueberry, magnified 8× relative to Fig. 3(B).

crete regions or tissues are clearly shown. The thin exocarp had a relatively high content, and sugar-(motion modified) water was also localized in the placenta and locules. Upon freezing/thawing the total inversion recovery signal decreased by 22%, (Fig. 4B) with the decrease confined primarily to placenta and locules. In addition to an overall decrease in sugar-(motion modified) water intensity, a relative increase of intensity occurred in the mesocarp, which indicates that upon freezing/thawing sugar migrated from the placental tissue. The proposed mechanism for this migration is that once the cell walls responsible for retaining the dissolved sugars were ruptured, the sugar diffused into other tissues.

CONCLUSIONS

MAGNETIC RESONANCE IMAGING was used to selectively map water and sugar distributions within fruit tissues. Images of water distribution were due primarily to water which was motion unmodified (i.e. no hydrogen bonding or chemical exchange). The images of sugar distribution also include a water component which has been motion modified, and as a result exhibited different relaxation behavior than the unmodified water component. Changes in tissue structure, caused by freezing/thawing could be mapped both as a function of changes in water T_1 and T_2 relaxation behavior and the redistribution of dissolved sugar.

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Activity of Softening Enzymes during Cherry Maturation

DIANE M. BARRETT and CARLOS GONZALEZ

ABSTRACT

Royal Anne and Bada cherries soften continuously throughout maturation and cold storage. However, major textural changes occurred during a 2-wk period which coincided with sharp increases in weight, volume, soluble solids, polygalacturonase and pectin methyl esterase activity. No difference occurred between cultivars in the activities of softening-associated enzymes at any sampling period. PME activity was detected during the first week of sampling, and PG and β-gal activity were detected following the second and fifth week, respectively. The integrated action of PG, PME and β-gal appear to be required for cherry softening.

Key Words: cherry, enzymes, softening, maturation

INTRODUCTION

TEXTURE is an important quality factor in both fresh and processed cherries, yet the mechanism by which cherries soften is not fully understood. Decrease in firmness of maturing fruits has been hypothesized to be due to alterations in both the cell wall and middle lamella (Huber, 1983). Stuctural changes which occur in the middle lamella and primary cell wall during ripening lead to cell separation and softening of tissue (Bartley and Knee, 1982). Such changes are presumably the results of enzymes such as polygalacturonase, pectin methyl esterase, β-galactosidase, cellulase and others.

Plant cell walls consist of cellulose microfibrils embedded in a complex matrix of pectic substances and hemicelluloses. These polysaccharides form the network of the cell wall and depolymerize to some extent during ripening (Huber, 1983; Eskin, 1979). The middle lamella (area between primary cell walls of adjoining cells), forms a continuous intercellular matrix. This layer is high in pectic substances and its solubilization has been correlated with fruit softening during ripening (Eskin, 1979).

The importance of enzymes in fruit softening has been investigated by many, but relatively few have reported on such activity in cherries. Polygalacturonase (PG) activity was related to the loss of firmness in dates (Hasegawa et al., 1969), peaches (Pressey et al., 1971), tomatoes (Wallner and Walker, 1975), apples (Knee, 1978), avocados (Awad and Young, 1979; Zauberman and Schiffmann-Nadel, 1972), pears (Bartley et al., 1982), papaya (Paull and Chen, 1983), green peppers (Jen and Robinson, 1984) and cucumbers (Miller et al., 1987).

Two types of PG have been identified (endo and exo), (Eskin, 1990). Endo-PG randomly hydrolyzes glycosidic bonds while exo-PG hydrolyzes bonds only on the terminal end of the pectin molecule. The activity of both generally increases during ripening, when pectic material in cell walls and middle lamella are hydrolyzed.

Pectin methyl esterase (PME) acts in concert with PG to increase cell wall solubilization (Pressey and Avants, 1981). De-esterification of cell wall galacturonans by PME may be required prior to hydrolysis by PG (Awad and Young, 1979). A close association between textural changes and PME activity during fruit ripening has been reported. In green peppers, maximum PME activity occurs at the light green stage and then declines during ripening (Jen and Robinson, 1984). Awad and Young (1979) found that avocado PME activity declined from its maximum at the time of harvest to a low level early in the climacteric. Proctor and Miesle (1991) reported increasing PME activity during blueberry development.

β-Galactosidase (β-gal), present in cell walls, hydrolyzes galactans (Bartley, 1974). Several studies have identified β-galactosidase activity in fruits and suggested a possible function of this enzyme in fruit softening. Wallner and Walker (1975) reported β-gal in ripe tomatoes. The increase in β-gal activity during tomato ripening was corroborated by Pressey (1983). Perhaps the most convincing evidence for the role of glycosidases in fruit softening has come from studies with apples (Bartley, 1974, 1976). The activity of β-gal increases during apple ripening and probably catalyzes the loss of galactose residues from the cell wall (Bartley, 1977).

Although the presence of PG, PME and β-gal in various fruits has been correlated with softening, reports of such activities in cherries are scarce. Cherries apparently contain no PG (Pilnik and Voragen, 1970). Early studies by Steele and Yang (1960) reported no evidence of the presence of PG in firm ripe cherries, but moldy cherries contained a high concentration of PG. However, Yang et al. (1960) reported that PG was responsible for the severe softening of brined cherries due to pectin degradation.

Horticulturists have long sought reliable, simple indicators of fruit maturity. Indices such as color, firmness, removal force, size, weight, and soluble solids are traditionally used to fruit maturity. All hypothesized that the physical changes in cherry firmness, or other maturity indices, may be related to increasing activity of softening enzymes. Our objective was to measure the activity of softening enzymes in cherries during development and storage, and attempt to correlate such changes with physical changes related to maturation.

MATERIALS & METHODS

ROYAL ANNE and Bada cherries were harvested from the Lewis Brown Farm, Oregon State University at weekly intervals, from June 6 (wk 1) to July 10, 1991 (wk 6), and stored at 1°C for a 2 wk period from July 10 (wk 6) through July 22, 1991 (wk 8). Enzymes were analyzed from the first week, but maturity indices (weight, volume, s.s., firmness and color) began on week two. Cherries were harvested randomly from the interior, exterior and tops of trees. Care was taken to pick only healthy, undamaged fruit. Cherries were placed in plastic bags and stored at 1°C and assays were performed the day of harvest.

Fruit were evaluated for changes in weight, volume, color, firmness, soluble solids and enzyme activities. Mean weight and volume of 100 cherries from each harvest period were determined. All characteristics were analyzed by one way analysis of variance with the date of harvest as the variable. Means were separated by the method of LSD at $p < 0.05$.

Soluble solids and firmness

Soluble solids were determined by the official method of analysis of the AOAC, using a Bausch & Lomb refractometer with a temperature compensator (VWR model 1160). For firmness, 10 cherries were selected from each sample. Flesh firmness was measured with a Hunter Spring mechanical force gage (Ametek Inc., Hatfield, PA), using a flat

Author Barrett is with the Dept. of Food Science & Technology, Univ. of California, Davis, CA 95616-8598. Author Gonzalez is with the Dept. of Chemistry, Universidad Autonoma de Chihuahua, Chihuahua, Mexico.

Table 1—Maturity indices for Royal Anne cherries

Harvest week	Weight (g)	Volume (mL)	S.S. (%)	Firmness (g)	Color a(*)	Hue
2	47.80[a]	48.0[a]	5.2[a]	>2000	—	−0.05[b]
3	68.90[b]	70.2[b]	8.4[b]	1691.2[a]	−2.44	−8.08
4	106.42[c]	107.4[c]	9.0[b]	675.0[b]	−0.50	0.83[c]
5	105.50[c]	103.6[c]	10.6[c]	506.5[c]	−0.31	−3.75
6	123.60[d]	120.8[d]	14.3[d]	300.7[d]	0.74[b]	−0.33
Storage						
7	123.68[d]	123.0[d]	15.8[e]	285.4[d]	2.80[c]	0.09[b]
8	121.30[d]	119.2[d]	16.8[e]	360.2[d]	2.70[c]	0.89[c]

[a-e] Means within a column separated by LSD = 5%. Means in columns with same letters not significantly different ($p < 0.05$).

Table 2—Maturity indicators for Bada cherries

Harvest week	Weight (g)	Volume (mL)	S.S. (%)	Firmness (g)	Color (a*)	Hue (a/b)
2	67.0[a]	66.0[a]	5.7[a]	—	—	−2.86
3	99.0[b]	93.2[b]	11.2[b]	752.3[a]	−2.34	−19.49
4	121.6[e]	125.4[d]	10.2[b]	391.0[b]	2.45[b]	−0.26
5	116.9[c]	115.6[c]	13.4[c]	277.0[c]	3.88[c]	0.90[b]
6	126.0[cd]	122.2[cd]	17.3[d]	245.3[d]	4.18[cd]	4.92[d]
Storage						
7	127.0[cd]	122.4[cd]	16.7[d]	215.0[d]	4.91[d]	1.50[c]
8	121.1[cd]	118.6[cd]	16.3[d]	268.4[c]	3.96[c]	1.60[c]

[a-e] Means within a column separated by LSD = 5%. Means in columns with same letters not significantly different ($p < 0.05$).

head attachment tip. Both cheeks of each cherry were tested after removal of the skin with a razor blade. Mean firmness was calculated from 20 readings.

Color

Hunter L* a* b* values were measured in the reflectance mode using a HunterLab D25-PC2 tristimulus colorimeter (Hunter Assoc. Inc., Reston, VA) equipped with optical sensor and IBM computer. Ten fruit were evaluated on both cheeks, those values were averaged and then averages of 10 cherries were recorded. Two indices of color change during maturation (a* scale and hue angle) were calculated. The a* scale indicates redness when positive, gray when zero, and greenness when negative. Hue angle, calculated from the arctangent of b*/a* (Clydesdale, 1991; McGuire, 1992), is a measure of the visual property normally regarded as color (red, yellow, green, etc.). In order to usefully evaluate hue angle, the method developed by McGuire (1992) for conversion of calculated values to those which remain positive between 0° and 360° was implemented. Means were separated by LSD at $p<0.05$.

Enzyme assays

Extraction. The method described by Buescher and Furmanski (1978) was used to extract all enzymes. Pectolytic enzymes were extracted by homogenizing cherry flesh in an aqueous solution containing 0.5M NaCl and 1% polyvinylpyrrolidone at 1°C. The homogenate was centrifuged at 12,000 × *g* for 15 min at 2°C in a RC-5 Superspeed refrigerated centrifuge (Sorvall, Dupont Instruments) and filtered through 4 layers of cheesecloth. This step was repeated once and the filtrate was used for enzyme assays.

Polygalacturonase. PG activity was assayed according to Pressey and Avants (1981). Enzyme extract (100 µL), 400 µL of 0.15M NaCl, and 100 µL of 1% polygalacturonic acid (PGA) in 0.1M sodium acetate buffer, pH 4.5, were incubated at 37°C for 30 minutes. After incubation, 500 µL of the mixture were analyzed for reducing groups (Milner and Avigad, 1967). A unit of activity was defined as the amount that catalyzed release of 1 micromole of reducing groups in 30 min.

PG activity was also tested by a modified procedure based on the "cup plate" technique developed by Dingle et al. (1953). A PGA substrate medium was prepared by slowly suspending 0.25% (w/v) of PGA (Sigma, St. Louis, MO, from orange) in distilled water with constant stirring. Then 0.25% ammonium oxalate, 1% potassium biphthalate and 0.1% thymol preservative were added and dissolved. The pH was adjusted to 4.0 with 0.1N sodium hydroxide. The medium was transferred to a bottle and 2% bacto agar medium (Difco) was added, then the mixture was sterilized by autoclaving for 15 min at ≈1000g/cm². Aliquots (25 mL each) were transferred to 15×150mm Petri dishes and allowed to solidify on a level surface. Wells were cut into the solidified agar with a sterile 6 mm internal diameter cork borer. Enzyme extract (60 µL) was dispensed into the wells, with one well designated as a negative control containing boiled enzyme extract, and one as a positive control containing a standard PG. After inoculation, plates were incubated for 24 hr at 35°C. PG activity was measured by a clear zone after flooding the plate with 6N HCl.

Pectin methyl esterase. PME activity was measured using a modification of the method of Rouse and Atkins (1955). The rate of citrus pectin demethylation was measured at room temperature (≈23°C) by titration with 0.025 N NaOH using a pH meter with a glass electrode and an automatic temperature compensator (Corning Model 125). Fifty mL of 1% pectin in 0.1M NaCl were the substrate, adjusted to pH 7.0 before addition of 25 mL of enzyme extract. Alkali was added at the rate required to keep the pH at 7.0 for 10 min. One unit of PME activity was defined as the amount of enzyme capable of catalyzing the consumption of 1 micromole of base/10 min under assay conditions.

β-Galactosidase. β-Gal activity was assayed using the method described by Labavitch and Rae (1977). The reaction mixture consisted of 1.0 mL of 50 mM sodium acetate buffer, pH 5.0, containing 0.05% p-nitrophenylgalacto-piranoside substrate (Sigma, St. Louis, MO), and 0.25 mL of enzyme extract. After 1 hr incubation at 37°C, the reaction was stopped by adding 2 mL of 1 N ammonium hydroxide containing 2 mM EDTA. The amount of liberated p-nitrophenol was determined by measuring the optical density at 400 nm. One unit of enzyme was defined as the amount of enzyme capable of catalyzing the release of 1 micromole of p-nitrophenol/hr under assay conditions.

CMC-Cellulase. CMC-cellulase activity was measured in a mixture containing 0.4 mL of a 0.5% CM-cellulose (CMC 7HOF, Aqualon Co., Hopewell, VA) solution in 50 mM sodium acetate, pH 5.0 and 0.1 mL of cherry extract. After incubation at 30°C for 1 hr, the reaction was stopped and reducing sugars were measured by addition of the Nelson Somogyi reagent. Samples were centrifuged and absorbance at 510 nm was read.

RESULTS & DISCUSSION

HARVEST DATE had a significant relationship to all fruit quality factors measured, many of which are typically used as maturity indices. Mean values for weight, volume, soluble solids, firmness and color (Table 1) were compared for Royal Anne and (Table 2) for Bada cherries. The major growth period for both cultivars occurred between wk 2 and 4. Neither weight nor volume changed during storage. Soluble solids levels in both Royal Anne and Bada cherries increased at a fairly constant rate during maturation and storage. Soluble solids content in fruit depends on the stage of maturity (Drake and Fellman, 1987), and Bada may have been more mature than Royal Anne cherries. By week 6, s.s. levels in Bada cherries were 17.3%, while in Royal Anne they were 14.3%.

Cherries softened continuously throughout maturation and harvest, but the most notable softening occurred between wk 2 and 4 (Tables 1 and 2). The firmness of samples harvested on June 12 was too great to be measured with the available penetrometer. Bada cherries softened faster than Royal Anne, another indicator that Bada matured earlier. Note that after 2 wk of refrigerated storage, firmness increased in the Bada cultivar to a level greater than that at harvest. Dehydration following harvest may have caused a toughening of the skin, hence higher firmness. This increase was only significant in Bada cherries and probably reflects earlier maturity.

Cherry firmness has been used as an indicator of fruit maturity by several investigators (Brown and Bourne, 1988; Facteau, 1982). Losses in firmness have been attributed to both enzyme activity and transpirational water loss. Drake and Fellman (1987) correlated the loss of sweet cherry firmness with increases of soluble solids. In our study, firmness may also be correlated to changes in soluble solids. Note that firmness changes were at their peak when major growth occurred.

In both cultivars, fruit color changed from green, as indicated by "a*" values and hue angle, at a steady rate during maturation. As noted by McGuire (1992), a hue angle between 0° and 45° indicates a red color, while the range of 135° to

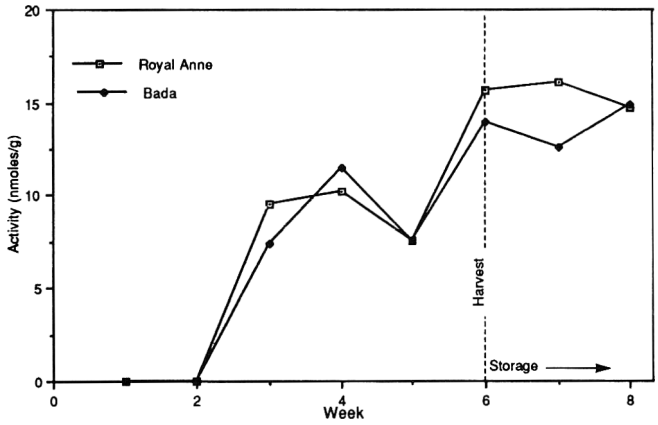

Fig. 1—Polygalacturonase activity in Bada and Royal Anne cherries.

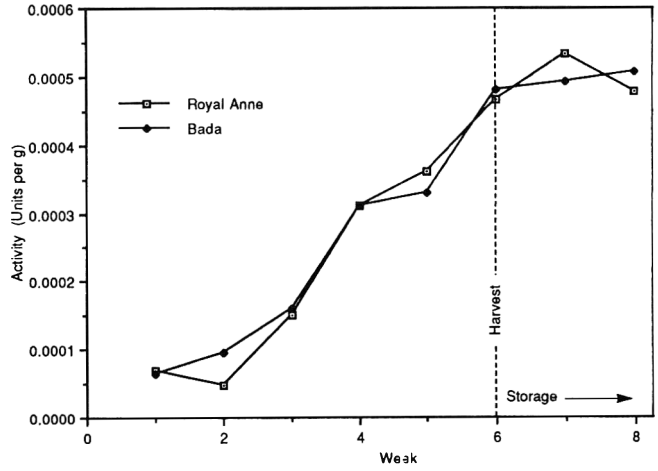

Fig. 2—Pectin methyl esterase activity in Bada and Royal Anne cherries.

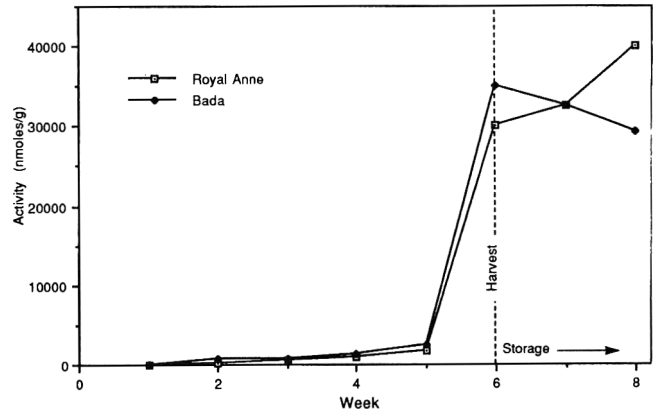

Fig. 3—β-Galactosidase activity in Bada and Royal Anne cherries.

180° would imply a yellow-green to green color. Bada apparently matured faster, with red color appearing much earlier. Red color in Bada fruit appeared to decline somewhat after 2 wk storage, while red color development in Royal Anne continued to proceed after harvest. Color is one of the most widely used maturity indicators, but it can vary tremendously among cherries on the same tree.

Cherry PG, PME, and β-gal activities were compared at different stages of maturation and storage (Fig. 1, 2, and 3). Cellulase activity was not detected at any time during maturation or storage. PG activity was not detected in either cultivar until wk 3 (Fig. 1). In general, PG activity increased during maturation and storage. No difference occurred in activity of the two cultivars until harvest and storage, but both showed the greatest increase in activity between June 12 and 26, 1991. PG activity in stored samples remained constant at about the same level as that of harvest, with activity in Royal Anne cherries slightly higher than Bada (Fig. 1).

Note that from wks 2 to 4 (the period of greatest increase in PG activity), firmness values showed the sharpest changes. Many investigators have associated textural changes with PG activity (Pressey et al., 1971; Wallner and Walker, 1975; Milner and Avigad, 1967). However, Pressey et al. (1971) also reported that peaches softened considerably prior to appearance of PG.

The level of PG activity in cherries was relatively low compared to that reported in other fruits. Previous studies have reported the following units/g fresh weight: avocado 0.8 (Awad and Young, 1979), peaches 4 to 6 (Pressey et al., 1971), tomatoes up to 30 (Gross and Wallner 1979), pears 20 to 70 units/mg protein (Ben-Arie and Sonego, 1979). We found from zero to ≈0.32 units/g fresh weight. Steele and Yang (1960) used the diffusion assay to evaluate cherry PG and reported activity in only a few samples. They did not report specific values for PG activity, but indicated that the enzyme was present. When the diffusion assay was used for our study, PG was not detectable.

PME activity (Fig. 2) was detected throughout maturation and storage, increased at a fairly constant rate and reached a maximum at harvest in both cultivars. PME was detectable even in the first 2 wk of sampling, before PG activity was detected. This supports the hypothesis that PME action is required before PG can be fully effective. No significant difference occurred in the activity of the two different cultivars at any time. PME activity was relatively constant after harvest and during storage.

Awad and Young (1979) also reported that PME activity was detectable long before that of PG in harvested avocados. They reported cellulase activity increasing at about the same time as that of PG. Note that both PG and cellulase activity were associated with the climacteric, whereas PME was not.

β-Galactosidase activity was also detected at the early stages of cherry ripening (Fig. 3). The β-gal activity from June 6 through July 3, 1991 was relatively low. However, activity was considerably higher in cherries picked on July 10. Values for β-gal at harvest and during storage were about nine times higher than values prior to July 10, 1991. Neither Royal Anne cherries nor Bada cherries showed any changes in β-gal during storage.

The sharp increase in β-gal activity occurred at a later date in both cultivars than the peaks in either PG or PME. There may be some association between the dip in PG activity on July 3 and the immediate burst of β-gal activity. Bartley (1977) reported that an increase in β-gal was not necessarily required for initiation of galactose residue hydrolysis from the cell walls of stored apples. Those results also indicated that β-gal activity increased after the decline in firmness, and suggested that either an increased rate of enzyme synthesis, decreased rate of degradation, activation of the enzyme or disappearance of an inhibitor may have been responsible. Some have suggested that β-galactosidase activity may be associated with the amount of soluble solids present, since it is involved with hydrolysis of galactans. The involvement of glycosidases in fruit softening has probably not been given enough consideration (Pressey, 1983).

In another study, the appearance of PG, PME and β-gal in blueberry fruit coincided with pectin solubilization (Procter and Peng, 1989). Peak PME activity occurred in red berries and preceded peak PG activity, which was observed in blue-red fruit (Procter and Miesle, 1991). In our study, both PME and β-gal activity were detectable before that of PG. The max-

imum activity of all 3 enzymes correlated with harvest, when cherries were most mature and had the greatest red color. The suggested mechanism for pectin metabolism in ripening blueberries (Proctor and Peng, 1989) involves at least two enzymes, PME and PG. Demethylation by PME results in a greater number of carboxyl groups which may facilitate PG activity. PG preferentially degrades deesterified pectic substances (Huber, 1983). Our results further support the proposed mechanism by which pectic substance changes occur at different stages of maturity.

Fruit ripening appears to be accompanied by a decrease in cell wall galactose content. This loss of galactose may represent a separate process of cell wall degradation. Declines in cell wall galactose content have been reported in tomatoes (Pressey, 1983) and apples (Bartley 1974, 1977). Our results indicated that β-gal is present in cherries and that its activity increases during ripening.

Koch and Nevins (1989) showed that the extent of tomato uronide release by PG depended on the stage of fruit development. When cell wall preparations from tomatoes of different maturities were pretreated with PME the release of uronides by PG was equivalent. Wallner and Walker (1975), evaluating tomato softening, suggested that the major modifications of the cell wall had already occurred by the turning stage, prior to the onset of greatest PG activity. They indicated that possibly the pronounced increase in PG activity was a consequence of the disruption of cellular organization and control. The correlation between onset of increased PG activity and ripening and softening has been noted by several investigators in tomatoes (DellaPenna et al., 1986; Grierson and Tucker, 1983).

In our study, changes in the physical state of cherries appeared to occur at about the same time that at least PG and PME activity were increasing. In cherries, as in tomatoes, PME activity increases precede and may be required for the most effective PG-initiated softening changes.

The integrated action of PG, PME and β-gal, and possibly other enzymes, appear to be required to break down the closely packed structure of fruit cell walls.

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Molecular Mass and Solubility Changes in Pectins during Storage of Satsuma Mandarin Fruits (*Citrus unshiu* Marc.)

J. NAOHARA and M. MANABE

ABSTRACT

Soluble pectins in satsuma mandarin fruits stored at 5°C were investigated by High Performance Gel Filtration chromatography. Pectins isolated from the flavedo, albedo, membranes and juice sacs were separated into four fractions. The hydrochloric acid soluble pectin fraction in each part was the highest and decreased slightly while the water-soluble pectin increased slightly. The avergae molecular weights of the fractionated soluble pectins in juice sacs were nearly constant but those in flavedo, albedo and membranes decreased as storage time increased. The ratios of higher molecular weight of water soluble and ammonium oxalate-soluble pectins in all the four parts and those of hydrochloric acid soluble pectin in flavedo and membranes decreased as ratios of lower molecular weight fractions increased.

Key Words: satsuma, oranges, mandarins, pectin, albedo

INTRODUCTION

SATSUMA MANDARIN FRUITS (*Citrus unshiu* Marc.) are produced high volume in Japan and usually harvested in November or December. To maintain freshness over several months, some of them are refrigerated. The properties of pectin (Tarutani and Manabe, 1963; Kawabata et al., 1974; Inoue et al., 1985; Manabe and Naohara, 1986) and its behavior (Miura et al., 1963; Daitou and Satou, 1984; Naohara and Manabe, 1987) during maturation in satsuma mandarin fruits have been reported, and the soluble pectin and neutral sugars in acidic pectin fractions have been analyzed. However, chemical changes, especially distribution of molecular weights of pectins, in fruit tissues during storage have not been reported.

Depolymerization of pectin is caused by the action of pectic enzymes in fruits. Although pectinesterase (PE) activity in citrus fruits is high, PE is not solely responsible for the depolymerization of pectin. Breakdown of pectin is initiated by both PE and polygalacturonase (PG), the activity of which in citrus fruits is not detectable or extremely low (MacDonnel et al., 1945; Pratt and Powers, 1953; Hobson, 1962; Mannheim et al., 1969; Rogers and Hurley, 1971; Riov, 1975; Robertson, 1976; Kunte and Ahastri, 1981). High endo-type PG activity was observed in stored satsuma mandarin fruits at low temperature and PG was assumed responsible for the depolymerization of pectin during cold storage (Naohara and Manabe, 1993).

Fundamental information on pectin content and distribution in satsuma mandarin fruits during storage can help maintain quality through development of circulation, storage and processing systems. Our objective was to investigate behavior of soluble pectins, changes in pectin molecules and distribution of pectins molecular weights in satsuma mandarin fruits during storage at 5°C.

MATERIALS & METHODS

Materials

Satsuma mandarin fruits (Owari var.) were harvested in a private orchard in Kagawa, Japan. Fruits were kept at room temperature a few days prior to storage up to 110 days at 5°C. Cold storage began on 12 December, 1992 and fruits were removed on 21 January, 1993 (40 days storage), 1 March 1993 (80 days storage) and 1 April, 1993 (110 days storage).

Author Naohara is with Environmental Resources Research Center, Okayama Univ. of Science, Okayama-shi, Okayama, 700 Japan. Author Manabe is with the Faculty of Agriculture, Kagawa Univ., Miki-cho, Kagawa, 761-07 Japan.

Extraction and qualitative analysis of soluble pectins

Alcohol insoluble solids (AIS) were prepared from satsuma mandarin fruits by the method of Manabe and Tarutani (1965a). Water soluble pectin (WSP), ammonium oxalate soluble pectin (ASP) and hydrochloric acid soluble pectin (HSP) were extracted successively from AIS according to the method of McColloch (1952). The sodium hydroxide soluble pectin (SSP) was extracted from the remaining materials insoluble in hydrochloric acid. The final residue was treated twice with 100 mL of 0.05N sodium hydroxide at 30°C for 2 hr. The soluble pectins were saponified with alkali and the uronic acids determined using carbazole-sulfuric acid were expressed as anhydrogalacturonic acids. The pectin content was measured three times and mean values determined. The coefficients of variation of pectin content values were ≤ 5%.

Molecular weights of pectins and distribution patterns

Molecular weight distribution patterns were obtained by high performance gel filtration (HPGF) chromatography and were average molecular weights (Mw) of extracted pectins calculated according to the method of Cazes (1966). The HPGF chromatography system included a Waters U6K injector, a Waters model 510 pump, a Waters model 401 differential refractometer, Waters ultrahydrogel linear columns heated with a Sugai model U620 heater and a Waters 805 data module. The mobile phase was 1% acetic acid and the flow rate was 1 mL/min. Eight pullulan standards (P-800, P-400, P-200, P-100, P-50, P-20, P-10 and P-5) purchased from Millipore were used to calibrate the HPGF chromatography columns. They represented molecular weights as follows: P-800, 853,000; P-400, 380,000; P-200, 186,000; P-100, 100,000; P-50, 48,000; P-20, 23,700; P-10, 12,200 and P-5, 5800. Mw was measured 3 times and a mean value for average molecular weight was determined. The coefficient of variation of each pectin content was ≤ 5%.

RESULTS & DISCUSSION

Changes in soluble pectins during storage

The AIS contents were 24% in albedo, 13% in membranes, 11% in flavedo and 4% in juice sacs immediately after harvest. The AIS content in the albedo decreased after storage for 40 days. AIS in juice sacs decreased during storage for 80 days but then increased during additional storage up to 110 days. AIS in flavedo did not change until 80 days but increased after that (data not shown).

Changes in concentrations of soluble pectins were compared (Fig. 1). The concentration of HSP was highest throughout storage in tall component parts, although the concentration of HSP in each part decreased with storage time. The extent of decrease differed in different fractions. HSP in flavedo decreased by 46%, which was the largest decrease in all fractions during storage for 110 days. However, only a 17% decrease occurred in HSP in membranes. Changes in WSP levels tended to be opposite those of the HSP. No sharp changes in ASP and SSP were observed.

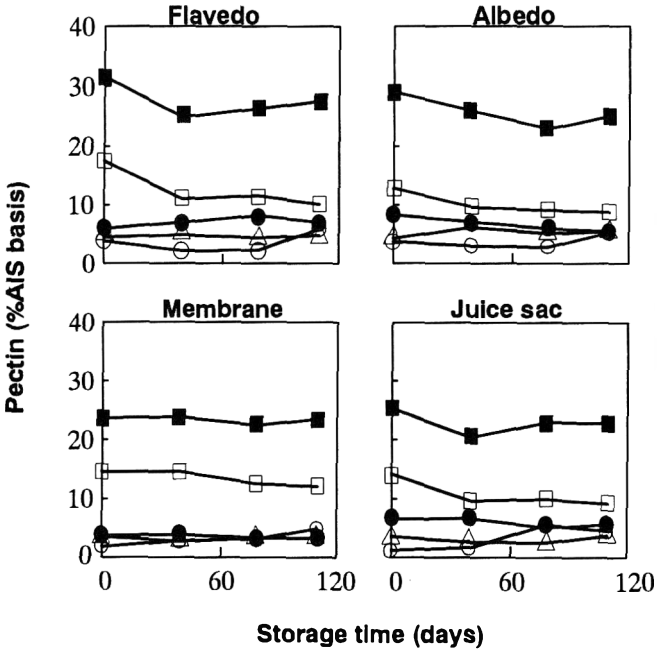

Fig. 1—Changes in soluble pectin in component parts of satsuma mandarin fruits during storage. ○ water-soluble pectin; △ ammonium oxalate-soluble pectin; □ hydrochloric acid-soluble pectin; ● sodium hydroxide-soluble pectin, ■ total pectin.

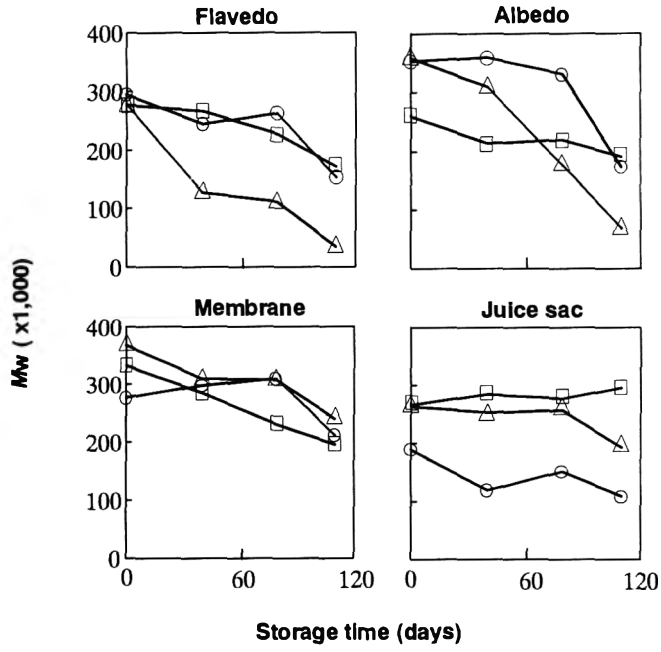

Fig. 2—Changes in *Mw* of water soluble pectin, ammonium oxalate soluble pectin and hydrochloric acid soluble pectin during storage. ○ water-soluble pectin; △ ammonium oxalate-soluble pectin; □ hydrochloric acid-soluble pectin.

Changes in Mw of soluble pectins during storage

The pullulan standards exhibited a linear relationship between the logarithm of molecular weights and retention times. Changes in Mws of soluble pectins in each fruit were composed (Fig. 2). Mw of WSP, ASP and HSP in all parts except juice sacs showed decreases during storage. Mw of WSP differed in fruit parts at the beginning of storage. They were : albedo, 353,000, flavedo, 295,000, membranes, 277,000 and juice sacs, 189,000.

Mw of the different soluble pectins in the parts during 80 days storage showed a slight change but decreased sharply after 80 days storage. Mw of ASP in the parts also appeared different at the beginning of the storage. They were: membranes, 368,000; albedo, 358,000; flavedo 279,000; and juice sacs, 262,000. While the fruits were stored for 110 days Mw of the ASP were reduced by 87%, in flavedo, 81%, in albedo, 35% in membranes and 27% in juice sacs. A large difference in reductions was observed between peel (flavedo and albedo) and pulp (membranes and juice sacs). Change in Mw of the HSP in albedo and juice sacs were small during storage. The decrease in Mw of the WSP tended to be the same as that in the HSP.

Molecular weight distributions of soluble pectins during storage

When a comparison was made between the HPGF chromatograms of WSP obtains after 80 days and 110 days storage, a peak at retention time 7.5 min (Mw 320,000), in the chromatogram of fruit stored for 80 days disappeared. New peaks at retention time 8.4 min (Mw 46,000) and at 10.5 min (Mw of monomeric galacturonic acid), appeared in the chromatogram of fruit stored 110 days. In the HPGF chromatograms of ASP, the retention time of peaks in each chromatogram lengthened as storage period lengthened. A peak at the void volume was observed in the HPGF chromatogram of HSP for 40 days storage but disappeared after 80 days storage. A new peak at retention time 10.8 min (Mw of monomeric galacturonic acid) appeared instead.

The molecular weight distributions of WSP, ASP and HSP in each part were compared (Fig. 3). For molecular weight distribution of WSP before storage, pectin with Mw > 853,000 were flavedo 25% of the fraction, albedo 46%, membranes 62% and juice sacs 39%. Pectins with Mw <100,000 were 30%, of the flavedo, 15% of the albedo, 20% of the membranes and 39% of the juice sacs fractions. Pectin with Mw > 100,000 decreased remarkably in flavedo, albedo and membranes parts between 80 and 110 days storage, and large size pectins (Mw ^ 853,000) decreased sharply. In juice sacs, pectins with Mw > 100,000 decreased gradually to 5% after 110 days storage.

For the molecular weight distribution of ASP before storage, pectin with Mw > 853,000 were 49% of the total in flavedo, 43% albedo, 52% membranes and 58% juice sacs and they were a little higher than those of WSP. On the contrary, pectin with Mw < 100,000 in all parts except albedo were lower than those of WSP. They were 24% in flavedo, 29% albedo, 10% membranes and 20% juice sacs. In flavedo and albedo, the pectin of Mw > 100,000 increased significantly as storage time increased. Levels of pectins with Mw > 853,000 were constant in membranes regardless of storage period.

For molecular weight distribution of HSP before storage, pectins with Mw > 853,000 were 32% in flavedo, 38% in albedo, 35% in membranes and 27% in juice sacs, and they were considerably lower than those of WSP and ASP. Pectins with Mw < 100,000 were 34% in flavedo, 38% in albedo, 21% in membranes, and 28% in juice sacs. In flavedo and membranes, pectin with Mw < 100,000 decreased, while pectin with molecular weights from 100,000 to 853,000 remained constant throughout storage.

A few investigations of pectin in satsuma mandarin fruits have involved fractionation and analysis of pectin in mature satsuma fruits (Tarutani and Manabe, 1963) and pectin concentrations in several strains of satsuma fruits (Manabe and Naohara, 1986). Changes occurred in pectin concentration (Daitou and Satou, 1984) and fractionation (Naohara and Manabe, 1987) during maturation, and soluble pectins were also analyzed (Naohara and Manabe, 1987). The total pectin content increased during maturation and the increase of HSP was especially notable. The behavior of pectin lemons during mat-

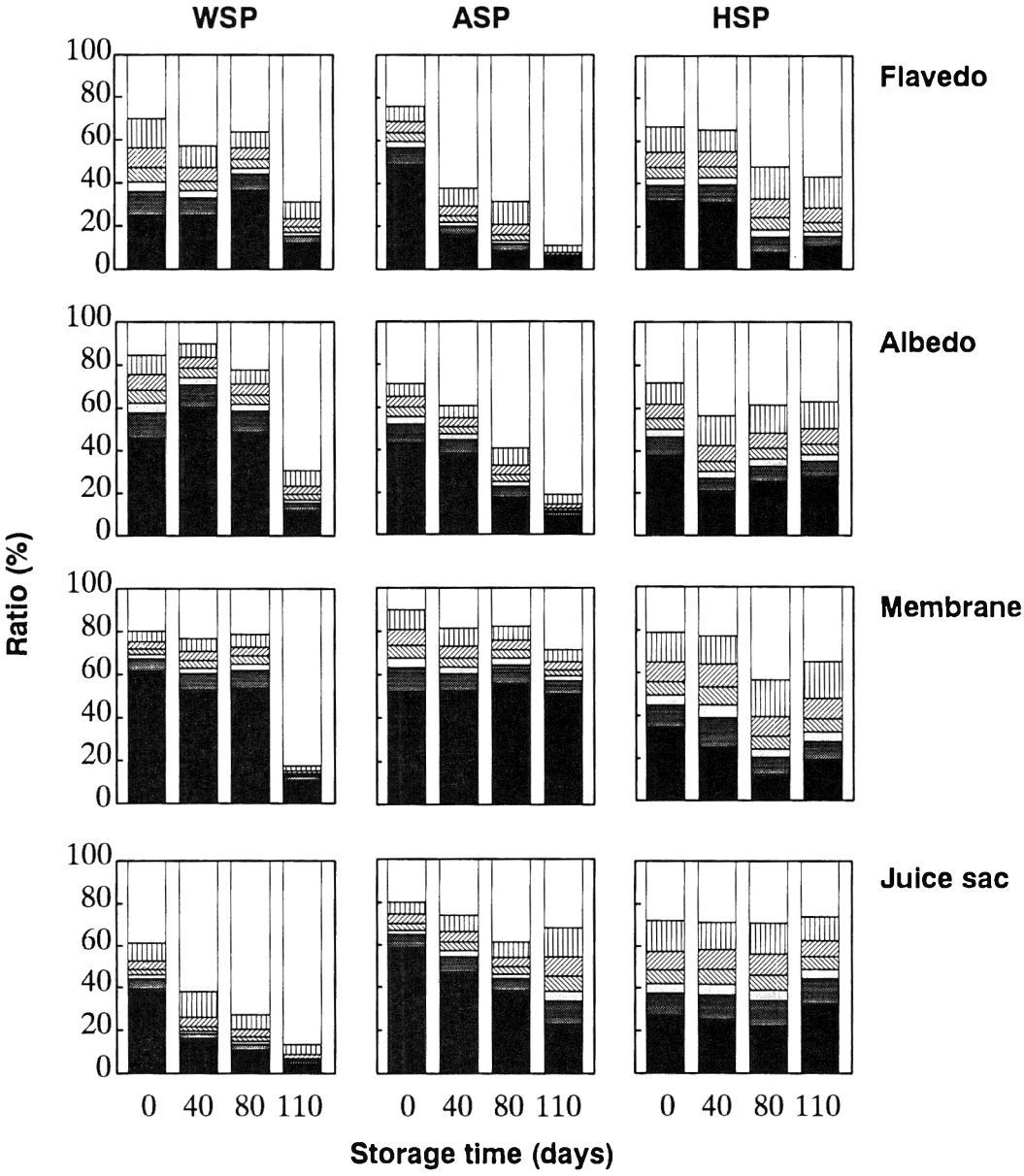

Fig. 3.—Molecular weight distribution of soluble pectin in flavedo part of satsuma mandarin fruits. WSP: water-soluble pectin; ASP: ammonium oxalate-soluble pectin; HSP: hydrochloric acid-soluble pectin. □ 100,000>, ▥ 100,000–200,000, ▨ 200,000–300,000, ▧ 300,000–400,000, □ 400,000–500,000, ■ 500,000–853,000, ■ 853,000<.

uration has been reported in detail (Rouse and Knorr, 1969, 1970).

In general, satsuma mandarin fruits are stored in a cold room up to 5-6 mon. The HSP in each component part of the satsuma mandarin fruits stored the low temperature decreased (Fig. 1) during storage while the WSP increased slightly. This tendency was more notable as the storage period exceeded 80 days . Mw of each pectin fraction decreased according to length of storage. The changes between 80 and 110 days of storage were more remarkable than in the initial stages of storage. The ASP, which was assumed to be pectin chelated with metal ions such as calcium and WSP declined more rapidly than HSP during storage. These results may indicate that the rate of depolymerization by the enzyme was dependent on the substrate structure (e.g., composition of neutral sugar, degree of esterification, etc.).

The peel of satsuma mandarin fruits after 80 days storage was considerably softened. This may be due to depolymerization of pectin by pectinases. PE activity in satsuma mandarin fruits was high during maturation as well as during storage (Manabe and Tarutani, 1965). Although little PG activity in mature satsuma mandarin fruits was observed, PG activity in fruits stored at low temperature for a long period was clearly found (Naohara and Manabe, 1993). PG as well as PE apparently induced the decrease in HSP and the increase in WSP, and depolymerized WSP, ASP and HSP during cold storage. However, whether the difference in depolymerization rate of soluble pectins was caused by different pectinases or different compositions of neutral sugars is not known.

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Superoxide Dismutase Activities in Senescing Apple Fruit (*Malus domestica* Borkh.)

ZHANYUAN DU and WILLIAM J. BRAMLAGE

ABSTRACT

Total superoxide dismutase (SOD:superoxide: superoxide oxidoreductase; EC 1.15.1.1) activity and individual activities of its three different metalloenzymes, CuZn-SOD, Fe-SOD, and Mn-SOD, were investigated during senescence of apples (*Malus domestica* Borkh.). Total SOD activity and relative activities of its three forms varied greatly among cultivars. Activities underwent considerable change during senescence of fruit, but changes were different among cultivars. Whether fruit senesced at 0°C or 20°C had little effect on SOD activities, and application of the antioxidant diphenylamine (DPA) did not alter activities. SOD activities increased with occurrence of the physiological disorder "senescent breakdown," but did not increase with occurrence of the disorder "superficial scald." In a given apple cultivar, changes in total SOD activity generally paralleled activities of the different SOD forms. Such activity may reflect changes affecting food and nutritive quality of the fruit.

Key Words: lipid peroxidation, superoxide dismutase, senescence, apples, enzymes

INTRODUCTION

THE SUPEROXIDE RADICAL is generated in all respiring cells, and can react with many cellular materials (Hassan and Scandilios, 1990). It can lead to damage and losses in many foods, and could be particularly important in fresh products awaiting processing. Superoxide dismutase (SOD: superoxide:superoxide oxidoreductase; EC 1.15.1.1) is a ubiquitous defensive enzyme against superoxide damage to aerobic organisms. It has been patented as an antioxidant for foods (Richardson and Hyslop, 1985). Three types of metalloenzymes of SOD exist in living organisms, namely CuZn-SOD, Fe-SOD, and Mn-SOD (McCord and Fridovich, 1969; Halliwell, 1974; Becana et al., 1989), and there are increasing reports that all three types of SODs exist in higher plants (Kwiatowski and Kaniuga, 1984; Kanematsu and Asada, 1989; Droillard and Paulin, 1990). The three SODs differ not only in their catalytic prosthetic metal, but also in their quantitative distribution in plant tissues and subcellular localization in cells (Polle et al., 1989; Jackson et al., 1978; Wingsle et al., 1991). All three types of SODs catalyze the dismutation of superoxide anions to produce hydrogen peroxide *in vivo*.

SOD activity has been linked to physiological stresses (Bowler et al., 1992; Malan et al., 1990; Monk et al., 1989). SOD activity increased during low temperature stress in *Chlorella ellipoidea, Zea mays,* and *Zea diploperennis* (Clare et al., 1984; Hodgson and Raison, 1991; Jahnke et al., 1991). SOD activity also decreased with senescence of *Nicotiana tabacum* and *Dianthus caryophyllus* (Dhindsa et al., 1981; Droillard and Paulin, 1990), and was inversely related to susceptibility to sunscald damage in *Lycopersicon esculentum* fruit (Rabinowitch and Sklan, 1980). Such results suggest that SOD may be involved in reactions to damage induced by oxygen, free radicals and ionizing radiations. In apple fruit, there is evidence of increased lipid peroxidation, including increased peroxidation products, peroxidation-induced membrane damage and lipoxygenase activity, during senescence (Feys et al., 1980; Paliyath et al., 1984; Feys, 1985). SOD activity could help protect cells from damage by peroxidation products. Baker (1976) reported that there were no major quantitative changes in total SOD activity of several ripening fruits, including Golden Delicious apples. Fruit SOD has not been separated into its metalloenzymes in crude fruit extracts, and relative activities under different physiological conditions have not been determined. We measured the activities of the three SODs in apple fruit during senescence, and tested effects of conditions that could influence occurrence of physiological damage in the fruit. Our objectives were to examine variability of SOD activity, and the relative contributions of the three SOD metalloenzymes to total activity in apple fruit.

Authors Du and Bramlage are with the Dept. of Plant & Soil Sciences, Univ. of Massachusetts, Amherst, MA 01003.

MATERIALS & METHODS

Plant materials

Apples were from mature apple trees (*Malus domestica* Borkh.) of three cultivars (Cortland, Empire, and Delicious) grown under standard horticultural conditions at the University of Massachusetts Horticultural Research Center, Belchertown, Mass. Individual trees served as sources of replications of fruit used in the experiments. A series of experiments was conducted during three years. Fruit were harvested at commercial maturity as indicated by starch hydrolysis or ethylene production. Harvested fruit were stored at 0°C in air except as otherwise indicated. At each sampling time, 10 fruit per replication were peeled with a mechanical peeler, and peels were frozen immediately in dry ice and stored at −25°C until use.

Enzyme activities

Extraction. Two grams of frozen tissue were ground with a mortar and pestle in 10 mL of 50 mM phosphate buffer (pH 7.0, 0.2% insoluble polyvinylpyrrolidone, 0.1 mM EDTA and 3 mM $MgCl_2$), and a small amount of washed sand. The homogenate was centrifuged at 15,000 × *g* for 15 min and the supernatant was used for assays of enzyme activities. All steps in preparation of the extract were carried out at 0–4°C. Enzyme activities were expressed as units/g fw.

Assays of SOD. SOD activity has been assayed by measuring ability of SOD to inhibit reduction of a chosen substrate by superoxide anion in a photochemical or xanthine non-photochemical system. Giannopolitis and Ries (1977) reported that interference by peroxidases or other enzymes and by non-enzymatic impurities in crude extracts of several plant species were insignificant in either photochemical or non-photochemical assay systems. They concluded that the photochemical system was more reliable than the xanthine/xanthine oxidase system for determining SOD activities in crude plant extracts. Therefore, we used a photochemical system for SOD measurement. The assays for activities of total SOD and the three individual SOD (i.e. CuZn-SOD, Fe-SOD and Mn-SOD) were essentially according to Beauchamp and Fridovich (1971) and Dhindsa et al. (1981). They measured the ability of SOD to inhibit the photochemical reduction of nitro blue tetrazolium (NBT). The assay mixture contained 50 mM phosphate buffer, pH 7.8, 13 mM methionine, 75 μM NBT, 2 μM riboflavin, 0.1 mM EDTA, and 100 μL of enzyme extract. The assay mixture (3 mL) in uniform transparent tubes was shaken and placed 50 cm below a light bank consisting of eight 15 W fluorescent lamps. The reaction was started by switching on the lights and was allowed to proceed for 10 min. The reaction was stopped by switching off the lights, and the absorbance by the assay mixture at 560 nm was read. There was no measurable effect of diffuse room light. Non-irradiated assay mixtures did not develop color and served as controls. The assay mixture lacking enzyme extract developed maximum color, which decreased with increasing

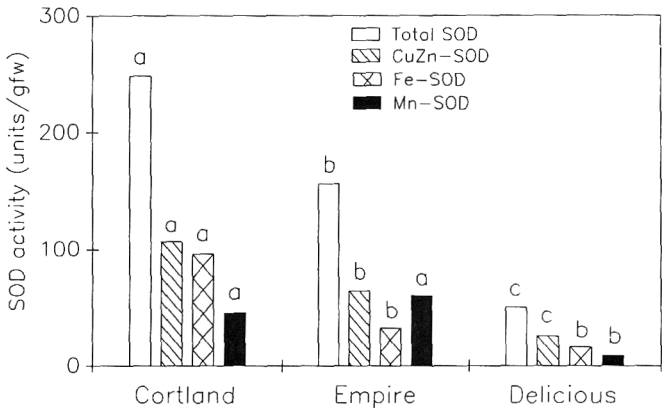

Fig. 1—Activities of SOD metalloenzymes in peel of three cultivars of apples at harvest. Cortland and Delicious fruit were harvested on Sept. 26 and Oct. 10, 1990, respectively, and each cultivar was replicated three times. Empire fruit were harvested on Sept. 27, 1991, and were replicated four times. Different letters for the same measurement among cultivars indicate significance at p = 0.05 separated by Duncan's New Multiple Range Test.

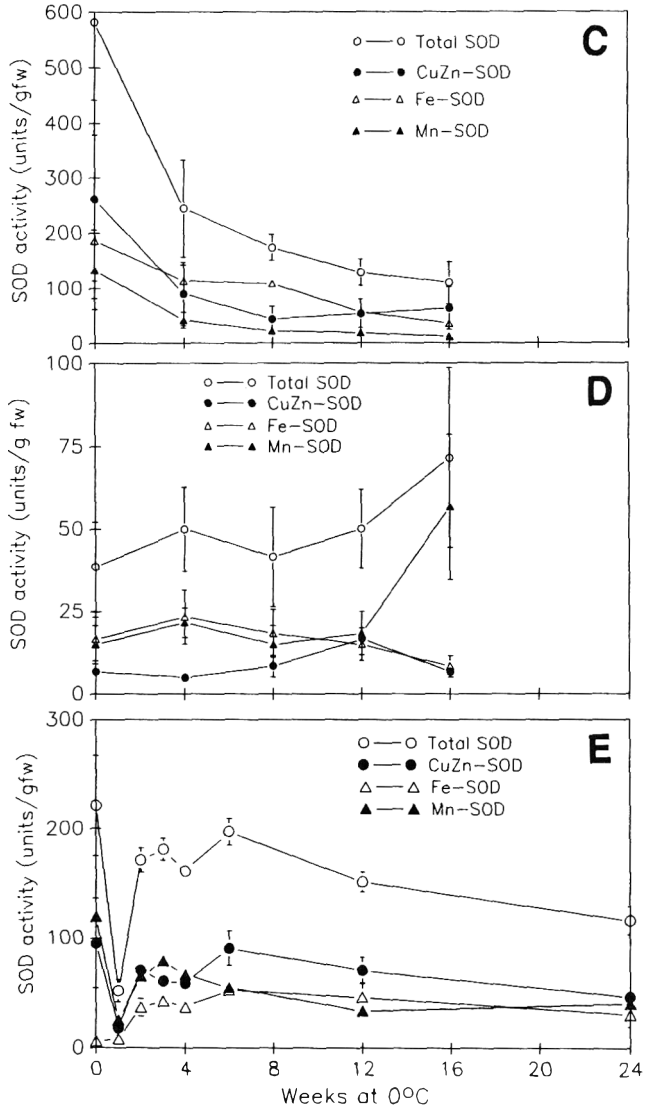

Fig. 2—Changes in SOD activities during storage at 0°C. Cortland and Delicious fruit were harvested on Sept. 26 and Oct. 10, 1990, respectively, and each cultivar was replicated three times. Empire fruit were harvested on Sept. 27, 1991, and were replicated four times. C: Cortland; D: Delicious; E: Empire.

amount of extract added. One unit of SOD activity was defined as the amount of enzyme that inhibited the NBT photoreduction by 50% under assay conditions. However, percent inhibition was not linear with SOD concentration. Thus, units of SOD activity were calculated from transformed data as described by Asada et al. (1974) and Giannopolitis and Ries (1977). SOD units/mL = (Vo/V − 1)·(dilution factor), where Vo is slope of the change in absorbance in the absence, and V in the presence, of enzyme extract. The transformation resulted in a linear correlation between SOD activity and the amount of extract used in this study (Vo/V = 0.989 + 0.48 [µl], R = 0.951***). According to Salin (1988), CuZn-SOD was inhibited by CN^- and by H_2O_2, Fe-SOD was resistant to CN^- but inactivated by H_2O_2, and Mn-SOD was resistant to both inhibitors. Thus, the total SOD activity was assayed in absence of both CN^- and H_2O_2, and the Mn-SOD in the presence of both 3 mM CN^- and 2 mM H_2O_2. The activity of Fe-SOD was calculated by subtracting the activity in the presence of both 3 mM CN^- and 2 mM H_2O_2 (Mn-SOD) from the activity in the presence of only 3 mM CN^{-1} (Fe-SOD + Mn-SOD). The CuZn-SOD activity was calculated by subtracting the activity in the presence of 3 mM CN^{-1} from the total SOD activity.

RESULTS & DISCUSSION

STRONG CULTIVAR DIFFERENCES occurred in total SOD activities and in distributions of total activity among metalloenzymes in apples (Fig. 1). Cortland apple peel contained the highest SOD activities, with higher percent of CuZn-SOD and Fe-SOD than Mn-SOD. Delicious apple peel contained the lowest total SOD activity, but again, CuZn-SOD and Fe-SOD activities were higher than that of Mn-SOD. In peel of Empire apples, total SOD activity was intermediate, and Fe-SOD activity was lower than that of CuZn-SOD or Mn-SOD.

SOD activities changed considerably during storage at 0°C (Fig. 2). However, the patterns of change were quite different among cultivars. In Cortland apples (Fig. 2C), total SOD activity and that of all three SOD types decreased greatly during storage, especially during the first 4 wk at 0°C. In Empire apples (Fig. 2E), SOD decreased during the first week, recovered to near-initial levels and then dropped gradually with time. In both Cortland and Empire, changes in total SOD activity with time generally reflected changes of individual SOD. However, in Delicious apples (Fig. 2D), activity of Fe-SOD and Mn-SOD were the same for 12 wk at 0°C and then fell slightly, but CuZn-SOD increased over time, especially late in storage, causing total SOD activity to increase late in storage.

Differences in total SOD activity among different plant species (Giannopolitis and Ries, 1977) and among different cultivars of potato (Spychalla and Desborough, 1990) have been reported. Our results showed that not only were there cultivar differences in total SOD activity, but also in relative activities of its components. Polle et al. (1989) reported that total SOD activity decreased with tree age of Norwegian spruce, but Spychalla and Desborough (1990) reported that it increased during cold storage of potato tubers. We found the pattern of change in SOD activities during senescence of apples was cultivar-related. This suggested that differences in genetic control are reflected in SODs between cultivars as well as between species. Perl-Treves and Galin (1991) and Tsang et al. (1991) have shown that SOD activity could be regulated at the gene level under environmental stresses.

Effects on SOD activities of temperature, use of antioxidant, and occurrence of senescence-associated disorders were also investigated. Storage temperature of 0°C vs 20°C had little effect on SOD activities in Empire apples (Fig. 3). Although changes in activity over time differed among the three SOD forms, differences between activities at 0°C and 20°C were small when measurements were compared at about the same senescence stages. For example, activities of total SOD and of individual forms measured at 2 and 4 wk at 20°C were similar to those measured at 12 and 24 wk at 0°C. Similar data for Cortland showed the same relationships except that Mn-SOD activity was slightly lower (p = 0.05) at 0°C than at 20°C (data not shown). Apples undergo chilling damage after long-

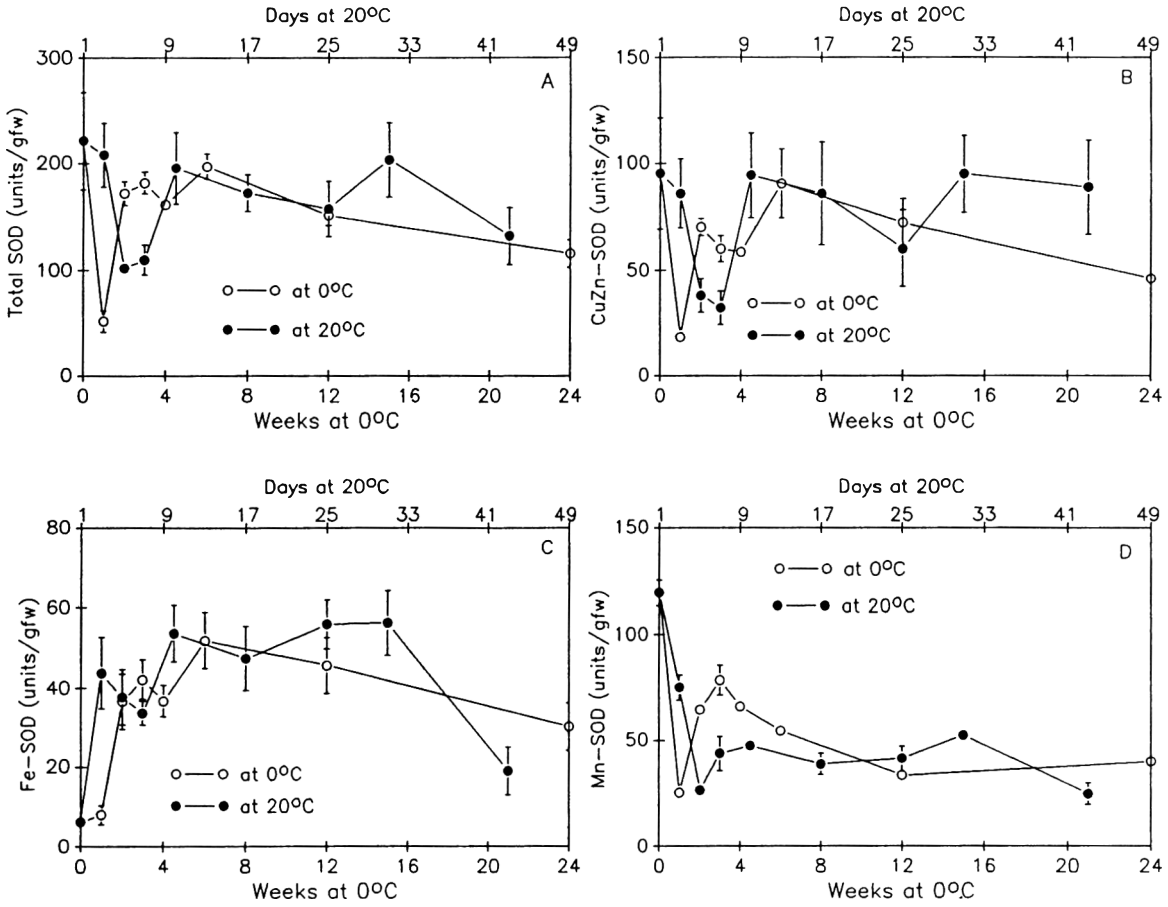

Fig. 3—Changes in SOD activity in Empire apples stored at 0°C or 20°C. Fruit were harvested on Sept. 27, 1991. Data are means of four replications.

term exposure to near-0°C (Bramlage and Meir, 1990), and these results indicate that chilling had little or no effect on SOD activities.

At both 0°C and 20°C, CuZn-SOD, Mn-SOD, and total SOD activities declined sharply shortly after harvest of Empire apples, and then returned to initial levels (Fig. 2 and 3). The decline corresponded with the onset of ripening in those fruit, during which ethylene production by the fruit increases logarithmically. In carnation flower, SOD activity declined as the ethylene climacteric occurred (Sylvestre and Paulin, 1990), as seen here in apples. This transient reduction in SOD activity might contribute to the beginning of fruit ripening (Meir et al., 1991). The absence of this transient decline in SOD from results of Cortland and Delicious may have been due to sampling at too wide (4-wk) intervals for those cultivars.

Diphenylamine (DPA) is an antioxidant used commercially on apples to reduce development of superficial scald, a peroxidation-mediated physiological disorder (Du and Bramlage, 1993). It also suppressed ethylene production by about 20% (Du and Bramlage, 1993), and can delay ripening and senescence of the fruit and reduce activities of some enzymes (Lurie et al., 1989). We applied 2000 ppm DPA to Cortland and Delicious apples before storage, and it had little effect on either total SOD activity or activities of individual forms of SOD, except for a slight reduction in Fe-SOD in DPA-treated Delicious apples ($p=0.05$) (Table 1). This suggested that SOD did not respond directly to exogenous antioxidant application, and it was consistent with the report that the antioxidant ethylenediurea did not act via SOD induction (Pitcher et al., 1992).

Both superficial scald and senescent breakdown are senescence-related disorders that can occur during storage of apples (Bramlage and Meir, 1990; Du and Bramlage, 1993). Activities of total SODs and CuZn- and Mn-SOD were much higher

Table 1—Effects of application of 2000 ppm diphenylamine at harvest on SOD activity in peal of Cortland and Delicious apples[a]

Treatment	Total SOD	CuZn-SOD	Fe-SOD	Mn-SOD
	(units/gfw)			
		Cortland		
−DPA	248.2	106.5	96.1	45.6
+DPA	267.3	117.0	104.3	46.7
F-test	ns	ns	ns	ns
		Delicious		
−DPA	50.5	25.4	16.4	8.7
+DPA	47.3	30.3	8.0	9.4
F-test	ns	ns	*	ns

[a] Cortland and Delicious fruit were harvested on 26 Sept., and 10 Oct., 1990, respectively, and stored for 16 wk at 0°C. Data are means of measurements taken every 4 wk during storage. There were three replications of each cultivar.
[b] * and ns are significance at $p = 0.05$ level, or non-significance.

in peel tissue taken from areas above visible symptoms of senescent breakdown (soft, moist, darkened areas in the flesh) on Empire apples than in peel from healthy tissue (Fig. 4). The increased SOD activities in senescent tissue may have been induced by tissue breakdown rather than by senescence or ripening, since senescing Empire fruit without symptoms of breakdown contained slowly declining activities of SODs during late storage (Fig. 2). This was consistent with the finding that SOD activity remained at the same level preceding and during senescence of Golden Delicious apples (Baker, 1976). Scald development also involves collapse of cells and formation of dark lesions on the fruit surface. However, when peel from fruit with and without scald, and with different intensities of scald, were compared, no differences occurred in any SOD form or total SOD activity (data not shown). Thus, change in

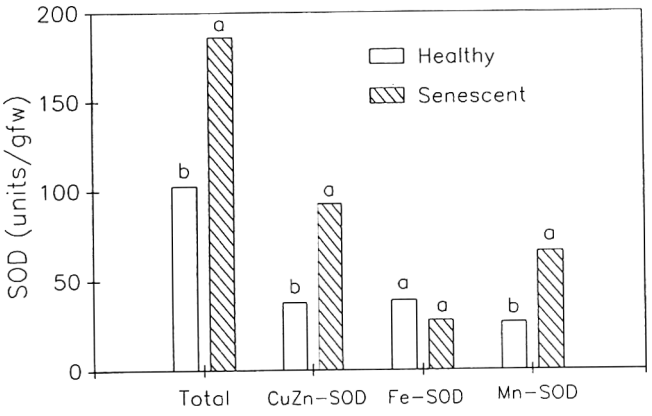

Fig. 4—SOD activity in peel from above areas with visible symptoms of senescent breakdown (senescent) and peel from breakdown-free (healthy) Empire apples. Fruit were harvested on Sept. 27, 1991, and stored at 0°C for 24 wk, and there were four replications. Different letters for the same measurement between 'senescent' and 'healthy' tissues indicate significance at p = 0.05 separated by Duncan's New Multiple Range Test.

SOD activity with cell collapse seemed to be specific to the cause of collapse. Such differences in SOD activity among cultivars also seemed not to be correlated with susceptibility to superficial scald. Both Cortland and Delicious are scald susceptible but Empire is scald resistant; however, Empire contained SOD activity higher than Delicious but lower than Cortland (Fig. 1). These differences among cultivars might be associated with their senescence properties, since Cortland senesces relatively rapidly and Delicious relatively slowly. High level of SOD may be protective against senescence-associated free radical damage. If so, manipulation of SOD activity (physiologically or genetically) might contribute to improved conservation of harvested fresh produce, since senescence-associated losses are common both on the fresh market and prior to processing.

CONCLUSIONS

SOD ACTIVITIES underwent considerable changes during senescence of apple fruit, but were scarcely affected by physiological factors. Both SOD levels and their changes during senescence were different among cultivars. Changes in SOD activity in apples under different physiological conditions usually reflected changes in activities of all 3 SOD metalloenzymes. Measurement of total SOD activity may be an adequate indication of functioning of SOD in uniform plant materials. However, genetic differences in SOD activity among different plant materials may affect their responses to environmental and cellular stresses.

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Purification and Characterization of an Endoprotease from Melon Fruit

K. NODA, M. KOYANAGI, and C. KAMIYA

ABSTRACT

An endoprotease was purified from melon fruit (*Cucumis melo* L.) by ammonium sulfate precipitation, gel filtration and ion-exchange chromatography using *t*-butyloxycarbonyl-Ala-Ala-Pro-Leu *p*-nitroanilide as a substrate. The molecular weight was estimated as 26,000 and isoelectric point pH 9.5. It preferentially hydrolyzed peptide bonds of the carboxyl terminal sides of Leu, Ala, His, Gln, and Asn. Activity was strongly inhibited by diisopropyl phosphofluoridate, indicating the serine protease nature of the enzyme. The migration distance on electrophoresis, molecular weight and substrate specificity differed from cucumisin, a known protease from melon. This unusual protease may have potential for special food treatment applications.

Key Words: melon, *Cucumis melo* L., endoprotease, synthetic peptide

INTRODUCTION

MELONS (*Cucumis melo* L.) are well known as fruits with high proteolytic activity. Kaneda and Tominaga (1975) isolated an endoprotease (cucumisin) from the sarcocarp of melon. The enzyme is a serine protease with broad substrate specificity (Kaneda et al., 1986; Kaneda and Tominaga, 1987).

We reported that L-aspartyl-L-phenylalanine methyl ester, a peptide sweetener, was hydrolyzed very rapidly to constituent amino acids by the action of protease(s) in melon juice (Noda et al., 1991). In order to clarify the properties of melon protease(s), we investigated the proteolytic activities in melon homogenate using a series of synthetic substrates having the sequence Boc-tetrapeptide-pNA (Boc, *t*-butyloxycarbonyl; pNA, *p*-nitroanilide) (Yoshida et al., 1988; Kadokami et al., 1990), and found an endoprotease activity hydrolyzing Boc-Ala-Ala-Pro-Leu-pNA along with trypsin-like and aminopeptidase activities. Our objective was to purify and characterize the Boc-Ala-Ala-Pro-Leu-pNA degrading emdoprotease. The purified enzyme differed from cucumisin in effects of electrophoresis, molecular weight and substrate specificity, and we labeled it C.m. protease to distinguish from cucumisin.

MATERIALS & METHODS

Materials

We used a type of netted melon, *Cucumis melo* L. var. Earls. Amino acid *p*-nitroanilides were synthesized according to the method described previously (Noda et al., 1990), and Boc-tetrapeptide *p*-nitroanilides were prepared by the conventional method of peptide synthesis (Yoshida et al., 1988). The following materials were purchased: cucumisin (Takara Shuzo, Tokyo); microbial protease inhibitors (Peptide Institute, Osaka); molecular weight markers for polyacrylamide disc gel electrophoresis (PAGE) and isoelectric focusing pI markers (Oriental Yeast, Tokyo); TSK gel Toyopearl HW-55 and CM-Toyopearl 650-S (Tohso, Tokyo); Sephadex PD-10 (Pharmacia, Uppsala). All other chemicals were analytical grade.

Purification of the enzyme

Purifications were carried out at 4°C.

Ammonium sulfate fractionation. Melon flesh (200g) was homogenized with 0.05M Tris-HCl buffer (pH 8.0, 200 mL) using an electric blender and the homogenate was filtered. Solid ammonium sulfate was added to the filtrate (200 mL) to 35% saturation. The mixture was stirred for 30 min and centrifuged at 10,000 × *g* for 15 min. The supernatant, in which the activity was observed, was brought to 70% saturation with ammonium sulfate. The mixture was stirred for 30 min, and the precipitate was collected by centrifugation. The pellets dissolved in 50 mM Tris-HCl buffer (pH 8.0, 10 mL). The solution was desalted using Sephadex PD-10 column equilibrated with the same buffer, and concentrated to 5 mL by membrane ultrafiltration.

Gel filtration on Toyopearl HW-55 column. The desalted concentrate was applied to a Toyopearl HW-55 column (1.9 × 100 cm) equilibrated with 50 mM Tris-HCl buffer (pH 8.0), and the column was eluted with the same buffer at 15 mL/hr. The active fractions were pooled, and concentrated by ultrafiltration.

Ion-exchange chromatography on CM-Toyopearl 650S. The buffer system of the concentrate was replaced with 10 mM Tris-HCl (pH 7.2) using Sephadex PD-10 column, and then the solution was loaded on a CM-Toyopearl 650S column (1.9 × 11 cm) equilibrated with the same buffer. After washing the column with the equilibration buffer, the absorbed enzyme was eluted with a linear gradient of NaCl (0 to 1.0M) in the buffer at 20 mL/hr. The active fractions were collected, concentrated, and stored at −10°C until use.

Enzyme activity

The protease activity was measured spectrophotometrically at each step of purification using Boc-Ala-Ala-Pro-Leu-pNA as substrate. The reaction mixture contained 0.1 M Tris buffer (pH 9.0), 0.2 mM substrate, 10% dimethylsulfoxide and enzyme in a total volume of 2 mL. After adding the enzyme sample (0.1 mL) and incubating the mixture at 37°C for 1 hr, the enzyme reaction was stopped by adding 30% acetic acid (1 mL), and the increase in absorbance at 410 nm was measured. A molar absorption coefficient of 8,800 at 410 nm was used for the liberated *p*-nitroaniline (Erlanger et al., 1961). Substrate specificity of the purified enzyme was tested under the described conditions using synthetic peptide substrates with the sequence Boc-Ala-Ala-Pro-X-pNA (X = amino acids). Protein concentrations were determined by Lowry's method (Lowry et al., 1951) using bovine serum albumin as standard, and the distribution of protein in column effluents was estimated from absorbance at 280 nm.

Electrophoresis

PAGE was performed according to the method of Davis (1964) in 7.5% polyacrylamide gel at pH 4.3. Electrophoresis was carried out at a constant current of 2.5 mA per gel tube for 1 hr. Proteins were stained with Coomassie brilliant blue G-250 in 7% acetic acid. Sodium dodecyl sulfate (SDS)-PAGE for molecular weight determination was carried out according to Laemmli (1970). In the presence and absence of 5% 2-mercaptoethanol, samples were treated with 2% SDS, heated at 80°C for 5 min, and subjected to electrophoresis under the constant current of 1 mA. Molecular weight markers used were horse heart cytochrome c and its oligomers (dimer to hexamer) cross-linked chemically. To determine the isoelectric point, purified enzyme was subjected to gel electrofocusing at 4°C in 5.0% polyacrylamide gels containing 2% pH 3-10 range Pharmalyte essentially according to the method of Wrigley (1971). The pI marker proteins were horse heart cytochrome c and its acetylated derivatives with pI values 10.6, 9.7, 8.3, 6.4, 4.9, and 4.1.

RESULTS & DISCUSSION

Purification of the enzyme

The protease was obtained easily in a homogeneous state after three steps of purification (Table 1). The gel filtration on Toyopearl HW-55 was very effective in removing inactive

Authors Noda, Koyanagi, and Kamiya are with the Laboratory of Biochemistry, Dept. of Food Science & Nutrition, Fukuoka Women's Univ., Higashi-ku, Fukuoka 813, Japan.

Table 1—Purification stages of C.m. protease

	Total protein (mg)	Total activity (μmol·min⁻¹)	Specific activity (μmol·min⁻¹·mg⁻¹)	Yield (%)	Purification (–fold)
Crude juice	450.0	144	0.29	100	1.0
Ammonium sulfate	65.5	132	2.0	92	7.0
Toyopearl HW-55	8.2	78	9.6	54	33.1
CM-Toyopearl 650S	5.6	68	11.2	47	39.1

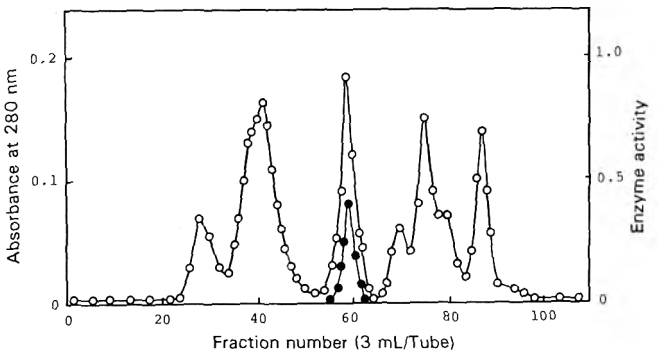

Fig. 1—Elution profile of C.m. protease on Toyopearl HW-55 gel chromatography. ○, protein concentration (absorbance at 280 nm; ●, enzyme activity (absorbance at 410 nm).

components (Fig. 1). Six major protein peaks were obtained by chromatography, and the Boc-Ala-Ala-Pro-Leu-pNA degrading activity was observed at the third protein peak. The second peak showed tryptic and aminopeptidase activities (data not shown), and was supposed to contain cucumisin, because it eluted at the volume (Rf) corresponding to cucumisin. Fractions corresponding to peak 3 were subjected to further purification by ion-exchange chromatography on CM-Toyopearl, which gave three protein peaks. The last peak, eluted at 50-60 mM NaCl, contained protease activity.

Enzymatic and physicochemical properties

The pH optimum of the protease for hydrolysis of Boc-Ala-Ala-Pro-Leu-pNA was in the range 8 to 10. The enzyme was relatively stable at pH 6-10, but was unstable under more acidic or basic conditions. The enzyme migrated faster than cucumisin on PAGE at pH 4.3, and was not detected on pH 9.5 gel, which indicates that C.m. protease was a strongly basic protein. Isoelectric focusing showed a single protein band, which appeared between reference protein markers with pI values 9.7 and 8.3, and its isoelectric point was calculated to be around 9.5 from their migration distances. The apparent molecular weight was estimated at about 24,000 by comparing migration distance with those of reference proteins on SDS-PAGE. The molecular weight of cucumisin was reported to be about 50,000 (Kaneda and Tominaga, 1975).

Substrate specificity

The substrate specificities of C.m. protease and cucumisin were examined at pH 9.0 and 37°C with a series of synthetic peptide *p*-nitroanilides, with the sequence of Boc-Ala-Ala-Pro-X-pNA with various amino acids (X) in the P_1 position (nomenclature of Schechter and Berger (1967)). As shown (Table 2) C.m. protease preferentially hydrolyzed the peptide *p*-nitroanilides containing leucine, alanine, histidine, glutamine and asparagine at P_1 position. Those containing aspartic acid and phenylalanine at P_1 were hydrolyzed slowly. The peptide bonds of glycine, glutamic acid, basic amino acids and hydroxyl amino acids were not hydrolyzed by the enzyme at a measurable rate. Note that aspartic acid was a residue at the P_1 position for hydrolysis of the peptide bond by the protease,

Table 2—Substrate specificity of C.m. protease and cucumisin toward peptide and amino acid p-nitroanilides

	Relative activity (%)	
Substrate	C.m. protease	Cucumisin
Boc-Ala-Ala-Pro-Leu-pNA	100	100
Boc-Ala-Ala-Pro-Ala-pNA	77	234
Boc-Ala-Ala-Pro-Gly-pNA	0	31
Boc-Ala-Ala-Pro-Lys-pNA	0	100
Boc-Ala-Ala-Pro-Arg-pNA	0	39
Boc-Ala-Ala-Pro-His-pNA	119	183
Boc-Ala-Ala-Pro-Phe-pNA	27	214
Boc-Ala-Ala-Pro-Ser-pNA	0	172
Boc-Ala-Ala-Pro-Thr-pNA	0	134
Boc-Ala-Ala-Pro-Glu-pNA	0	157
Boc-Ala-Ala-Pro-Asp-pNA	61	195
Boc-Ala-Ala-Pro-Gln-pNA	116	219
Boc-Ala-Ala-Pro-Asn-pNA	127	216
Leu-pNA	0	0
His-pNA	0	0

Table 3—Effect of various additives on Boc-Ala-Ala-Pro-Leu-pNA hydrolyzing activity of C.m. protease

Additive	Concentration	Residual activity (%)
None	—	100
DFP	1 mM	5
TPCK	1 mM	66
Chymostatin	0.2 mg/mL	57
Leupeptin	0.2 mg/mL	93
Antipain	0.2 mg/mL	102
Elastatinal	0.2 mg/mL	100
E-64	0.2 mg/mL	105
Pepstatin	0.2 mg/mL	98
Iodoacetamide	1 mM	109
N-Ethylmaleimide	1 mM	92
PCMB	1 mM	97
EDTA	1 mM	110
o-Phenanthroline	1 mM	97
Dithiothreitol	1 mM	106

while the other acidic amino acid, glutamic acid, was not. The results also showed that the protease lacked aminopeptidase activity toward Leu-pNA or His-pNA, indicating that the chromophore was released from the substrates by direct hydrolysis of the peptide bond between the chromogenic group and the adjacent amino acid.

On the other hand, cucumisin hydrolyzed more or less all the peptide substrates tested (Table 2), which agreed with results reported by Kaneda et al. (1986). Susceptibilities of substrates containing lysine, arginine, serine, threonine and glutamic acid at P_1 toward the two enzymes were quite different. This indicated that C.m. protease was a different enzyme, distinct from cucumisin.

Effect of chemical reagents and inhibitors

Table 3 shows the effect of protein modifying reagents and protease inhibitors on the Boc-Ala-Ala-Pro-Leu-pNA hydrolyzing activity of C.m. protease. The enzyme was almost completely inactivated by 1 mM diisopropyl phosphofluoridate (DFP), indicating that it was a serine protease. The activity was partially inhibited by tosyl-L-phenylalanine chloromethylketone (TPCK) and chymostatin. Other chemicals and inhibitors, including leupeptin, antipain, iodoacetamide,

p-chloromercuribenzoate (PCMB) and ethylenediaminetetraacetic acid (EDTA) had no effects on enzyme activity.

Most of the proteases isolated from plant sources (e.g. papain, bromelain and ficin) have been thiol enzymes. Note that C.m. protease and cucumisin are serine proteases. A highly unusual example of such a protease is the proteolytic enzyme of white gourd (*Benincasa criapra*) which was reported to be unaffected by reducing compounds (Deb-Sarma, 1942). The proteolytic enzymes of melons and gourds are probably both comprized of serine proteases. Such unusual enzymes from melon may have potential for special applications in food products.

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Production of Anthocyanin from Strawberry Cell Suspension Cultures; Effects of Sugar and Nitrogen

TSUKASA MORI and MIEI SAKURAI

ABSTRACT

Production of anthocyanins was investigated, controlling sugar concentration and ratio of ammonium: nitrate in culture medium of strawberry, *Fragaria ananassa* cv Shikinari. Anthocyanins were produced under 8000 lux for 2 wk using calli derived from suspension cultures of leaf tissues. Yield was greatest in modified LS medium containing 5% sucrose (W/V), a ratio of NH_4^+ (2 mM):NO_3^- (28mM), 2,4-dichlorophenoxyacetic acid and benzyladenine. Total anthocyanin was about 15 mg/100 mL of culture medium, almost six times greater than that in MS medium. Effects of sugars were also studied using eight sugars. Cell growth and anthocyanin accumulation were enhanced by glucose, sucrose, and fructose, but anthocyanin compositions were not affected. Major anthocyanins were peonindin-3-glucoside and cyanidin-3-glucoside. Peonidin-3-glucoside increased with an increase in the NH_4^+: NO_3^- ratio at nitrogen concentration 30 mM, while that of cyanidin-3-glucoside changed *vice versa*.

Key Words: strawberries, anthocyamins, sugar, ammonium:nitrate

INTRODUCTION

ANTHOCYANINS are widely distributed pigments in plant species, especially flower petals and fruits. Obtaining stable pigments in large quantities throughout all seasons is difficult. Tissue culturing has become an important biotechnological technique to produce secondary metabolites on mass scale. Reports have been published production of secondary metabolites such as alkaloids, terpenes, quinones, and flavonoids in plant tissue cultures. Anthocyanins have also been produced in plant tissue cultures (Mori et al., 1993). Culture conditions were studied in detail in *Vitis hybrida* (Yamakawa et al., 1983a, b), *Daucus carota* (Kinnersley and Dougall, 1980; Dougall and Weyrauch, 1980; Ozeki and Komamine, 1985), and *Euphorbia millii* (Yamamoto et al., 1989). Studies on anthocyanin production are concerned not only with improving productivity for large-scale production, but also with factors influencing anthocyanin accumulation. Such factors are UV (Wellmann et al., 1976), nitrogen sources (Do and Cormier, 1991a,b), types of sugar (Nakajima et al., 1989), and osmotic stress (Do and Cormier, 1991c). However, little work has been reported concerning anthocyanin production using cultures of callus cells from strawberry plants suspended in liquid.

Hong et al. (1989a) reported anthocyanin production in suspended callus cultures of immature strawberry fruits. The report revealed that cultivars were different in abilities to initiate callus formation and produce anthocyanins, and that inoculum size influenced cell growth. Various factors influencing cell growth and anthocyanin synthesis in suspended cultures of strawberry cells remain to be elucidated. For example, the relationship between the ratio of NH_4^+:NO_3^- and cyanidin and peonidin-3-glucoside production has never been reported in cultured strawberry cells. The formation of anthocyanins using cells cultured from three different parts of a strawberry plants was studied by Mori et al. (1993). In that report, peonidin-3-glucoside and cyanidin-3-glucoside were the main pigments produced by strawberry cells, and callus derived from leaf tissue was suitable to obtain such pigments.

Our current objective was to study the production of anthocyanins in strawberry cells using suspended cultures, and to show how the ratio of ammonium: nitrate and the total nitrogen in the medium affects anthocyanin production and composition of strawberry cell cultures.

Author Mori is affiliated with the Faculty of Fisheries, Hokkaido Univ., 3-1-1 Minato, Hakodate 041, Japan. Author Sakurai is with the Biotechnology & Environmental Engineering Dept., Research Institute, Ishikawajima Harima Heavy Industries, Co., Ltd., Yokohama 235, Japan.

MATERIALS & METHODS

Plant materials and callus formation

Callus tissues were induced from the leaf of *Fragaria ananassa* cv Shikinari. Leaves were obtained from aseptically regenerated plants (Mori et al., 1993) and placed on LS medium (Linsmaier and Skoog, 1965) containing 3% sucrose, 0.2% Gellangum (Wako Chemical), 0.1mg/L of benzyladenine (BA), and 1mg/L of 2,4-dichlorophenoxyacetic acid (2,4-D). Tissues were then incubated at 25°C under a 16 hr-light, 8 hr-dark cycle with light intensity 800 lux. Callus tissues were transferred to a freshly prepared LS medium every 3 wk.

Cell suspension cultures

Suspended cell cultures were initiated by transferring about 2g (fresh weight) of friable callus tissue to 100 mL of liquid LS medium supplemented with 3% sucrose, 2,4-D (1 mg/L) and BA (0.1 mg/L) in 500 mL flasks. They were incubated on a rotary shaker (80 rpm) under continuous light of 800 lux at 25°C. Suspension cultures were grown for 3 wk, during which time the medium was changed every week. The inoculation rate was about 10%. Resulting cell suspensions (2 g fresh) were transferred to freshly prepared basal liquid media and then incubated under continuous light of 8000 lux at 25°C on a rotary shaker (80 rpm) for anthocyanin production. After 2 wk incubation, cells were harvested and measured for cell weight and anthocyanin content.

Various sugars (xylose, D-mannose, L-rhamnose, D-arabinose, D-galactose, D-glucose, sucrose, and fructose) and sucrose concentrations (2%, 5%, 8%, 10%, and 12%) were changed and cells were cultured for 2 wk in B5 medium (Gamborg et al., 1976) under continuous light of 8000 lux. To examine nitrogen source influence on cell growth and anthocyanin $(NH_4)_2SO_4$ and KNO_3 were added to a nitrogen-free LS medium containing 2% (W/V) sucrose, 2,4-D (1mg/L), and BA (0.1mg/L) at 30mM or 60mM NH_4^+:NO_3^- (0:30, 2:28, 4:26, 10:20, 20:10, 30:0, 0:60, 2:58, 10:50, 20:40, 40:20, and 60:0). Cells were cultured 2 wk under continuous light of 8000 lux.

HPLC analysis of major anthocyanins

Solution A (acetic acid: acetonitrile: water-20:25:55), diluted to 35% and containing 0.1% trifluoroacetic acid (TFA) at 4°C was used to extract anthocyanins from fresh callus tissues of leaves for HPLC analysis. The extract was then diluted with 35% solution A to a concentration of 1 OD at 528 nm. After filtration, 2 μL of sample was injected and analyzed with HPLC (Waters 600E) using an octadecyl silica (ODS) column (Develosil ODS-5 4.6 mm ϕ X 250 mm, Nomura Chemical), eluted with 35% solution A diluted with H_2O containing 0.1% TFA and methanol solution (water: acetic acid: methanol - 80: 15: 5) containing 0.1% TFA at 40°C. Chromatograms were recorded on a Shimazu C-R2A and calculated for percentage of cyanidin-3-glucoside and peonidin-3-glucoside from peak areas.

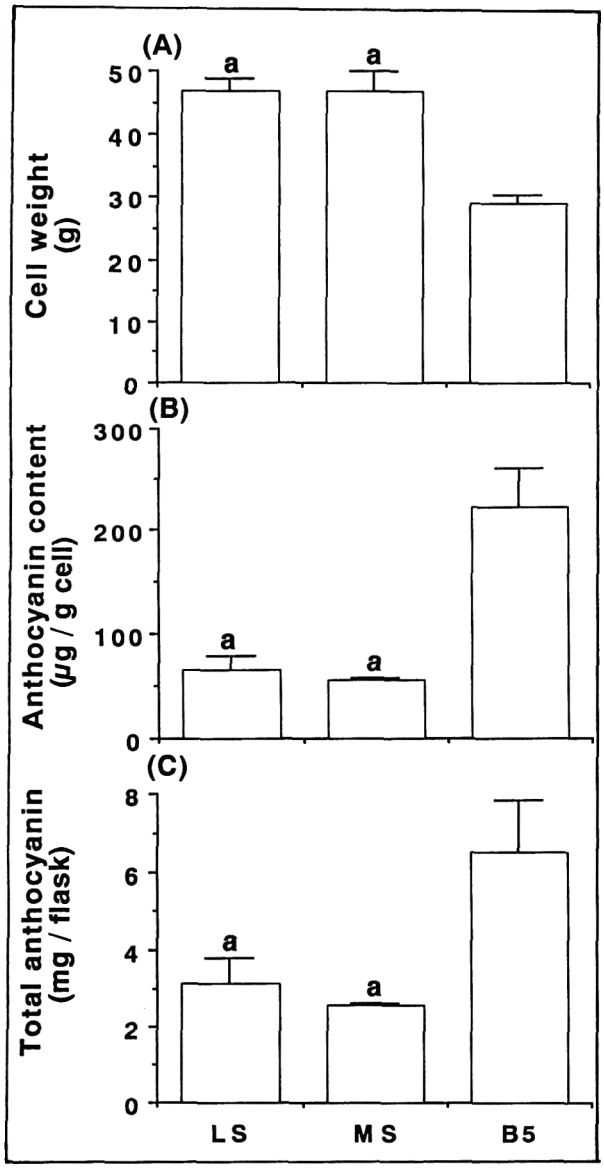

Fig. 1—Effect of basal medium on growth and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained with (1 mg/L), 2,4-D and (0.1 mg/L) BA, and 2% (W/V) sucrose on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical bars represent the standard error of three replicates. [a]Means with same letter do not show significant difference at P = 0.05.

Determination of anthocyanins and cell growth

Anthocyanins were extracted overnight from using a solution containing 0.1% HCl-MeOH at 4°C. After centrifugation at 1000 × *g* for 5 min, absorbance of clear supernatant was measured at 528 nm. Anthocyanin content was calculated with the extinction coefficient ($E^{1\%}_{1cm}$ =680 at 528 nm) obtained by using purified peonidin-3-glucose from cultured strawberry cells as a standard. Major anthocyanins had been previously identified by FAB-MS and NMR (Mori et al., 1993). Total anthocyanin yield was expressed as mg/100 mL of liquid LS medium in a 500 mL cell culture flask. Cell were separated from the culture medium by filtration through a nylon filter (30 μm) and weighed. Results were expressed as fresh cell weight per flask.

Statistics

Data were presented as means ± SE. Statistical analyses were made using one-way analysis of variance (ANOVA). Differences with P <0.50 were considered statistially significant.

RESULTS & DISCUSSION

Basal culture media

Strawberry cells derived from leaf tissues were cultured in three different liquid basal media (MS, LS, and B5) to examine the influence of medium compositions on cell growth and anthocyanin production. LS and B5 media contained only thiamin as a vitamin source and low levels of ammonium ion, respectively. MS medium contained high levels of ammonium, four vitamins, and glycine. Cell weight in "Shikinari" cell suspension cultures in each medium was compared (Fig. 1a). Cell weight in both LS and MS media was significantly larger than that of B5 (P <0.01). No difference was found between LS and MS.

Hong et al. (1989a) reported on cell growth in liquid suspension using "Brighton" strawberry fruits and four different culture media: MS, LS, B5, and E&R. The report revealed that cell growth in MS medium was slower than that in other media during the first week, but no difference was found among cultures after the second week of incubation. The difference between our results and those of Hong et al. may be related to variations in parts of plants and cultivars. Hong et al. (1989b) also reported growth kinetics of strawberry cell suspension cultures and revealed specific oxygen consumption rates of strawberry suspension cultures in various culture systems. They showed the oxygen transfer coefficient in the shake flask was higher than in the roller bottle type bioreactor; however, the cell growth rate in the shake flask was lower than in the roller bottle. This suggests that the aeration rate due to shapes of culture bottle, volume of medium and rotation speed are important for cell growth. Therefore the difference in size of flask and volume of medium used in our experiments may be the reasons for different cell growth kinetics compared to the results of Hong et al.

Anthocyanin content in the B5 medium was highest, reaching about four-fold higher than LS and MS media (Fig. 1b). Total anthocyanin production was also highest (P <0.05) in B5 medium. It was about 2.5-fold higher than those of LS and MS media (Fig. 1c).

There are few reports on cell growth and anthocyanin production in different basal media. The B5 medium yielded the highest anthocyanin production in *Daucus carota* (Nagarajan et al., 1989) and *Euphorbia millii* (Yamamoto et al., 1989). Overall results show that high ammonium in LS and MS stimulated cell growth, but reduced anthocyanin accumulation.

Sugar type effect on cell growth and anthocyanin production

The effect of eight carbon sources (xylose, D-mannose, L-rhamnose, D-arabinose, D-galactose, D-glucose, sucrose, and fructose) on cell growth and anthocyanin production were studied in B5 medium containing 2% sugar each. Addition of sucrose resulted in maximum cell yield (Fig. 2a). Cell weight was higher (P <0.01) in glucose, sucrose, and fructose media than the others. Cell weight increased and anthocyanin production did not occur on xylose, D-mannose, L-rhamnose, and D-arabinose media. These sugars did not support cell growth. Anthocyanin content and total anthocyanins were higher (P <0.01) in glucose, sucrose, and fructose media than the others. There was no difference among them (Fig. 2b,c).

Nagarajan et al. (1989) reported anthocyanin production from *Daucus carota* using four carbon sources (sucrose, fructose, glucose, and lactose) in suspended cultures. They obtained maximum cell yield and anthocyanin content using 20g/L of galactose and ranked the production capacity as galactose, fructose, glucose, sucrose, and lactose in descending order. A comparison of results with our data indicates that strawberry cells are preferable to carrot cells for producing anthocyanins since they show better growth and anthocyanin accumulation

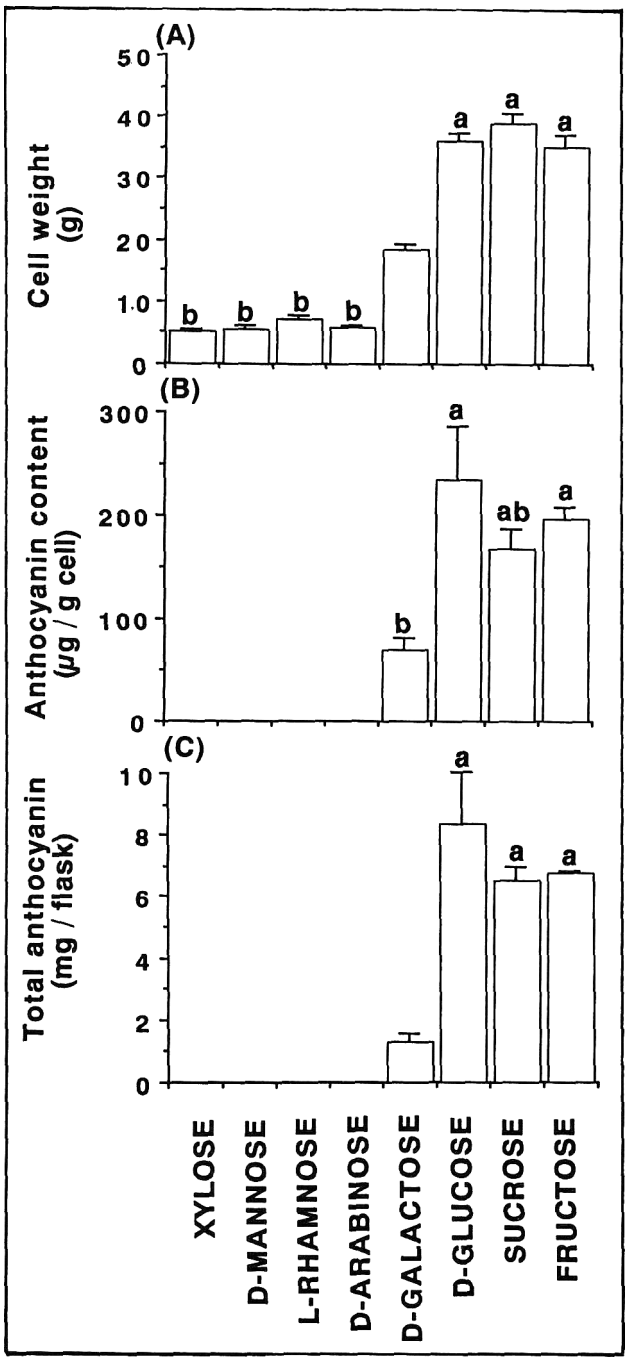

Fig. 2—Effect of various sugar type (2%) on cell weight and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained in B5 medium with (1mg/L) 2,4-D and (0.1mg/L) BA on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical bars represent the standard error of three replicates. [a-b]Means with same letter do not show significant difference at P = 0.05.

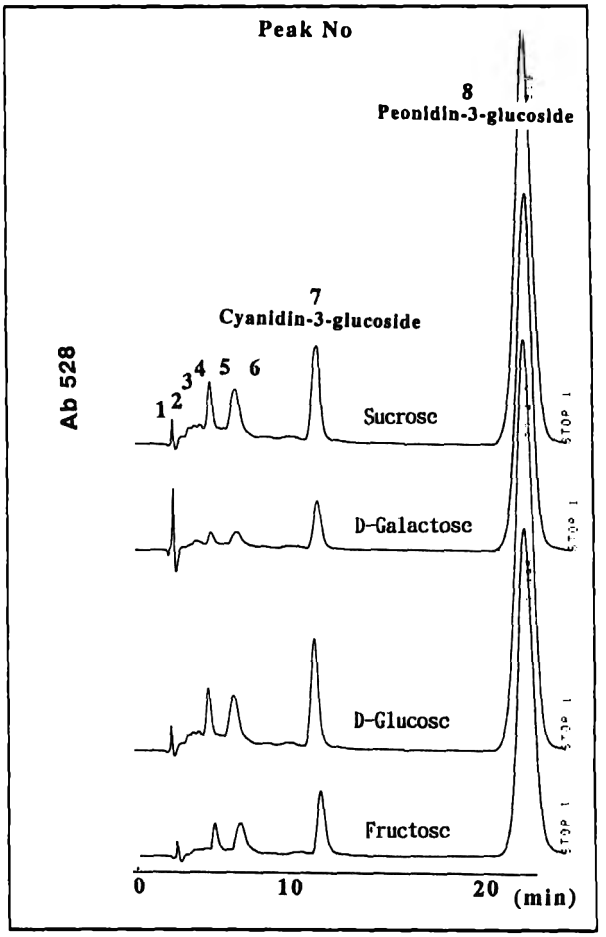

Fig. 3—HPLC profiles of anthocyanins from strawberry cells cultured with various sugars. Samples were separated using 35% solution A containing 0.1% TFA.

with lower cost carbon sources (sucrose, glucose, and fructose).

The effects of sugar type on anthocyanin composition (Fig. 3 and Table 1) showed about eight kinds of anthocyanins were detected with HPLC at 528 nm in all cultured callus. These data suggest that the major anthocyanins produced were identical to those produced on sucrose. We had identified the major anthocyanins produced in strawberry suspension cells cultured in LS medium containing sucrose as peonidin-3-glucoside and cyanidin-3-glucoside (Mori et al., 1993). These results show none of the carbon sources affected the key enzymes for attaching the aglycone to anthocyanidin, resulting in the production of cyanidin-3-glucoside and peonidin-3-glucoside as major pigments. Moreover, pelargonidin-3-glucoside was not detected from these cells, indicating that the callus cells may lack an enzyme changing dihydrokaempferol into kaempferol.

Sucrose concentrations

The optimum concentrations in B5 medium for cell growth was 2~8% (W/V) and anthocyanin content 5% (Fig. 4). Ten percent sucrose diminished cell growth. Total anthocyanin production using 5% sucrose was also the highest. A high concentration of sugar promotes production of secondary metabolites, such as anthocyanin, in cultured cells. For example, 9.9% sucrose greatly increased anthocyanin production in *Vitis* (Yamakawa et al., 1983b), and 5% sucrose in Populus (Matsumoto et al., 1970) and *Euphorbia millii* (Yamamoto et al., 1989). The use of high sucrose concentration to produce anthocyanin was more effective in medium with low ammonium concentrations, such as B5 medium.

Ammonium and nitrate as nitrogen source

In the LS medium containing about 60 mM nitrogen as KNO_3 and NH_4NO_3; the molar ratio of NH_4^+ to NO_3^- was 1:2, while B5 medium contained about 30 mM nitrogen, with a ratio of 2:25. The main difference between LS and B5 consisted in total nitrogen concentrations and ratios of NH_4^+:NO_3^-. We have found that B5 medium was best for anthocyanin production in strawberry cells (Fig. 1). It may therefore be possible to increase anthocyanin production in LS medium by controlling the total nitrogen amount and ratio of NH_4^+:NO_3^- in

Table 1—Chromatographic mobility of anthocyanins isolated from strawberry suspended callus

Sugar	(A)[a] Peak No. RT (min)								(B)[b] Peak No. RT (min)							
	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Sucrose	1.8	2.2	2.8	3.6	4.1	6.1	11.5	23.2	1.2	4.0	4.3	4.8	5.0	6.0	9.6	15.1
Galactose	1.7	nd	nd	3.6	4.1	6.3	11.4	23.5	1.2	nd	nd	4.8	5.0	5.9	9.6	15.1
Glucose	1.7	2.2	2.8	3.6	4.0	6.2	11.3	23.4	1.2	4.0	4.3	4.7	5.0	6.0	9.6	15.1
Fructose	1.8	nd	nd	nd	4.0	6.1	11.6	23.6	1.2	nd	4.3	4.8	5.2	6.1	9.5	15.0

[a] (A): Samples were separated with HPLC using 35% solution A containing 0.1% TFA.
[b] (B): Samples were separated using methanol solution containing 0.1% TFA.

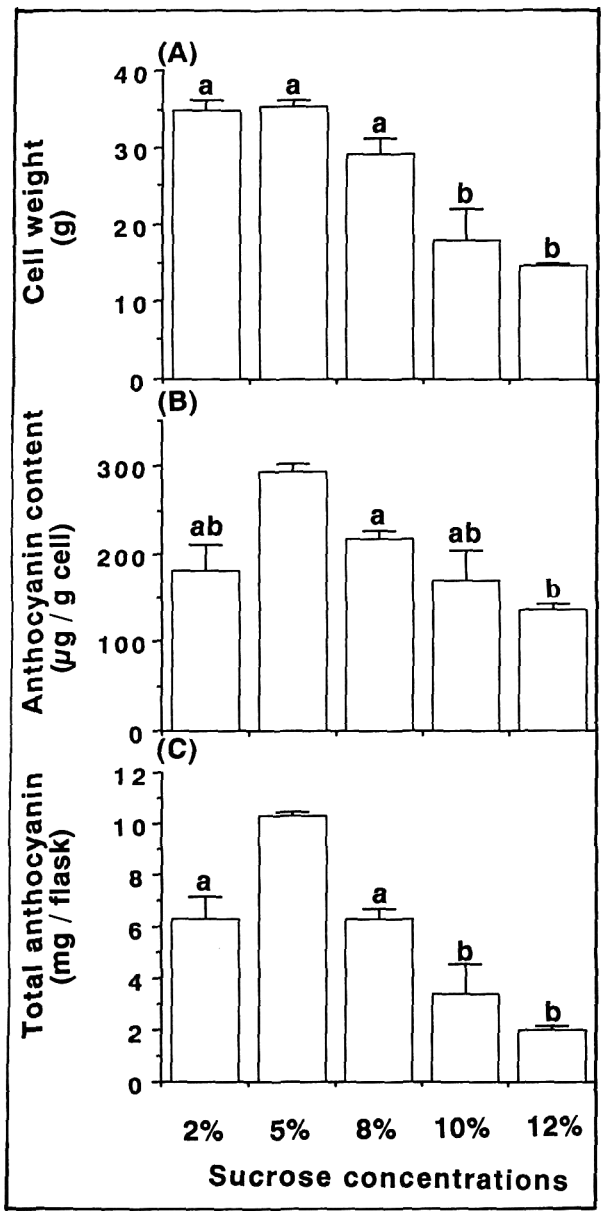

Fig. 4—Effects of sucrose concentrations on cell weight and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained in B5 medium with 2,4-D (1mg/L) and BA (0.1mg/L) on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical bars represent standard errors for three flasks. [a-b]Means with same letter do not show significant difference at P = 0.05.

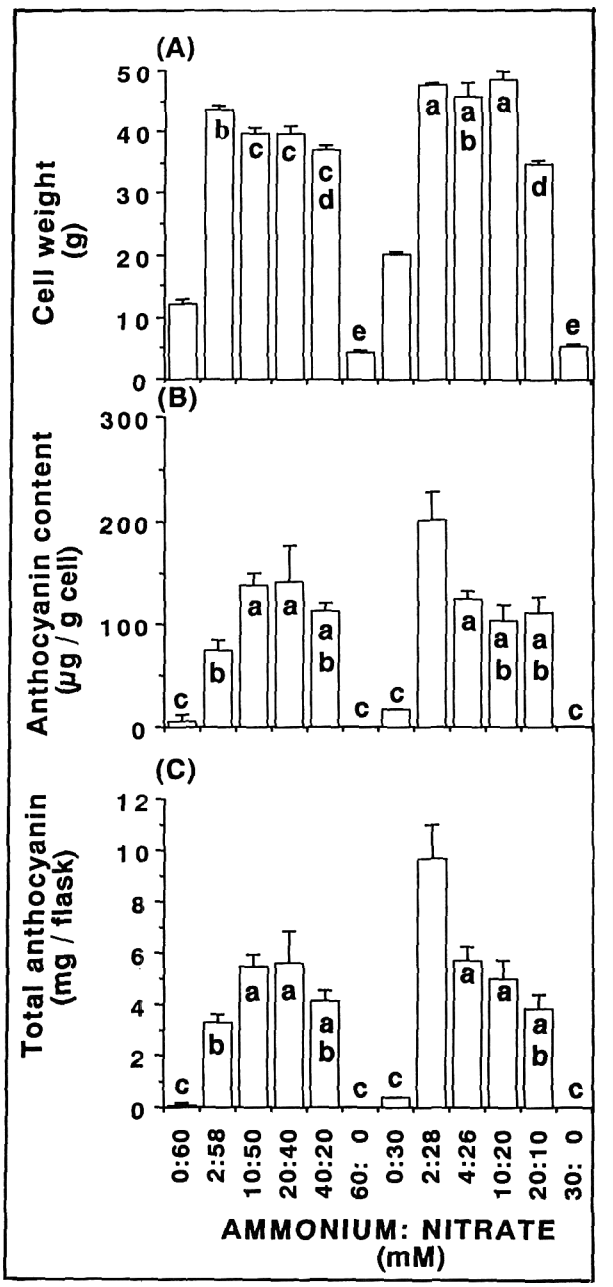

Fig. 5—Effects of ratios of ammonium and nitrate on cell weight and anthocyanin production in suspension cultures of strawberry cells. Cultures were maintained in LS medium with various ammonium and nitrate concentrations, 2,4-D (1mg/L), BA (0.1 mg/L), and 2% (W/V) sucrose on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical bars represent standard errors for three flasks. [a-e]Means with same letter do not show significant difference at P = 0.05.

the LS medium. As shown (Fig. 5) the total nitrogen and ratio of NH_4^+ to NO_3^- both had a strong effect on anthocyanin production. It was promoted more by 30 mM total nitrogen than by 60mM (Fig. 5c). This concentration almost equalled that in the standard B5 medium. However, the optimal ratio of NH_4^+ to NO_3^- for anthocyanin production was 2mM:28mM (Fig. 5c), slightly different from that of B5 medium. Anthocyanin production and cell growth were inhibited by high NH_4 (Fig. 5a,b).

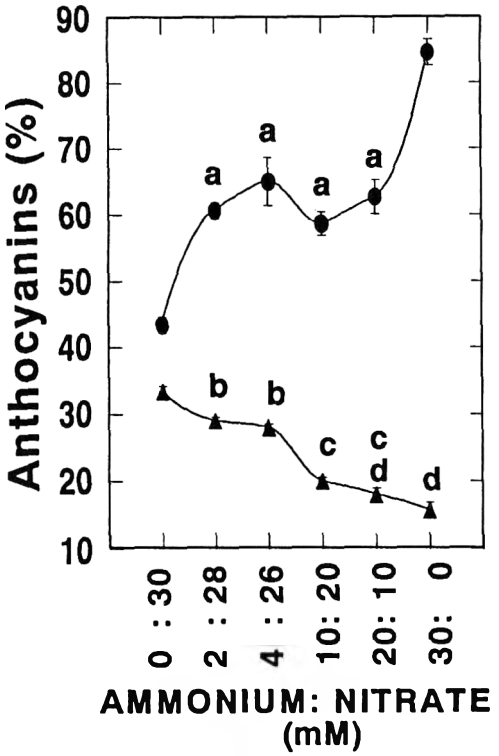

Fig. 6—Percentages of peonidin-3-glucoside (●) and cyanidin-3-glucoside (▲) in LS medium with various ammonium and nitrate concentrations (total nitrogen 30mM), 2,4-D (1mg/L), BA (0.1mg/L), and 2% (W/V) sucrose. Cultures were maintained on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical bars represent standard errors for three flasks. [a-d]Means with same letter do not show significant difference at P = 0.05.

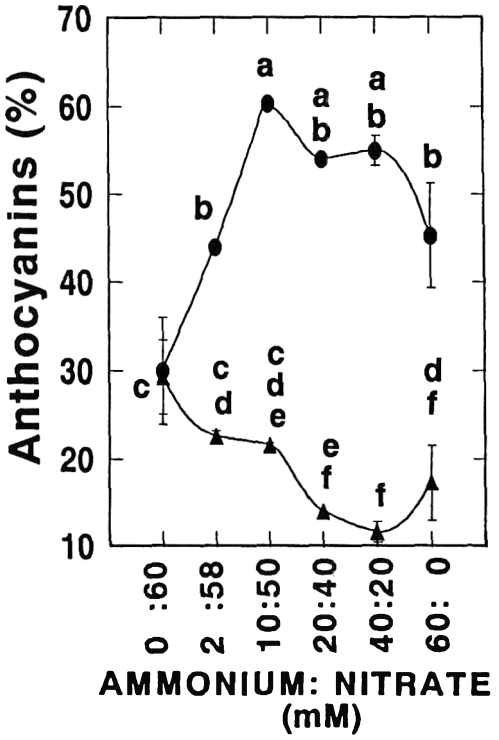

Fig. 7—Percentages of peonidin-3-glucoside (●) and cyanidin-3-glucoside (▲) in LS medium with various ammonium and nitrate concentrations (total nitrogen 60 mM), 2,4-D (1mg/L), BA (0.1mg/L), and 2% (W/V) sucrose. Cultures were maintained on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical bars represent standard errors for three flasks. [a-f]Means with same letter do not show significant difference at P = 0.05.

Using cultured *Vitis* and *Euphorbia millii*, Yamakawa et al. (1983b) and Yamamoto et al. (1989) also succeeded in increasing cell growth and anthocyanin production by varying the ratio of NH_4^+ to NO_3^- in MS and B5 media. Yamakawa et al. (1983b) reported that the best ratio of NH_4^+ to NO_3^- was 1:1 at 60 mM total nitrogen. Yamamoto et al. (1989) obtained the best cell growth and anthocyanin production at a ratio of NH_4^+ (1):NO_3^- (16) at 30 mM total nitrogen. We suggest that it may be necessary to investigate the optimum ratio for particular cells to obtain maximum anthocyanin production. In our current study, the best nitrogen conditions for anthocyanin production were NH_4^- (2 mM):NO_3^- (28 mM).

We also studied the effect of total nitrogen and ratios on anthocyanin composition. To compare composition of anthocyanin produced under different concentrations and ratios at 2 wk, the contents of two major anthocyanins, cyanidin-3-glucoside and peonidin-3-glucoside, were measured by HPLC. The percentage of cyanidin-3-glucoside decreased with a rise of NH_4^-:NO_3, while peonidin-3-glucoside increased (Fig. 6). This tendency was clearly seen at 30 mM total nitrogen. The percentage of cyanidin-3-glucoside was higher ($P < 0.01$) at 0:30 than at the other ratios. The cyanidin-3-glucoside content gradually decreased with an increase in NH_4^+:NO_3^- (Fig. 6). Peonidin-3-glucoside content was also higher ($P < 0.01$) at 30:0 than at other ratios. Similar effects were seen in 60 mM total nitrogen except 60:0 (Fig. 7), where the contents of both cyanidin-3-glucoside and peonidin-3-glucoside were directly opposite to the result of NH_3^- (30 mM):NO_3^- (0 mM).

Stanko and Bardinskaya (1963) reported that, at the start of formation of anthocyanins, the culture of *Parthenocissus tricuspidata* callus tissues mainly contained substances from the group of cyanidin-3,5-diglucoside. However, by the end of growth and at the start of necrosis, primarily methylated groups of delphinidin-malvidin-3-monoglucoside and malvidin-3,5-diglucoside were present. Since nitrogen source affected cell growth remarkably, the effect of nitrogen source on anthocyanin composition in strawberry suspension culture may be related to the results of Stanko and Bardinskaya (1963).

We have reported that 8 kinds of anthocyanins were detected in cultured strawberry cells using HPLC (Mori et al., 1993). Of these, the major anthocyanins were peonidin-3-glucoside and cyanidin-3-glucoside, and the other six were minor pigments that accounted for ≈30% of the total anthocyanins from LS medium. The medium containing NH_4^+ and NO_3^- at a 20:40 ratio was closest to the composition of LS medium, and the medium at 2:28 was closest to B5 medium. Cyanidin-3-glucoside produced at 2:28 was about 2 times greater than that of 20:40. Cells growth in B5 looked more red visually than those grown in LS.

Anthocyanin production in suspended cultures of *Vitis vinifera L.* was influenced by both low nitrate and high sugar concentration, and high ammonium and high sugar concentration by Do and Cormier (1991a,b). They demonstrated that high sucrose and low nitrate repressed cell growth but enhanced intracellular accumulation of anthocyanins, especially peonidin-3-glucoside. Increasing ammonium concentrations in the production of medium from 2 to 8-16 mM stimulated cell growth and decreased accumulation of both cyanidin-3-glucoside and peonidin-3-glucoside, while accumulation of peonidin-3-p-coumaroylglucoside was progressively enhanced. Since anthocyanin compositions were monitored with fixed values of ammonium or nitrate in the medium, comparison of their data with ours are not reliable. A ratio of ammonium and nitrate obviously affects anthocyanin composition in cultured

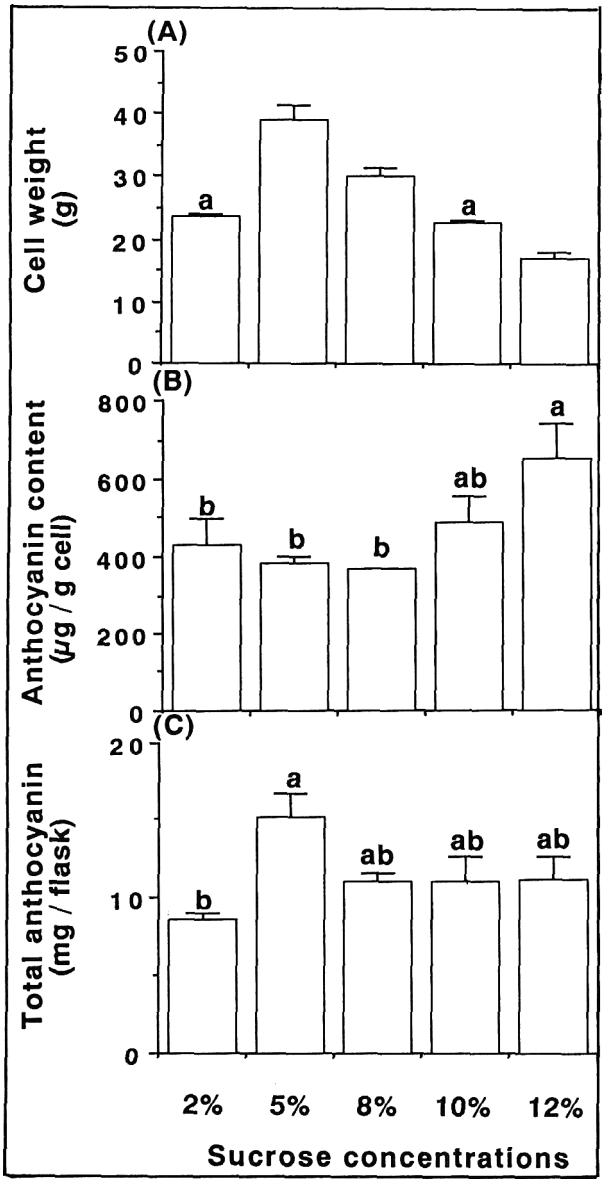

Fig. 8—Effects of sucrose concentrations on cell growth and anthocyanin production of strawberry cells in LS medium with ammonium and nitrate concentrations (2mM:28mM), 2,4-D (1mg/L), BA (0.1mg/L), and various sucrose 2~12% (w/v) 2% (W/V) sucrose. Cultures were maintained on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical bars represent standard errors for three flasks. [a-b]Means with same letter do not show significant difference at P = 0.05.

cells. Therefore, by altering the ratio of ammonium and nitrate in a medium, regulation of the color balance of anthocyanins produced in suspended cells should be possible.

Sugar concentrations for anthocyanin production

Since anthocyanin production was most active at a ratio of ammonium (2 mM): nitrate (28 mM) (Fig. 5) we studied the effect of high sucrose concentrations on cell growth and anthocyanin accumulation using a modified nitrogen source with LS medium. Cell growth was enhanced and higher ($P < 0.01$) with 5% sucrose, and sucrose concentrations <5% depressed cell growth (Fig. 8a). Anthocyanin content was highest in 12% sugar concentration. High sucrose levels depressed cell growth but promoted anthocyanin accumulation as sucrose concentration rose (Fig. 8b). These data suggest that ≈5% sucrose was suitable for anthocyanin production (Fig. 8c). Total anthocyanin production with 5% sucrose reached about 15 mg/100 mL, about 6 fold that of MS medium. However, cell growth in 2% sucrose was very low although it was the same medium in which 2:28 ammonium : nitrate was used (Fig. 5). Probably the true amount of anthocyanin production using 5% sucrose concentration was greater than our data indicated (Fig. 8). We optimized medium components by changing sugar concentrations and the ratio of ammonium : nitrate to increase anthocyanin production. Recently, Kamei et al. (1993) reported that anthocyanins inhibited growth of cancer cells. Anthocyanins are becoming more important, not only as food additives but also for therapeutic reasons.

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Heat Induced Gelation of Pea (*Pisum sativum*) Mixed Globulins, Vicilin and Legumin

PUSHKAR S. BORA, CLARK J. BREKKE, and JOSEPH R. POWERS

ABSTRACT

Mixed globulins (MG) were extracted from ground dry peas (*Pisum sativum*, B-160) with 0.5M NaCl, 50 mM potassium phosphate, pH 7.2, and isolated by precipitation at pH 4.5. Crude vicilin and legumin were fractionated from the MG by dialysis against 0.2M NaCl, pH 4.8, and centrifugation, then further purified using DEAE-cellulose chromatography. Conditions for maximum gel hardness of heat induced MG gel, as determined with an Instron Universal Testing Machine, were heating for 20 min at pH 7.1 at 87°C. Purified vicilin, but not legumin, formed heat induced gels. The relationship was linear between protein (globulin) concentration and log gel hardness. At all protein concentrations studied, as proportion of legumin decreased, gel hardness increased.

Key Words: pea protein, globulins, gelation, vicilin, legumin

INTRODUCTION

PROTEIN GELATION is an aggregation in which polymer to polymer and polymer to solvent interactions (both attractive and repulsive) are balanced, and an ordered network or matrix capable of holding water is formed (Schmidt, 1981). The capacity of gels to act as a matrix for holding water, lipids, sugars, flavors and other ingredients is useful in food applications and for development of new products (Kinsella, 1979).

Thermal gelation of soybean protein has been studied extensively (Mori et al., 1982a; Nakamura et al., 1984a, 1985a). Mori et al. (1982b) and Nakamura et al. (1984b, 1985b) have reported the contributions of 7S and 11S soy globulins in the process of gelation. Utsumi and Kinsella (1985) indicated that electrostatic interactions and disulfide bonds are involved in the formation of 11S globulin gel, mostly hydrogen bonding in 7S globulin gel, and hydrogen bonding and hydrophobic interactions in soy isolate gel. Similar studies on the involvement of molecular forces in the process of gelation of soy protein were reported by Nakamura et al. (1986a,b) and Mori et al. (1986). The 11S and 7S globulins may interact with each other in gels made from soy isolate proteins (Babajimopoulos et al., 1983; Utsumi and Kinsella, 1985).

Pea seeds contain 20 to 30% protein, most of which are storage proteins. The two major proteins, legumin and vicilin, are classical globulins and represent 65 to 80% of the total buffer-extractable protein (Schroeder, 1982). Legumin and vicilin proteins are similar to the 11S and 7S proteins of soybean, respectively (Derbyshire et al., 1976), and, thus, potentially may be important as functional protein ingredients in processed foods. However, information on the gelation behavior of such proteins is limited (Bacon et al., 1990; Gueguen and Lefebvre, 1983). Our objective was to examine thermal gelation of mixed pea globulins at various conditions and compare the results to those for crude and column purified legumin and vicilin at standardized conditions.

Authors Brekke and Powers are with the Dept. of Food Science & Human Nutrition, Washington State Univ., Pullman, WA 99164-6376. Author Bora's present address: Universidade Federal da Paraiba, Departmento de Tecnologia Química e de Alimentos, Centro de Tecnologia, Campus Universitário I, 58.059 - Joao Pessoa/PB, Brasil. Address inquiries to Dr. C. J. Brekke.

MATERIALS & METHODS

Extraction of pea globulins

The extraction method was that of Koyoro and Powers (1987). Whole dry green peas (*Pisum sativum*, B-160) from a single bag, provided by Dumas Seed Co., Pullman, WA, were ground into flour using a cyclone sample mill equipped with a 0.5 mm screen. The flour was extracted at room temperature (≈23°C) with 0.5 M NaCl, 50 mM potassium phosphate buffer, pH 7.2, in a ratio of 6 mL buffer/g of flour for 1 hr with constant agitation using a propeller stirrer. The extract was centrifuged (19,000 × *g*, 15 min, 4°C), and the supernatant was filtered through pads of glass wool. This filtrate was diluted with 5 vol of cold distilled water (4°C) and pH adjusted to 4.5 with 2N HCl to precipitate salt soluble proteins. The protein floc was allowed to settle overnight at 4°C. After decanting much of the supernatant, the remaining suspension was centrifuged, the pellet resuspended and washed with water, and re-centrifuged. The resulting pellet was re-extracted twice with extraction buffer in a ratio of 5 mL buffer/g of pellet for 1 hr at 4°C, followed by re-centrifugation. The supernatants resulting from the re-extraction were combined and divided into three parts. The pH of one-third of the supernatant was adjusted to 4.5 to precipitate mixed globulins, which were recovered by centrifugation (19,000 × *g*, 15 min, 4°C), washed with water, centrifuged and freeze-dried. The remaining two-thirds of the supernatant was dialyzed against McIlvaines buffer, 493 mL 0.2M $Na_2HPO_4 \cdot 2H_2O$ + 507 mL 0.1M citric acid, containing 0.2M NaCl, pH 4.8 (Scholz et al., 1974), at 4°C for 42 hr with two changes of buffer in a ratio of 10 mL of buffer per mL of original supernatant. The precipitate (legumin) was collected by centrifugation, suspended in distilled water at a ratio of 1:10, agitated, re-centrifuged (19,000 × *g*, 15 min) and the precipitate freeze-dried. The supernatant from dialysis was adjusted to pH 4.5 and the precipitate (vicilin) recovered, washed with distilled water, centrifuged and freeze-dried. The freeze-dried protein samples, representing several separate protein extractions, were stored in screw cap glass scintillation vials at −20°C until used.

Chromatography of legumin and vicilin fractions

Legumin and vicilin preparations were further purified by DEAE cellulose column chromatography using 35 mM potassium phosphate, 0.075M NaCl, 0.02% sodium azide buffer, pH 8.0. Freeze-dried protein was re-solubilized in column buffer at a concentration of 12 to 15 mg/mL by sonication for 1 min and placed on the column. Vicilin and legumin were eluted stepwise with buffer containing 0.15M and 0.4M NaCl, respectively (Grant and Lawrence, 1964). Eluted fractions were isoelectrically precipitated, centrifuged, washed with distilled water, centrifuged and frozen (−20°C).

Elution patterns of mixed globulin, crude legumin and crude vicilin samples from the DEAE column were also used as a measure of the composition of those preparations. Thus, total protein eluting at 0.15M NaCl was recorded as a measure of vicilin and that at 0.4M NaCl was as legumin content.

Denaturation temperature of mixed globulins by differential scanning calorimetry (DSC)

DSC was performed in a Perkin-Elmer DSC 4 fitted with a 3600 Thermal Analysis Data Station. Protein samples in 35 mM phosphate buffer, 0.4M NaCl, pH 7.2, were hermetically sealed in aluminum pans (Perkin-Elmer kit No. 219-0062) and scanned at 10°C/min over the range 25–100°C at a sensitivity of 1.0 mW using phosphate buffer in the reference pan. The instrument was calibrated for temperature using indium (m.p. 156.60°C). Tm was determined with data analysis programs supplied by Perkin-Elmer.

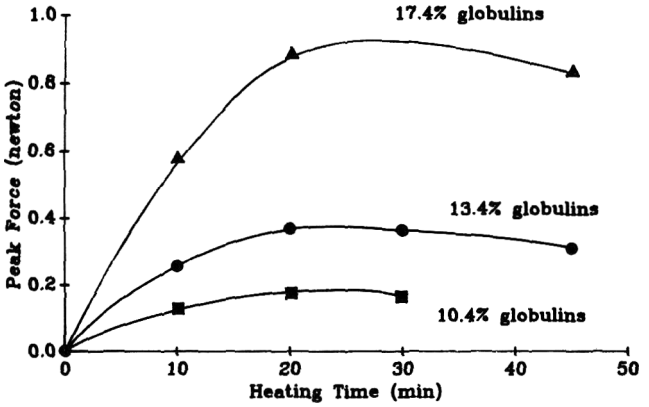

Fig. 1—Heating time and protein concentration effects on peak force of gels prepared from pea mixed globulin at 87°C and pH 7.2.

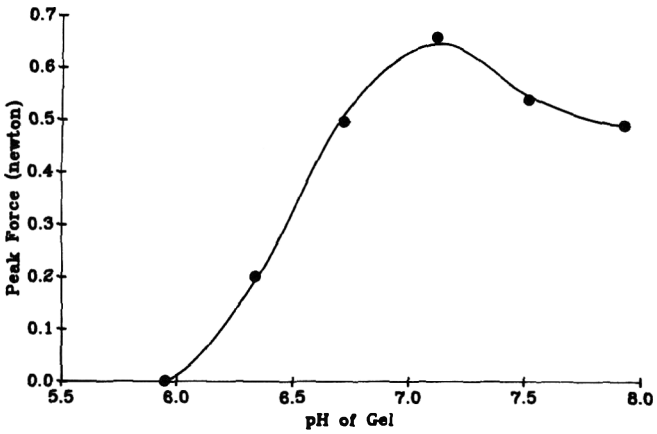

Fig. 2—Effect of pH on peak force of gels prepared from pea mixed globulins. Gels were made by heating 15% mixed globulin solutions 20 min at 87°C in 30 mM Tris-HCl buffers.

Preparation of gels

Gelation of protein samples was carried out according to the method described by Utsumi and Kinsella (1985). Freeze-dried protein was suspended in 30 mM Tris-HCl buffer, pH 7.2, in a test tube, and the suspension was sonicated for 1 min. The suspension was centrifuged for 30 min, and the supernatant (protein solution) was used for preparation of the gels. Aliquots of protein solution (25 to 30μL) in 30 mM Tris-HCl buffer were transferred to Caraway blood tubes (75 mm × 2.5 mm i.d) (Fisher Scientific), the tube ends were sealed with polyvinylidene chloride film and O-rings, and centrifuged at 500 × *g* for 10 min to remove air bubbles. Heating time of the tubes ranged from 0 to 45 min in a water bath at 87°C followed by immersion in cold water. The tubes were then held at 4°C for 24 hr, prior to gel peak force determinations. In a second experiment, pH was varied from 5.95 to 8.60, with heating for 20 min at 87°C in the water bath. All other procedures and conditions were as before.

Gel peak force and protein

The gels formed in blood tubes were carefully removed by blowing on the narrow end, taking care to collect the expelled gel on a glass slide without damage, cut into 1 cm pieces, a piece laid lengthwise on the stage of an Instron Universal Testing Machine, Model 1350 (Instron Corp., Canton, MA), and the gel peak force was determined. The speed of the plate (76 mm diam) was adjusted to 4 cm/min and the compression to 70%. Peak force refers to that obtained during the first compression cycle (Bourne, 1978). Two to five gels were used for replicate determinations. Protein was determined by the biuret method (Gornall et al., 1949), using bovine serum albumin as standard.

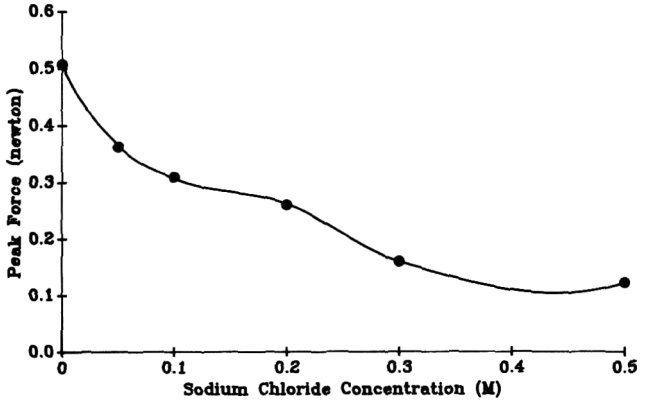

Fig. 3—Sodium chloride concentration and peak force of gels made with pea mixed globulin. Gels were prepared by heating 15% mixed globulin solutions 20 min at 87°C in 30 mM Tris-HCl, pH 7.1, with variable concentrations of NaCl.

Table 1—Composition of crude globulin fractions as calculated from DEAE cellulose chromatography profiles according to Grant and Lawrence (1964)

Fraction	Legumin (%)	Vicilin (%)
Mixed globulins	35.7	64.3
Crude legumin	68.5	31.5
Crude vicilin	9.1	90.9

RESULTS & DISCUSSION

DSC OF MIXED GLOBULINS indicated one transition between 74 and 95°C with a maximum at 86.2°C (thermogram not shown). This was essentially the same temperature (86°C) previously reported for field peas by Arntfield and Murray (1981), although the solvent system they used was not specified. Time of heating at 87°C had a marked effect on mixed globulin gel peak force (Fig. 1). At pH 7.2, gel strength increased with heating time up to 20 min, but heating beyond that period caused a decrease in gel peak force. Nakamura et al. (1985a) noted similar behavior for soybean glycinin (11S) when heated at 100°C for different times. They also reported maximum gel strength at 20 min heating. We adopted 20 min heating time at 87°C as our standard gelation conditions, based on observed maximum transition of 86.2°C and data presented in Fig. 1.

To study the effect of pH on gel formation and gel peak force, and further define standard conditions, pH of the mixed globulin solutions was varied from 5.95 to 8.60 (Fig. 2). We observed that at pH 5.95 no gel formed. Gel formation occurred at ≥ pH 6.4, with the greatest gel peak force at pH 7.1. Increasing pH to > 8.0 resulted in a translucent and sticky gel which was difficult to remove from the tubes.

Sodium chloride had an adverse effect on mixed globulin gel peak force. Within the concentration range 0 to 0.5M NaCl, gel strength was maximum at 0M NaCl and decreased with increasing NaCl concentration (Fig. 3). Utsumi and Kinsella (1985), using soybean isolate and 11S protein, also reported a decrease in gel strength with increase in NaCl concentration. Wang and Damodaran (1991) noted that in the presence of 0.5M NaCl, soy 11S did not form a gel when heated at 90°C, and the peak force of soy isolate gels was reduced. Conversely, they did not observe a reduction in peak force of soy 7S gels when 0.5M NaCl was added.

The make-up of the mixed globulin, crude legumin and crude vicilin preparations, based on elution patterns from the DEAE column was determined (Table 1). The data are based on the definition of Grant and Lawrence (1964) that vicilin elutes at 0.15M NaCl and legumin at 0.4M NaCl from DEAE cellulose under the conditions used. At the conditions used for gelation, i.e. pH 7.1, 20 min at 87°C, and at the protein concentrations studied, legumin fractions, either crude or further

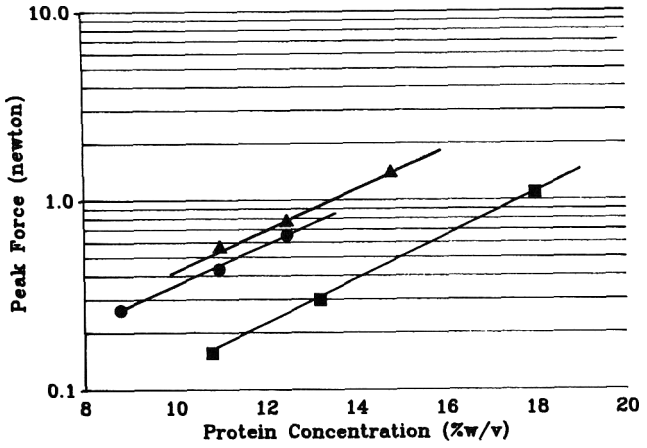

Fig. 4—Peak force of gels from solutions of several pea protein fractions of variable protein concentration. Gels were made by heating protein solutions for 20 min at 87°C at pH 7.01–7.04. ■ = mixed globulins, ● = crude vicilin, ▲ = DEAE-purified vicilin.

purified by DEAE cellulose column-chromatography, did not gel. At the highest concentration, neither of these fractions showed any evidence of a gel hard enough to measure. However, further-purified vicilin from the DEAE column formed a gel of greater peak force than for gels from crude vicilin or mixed globulins. While both 7S and 11S soy globulin fractions have the capacity to form gels, the pea vicilin (7S) fraction had that capacity while pea legumin (11S) did not. Saio and Watanabe (1978) reported that the 11S soy protein produced a much harder gel than the 7S soy protein.

Data for vicilin fractions and mixed globulins demonstrated a linear relationship (semi-log scale) between gel peak force and protein concentration (Fig. 4). At equal protein concentrations, peak force of the gel from column purified vicilin was greatest, followed by the gels made from crude vicilin and mixed globulins. That is, the greater the proportion of vicilin, the greater was the gel peak force. That all three lines (Fig. 4) were nearly parallel indicated that the effect of legumin on gel peak force was not protein concentration dependent. The decrease in gel peak force with increase in legumin may not be simply a dilution or vicilin sparing effect, but may be due to protein to protein interaction. For example, with soybean 7S and 11S globulins, the 7S protein interacts electrostatically with the basic subunits of 11S globulin to form soluble complexes during gelation (Damodaran and Kinsella, 1982; Utsumi et al., 1984). However, unlike vicilin and legumin in our study, the interaction of soybean 11S and 7S proteins was affected by the proportion of globulins.

CONCLUSIONS

VICILIN, the 7S component of pea globulin, undergoes heat gelation in a model system while legumin, the 11S component, does not gel under the same conditions. The optimal conditions for gelation of a pea mixed globulin system, as assessed by gel hardness, were pH 7.1 and heating time 20 min at 87°C. Addition of NaCl at concentrations of 0.05M or greater resulted in mixed globulin sets of reduced hardness. Legumin concentration in a range of pea globulin preparations, as estimated by DEAE ion exchange chromatography elution profile, was inversely related to gel hardness in the model system.

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Membrane Lipid Metabolism, Cell Permeability, and Ultrastructural Changes in Lightly Processed Carrots

G.A. PICCHIONI, A.E. WATADA, S. ROY, B.D. WHITAKER, and W.P. WERGIN

ABSTRACT

We monitored changes in phospholipid (PL), steryl lipid, and glycolipid classes, cell permeability, and ultrastructure in wound-stressed tissues (shreds and disks) of carrots (*Daucus carota* L. 'Apache'), stored up to 10 days at 10°C and 95% RH. Total PL rose 47% ten days after shredding, with phosphatidylcholine decreasing and phosphatidic acid increasing in relative abundance. Acylated sterol glycoside doubled after 2 days. Leakage of UV-absorbing metabolites from disks increased by 45% between 1 and 3 days storage. Extensive, storage-dependent accumulation of endoplasmic reticulum and attached ribosomes within vascular parenchyma cells occurred 10 days after wounding. Thus net synthesis of membrane lipid components occurred together with increases in permeability and the accumulation of phosphatidic acid. Membrane degradation and repair processes likely coexist during prolonged storage of lightly processed carrots.

Key Words: membrane repair, senescence, phospholipids, glycolipids, endoplasmic reticulum

INTRODUCTION

CONSUMER DEMAND for lightly processed or precut vegetables has increased, but limited shelf life has slowed development of new markets (Bolin and Huxsoll, 1989). Lightly processed tissues experience injury upon cutting or slicing and thus are more perishable and senescence-prone than the intact organs from which they were obtained (Huxsoll et al., 1988). Tissues of quiescent plant storage organs (fleshy roots, tubers, etc.) become highly activated after cutting or slicing and incubation in a moist environment (Kahl, 1974). Wounding likely leads to rupture of membranes and activation of membrane lipid catabolism (Kahl, 1982), the extent of which is species-related. For example, in potato tubers, up to 30% of membrane phospholipid (PL) is lost within 2 hr of cutting whereas in carrot roots, no measurable PL breakdown occurs (Theologis and Laties, 1980), despite the considerable disorganization of cells which may occur at the cut surface (Tatsumi et al., 1991).

Wounding also initiates a complex series of slower metabolic changes which are related to *de novo* synthesis of proteins and which comprise cellular "repair" processes. The biosynthesis of new membranes is an important component of this response. In potato, a marked increase in lipid synthesizing ability in wounded tuber tissue within hours of excision (Tang and Castelfranco, 1968) is associated with induction of key enzymes in PL synthesis (Kahl, 1983) as well as fatty acid synthesis (Willemot and Stumpf, 1967). Ultrastructural changes documented in such tissues are consistent with increased protein synthesis, membrane repair, and secretory phenomena. These include development of endoplasmic reticulum (particularly lamellae with bound ribosomes), appearance of polyribosomes, nuclear enlargement, and increased number of well-developed dictyosomes (Leaver and Key, 1967; Sparkuhl et al., 1976; Barckhausen, 1978).

Many reports have been published describing metabolic and ultrastructural changes within cells of wounded storage organs (Kahl, 1974, 1982, and 1983; Barckhausen, 1978; Mazliak and Kader, 1978; Stanley, 1991). Most studies have monitored changes in excised tissue segments (often from potato tuber) up to 24 hr after wounding. However, very little is known about membrane lipid metabolism and ultrastructural modifications of lightly processed organs which must necessarily withstand more prolonged storage (e.g., days). This may be because interest in lightly processed foods has emerged only recently (King and Bolin, 1989).

Better understanding of membrane lipid metabolism in cells is needed in order to determine how senescence is regulated, particularly in lightly processed fruits and vegetables. Our objective was to monitor levels of membrane lipids and examine permeability and ultrastructural changes of stored, lightly processed carrots.

Author Picchioni is associated with the Dept. of Agricultural Sciences, Technology, and Education at Louisiana Tech Univ., P.O. Box 10198, Ruston, AL 71272. Authors Watada, Roy and Whitaker are with the Horticultural Crops Quality Laboratory and Authors Roy and Wergin are affiliated with the Electron Microscopy Laboratory, USDA-ARS Beltsville Agricultural Research Center, Beltsville, MD 20705.

MATERIALS & METHODS

Test material and experimental conditions

Whole 'Apache' carrots were obtained from a wholesale distributor, and after peeling, 5-cm root sections were shredded using a food processor. Shredded samples (25g fresh weight) were either frozen immediately in liquid N_2 and kept sealed under N_2 gas at −80°C (0 days storage), or stored to simulate conditions in retail markets. During storage, shreds were placed on layered plastic grids within a 10-L plastic container (≈300g total fresh weight) covered with a polyethylene bag and aerated with humidified air at 15 mL/min. The container was stored in a controlled room at 95% RH and 10°C. After 2, 5, and 10 days, samples were frozen and stored at −80°C as described.

For cell permeability evaluation (see below), carrot disks were placed atop 2.5 cm^2-wide plastic cups and stored within sealed 1-L glass jars (≈6.5g total fresh weight tissue/jar) aerated at 3–5 mL/min.

Sample preparation and lipid analyses

Frozen tissue was lyophilized, ground, and a 200-mg dried subsample was homogenized in $CHCl_3$:MeOH (2:1) with three 15-s bursts of a Polytron homogenizer. Homogenates were filtered through a sintered glass funnel and the residue was re-extracted with $CHCl_3$:MeOH (2:1). Combined extracts were washed sequentially with 0.85% (w/v) NaCl and MeOH:H_2O (1:1). The $CHCl_3$ phase containing total lipids was evaporated to dryness under N_2 and redissolved in 1 mL $CHCl_3$. The total lipid extract was passed through a silica Sep-Pak cartridge (Waters, Milford, MA) to sequentially elute neutral lipid (NL), glycolipid (GL), and phospholipid (PL) fractions after modifying the method of Glass (1990). Only a single cartridge was used per sample and it was preconditioned with 3 mL $CHCl_3$. Four mL $CHCl_3$ eluted pigments and steryl esters (the latter were typically below detection limits); 8 mL $CHCl_3$:Me_2CO (9:1) eluted free sterols (FS) and pigments of greater polarity; 8 mL Me_2CO eluted glycolipids (GLs), including acylated sterol glycoside (ASG), sterol glycoside (SG), and monogalactosyldiacylglycerol (MGDG); finally, phospholipids (PLs) and digalactosyldiacylglycerol (DGDG) were eluted with 12 mL MeOH:H_2O (9:1). Each collected fraction was evaporated to dryness under N_2, redissolved in 1 mL $CHCl_3$ for NL and GL, or 1 mL $CHCl_3$:MeOH (1:1) for PL, sealed under N_2, and stored at −80°C until time of analyses.

Table 1—Phospholipid concentrations (mg/100g dry wt) in 'Apache' carrot shreds during 10 days storage at 10°C and 95% RH[x]

Storage (days)	Phospholipid class[y]					
	PC	PE	PI	PA	LPC	Total
0	423 ± 27	210 ± 15	75 ± 17	76 ± 24	9 ± 0.2	793 ± 83
2	464 ± 11	226 ± 6	71 ± 3	79 ± 7	6 ± 0.1	847 ± 23
5	505 ± 29	247 ± 9	84 ± 7	114 ± 15	8 ± 0.5	958 ± 60
10	522 ± 18	266 ± 11	94 ± 8	151 ± 10	7 ± 0.7	1039 ± 47

[x] Each value is the mean ± standard deviation of three sample replicates.

[y] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PA, phosphatidic acid; LPC, lysophosphatidylcholine. Phosphatidylglycerol and lysophosphatidylethanolamine were below detection limits.

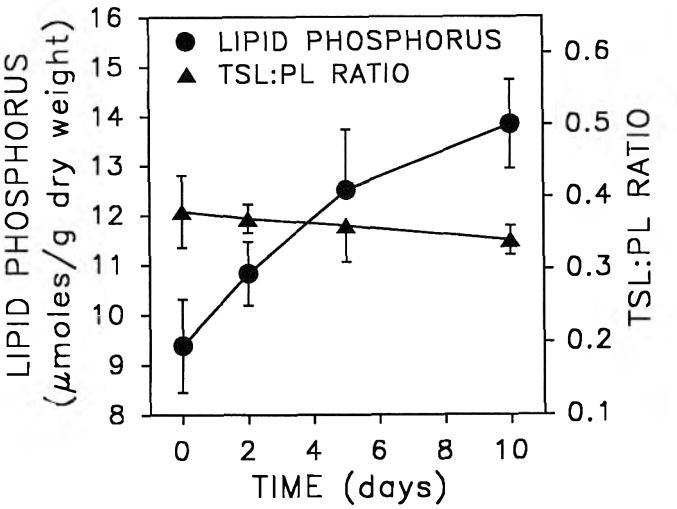

Fig. 1—Changes in total lipid-P and in the mole ratio of total steryl lipid:phospholipid (TSL:PL) in 'Apache' carrot shreds during 10 days storage at 10°C and 95% RH. Each point is the mean ± standard deviation of three sample replicates. Molar values for steryl lipids (moles/g dry weight of tissue) were calculated from data in Table 4 using the following molecular weights: FS = 410; ASG = 825; and SG = 572. Molar values for total PL (moles/g dry weight) were obtained by phosphate assay.

Free sterols (FS) in the NL fraction were isolated and quantified by GLC (Whitaker and Lusby, 1989) using lathosterol (cholest-7-en-3β-ol; Sigma, St. Louis, MO) as an internal standard (10 μg added to total lipid extract). Total lipid phosphorus (lipid-P) was determined on duplicate, 20-μl aliquots taken from the final PL fraction using the method of Ames (1966).

Component classes in GL and PL fractions were resolved within separate samples (injections) by normal phase HPLC using a 10 cm × 3 mm ChromSep LiChrosorb Si 60 (5-μm) silica cartridge system (Chrompack, Raritan, NJ). The HPLC instrument was equipped with a WISP 712 programmable injector, 600E quaternary solvent delivery system/gradient controller, and Maxima 820 software with personal computer to determine peak areas and automate analyses (all hardware from Waters, Milford, MA; software from Dynamic Solutions, Millipore Corp., Ventura, CA).

Prior to injection, aliquots of GL and PL fractions were taken to dryness, redissolved in HPLC mobile phase (1:1 mixture of 2-propanol:hexane for GL, or 58:40:2 mixture of 2-propanol:hexane:H_2O for PL), and passed through a 0.2-μm PTFE membrane filter (Gelman Sciences, Ann Arbor, MI) using a gas-tight syringe. The syringe and filter were flushed twice with mobile phase to recover held-up sample volume, and the combined filtrates were dried and again redissolved in a known volume (250–500 μL) of the same solvent. The injection volume (100 μL) represented 14% or 20% of the total PL or GL fraction, respectively.

GL and PL components were detected using a Varex IIA evaporative light scattering detector (Varex Corp., Burtonsville, MD) with N_2 flow rate at 45 mm (2.5 L/min) and the drift tube temperature at 90°C. Individual GL and PL classes were quantified by external standardization using calibration curves generated using authentic standards. MGDG, DGDG, SG, and ASG standards were purchased from Matreya (Pleasant Gap, PA). PL standards were obtained from Sigma (St. Louis, MO). HPLC grade solvents were obtained from Mallinckrodt Specialty Chemicals, Inc. (Paris, KY).

The mobile phase was similar to that used by Moreau et al. (1990) and Letter (1992) with modifications in elution times and flow rate. PLs (including the GL DGDG) were eluted with a mobile phase of 2-propanol:hexane:H_2O using a logarithmic gradient from 58:40:2 to 52:40:8 in 20 min, a hold for 40 min, a reverse linear gradient to the starting solvent mixture in 15 min, and a 40-min hold for re-equilibration. GLs were eluted using linear gradients (2-propanol:hexane without H_2O) from 5:95 to 20:80 in 15 min, to 40:60 in 10 min, a 25-min hold, a reverse to the starting solvent mixture in 5 min, and a 40-min hold for re-equilibration. The flow rate was 0.5 mL/min throughout all analyses. Lipid class data represent the mean ± standard deviation of three sample replicates, each derived from two roots.

Cell permeability

In a second experiment, carrot slices were prepared by first using the food processor (equipped with single blade), then disks (7 mm wide × 4 mm thick) were obtained from secondary vascular tissue of the slices using a cork borer. Disks (averaging 180 ± 10 mg fresh weight) were randomized, then immersed for 15 min in solutions containing deionized water or $CaCl_2$ (45 or 90 mM) on an orbital shaker. An earlier study showed that exposure to water did not increase cell permeability from carrot tissue when compared to hypertonic solutions (Simon, 1977). The pH of all solutions ranged from 5.8 to 6.0. Immediately following treatment, disks were placed in a salad spinner and spun for 30 sec at 200 rpm to remove residual treatment solution. Tissues were then held in storage for 1, 3, or 5 days. Each treatment was placed in a storage jar and was composed of four, 3-disk replicates per storage interval.

Following storage, the leakage of UV-absorbing solutes was monitored using methods reported by Picchioni et al., (1991) with slight modifications. Three disks per treatment were removed from storage and incubated in 7.5 mL deionized water on an orbital shaker for 4 hr at 25°C. Three-ml volumes of incubation medium were centrifuged at 1300× g for 10 min, and leakage was expressed as absorbance of the clarified solution measured at 260 nm (A_{260}) using a Shimadzu UV-160 spectrophotometer (Shimadzu Corp., Kyoto, Japan).

Transmission electron microscopy (TEM)

In a third experiment, carrot shreds were prepared and stored as described above. Samples (2 to 3 mm^3) of carrot were randomly taken from shreds that were freshly prepared and from others that had been stored for 10 days. The excised samples were chemically fixed with 2.5% glutaraldehyde in 0.1M cacodylate buffer at pH 7.2 for 5 hr, washed in cacodylate buffer, and postfixed overnight in 1% OsO_4. After dehydration in an alcohol series, samples were embedded in Spurr's resin as described by Roland and Vian (1991). Ultrathin sections were cut with a diamond knife, stained with 1% uranyl acetate for 10 min and 2% lead citrate for 2 min, and then observed with a Hitachi H500H transmission electron microscope operating at 75 keV.

RESULTS & DISCUSSION

Lipid composition and cell permeability

PL composition of carrot roots showed a pattern typical of higher plants in that phosphatidylcholine (PC) was the dominant component followed by phosphatidylethanolamine (PE) (Table 1). The total PL mass in each sample was calculated both by lipid-P determination (using average MW of 750) and by summation of individual PL classes determined by integration of HPLC peaks. Total PL values by the 2 methods were in good agreement (lipid-P values = 96% ± 5% of HPLC values). Total lipid-P increased by 15%, 33%, and 47% within

Table 2—Distribution of individual PL classes expressed as the weight percent of total PL in 'Apache' carrot shreds during 10 days storage at 10°C and 95% RH[x]

Storage (days)	Phospholipid class[y]				
	PC	PE	PI	PA	LPC
0	54 ± 2	27 ± 1	9 ± 1.1	9 ± 2	1.0 ± 0.1
2	55 ± 1	27 ± 0.3	8 ± 0.1	9 ± 1	0.7 ± 0.01
5	53 ± 0.4	26 ± 1	9 ± 0.3	12 ± 1	0.8 ± 0.1
10	50 ± 1	26 ± 0.1	9 ± 0.3	15 ± 0.5	0.7 ± 0.04

[x] Each value is the mean ± standard deviation of three sample replicates. For abbreviations see Table I.

Table 3—Leakage of solutes from 'Apache' carrot disks during 5 days storage at 10°C and 95% RH[x]

$CaCl_2$ conc (mM)	Time in storage (days)		
	1	3	5
0	0.029 ± 0.003	0.042 ± 0.002	0.036 ± 0.008
45	0.031 ± 0.003	0.030 ± 0.005	0.025 ± 0.002
90	0.032 ± 0.001	0.030 ± 0.004	0.024 ± 0.003

[x] Each value is the mean ± standard deviation of 4, three-disk replicates. Prior to storage, disks were pretreated for 10 min with water (0 mM $CaCl_2$) or with Ca-containing solutions (all at pH 5.8–6.0). Following storage, leakage was measured and expressed as UV absorbance of released solutes (A_{260}). Data are the A_{260} values obtained following a 4-hr incubation period in water at 25°C.

Table 4—Steryl lipid concentrations (mg/100g dry weight) in 'Apache' carrot shreds during 10 days storage at 10°C and 95% RH[x]

Storage (days)	Steryl lipid[y]			
	FS	ASG	SG	Total
0	110 ± 5	49 ± 3	14 ± 0.3	174 ± 5
2	107 ± 7	98 ± 14	14 ± 1.0	219 ± 8
5	117 ± 2	115 ± 9	14 ± 0.7	246 ± 11
10	121 ± 4	119 ± 9	15 ± 2.5	255 ± 14

[x] Each value is the mean ± standard deviation of three sample replicates.
[y] Abbreviations: FS, free sterol; ASG, acylated sterol glycoside; SG, sterol glycoside.

2, 5, and 10 days of storage, respectively (Fig. 1). All PLs except lysophosphatidylcholine (LPC) increased in concentration from 0 to 10 days. PC, PE, and phosphatidylinositol (PI) rose 23% to 27% by 10 days, whereas phosphatidic acid (PA) increased 99% by day 10. Thus, PA increased at the highest rate and represented a significantly greater proportion of the total PL fraction after 5 and 10 days storage.

The proportions of individual PL classes (from Table 1) varied little during storage, except that the relative amounts of PC and PA changed inversely over time (Table 2). PA is generally regarded as a membrane degradation product resulting from action of phospholipase-D (Larsson et al., 1990). This enzyme affects membrane-bound PLs in disrupted or senescing plant tissue (Galliard et al., 1976; Chéour et al., 1992) and can be specific for PC over other PLs (Mounts and Nash, 1990). Phospholipase-D activity in carrot storage root extracts ranked relatively high in a survey among many plant species and organs (Quarles and Dawson, 1969). Thus, the inverse changes in proportions of PC and PA (decrease in PC offset by increase in PA) probably reflects PL catabolism.

Such an accumulation of PA (Table 1) is atypical, because PA is usually rapidly hydrolyzed to diacylglycerol by phosphatidate phosphatase in the pathway of membrane lipid degradation associated with senescence (Paliyath and Droillard, 1992). Possibly, the extraction method used did not completely inactivate phospholipase-D, which could have contributed to the high PA levels. Total inactivation could have led to lower PA values on day 0 and, quantitatively, a more substantial increase during storage (C. Willemot, personal communication).

PA is a central precursor in PL synthesis (Joyard and Douce, 1979; Moore, 1982). Thus, whether the increase in PA was related to the overall increase in PL content, or whether it was a consequence of lipolytic activity, is unclear. Isotopic labelling studies are needed to elucidate this question. The indication that total PL began to increase before PA (Table 1) appears to support the lipolytic activity explanation.

Cell permeability (leakage) of all disks (pretreated with water or Ca-containing solutions) was similar following one day of storage (Table 3). However, a 45% increase in leakage from disks pretreated with water only occurred between 1 and 3 days storage, providing further evidence for membrane degradation. Between 3 and 5 days storage, average leakage from water-treated disks decreased, but was variable. Leakage of electrolytes from water-treated carrot slices increased to a similar degree during storage (data not shown).

In contrast to water-treated disks, leakage from Ca-treated disks remained constant between 1 and 3 days storage, then decreased 17–20% by the fifth day. This indicates that Ca pretreatments may increase membrane integrity and thus reduce the rate of senescence of cut and stored carrot tissues. Enoch and Glinka (1983) reported a similar finding using carrot disks during shorter experimental periods (4 hr in aqueous media).

Total steryl lipids (TSL = FS + ASG + SG) increased by 48% between 0 and 10 days storage (Table 4). This partly resulted from the marginal, 10% increase in total FS. It was mainly from the large increase in ASG, which doubled in only 2 days and showed further increases after 5 and 10 days. The increase in ASG was coincident with that of PL (predominantly PC and PE). This is consistent with the findings that, in higher plants, ASG synthesis is directly stimulated by PC (Forsee et al., 1974) or PE (Péaud-Lenoël and Axelos, 1972), which presumably serve as fatty acid donors.

The physiological basis of the ASG increase is not known, but sterol synthesis and conjugation could have a major influence on membrane properties (Benveniste, 1978). Reversible esterification/de-esterification and glycosylation/de-glycosylation have been suggested to exert modulatory effects on membrane organization and function (Wojciechowski, 1980). Moreau and Preisig (1993) demonstrated that ASG accumulates in plant cells during stress acclimation. Thus, the increase in ASG that we observed (following wounding stress) may be an indication of cell viability (R. Moreau, personal communication).

SG concentrations were comparatively low, which may reflect continued high levels of SG-6'-O-acyltransferase activity (Hartmann and Benveniste, 1987). Accumulation of ASG without depletion of FS and SG pools (Table 4) suggests continued FS synthesis and glycosylation during storage, although no substantial increase occurred in concentrations of FS and SG. However, the FS composition was altered following 10 days storage. The relative content of the two dominant sterols (stigmasterol and sitosterol) shifted, such that the ratio of stigmasterol:sitosterol increased from 0.30 to 0.45 during the 10-day period (Table 5). This specific change in FS composition has occurred during fruit ripening (Whitaker, 1988), and would be expected to result in decreased ordering and increased permeability of plant PL bilayers (Schuler et al., 1991).

The TSL:PL molar ratio decreased marginally during the 10-day storage (Fig. 1), but averages were not significantly different (statistical data not shown). The relative stability of this ratio may be adaptive. Coordinated regulation of sterol and PL pathways is believed to ensure optimal sterol:PL interaction in membranes (Burden et al., 1990). The free sterol:PL molar ratio decreased from 0.29 to 0.22 during 10 days storage (data not shown). This contrasts with earlier findings involving cabbage leaf disks aged at 15°C for up to 14 days

Table 5—Free sterol (FS) composition (weight percent of total FS) and ratio of stigmasterol to sitosterol in 'Apache' carrot shreds during 10 days storage at 10°C and 95% RH[a]

Storage (days)	Sterol moiety			Stigmasterol: sitosterol ratio
	Campesterol	Stigmasterol	Sitosterol	
0	12 ± 0.9	20 ± 5	68 ± 5	0.30 ± 0.09
2	11 ± 1.4	20 ± 3	69 ± 4	0.30 ± 0.06
5	10 ± 0.8	19 ± 2	70 ± 3	0.28 ± 0.04
10	10 ± 0.2	28 ± 3	62 ± 3	0.45 ± 0.07

[a] Each value is the mean ± standard deviation of three sample replicates.

Table 6—Galactolipid concentrations (mg/100g dry weight) in 'Apache' carrot shreds during 10 days storage at 10°C and 95% RH[a]

Storage (days)	Galactolipid[b]	
	MGDG	DGDG[c]
0	185 ± 8	366 ± 21
2	168 ± 1	323 ± 33
5	200 ± 42	337 ± 29
10	211 ± 17	361 ± 41

[a] Each value is the mean ± standard deviation of three sample replicates.
[b] Abbreviations: MGDG, monogalactosyldiacylglycerol; DGDG, digalactosyldiacylglycerol.
[c] DGDG was collected in the phospholipid fraction during preparative chromatography (thus quantitated with the phospholipid classes), but is included in this table for simplicity.

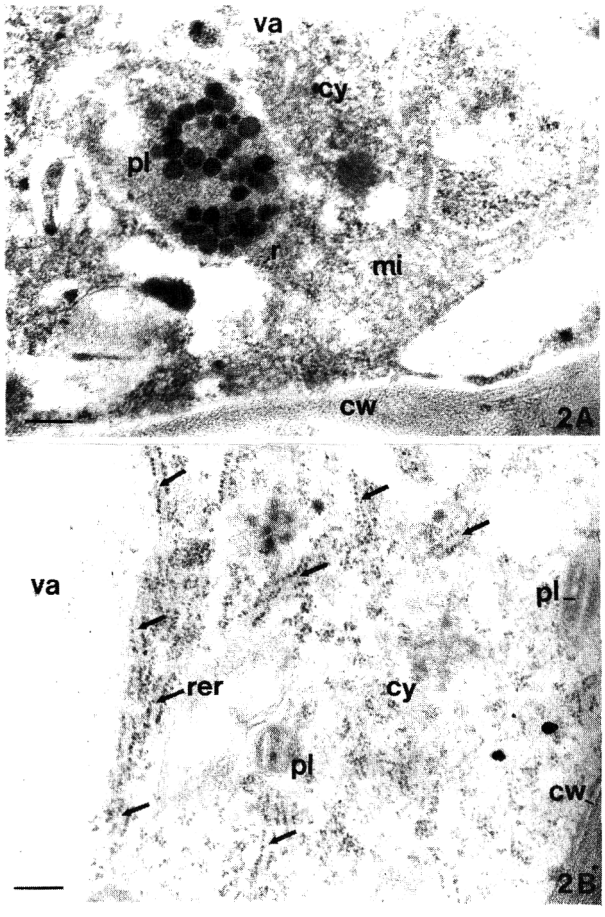

Fig. 2—TEM of thin sections showing typical vascular parenchyma cells in 'Apache' carrot shreds. Fig. 2A, portion of a parenchyma cell from a freshly shredded carrot. Fig. 2B, portion of a parenchyma cell from a carrot shred that had been stored for 10 days at 10°C and 95% RH. Cisternae of the ER (RER shown by arrows) are longer and more abundant in the stored tissue than in fresh tissue. Abbreviations used are as follows: cy, cytoplasm; cw, cell wall; mi, mitochondrion, pl, plastid; r, ribosome; rer, rough endoplasmic reticulum; va, vacuole. Bars represent 0.30 μm. (Magnification × 25000)

(Chéour et al., 1992), during which time a measurable increase in free sterol:PL was attributed to membrane degradative processes.

Slicing and cutting may advance the onset of senescence in plant organs (Huxsoll et al., 1988). In ripening fruit, measurable losses in membrane lipids and increases in the proportion of steryl lipids to PL are commonly reported (Thompson, 1988; Stanley, 1991). Thus, the coincident increases in PL and TSL and the lack of significant change in TSL:PL ratio in our study are contrary to known changes during genetically-programmed senescence. This suggests that, under the storage conditions we used, lightly processed carrots undergo a prolonged period of active lipid synthesis necessary for biogenesis of new membranes.

Previous studies involving plant storage organs (largely potato tuber) have demonstrated increases in lipid synthesis in wounded tissue, but typically only during the first several hours (e.g., < 24 hr) subsequent to wounding (Mazliak and Kader, 1978 and references cited therein). However, De Siervo (1990) reported a large reduction in PE concentration over an 8-day period in wounded tuber tissue of 2 potato cultivars. This indicates a major difference in membrane lipid metabolism of wounded potato tuber and wounded carrot storage root, 2 hr after wounding (Theologis and Laties, 1980) as well as following long-term incubation or storage (5–10 days).

On average, DGDG and MGDG were present in a mass ratio of almost 2:1. There was little or no indication that their concentrations changed over time (Table 6), demonstrating that storage did not result in a general increase in all lipids. Galactolipids are most abundant in plastidic membranes (Miernyk, 1985; Mudd, 1967), such as chromoplasts and amyloplasts of the carrot storage root (Grote and Fromme, 1984). Thus, the relatively constant MGDG and DGDG concentrations suggests the probability of site-dependent changes in membrane lipid metabolism.

Ultrastructural changes during storage

The mature carrot storage root primarily consists of secondary vascular tissue that contains the xylem and phloem parenchyma cells (Esau, 1940). Observations were limited to these cells, which appeared to be the most ultrastructurally and metabolically active. Parenchyma consisted of polygon-shaped cells, each with a large central vacuole and a thin parietal layer of cytoplasm that was bounded by the plasma membrane and pressed to the cell wall. In freshly shredded carrots, the cytoplasm contained a dense ribosome population, mitochondria, and plastids (primarily chromoplasts). Rough endoplasmic reticulum (RER) was only occasionally present (Fig. 2A). After 10 days storage, however, RER was the most conspicuous organelle. Several parallel layers of cisternae were found along the vacuole, with more randomly oriented membranes common in the cytoplasm (Fig. 2B). In order to establish with certainty that variations in RER lamellae occurred between fresh and stored tissues, large numbers or cells were examined. Observed changes in frequency or structure of cellular organelles (besides RFR) during storage were inconclusive.

The proliferation of RER supports the conclusion that membrane repair processes were active up to 10 days after wounding, since in plant cells, the ER is the primary site of PL and FS synthesis (Yamada et al., 1980; Moore, 1982; Browse and Somerville, 1991; Hartmann and Benveniste, 1987). In addition, the constant TSL:PL ratio suggests the synthesis of complete membranes with all their components, as indicated by the increase in RER. Such repair processes may be intensified following Ca pretreatments (Table 3), which would likely result in improved shelf life of lightly processed carrots. However, further study is necessary to verify this.

The greater abundance of RER in stored compared to freshly-cut tissue was somewhat expected, since only hours

after excision, the proliferation of RER is characteristic of wound tissue (Asahi, 1978). Also, ER strands are seen infrequently as part of cells in the resting state (Kahl, 1982). However, ER biogenesis in wounded plant storage organs has received very little evaluation during long-term periods (e.g., 10 days). Jackman and Van Steveninck (1967) reported a similar finding in beetroot disks aged up to 8 days at 24°C. In their study, ER lamellae were reduced to vesicles 2 hr after excision, but formed a near continuous layer within the cytoplasm 2 days later, which persisted up to 8 days.

Presumably, a buildup of RER would be of major importance in the wound repair mechanism. In addition to its role in membrane lipid synthesis, ER (RER) would be essential in the enzyme induction processes known to occur within 12 hr of wounding in storage organs (Kahl, 1983). Also, chemical modification of cell walls during wound healing, such as transport and secretion of phenolics and other precursors in lignin and suberin synthesis (Kahl, 1983; Kolattukudy, 1978), probably depends on ER vesiculation (Benveniste, 1978). For example, Babic et al. (1993) showed that, among four cultivars, storage stability of carrot shreds correlated with the rate of chlorogenic acid accumulation in the tissue 24 hr after wounding.

Cell walls of injured carrot root tissue (disks) are also known to accumulate large amounts of hydroxyproline-containing polypeptides/glycoproteins (e.g., extensin), even when aged as long as 6 days at 30°C (Chrispeels, 1969). In carrot disks, extensin biosynthesis and cell secretory capacity are specifically enhanced in response to excision and incubation (Chrispeels et al., 1974). Furthermore, ER lamellae or bound ribosomes may be involved in the extensin glycosylation process (Sadava and Chrispeels, 1978), in translation of extensin mRNA (Barckhausen, 1978), and in the secretion of extensin (Brodl and Ho, 1992).

Further study is needed to determine specific cellular membranes affected by these metabolic changes and to identify key enzymes involved in the repair process. Possibly, such enzymes can be genetically manipulated in the development of new cultivars with extended shelf life.

CONCLUSION

MEMBRANE LIPID METABOLISM of lightly processed (wound-stressed) carrots undergoes major modifications during 10-days storage. The most pronounced changes appear to reflect net synthesis of PL and ASG, development of RER, and membrane biogenesis. The simultaneous accumulation of PA and an increase in cell permeability, however, suggest coordinated involvement of catabolic and anabolic reactions. Also, an increase in proportion of stigmasterol relative to sitosterol during storage may indicate a senescence relationship.

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Cultivar/Location and Processing Methods Affect Yield and Quality of Sunflower Pectin

K.C. CHANG, N. DHURANDHAR, X. YOU and A. MIYAMOTO

ABSTRACT

The influence of cultivar/location of sunflower and grinding, blanching, water washing, and re-extraction of sunflower head residues were studied for yield and quality of pectin. Sunflower head residues from seven varieties/locations were used. The Interstate cultivar from Ardock (North Dakota) and Agri-Pro from Carrington (North Dakota) had highest yields (9.14–9.47%). Galacturonic acid content of the pectin did not differ significantly among cultivars. Grinding sunflower heads to particle size <60 mesh did not increase yield or quality of pectin. Peroxidase in head residues was completely inactivated after heat treatment at 75°C for 15 min. Higher shear blending did not increase yield, but decreased molecular mass and firmness of pectin gels. Extracting pectin twice yielded a total 13–14% pectin without lowering pectin quality.

Key Words: sunflower head, pectin, extraction, cultivar peroxidase

INTRODUCTION

THE WORLD CONSUMPTION OF PECTIN is 18,000–19,000 metric tons (Axinn, 1991) and is important in the food industry. Most pectin consumed in the U.S. is imported (Axinn, 1991; Crandall, 1991). Although apple pomace and citrus peels are traditional sources for commercial pectin, sunflower heads could be used for commercial pectin (Miyamoto and Chang, 1992). The pectin from sunflower heads has good gelling properties, high molecular mass, and high viscosity (Lin et al., 1976; Miyamoto and Chang, 1992; Chang and Miyamoto, 1992). Developing the full potential of sunflower as a commercial source of pectin requires an understanding of the physicochemical characteristics of raw materials. Knowledge of physical properties and mechanical requirements related to raw material and the extraction process are necessary for optimum quality and maximum yield of pectin. The sunflower cultivar can affect yield and quality of pectin (Lin et al., 1975). Variations in yield and quality with different parts of the sunflower plant (head and stalk), also have been reported (Lin et al., 1975, 1978; Sabir et al., 1976). The effect of grinding, blanching, water washing methods and re-extraction of residues has not been reported. Our objective was to investigate the yield and quality of pectin from head residues of several sunflower cultivars, and determine effects of processing steps, (grinding, blanching, water washing and re-exraction), on yield and quality of pectin.

MATERIALS & METHODS

Sunflower heads and proximate analysis

Seven sunflower head residue samples (cultivar/location), Interstate cultivar from Ardock, Fargo, and Enderlin; Cargill cultivar from Fargo, and Oil Hybrid 1 and 2, and Agri-Pro from Carrington (all from North Dakota), were harvested in the last week of October, 1991. The head residues were dried in a cabinet oven with air circulation at 55°C. The dried head residues were crushed, ground by a hammer mill to pass a 60-mesh screen, and stored at −20°C until used. These samples were hydrolyzed with sulfuric acid for 2 hr (Englyst et al., 1988) and analyzed for pectin as galacturonic acid content (Scott, 1979). Moisture content was determined by air-oven method (AACC method 44.15A, 1983). Ground sunflower samples and pectin were dried in an air-circulated oven at 135°C for 2 hr. Ash content was measured by incinerating the sample overnight in a muffle furnace at 550°C (AOAC Method 14.006, 1984).

Authors Chang, Dhurandhar, and Miyamoto are with the Dept. of Food & Nutrition and Dept. of Cereal Science & Food Technology, North Dakota State Univ., Fargo, ND 58105. Author You is with the Dept. of Food Science, Purdue Univ., West Lafayette, IN 47907.

Pectin isolation and analysis

Pectin was extracted from sunflower heads using a modification of the method of Sosulski et al. (1978). Nitric acid was used to replace hydrochloric acid for pectin precipitation. Instead of using a laboratory hydraulic press, the precipitated pectin was pressed by hands as much as possible. Galacturonic acid was determined by a colorimetic procedure, using 3, 5-dimethylphenol as a color reagent (Scott, 1979). The methyl ester content was determined, using an alcohol oxidase method (Klavons and Bennett, 1986), and was expressed as percent of the number of anhydrogalacturonic acid molecules. The weight averaged molecular mass was determined, using gel filtration (Miyamoto and Chang, 1992). Jellies of pectin were prepared at pH 3.0 and 5.4 (Chang and Miyamoto, 1992). At pH 3.0, jellies were prepared with 1.0% pectin, 30% sugar, and 25 mg calcium/g pectin. At pH 5.4, concentrations were 1%, pectin, 20% sugar, and 50 mg calcium. Firmness of the jellies was determined using the Instron method (Friedman et al., 1963; Chang and Miyamoto, 1992), and expressed as g force. A standardized citrus low methoxyl pectin (Genu LM12CG) was used as reference (Hercules Inc., Middletown, NY).

Grinding

The Interstate cultivars from Ardock and Enderlin were used for this experiment. Four ground sunflower head samples were prepared using a hammer mill to pass through (1) a 2.3 mm screen (1-cycle grinding); (2) a 2.3 mm screen and then through a 0.6 mm screen (2-cycle grinding); (3) a 2.3 mm screen and twice through the 0.6 mm screen (3-cycle grinding); and (4) a 2.3 mm screen and three times through the 0.6 mm screen (4-cycle grinding). The particle size distributions of the four samples were determined, using a Rotap (Testing Sieve Shaker, Model B, Tyler W-S Inc., OH) equipped with screens of various mesh sizes (10, 20, 30, 40, 60, 80, and 100 mesh). To determine the concentration effect of fractionation, the pectin content as total galacturonic acid content of various size fractions was determined using the method of Scott (1979).

The low-methoxyl pectin in the four ground samples was extracted. The yield and chemical compositions, including moisture, galacturonic acid, methoxyl content, molecular size, and gelling quality were determined.

Blanching

Sunflower head residues of the Interstate cultivar from Ardock and Enderlin, and the Cargill cultivar from Fargo were used for blanching studies to determine the effects of various temperature and time treatments on peroxidase activity of head residues. Samples were suspended in water and heated in a water bath to 75°, 85°, and 90°C for various times up to 20 min. For treatments at 110° and 120°C, samples were hydrated with water (head residue weight/water = 1:5, g/v) and retorted for up to 3 min. after the retort had reached the desired temperature. After heating, a 1 mL portion of water suspension was mixed with a guaicol-hydrogen peroxide solution to react with peroxidase in the sample (Luh and O'Neal, 1978). An orange-brown color indicated

Table 1—Pectin (galacturonic acid) content of sunflower head residues

Cultivar—Location	Pectin content (%)[a]
Interstate—Ardock	24.70[a]
Interstate—Fargo	23.45[b]
Cargill—Fargo	23.35[b]
Interstate—Enderlin	13.47[d]
Oil hybrid 1—Carrington	22.01[c]
Oil hybrid 2—Carrington	22.10[c]
Agri-Pro—Carrington	22.20[c]

[a] Dry weight basis.
[b-d] Means followed by different letter differ at $p < 0.05$.

Table 2—Effect of various cultivar/locations on yield, composition, and quality of sunflower pectin[a]

	Interstate Ardock	Interstate Enderlin	Interstate Fargo	Cargill Fargo	Agri-Pro Carrington
Yield, %	9.47[a]	3.50[c]	6.18[b]	5.17[b]	9.14[a]
Moisture, %	4.11[a,b]	2.79[b]	5.27[a]	3.58[a,b]	4.41[a,b]
Galacturonic acid, %	91.4[a]	64.83[b]	84.02[a]	90.82[a]	86.89[a]
Ash, %	0.90[b]	11.01[a]	1.18[b]	1.53[b]	1.39[b]
DM,[c] %	22.50[c]	29.68[b]	30.20[b]	32.77[a]	14.40[d]
Mol. wt., g	1,046,400[a]	659,500[b]	882,900[a]	644,500[b,a]	456,500[c]
Gel firmness pH 3, g force	299[b]	253[b]	522[a]	543[a]	258[b]
Gel firmness pH 5.4, g force	822[a]	513[d]	655[c]	459[e]	730[b]

[a-e] Means in a row followed by different letters differ at $p < 0.05$.
[c] DM = degree of methylation expressed as % of anhydrogalaturonic acid residues that were esterified.

a positive reaction. A negative reaction indicated adequate heat treatment to inactivate undesirable enzymes.

High-shear water washing and re-extraction

The Interstate cultivar from Ardock was used for these experiments. High speed washing at 75°C with a commercial (≈ 4L) Waring Blendor at low speed (15,000 rpm) for 1, 3, and 5 min was used to compare with the standard water washing, which used a motorized stirrer (700 rpm) at 75°C for 10 min. In the re-extraction experiments, the residue after 0.75% sodium hexametaphosphate (SHMP) extraction was subjected to re-extraction with 15/1 (volume/dry wt) ratio of 0.5% SHMP solution for 60 min at 75°C. The re-extracted pectin was precipitated, pressed, washed and dried, as described. The effects of high-shear washing and re-extraction on yield and quality of isolated pectin were determined. Moisture, galacturonic acid, methoxyl content, molecular size, and gelling property were determined.

RESULTS & DISCUSSION

Cultivar/location effects

Galacturonic acid contents of the seven sunflower head samples were compared (Table 1). The Interstate cultivar from Ardock contained the highest galacturonic acid content, (≈ 25% dry weight basis). For this reason, this head residue sample was used in most of the other studies. The yields, chemical composition, and gelling property of pectin from five sunflower head samples were compared (Table 2). The Interstate from Ardock and Agri-Pro from Carrington had higher yields than the other three cultivars. The Interstate head residue from Enderlin had the lowest pectin yield. Methoxyl content was highest in the Cargill cultivars from Fargo. Agri-Pro had the lowest molecular size. However, all pectins formed jellies at pH 3 and pH 5.4. At pH 3, Cargill and Interstate from Fargo had higher firmness than the other three samples, and Interstate from Ardock and Agri-Pro from Carrington had the highest firmness at pH 5.4. The mean firmness of the commercial citrus low methoxyl pectin jellies was 987 at pH 3.0 and 479 at pH 5.4. Generally, the firmness of sunflower pectin jellies at pH 3 was lower than that of commercial citrus low methoxyl pectin. This agreed with the reports of Chang and Miyamoto (1992). However, the firmness of the sunflower pectin jellies at pH 5.4 was higher than that of the commercial citrus low methoxyl pectin. Variation in the pectin content of four different cultivars (one confectionery and three oil seed) and the effects of maturity of plant development were reported by Lin et al. (1975) in Saskatchewan, Canada. Total pectin in the head residues of the cultivars varied between 14.9% and 24.2%, influencing the yield of pectin. However, galacturonic acid content of pectins isolated from different cultivars did not differ significantly (Lin et al, 1975). In our present study, the

Table 3—Effects on particle size distributions of Interstate cultivar Pectin samples (Ardock, North Dakota) ground through one to four cycles[a]

Sieve No.	g/100 g total sample 1st cycle	2nd cycle	3rd cycle	4th cycle
>10	0.07 (0.02)	0.00 (0.0)	0.00 (0.0)	0.00 (0.0)
>20	7.15 (0.24)	0.20 (0.0)	0.27 (0.07)	0.23 (0.0)
>30	10.49 (0.76)	0.35 (0.07)	0.59 (0.36)	0.41 (0.0)
>40	9.43 (0.60)	0.40 (0.0)	0.21 (0.07)	0.21 (0.03)
>60	19.40 (0.24)	6.65 (0.07)	3.66 (0.67)	2.63 (1.27)
>80	12.14 (0.07)	13.65 (0.07)	12.03 (1.03)	9.50 (0.10)
>100	6.45 (0.02)	9.60 (2.2)	8.12 (0.06)	6.52 (0.41)
Bottom	36.43 (1.73)	71.4 (1.69)	76.83 (1.05)	80.99 (1.60)
Sum of residue >10–60 mesh	46.54[d] (1.87)	7.60[b] (0.14)	4.73[b,c] (0.37)	3.49[c] (1.24)
Sum of residue ≤ 60 mesh	55.02[b] (1.83)	94.65[d] (0.49)	[68.99d] (0.08)	97.02[d] (1.90)

[a] Numbers in parentheses are standard deviation.
[b-d] Means in a row followed by different letters differ at $p < 0.05$.

Table 4—Pectin (galacturonic acid) content of ground sunflower head residues of different particle size fractions[a]

Particle Size-Sieve Number	Interstate-Enderlin	Interstate-Ardock
Over #40	10.54 (0.62)	—
Over #60	12.96 (0.46)	18.15 (0.95)
Over #80	12.15 (1.28)	19.86 (0.86)
Over #100	12.19 (0.12)	24.95 (0.31)
Bottom	11.36 (0.43)	22.22 (1.27)

[a] Pectin content is expressed on dry weight basis. Numbers in parentheses are standard deviation.

Table 5—Effect of grinding of sunflower head residues on pectin from the Interstate cultivar from Ardock, North Dakota[a]

	Grinding cycles 1	2	3	4
Yield, %	9.45[a]	9.40[a]	9.47[a]	9.69[a]
Moisture, %	5.24[a]	4.33[b]	4.11[b]	4.18[b]
Galacturonic acid, %	85.90[a]	91.94[a]	91.40[a]	93.14[a]
Ash, %	0.98[a]	0.65[b]	0.90[a]	0.94[a]
DM[c], %	22.96[a]	22.48[a]	22.50[a]	21.54[a]
Mol. wt., g	545,300[b]	560,400[b]	1,046,400[a]	543,900[b]
Gel firmness at pH 3, g force	320[a]	268[a,b]	299[a,b]	254[b]
Gel firmness at pH 5.4, g force	670[b]	630[b]	822[a]	686[b]

[a-b] Means in a row followed by different letters differ at $p < 0.05$.
[c] DM = degree of methylation expressed as % of anhydrogalacturonic acid residues that were esterified.

Table 6—Peroxidase reactivity on sunflower head residues after blanching treatments

Treatment[a]	Cultivar—Location	Duration of blanching (min)[b]						
		5	10	15	20			
A. 75°C	Interstate Ardock	++	+	–	–			
	Cargill Fargo	++	+	–	–			
	Interstate Enderlin	++	+	–	–			
B. 85°C	1	2	3	4	5	6	7	8
	Interstate Ardock +++	+++	++	+	+	–	–	–
	Cargill Fargo +++	+++	++	+	+	+	–	–
	Interstate Enderlin ++	+	+	+	–	–	–	–
C. 95°C		1	2	3				
	Interstate Ardock	++	++	–				
	Cargill Fargo	++	+	–				
	Interstate Enderlin	+	–	–				
D. 110°C		1	2	3				
	Interstate Ardock	+	+	+				
	Cargill Fargo	++	++	++				
	Interstate Enderlin	+++	+++	+++				
E. 120°C		1	2	3				
	Interstate Ardock	–	–	–				
	Cargill Fargo	++	+	–				
	Interstate Enderlin	++++	++	–				

[a] Treatment conditions A, B, and C were blanched by immersing test tube in hot water, and D and E were blanched in retort.
[b] Peroxidase activity, ++++: very strong (very dark color); +++: strong (dark color); ++: medium (brown); +: weak (light brown); and, –: negative (no color change).

Table 7—Effect of high-shear blending on yield, composition, and quality of sunflower pectin from the Interstate cultivar from Ardock, North Dakota

	Regular stirring 75°C 15 min	Blending treatment		
		Room temp 1 min	Room temp 3 min	Room temp 5 min
Yield, %	9.47[a]	8.93[a]	9.39[a]	9.94[a]
Moisture, %	4.11[a,b]	4.24[a]	4.35[a]	3.91[b]
Galacturonic acid, %	91.4[a]	89.81[a]	92.85[a]	94.75[a]
Ash, %	0.90[a]	0.41[b]	0.78[a,b]	0.57[b]
DM[d], %	22.50[a]	19.60[a]	18.03[a]	19.57[a]
Mol. wt., g	1,046,400[a]	687,700[b]	597,800[b,c]	367,100[c]
Gel firmness pH 3, g force	299[a]	300[a]	270[b]	209[c]
Gel firmness pH 5.4, g force	822[a]	524[b]	515[b]	348[c]

[a–c] Means in a row followed by different letters differ at $p < 0.05$.
[d] DM = degree of methylation, expressed as % of anhydrogalaturonic acid residues that were esterified.

Interstate from Ardock, which had highest galacturonic acid (Table 1), gave the highest pectin yield (Table 2). Except for the Interstate cultivar from Enderlin, galacturonic acid contents of the isolated pectins of the other four cultivars did not differ significantly (Table 2). Molecular sizes of the sunflower pectins of all cultivars were high, with Interstate from Ardock having the highest molecular sizes. Pectin from Interstate (Fargo) and Cargill (Fargo) had higher methoxyl contents than the others. The higher content of methoxyl groups in these two samples seemed to correspond with the higher gel firmness at pH 3.0. Although, Interstate (Enderlin) had ≈ 30% methoxyl content, the gel firmness at pH 3.0 was low, possibly because of the high ash content. Why the pectin from Interstate (Enderlin) had such a high ash content is not known.

Grinding

The particle size distributions of samples ground through one to four cycles by the hammer mill (Table 3) showed amounts of sizes through the 60 mesh screen did not differ ($p < 0.05$) for the 2, 3, and 4 cycle samples. The 1-cycle ground sample contained only ≈ 55% of the residues that passed through the 60-mesh screen. The pectin contents as galacturonic acid on a dry weight basis of the major fractions for two residue samples, Interstate from Enderlin and Ardock were similar (Table 4). There was a difference among fractions of the head residue for Interstate from Ardock. However, for Interstate from Enderlin, there was no substantial concentration effect by grinding and separating.

The effect of grinding on yield and quality of pectin after extraction (Table 5) showed galacturonic acid yield and methoxyl content did not differ significantly ($p < 0.05$). Molecular size was not reduced by grinding up to four cycles, although the three-cycle sample had a higher molecular size than the others. The firmness of jellies at pH 3 was slightly reduced by grinding four-cycles. However, the firmness of the jellies prepared at pH 5.4 was not reduced by grinding. Therefore, grinding to particle size <60 mesh did not increase yield and quality of pectin. Turmucin et al. (1983) reported an increase in pectin yield as particle size of sunflower heads decreased from 20 to 60 mesh. Turmacin et al. (1983) did not report gelling quality of pectin isolated from various mesh sizes. Our results implied that sunflower head residues do not need to be ground to <60-mesh size.

Blanching

After heating for 10 min at 75°C, little peroxidase activity remained (Table 6). After heating for 15 min or longer at 75°C, the enzyme was completely inactivated. The times required to remove peroxidase activity at 85° and 95°C were 7 and 3 min, respectively. Retorting the hydrated head residues with 110°C steam required >3 min to inactivate the enzyme; heating with 120°C steam inactivated the enzyme in 3 min.

Undesirable enzymes in the plant cells, including methyl esterase, polygalacturonase, phenolases, and lipoxygenase, should be inactivated in the beginning stages of extraction. Pectin in the plant is vulnerable to attack by deesterifying and degrading enzymes. The raw material should be heated under conditions to destroy undesirable enzymes, without destroying heat labile pectin (May, 1990). Peroxidase is a heat-resistant enzyme. When peroxidase was inactivated, all other plant enzymes were also inactivated. Our data could be used as a reference for inactivating enzyme activities of head residues for pectin extraction.

Water washing and high-shear blending

Water washing removes dust and some pigments in head residues. The effects of high shear blending were compared with the standard water washing procedure (stirring for 15 min at 75°C) (Table 7). High shear blending increased the speed of water washing. Ash content in all samples was low and did not differ significantly ($p<0.05$). High shear blending did not increase pectin yield and did not change the methoxyl content. However, with the increase of high-shear blending time up to 5 min, the molecular size of pectin was significantly ($p<0.05$) reduced. The firmness values of most of the jellies prepared at both pH of 3 and 5.4 also were decreased ($p<0.05$). Thus, high-shear blending for 5 min caused physical degradation of pectin polymers.

Re-extraction

The second extractions yielded 3–4% additional pectin (Table 8). Therefore, the total yield from both the first and second extractions ranged from ≈13–14%, which represents ≈55% of the pectin in the head residues. The yield from first-time extractions of head residues was >7.3% yield reported earlier (Miyamoto and Chang, 1992). This may be due to the different cultivars.

The re-extracted pectin also formed jellies. Except for one sample, re-extracted pectin did not decrease in molecular size. Methoxyl content also did not decrease in the re-extracted pec-

Table 8—Comparison of regular and re-extracted sunflower pectins from the Interstate cultivar from Ardock, North Dakota

	I[a]		II[a]		III[a]	
	Regular	Re-extract	Regular	Re-extract	Regular	Re-extract
Yield, %	8.93[c]	4.35[c]	9.39[c]	3.95[b]	9.94[c]	3.32[b]
Moisture, %	4.24[c]	1.70[b]	4.35[c]	ND[e]	3.91[f]	2.44[b]
	(0.01)	(0.05)	(0.09)		(0.19)	(0.13)
Galacturonic acid, %	89.8[c]	85.19[c]	92.85[c]	79.90[b]	94.75[c]	70.08[b]
	(0.95)	(1.18)	(2.43)	(2.71)	(3.56)	(0.40)
DM[d], %	19.60[c]	19.39[c]	18.03[c]	21.03[c]	19.57[c]	23.16[c]
	(2.9)	(1.64)	(0.39)	(1.71)	(1.88)	(1.71)
Mol. wt., g	687,700[c]	400,700[b]	597,850[c]	428,000[c]	367,100[c]	540,200[c]
Gel firmness pH 3, g force	300[b]	450[c]	270[c]	233[b]	209[b]	357[c]
Gel firmness pH 5.4, g force	524[c]	440[b]	515[b]	635[c]	348[b]	910[c]

[a] Numbers in parentheses are standard deviations.
[b-c] Mean in each row within regular and re-extract, of each experiment, respectively, followed by different letters differ at $p < 0.05$.
[d] DM = degree methylation, expressed as % of anhydrogalaturonic acid residues that were esterified.
[e] ND = not detectable.

tin. Results indicate that re-extraction of the head residues could increase pectin yield without lowering jelling quality. The combined yield, 13–14%, for the two extractions was higher than the 10% yield Lin et al. (1978) reported.

CONCLUSION

SIX SUNFLOWER HEAD RESIDUE SAMPLES from different cultivars/locations contained >22% pectin. Several blanching methods inactivated enzymes in the head residues. Grinding to particle sizes <60-mesh did not improve yield or quality of the pectin. High shear water washing in a blender could damage pectin quality. More than 55% of the pectin in the head residues could be extracted by two successive extractions. These results could be applied to develop a commercial process for sunflower pectin.

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Surface Properties and Emulsification Behavior of Denatured Soy Proteins

I. NIR, Y. FELDMAN, A. ASERIN, and N. GARTI

ABSTRACT

Soy protein isolate was physically (heat pretreatment) and chemically (urea, guanidine·HCl, and cleavage of SS bonds) modified in order to dissociate subunits, unfold the protein and improve surface properties: hydrophobicity, emulsification capability, and stability. Heat pretreatment as well as chemical treatment with urea or guanidine·HCl or reduction of disulphide bonds, will improve emulsification performance in comparison to native soy protein isolate. Significant differences in reduction of surface tension of water in the presence of native and modified proteins were observed (45 and 35 dynes/cm respectively). Measurements of fluorescence indicated that the relative hydrophobicity of the soy protein was also improved (from 600 to 1360) after heat pretreatment or contacting the soy protein with 8M urea solution.

Key Words: soy, protein, emulsification, denaturation, rheology

INTRODUCTION

PROTEINS have been recognized as good emulsifiers from the earliest days of surface and colloids science. Protein adsorption at an interface is important for the food industry because they provide stability against phase separation in food systems such as dairy products, baked goods, ice cream, meat and mayonnaise (Berger, 1976; Kinsella, 1981; Foegeding, 1989). Emulsifying and surface properties are useful functions which are important in development of soy protein products for food use. Emulsifying properties of soy protein have been long utilized (Kinsella, 1979; Pearson et al., 1965; Inklaar and Fortuin, 1969; Hutton and Campbell, 1977; McWatters and Holmes, 1979). Surface and emulsification properties of proteins are strongly correlated to their spatial structure (Kinsella, 1981, 1982; Mitchell, 1986; Kinsella and Whitehead, 1986, 1988; Hobbet and Brash, 1986; Dickinson et al., 1988; Damodarn, 1989). Chemical modification of food proteins has indicated that alteration of specific structural features (e.g. net charge, disulfide bonding and size influences film formation, foaming, and emulsifying properties (Kinsella, 1982; Kinsella and Whitehead, 1986). Such modifications have included succinylation (Franzen and Kinsella, 1976a,b; MacRitchie, 1978; Shimizu et al., 1985), acetylation (Franzen and Kinsella, 1976a,b; Kim and Kinsella, 1986a), phosphorylation (Woo et al., 1982; Woo and Richardson, 1983), glycosylation (Waniska and Kinsella, 1979; 1984), reduction of disulfide bonds (German et al., 1985; Kim and Kinsella, 1986b), enzymatic modifications (Puski, 1975; Monti and Jost, 1978; Kim and Kinsella, 1987) and thermal denaturation (Kato and Nakai, 1980; Kato et al., 1983; Townsend and Nakai, 1983; Shimizu et al., 1986). Interactions of urea and guanidine·HCl with proteins caused the dissociation and unfolding of subunits (Wolf and Brigges, 1958; Kelley and Pressey, 1966; Puski and Melnychyn, 1968).

The protein surface hydrophobicity has received much attention since hydrophobic interactions are considered important in functional properties of food proteins (Kinsella, 1979; Voutsinas et al., 1983). Keshavarz and Nakai (1979) reported a significant correlation between surface hydrophobicity (determined by hydrophobic chromotography and hydrophobic partition techniques) and interfacial tension of proteins. Kato and Nakai (1980) subsequently reported that the surface hydrophobicity (determined fluorometrically) showed significant correlations with interfacial tension and emulsifying activity of proteins. Results suggested that the emulsification of oil with protein could be explained using a concept of protein hydrophobicity. Nakai et al. (1980) also reported that the effective surface hydrophobicity showed good correlations with interfacial tension and emulsifying activity of plant proteins. Voutsinas et al. (1983) studied the effect of heating on emulsifying properties of selected food proteins and protein surface hydrophobicity (So) as a predictor of such properties. They reported that the emulsifying activity, emulsion stability and fat binding capacity of the proteins could be explained using the surface hydrophobicity concept.

Our objective was to examine the effect of several modifications on surface and emulsification properties of isolated soy protein. This would enable us to compare the effectiveness of different treatments on improving surface properties of commercial type protein.

The authors are affiliated with Casali Institute of Applied Chemistry, School of Applied Science & Technology, The Hebrew Univ. of Jerusalem, 91904 Jerusalem, Israel. Address inquiries to Dr. N. Garti.

MATERIALS & METHODS

ISOLATED SOY PROTEIN (Purina 500E) was purchased from Purina Ralston (Cleveland, OH) and served as a reference native protein; Cisparinaric acid was obtained from Molecular Probe (Roseville, MN). All other reagents were purchased from Sigma Chemicals (USA). Orange oil was purchased from Aromor (Israel) and coconut oil was supplied by Etz-Hazait (Israel) and was used without further purification.

Oil-in-water emulsions, with a total of 12 wt% of coconut oil/orange oil (3:1,wt/wt), 5 wt% protein, and 0.1wt% sodium azide were prepared at room temperature using an homogenizer (Silverson, England) at 2000 rpm for 20 min. No buffer solutions were used (emulsions pH was 6.7). Droplet size distributions were determined using a Coulter counter (model TAII, Coulter Electronics, England) with a 50 μm orifice tube and 0.9 wt% NaCl as electrolyte. The relative size of oil droplets in emulsions was measured by using the "R-index" (Kaufman and Garti, 1981) derived from measurements of emulsion turbidity (OD of 1:1000 dilution) at 800 and 400 nm, and obtaining the optical density ratio (OD 800/OD 400) vs time using a spectrophotometer (Spectronic 2000, Bausch & Lomb, Rochester, NY).

Surface tensions were measured with Wilhelmy platinum plate (Lauda Tensiometer, Germany). Each sample was centrifuged (2 hr, 7000×*g*) to remove possible solid impurities. Samples were equilibrated for a minimum of 24 hr to reach equilibrium and constant value readings in dyne/cm. The solutions were of 0.5 wt% soy protein at pH 6.7 and 25°C. The viscosities (Pa·sec) of the protein solutions in water (5 wt%, pH 6.7 at 25°C) were measured with a viscometer (model DV-I, Brookfield, USA). Denaturation was achieved by heating the protein solution (pH 6.7, 1-7 wt% protein, 0.1 wt% sodium azide) to 85°C for 45 min. Protein surface hydrophobicity was fluorometrically determined according to Kato and Nakai (1980). The hydrophobicity of the untreated protein was established as 100, and the hydrophobicities of the others were related to it. Reduction and cleavage of the proteins' SS bonds was carried out by treating 5 wt% protein in urea solution (8 M) in the presence of Tris buffer (pH 7.4, 0.02M) with sodium sulfite at 38°C for 1 hr. The sodium sulfite was added at 0.8 g for 1 g protein (Cole, 1967).

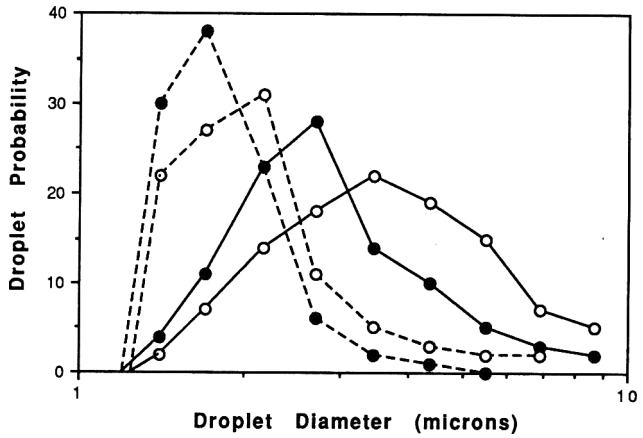

Fig. 1—Droplets size distribution of o/w emulsions of 12 wt% oil and 5 wt% soy protein. The volume weighed droplet probability was measured by volume (%). — crude 500E soy protein, --- preheated 500E soy protein at 85°C for 45 min, • fresh emulsion, o aged 2 mo. The emulsions were aged after (1:50) dilution which was done immediately after preparation.

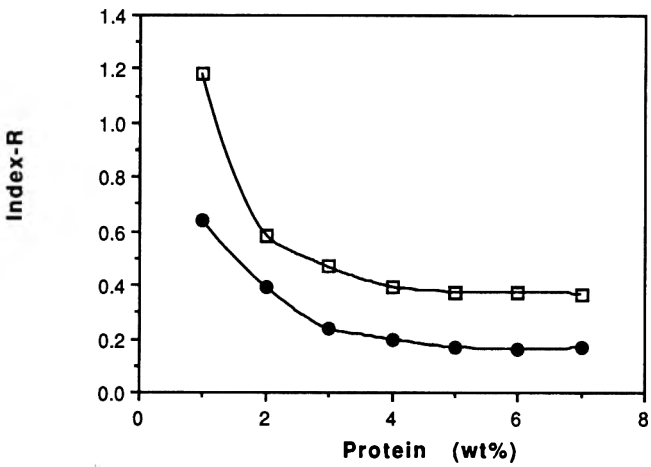

Fig. 2—Index-R changes with protein concentration in freshly prepared (12% wt oil, pH 6.7) and diluted (1:1000) emulsions. □ crude 500E soy protein, • preheated 500E soy protein at 85°C for 45 min.

Reduction of the SS bonds was also achieved by a mercaptoethanol technique with a slight modification to the procedure of Crestfield et al., (1963). Soy protein, (5 wt% in 8M urea solution) was dispersed in the presence of Na_2EDTA (50 mg for every 1 mL protein solution) and Tris buffer (pH 8.0, 0.03M). 2-Mercaptoethanol was added (1 μL/mg protein) to the protein solution (pH 8.0, room temperature ≈23°C) and was allowed to react for 8 hr while stirring.

The solubility of native and treated protein was measured by mixing 5% soy protein in distilled water at pH 6.7 and room temperature. The suspension was centrifuged at 30,000 × *g* for 20 min. The protein concentration was determined by the Lowry method (Lowry et al., 1951), and the relative solubility of the protein was determined by:

$$\text{Relative solubility} = \frac{\text{Conc of protein in supernatant (wt\%)}}{5\ \text{wt\%}} \times 100$$

Reproducibility of results

Each measurement was repeated three times. Results are averages of the three measurements. The differences in each measurement, in Index-R and viscosity, were all ≤10%, while differences in hydrophobicity measurements were ≤40%. The maximum fluctuation in surface tension, as measured three times, did not exceed 1 dyne/cm.

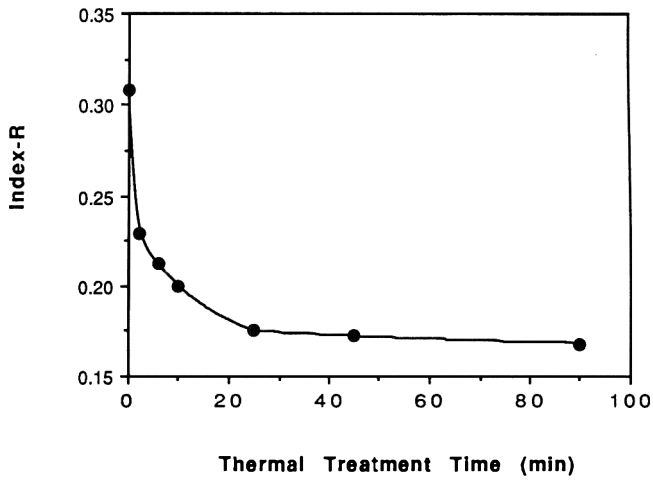

Fig. 3—Index-R of the o/w (12 wt% oil, pH 6.7) emulsions as affected by preheating time treatment of 5 wt% protein solutions. The heat treatment was done at 85°C.

Statistical analysis

The stability of the emulsions with time was checked by changes in average droplet sizes and in droplet size distribution, calculated by mean square deviation.

RESULTS & DISCUSSION

Heat pretreatment

The freshly made emulsion composed of heat pretreated proteins had a small droplet size (average 1.84 μm Fig. 1) and a narrow size distribution (mean square deviation of 0.05 μm) compared to emulsion stabilized by crude isolate Purina 500E (average size 3.07 μm and mean square deviation 1.45 μm). Emulsion stability was also significantly improved with the pretreated protein. At 5 wt% protein, both emulsions were stable for long periods of time as indicated by very small changes in droplet size. However, when diluted to 1:50, the preheated emulsion tended to coalesce, at lower rates than crude proteins (Fig. 1). This can be seen from the smaller changes in droplet sizes with respect to time in preheated emulsions. In the emulsion composed of heat pretreated protein, average size increased from 1.84 to 2.22 and standard deviation changed from 0.05 to 1.05 respectively, after 2 months. In the emulsion stabilized by crude isolate Purina 500E changes in droplet size were greater: the average size increased from 3.07 to 3.81 and the mean square deviation changed from 1.45 to 1.89. Moreover the droplet size was extremely important for stability of the emulsions with regard to creaming. According to Stock's Law, the creaming rate decreases as droplets are smaller. Consequently, pre-heating of the protein apparently caused a decrease in droplet size and better stability with respect to coalescence and creaming.

However, note that Purina 500E was also a partially pretreated protein (by manufacturer); therefore, the stability of the emulsion was affected less than expected for crude proteins. The 4–5 wt% of protein are essential for stabilizing the oil droplets (Fig. 2) and very little change was observed at higher protein concentration. The heat-treated protein solution (85°C) always gave emulsions with smaller droplets sizes than the untreated protein.

Most temperature effects at 85°C occurred during the first 2 min. Further preheating had only a limiting effect, (Fig. 3). Improvement of surface activity and emulsifying properties of the denatured protein was caused by changes in tertiary and quaternary structures of the protein. The heat provides the protein with sufficient thermal energy, which causes breakage of the hydrophobic interactions and disassociation of subunits which compose the protein molecule (Iwabuchi et al. 1991a,

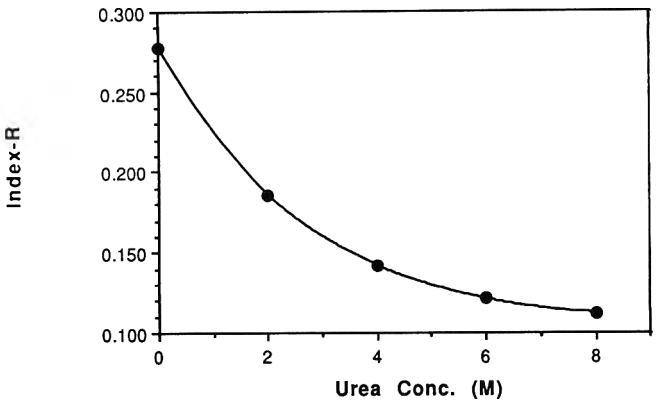

Fig. 4—Changes in index-R of the o/w emulsions (12 wt% oil, pH 6.7) immediately after preparation, as affected by urea concentration (added to aqueous phase).

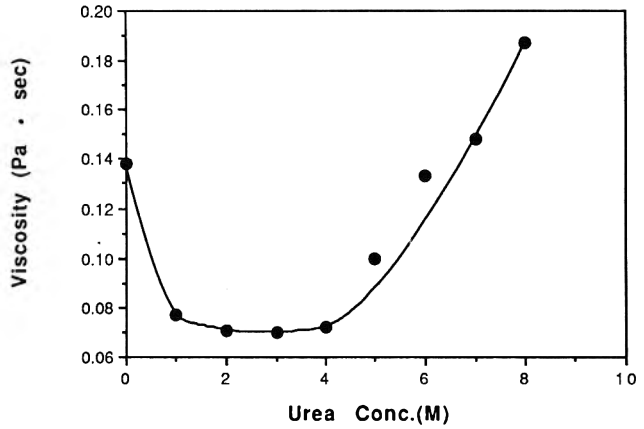

Fig. 5—Changes in viscosity (Pa·sec) of dispersions of 5 wt% 500E soy protein and 0.1wt% sodium azide in water (pH 6.7, 25°C) with different concentrations of urea.

Table 1—Surface tensions, relative surface hydrophobicity and solubility of native and modified soy protein. The surface tension was measured at a concentration of 0.5 wt% of protein, pH 6.7 and 25°C

Protein modification	Air/water surface tension (dyne/cm)	Relative hydrophobicity*	Solubility (wt%)
Untreated	45.0	100	45
4M urea	41.5	870	75
8M urea	38.7	1360	98
8M urea + Na_2SO_3	35.0	—	98
Heating (80°C for 90 min)	43.3	600	

* Determined by fluorescence method of Kato and Nakai (1980).

b; Nissen, 1976). Iwabuchi et al. (1991a) also showed that the separate subunits unfold. The disassociation and unfolding cause exposure of hydrophobic groups, previously enclosed within the contact area between subunits or in the interior of the folded molecule. The exposure of the hydrophobic sites of the protein causes an increase in surface activity and increases adsorption on the interface, with a strong protein-oil interaction. A fast and strong adsorption of protein on the interface enables the creation of smaller droplets during homogenization.

The exposure of hydrophobic sites to the protein's surface is indicated by the increase of surface hydrophobicity of the protein. Relative hydrophobicity increased from 100 for the native Purina 500E to 600 for the thermally treated one (Table 1). This effect was shown for different kinds of proteins by Kato and Nakai, (1980) and by Voutsinas et al. (1983).

The reversibility of denaturation is of great importance. If the changes as a result of heat treatment are reversible, after cooling the protein would return to its original structure, i.e. refolding and reassociation of subunits. As a result, the surface activity should decrease to its original level. Iwabuchi et al. (1991a) and Damodarn (1988) found that the denaturation of soy protein was only partially reversible. At low ionic strength the disassociated subunits stayed separate after cooling. However, concerning unfolding, denaturation was reversible, i.e. the protein refolded upon cooling.

Iwabuchi et al. (1991a) reported that the refolding of 7S soy protein was complete and that the subunits refolded to their original structure without reassociation. The unfolding of 11S soy protein is partially reversible, i.e. cooling causes the subunits to refold almost to their compact structure. Because of its irreversibility, the heat denatured protein after cooling, is different from the original in spatial structure.

Treatment with urea

In addition to heat-treatment, it is possible to denature protein by reagents such as urea, guanidine·HCl and different organic solvents, Nielsen (1985) and Tanford (1962). The action of the urea on the protein is complicated and unclear. The effects of urea on emulsification and surface properties of proteins have not been reported.

Changes of the R-index were studied (Fig. 4) as a function of the urea concentration (added to aqueous phase prior to homogenization). As with heat pre-treatment the droplets sizes in fresh emulsions were smaller (smaller R-index) than untreated protein and the effect seemed to be gradual (best at 8 M urea).

The decrease in droplet size is explained by a faster and stronger adsorption of treated protein onto the oil droplets. This could be attributed to: (1) Dissociation of the subunits composes the 7S and 11S soy protein fraction as shown by Wolf and Briggs (1958) and Kelley and Pressey (1966). Badley et al. (1975) showed that in the contact area of the associated subunits, hydrophobic areas exist. Thus, dissociation of the subunits would probably expose hydrophobic domains to the protein surface. (2) Unfolding of separate subunits of the soy protein molecule. Most of the hydrophobic domains are enclosed in the interior and the subunits contact regions (Kim and Kinsella, 1986; Iwabuchi et al., 1991a, b). Thus unfolding of the subunit would cause exposure of hydrophobic domains previously covered in the interior of the subunits. The urea presence caused an increase in the protein relative surface hydrophobicity (Table 1) from 100 for native protein to 800 for protein treated with 8M of urea. (3) Increase of protein solubility (Table 1). This increasing of solubility is a major factor of film formation on oil droplets. A rapid migration of the protein to the interface is critical prior to its adsorption. Any significant change in solubility would affect the migration of the protein. This effect was stressed by Chobert et al. (1988).

The urea effect may be better understood in view of the viscosity measurement of the protein solutions pretreated with urea (Fig. 5). Viscosity appears to be at a minimum at 4M urea, and increases from that point with additional urea concentration. The decrease in viscosity has been assumed to be caused by a reduction in protein molecular weight resulting from a subunit disassociation from the protein complex. At high urea concentrations (>4M) the process of unfolding becomes significant and dominant, leading to an increase in protein volume, manifested in a viscosity increase. The two effects of unfolding and disassociation are opposing forces and the unfolding becomes dominant only after exposure of the protein to high urea concentrations. The dissociation and unfolding of the protein subunit in the presence of urea was shown by Wolf and Briggs (1958), Kelley and Pressey (1966), and Catsimpoolas et al. (1969).

The influence of guanidine·HCl was followed (Fig. 6) on the viscosity of the protein dispersion. Results were similar to those observed in urea-treated solutions. Guanidine·HCl is a

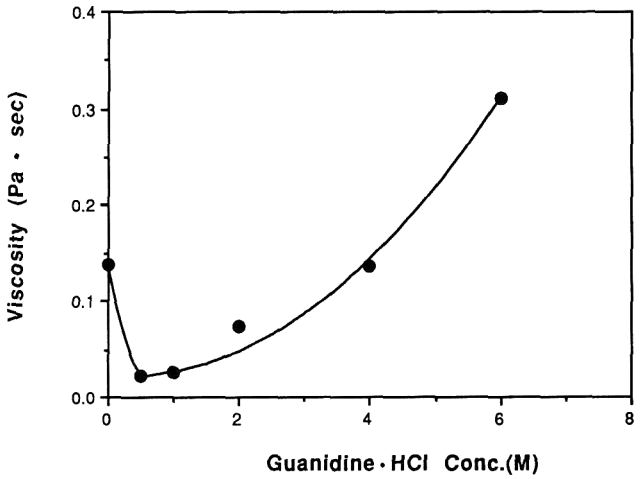

Fig. 6—Changes in viscosity (Pa·sec) of dispersions of 5 wt% 500E soy protein and 0.1wt% in water (pH 6.7, 25°C) with different concentrations of guanidine·HCl.

more active reagent than urea (Liener, 1958). The unfolding process is significant in concentrations >1M (vs 4M urea). At a concentration of 6M guanidine·HCl, the viscosity of the protein dispersion was about 0.32 Ps, while a dispersion of the protein with 8M of urea showed viscosity 0.19 Ps. This difference between guanidine·HCl and urea suggested that the unfolding of the protein molecule was stronger with guanidine·HCl (Liener, 1958).

Reduction of disulfide bonds

In every emulsion the reduction of SS bonds (by Na_2SO_3 or β-mercaptoethanol) caused a decrease in R-index (Table 2), i.e. a decrease in the emulsion droplet sizes. Shibasaki and Okubo (1966) showed that the urea separated the glycinin 11S molecules into subunit pairs. In each pair the two subunits are held together by a single SS bond. The cleavage of the disulfide bond causes each pair of subunits to separate into singles. As a result, hydrophobic subunits, which were previously enclosed in the contact area between every pair of subunits, are exposed on the surface (Kim and Kinsella, 1986b). In addition, Kato and Nakai (1980) have shown that the exposure of hydrophobic groups increased the protein ability to adsorb on the interface. Moreover, the cleavage of the disulfide bonds causes the glycinin residues, whose rigidity and compatibility are maintained by the SS bond (Kinsella, 1979; Kim and Kinsella, 1986b, 1987) to become more flexible and to unfold. Flexibility and an unfolded structure facilitate adsorption (Kim and Kinsella, 1986b, 1987). Improved adsorption will explain the improvement in droplet size and stability of emulsions we found.

Kim and Kinsella (1986b) found that the reduction of S-S intermolecular bonds by dithiothreitol (DTT) caused an increase of hydrophobicity of the soy glycinin molecule. In addition, Kim and Kinsella (1987) found that the reduction of SS bonds improved the protein rheological properties on the film at the interface. A strong and elastic film facilitates the emulsifying activity of the protein. The reduction of SS bond by sodium sulfite caused a decrease of surface tension (Table 1) of a solution of 0.5 wt% soy protein, 8M urea to 35 dyne/cm compared to 38.5 without reduction. This was in accordance with the decrease of droplet size (Table 2) and the increase of hydrophobicity of soy glycinin due to reduction of the disulfide bond (Kim and Kinsella, 1986b).

Surface tension and hydrophobic measurements of native and modified protein

The chemical or physical modifications of protein (Table 1) caused a decrease in surface tension and an increase in hydrophobicity of the protein (from 100 to 1360 for untreated protein and urea-treated protein respectively). Both surface properties and hydrophobicity of the protein influence the emulsifying activity of the protein. These results were in accordance with the decrease in droplet size in the emulsions resulting from premodification of the protein. Kato and Nakai (1980) showed a close connection between hydrophobicity-interfacial tension and emulsion activity index (EAI), in different native and heat-pretreated proteins. In many cases higher hydrophobicity resulted in a higher EAI and lower interfacial tension. Our findings are in accordance with those results. The modified protein showed higher hydrophobicity, higher EAI (equivalent to lower R-index) and lower interfacial tension of protein solutions in comparison to active soy protein.

Table 2—Index-R in 12 wt% oil in water emulsions and diluted (1:1000), prepared with 5 wt% soy protein, pretreated thermally, with urea and by reduction of SS bonds (pH 6.7)

Reagent (added to aqueous phase)	Aqueous phase of emulsion	Index-R without SS bonds reduction	Index-R after SS bonds reduction
β-mercaptoethanol	Protein + 4M urea	0.150	0.118
β-mercaptoethanol	Protein + 8M urea	0.110	0.084
β-mercaptoethanol	Protein + 8M urea + heat to 85°C	0.122	0.099
Na_2SO_3	Protein + 4M urea	0.143	0.118
Na_2SO_3	Protein + 8M urea	0.114	0.090
Na_2SO_3	Protein + 4M urea + heat to 85°C	0.122	0.096

CONCLUSION

BOTH SURFACE PROPERTIES and emulsification effectiveness of soy proteins can be improved significantly not only by heat treatment but also by cleavage of some of the SS bonds on the globular protein and by pretreating the protein with urea or guanidine hydrochloride. The urea pretreatment was more efficient than heat pretreatment with regard to interfacial activity, hydrophobicity, emulsification capacity and droplet size distribution. Similar results can be obtained with guanidine·HCl at lower reagent concentrations. The only important disadvantage is that their action is reversible and any attempt to remove the reagents (urea or guanadine) from the emulsification mixture would prompt negative effects on stability of the emulsions. Therefore, the reducing agents must be present during and after emulsification which is a strong disadvantage in food emulsions.

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Rapid Rehydration Methods for Dried Beans

M.O. OGWAL and D.R. DAVIS

ABSTRACT

Rapid methods of rehydrating dried kidney, pinto or navy beans by soaking at 82°C or 93°C for 5, 10 or 30 min were compared to standard 18 hr soaking at ambient temperature. Canned beans processed 21 min at 121°C had higher drained weights and softer texture with fewer split beans than those processed 41 min at 116°C. Kidney, pinto and navy beans soaked 30 min at 82°C had higher drained weight than those soaked 30 min at 93°C. Hydration coefficient (2.07) of controls (18-hr soak) and beans soaked 82°C (1.94) or 93°C for 30 min were not different. Pre soaking 30 min at 82°C provided adequate rehydration prior to canning.

Key Words: beans, rehydration, blanching, soaking, texture

INTRODUCTION

PROCESSORS usually soak dry beans 12 to 18 hr at ambient temperature prior to canning. This requires advanced production scheduling and large holding tanks. Scheduling would be improved if dry beans could be rapidly rehydrated for canning the same day. Pre-soaking is necessary for shortening the thermal processing time and to reduce intrinsic toxic components (Uebersax et al., 1991). Rapid rehydration methods would reduce labor costs and floor space requirements, and allow efficient production scheduling. Potential flat sour problems associated with long soaking periods would be eliminated by fast rehydration. Hydration to tenderize beans has been accelerated by vacuum, ultrasonics, additives in soak-water and gamma irradiation (Uebersax et al., 1991). Rockland and Metzler (1967) quickly cooked beans by vacuum-infiltration to loosen the seed coat and boiling less than 15 min in a solution containing either NaCl, $Na_5P_3O_{10}$ or $NaHCO_3$. This method has not been broadly used commercially, partly because of excessive sodium and the labor and equipment cost when using vacuum (Uebersax et al., 1991).

Rockland and Gardiner's (1969) study on infusion of beans with inorganic salts produced variable results: Soaking in sodium tripolyphosphate improved color, and sodium bicarbonate decreased soaking time. Both sodium carbonate and bicarbonate discolored and produced undesirable flavor in canned beans. However, 1% bicarbonate (pH 6.5 and pH 8.5) reduced shear press values of beans. In a similar study, Al-Nouri and Siddiqi (1982) blanched broad beans 7 min in boiling water followed by soaking in distilled or tap water containing 0.1% Na_3PO_4, 0.1% Na_2Co_3 and $NaHCO_3$ for 6 to 12 hr. Cooking time was reduced to 20 min. Sastry et al. (1985) showed that vacuum soaking of dry beans lowered the incidence of splitting in canned beans. Uebersax (1985) and Uebersax and Bedford (1980) reported that inclusion of $CaCl_2$ in soak solution decreased the percentage of split beans and drained weight, and increased firmness relative to untreated beans. The rate of soak water uptake (rehydration rate) decreased with increased calcium chloride.

Yoshida and Kajimoto (1986) demonstrated that 8 to 10 min exposure of 25% moisture presoaked soybeans to microwave inactivated antinutritional components, and provided a preconditioning treatment to reduce processing time. Earlier studies by Dawson et al. (1952) and Morris et al. (1950) indicated that extended soaking times may not be necessary. Beans soaked for 1 hr or 16–18 hr exhibited no differences in hydration ratios or shear press readings after processing. Davis (1976) showed that soaking 14 hr in tap water and 5 min steam blanching, resulted in larger drained weights and smaller percentage splits than 82°C 8 min. Water-blanched pinto, kidney and navy bean rehydration generally results in beans with a final moisture range 53 to 57% (Uebersax et al., 1991).

Our objective was to compare rapid rehydration methods to the standard overnight soaking method for three dry bean types.

Authors Ogwal and Davis are with the Dept. of Food Science, 272 Young Ave., Univ. of Arkansas, Fayetteville, AR 72702.

MATERIALS & METHODS

TEN KILOGRAMS each of dried pinto, kidney and navy beans were obtained from Allan Canning Food Industry at Siloam Springs, AR. The dry beans containing 12% moisture were stored at room temperature (22°C) until needed for processing.

Moisture determination

Dry moisture contents were determined with an OHAUS moisture balance. Beans (≈25g) were placed in a small osterizer blender receptacle and chopped 3 min. Three 10-g replicates of each bean type were weighed onto the pan of the 18 OHAUS moisture balance. The percentage moisture was read on a scale after the lamp height was set at 1.5, the heat at 2, and the timer at 30 min.

Bean soaking

Samples (200g) of whole pinto, kidney, and navy beans were soaked 18 hr at room temperature (22°C) in 1000 mL deionized water. They were blanched 5 min in 82°C water then cooled 5 min in running tap water. These beans served as controls. For other treatments, 200g of each type were weighed and soaked (blanched) in hot water at 82° or 93°C for 5, 10 or 30 min. The soaked beans were cooled 5 min in running tap water.

Thermal processing

For controls, three samples each of 220g of soaked pinto, 213g of kidney, or 217g of navy beans were filled into "C" enameled 303 × 406 cans. Also in triplicates, appropriate weights of beans soaked at 82°C or 93°C for 5, 10 or 30 min were weighed into the cans. One salt-EDTA tablet was added, and the cans were steam sealed. The canned beans were processed 121°C for 21 min or at 116°C for 41 min. After storing 8 wk at room temperature analyses were conducted.

Instrumental analyses

Rehydration weights (RHWT) were determined. Hydration coefficients (HC) were calculated as a formula (hydration coefficient = rehydrated weight/dry weight). The number of split beans were counted in randomly selected 50 seeds and multiplied by 2. The percentage change firmness or softness of beans relative to controls was determined from the compression force (kg) of the soaked or processed beans using the Texture Technologies TA.XT2 Instrument.

Experimental design

A factorial arrangement of 126 soaking treatments with three replicates of each was designed. It consisted of three levels of soaking temperatures 22°, 82° and 93°C, pinto, kidney and navy beans, and 5, 10, 30 min, and 18 hr soaking times. The experiments were ar-

Table 1—Rapid rehydration methods and the quality of canned dry kidney, pinto and navy beans[a]

Soak treatment °C time	HC*	% RMC	Process 116°C, 41 min[1] % Splits	g DW	kg/100 CPF	Process 121°C, 21 min[2] % Splits	g DW	kg/100 CPF
				Kidney beans				
22 18 hr	2.07[a]	55[d]	3.33[d]	305[d]	0.48[d]	1.67[d]	313[d]	0.38[d]
82 5 min	1.61[b]	33[b]	7.00[b]	328[b]	0.76[b]	0.67[b]	330[b]	0.66[b]
82 10 min	1.77[b]	47[b]	5.00[c]	338[c]	0.60[b]	0.67[b]	343[b]	0.50[b]
82 30 min	1.94[a]	51[d]	4.67[a]	365[a]	0.55[c]	1.33[c]	379[c]	0.53[c]
93 5 min	1.41[a]	48[d]	5.33[d]	323[d]	0.68[d]	1.00[d]	329[d]	0.58[d]
93 10 min	1.59[a]	48[d]	7.00[b]	333[d]	0.68[d]	1.00[d]	337[d]	0.58[d]
93 30 min	1.94[b]	55[b]	5.67[d]	347[b]	0.67[d]	1.00[d]	367[b]	0.54[d]
				Pinto beans				
22 18 hr	2.09[a]	57[d]	4.67[d]	350[d]	0.54[d]	0.67[d]	354[d]	0.44[d]
82 5 min	1.43[b]	37[b]	5.00[d]	330[b]	0.71[b]	0.67[d]	335[b]	0.61[b]
82 10 min	1.64[b]	44[c]	6.00[b]	338[c]	0.68[b]	1.00[b]	342[b]	0.58[b]
82 30 min	1.91[a]	53[d]	7.00[c]	371[a]	0.62[c]	1.67[c]	367[c]	0.57[c]
93 5 min	1.68[a]	47[d]	2.17[d]	332[d]	0.61[d]	0.33[d]	334[d]	0.56[d]
93 10 min	1.80[b]	48[d]	3.00[b]	336[d]	0.61[d]	1.33[d]	339[d]	0.56[d]
93 30 min	1.94[c]	57[b]	3.50[b]	360[b]	0.62[d]	1.33[d]	363[b]	0.55[d]
				Navy beans				
22 18 hr	2.07[a]	54[d]	2.67[d]	360[d]	0.53[d]	1.00[d]	364[d]	0.43[d]
82 5 min	1.64[b]	29[b]	6.33[b]	331[b]	0.74[b]	1.67[b]	339[b]	0.64[b]
82 10 min	1.74[c]	39[c]	5.33[b]	340[b]	0.63[c]	1.00[d]	346[b]	0.53[b]
82 30 min	1.85[d]	48[a]	4.67[c]	381[c]	0.57[d]	1.67[c]	390[c]	0.51[b]
93 5 min	1.70[a]	39[d]	3.33[d]	331[d]	0.67[d]	1.33[d]	334[d]	0.57[d]
93 10 min	1.70[a]	39[d]	3.67[d]	331[d]	0.67[d]	1.00[b]	336[d]	0.57[d]
93 30 min	1.94[b]	47[b]	6.00[b]	347[b]	0.63[d]	1.00[b]	370[b]	0.53[d]

[d] RMC = mean rehydration moisture content; DW = mean drained weight; CPF = mean compression force; HC = mean hydration coefficient; [1] = soaked beans processed 41 min at 116°C; [2] = soaked beans processed 21 min at 121°C; [3] = control beans soaked at 18 hr at 22°C.

[b-a] Where letters differ, means are significantly different (Pr > F = 0.05) from the specified treatment.

* = Mean hydration coefficient = (Mean rehydration bean wt/dry bean wt).

ranged in a randomized complete block design. Data were collected on rehydration weights, rehydration ratios, percentage splits, rehydration moisture contents, dry moisture contents, drained weights and compression forces. Data were analyzed on a linear regression model with a SAS Institute, Inc. program.

RESULTS & DISCUSSIONS

SOAKING any of the three bean types at 82°C for 30 min and processing at 121°C for 21 min resulted in beans with heavier drained weights (DW), softer textures (compression force—CPF) and fewer split beans than controls (soaked 18 hr at 22° and processed at 116°C for 41 min; Table 1). Beans soaked at 93°C for 30 min were also heavier, softer and had less splits than controls ($pr > F = 0.05$). A comparison between those soaked at 82°C and 93°C showed an increase in DW and softness at 82°C compared to 93°C. No significant differences occurred in number of split beans between the two temperatures (Table 1). No significant differences occurred in hydration coefficient (HC) between controls (2.07) and those beans soaked at 82°C or 93°C for 30 min (1.94). No significant differences occurred in rehydration moisture content (RMC) between controls and those from other treatments.

However, with 93°C soaking the RMC indicated that moisture content was near equilibrium. Of the total 97% variations resulting from multiple effects of the 3 variables, temperature alone accounted for 48% while time accounted for 49% of the variations. Type of bean was not significant.

According to Uebersax et al. (1991), this process for dry beans is designed to provide beans with a final moisture of 53 to 57%. Our results (Table 1) for kidney and pinto beans agreed with findings of Uebersax et al. (1991), but for navy beans moisture ranged 47 to 48% for the 8° and 93°C after 30 min soaking. Compared with controls, the higher temperature and shorter soaking times significantly increased the RMC. According to Swanson et al. (1985), the microscopic observation of bean seeds showed that there were three entrance routes for water: the micropyle, the hilum and the raphe. As reported by Hsu et al. (1983), variation in sizes of these "valves" is partly a function of temperature and ionic concentration. The water passes through these entrances due to the osmotic pressure from the solutes, soluble carbohydrates, protein and metallic ions such as Mg^{2+} and Ca^{2+} found within and beneath the seed coat. High temperatures tend to increase solubilities of these compounds within the bean. Increased solubility indicates higher osmotic pressure beneath the seed coats and therefore a faster rate of water imbibition. Likewise, the permeability of the seed coat is improved as the waxy cuticle is removed by high temperature, thus increasing the surface area for water imbibition. In our experiment the compression values were constantly high when drained weights were small. At 82°C for 30 min the largest (0.57 kg for pinto) and the smallest (0.51 kg for navy) compression values corresponded to the lightest (367g) and heaviest (390g) drained weights. Similar results were reported by Nordstrom and Sistrunk (1979). Soaking times of 5 and 10 min produced no significant differences in HC, RMC, DW, CPF and splits (Table 1) for beans soaked at 82 or 93°C. Increases in these properties were evident with soakings between 10 and 30 min. The data indicated that the HC for the dry beans increased with time of soaking, from 1.85 to 1.94 for beans soaked at 82° or 93°C for 30 min. This compares with 2.07 to 2.09 for the control treatment. Nordstrom and Sistrunk (1979) reported HC of 1.8 to 2.0 for dry beans soaked in cold water 2 to 14 hr. They also noted that to reach a value of 2.0 the beans were soaked for 18 hr. In our study, soaking the dry beans for 30 min at 82°C or 93°C produced a range of 1.85 to 1.97, not significantly different from the HC of controls. These values are considered normal at ($Pr > F = 0.05$). This theory is indirectly supported by the work of Swanson et al. (1985) on "paths of water uptake" that demonstrated that intact beans fully hydrated in about 1½ hr, while beans with seed coats removed required only 2 hr to become fully hydrated. Hosfield (1991) pointed out that an HC of 1.8 was considered optimum for soaked beans prior to canning. The

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Microwave Heating Affects Composition and Oxidative Stability of Sesame (*Sesamum indicum*) Oil

HIROMI YOSHIDA and GORO KAJIMOTO

ABSTRACT

The effects of microwave heating on antioxidative components and lipid quality of sesame seeds were studied. Amounts of antioxidative components were 576, 18, and 8 mg/kg oil for γ-, δ-, and α-tocopherols; 6,824, 5,642, and 54 mg/kg oil for sesamin, sesamolin, and sesamol, respectively. During microwave treatments, concentrations of tocopherols, sesamin and sesamolin gradually decreased, and ca. 20% of these dominant components was lost after 30 min heating. However, microwave treatments for 16 to 20 min, which would be optimal to prepare sesame oil with better quality, still retained over 85% of the antioxidative components (with few exceptions) and caused no significant chemical changes in lipids.

Key Words: fatty acids, microwave heating, tocopherols, sesame-oil, sesame seeds, antioxidative components

INTRODUCTION

SESAME (*Sesamum indicum L.*) is one of the world's most important oil seed crops. The oil has a mild, pleasant taste and is used as a salad oil requiring little or no winterizing. An important characteristic of sesame oil is its resistance to oxidative deterioration. Its remarkable stability may be due to endogenous antioxidants (sesaminol and sesamol) together with tocopherols. Sesame oil from roasted sesame seeds has a distinctive flavor and long shelf life (Manley et al., 1974; Kikugawa et al., 1983), and is used as a cooking oil, in shortenings and margarines.

The conventional method for preparation of sesame oil involves cleaning, roasting, grinding, cooking, and pressing but not refining (Fukuda and Namiki, 1988). Roasting is the key step because color, composition and quality of the oil are influenced by conditions of roasting. Yen and Shyu (1989) reported that the oxidative stability of sesame oil depended on roasting temperature. A higher roasting temperature used to provide a strong flavor, results in oil of lower quality (Yen et al., 1986). To make better quality sesame oil, optimum roasitng conditions should be established. Few studies concerning the influence of roasting on composition and quality of sesame oil have been reported. Microwave ovens are an energy-efficient means of heating, and a rapid method for reheating foods (Tsuyuki, 1982). Microwaves have great penetrating power, and food products heated by them have little temperature gradient. Foods containing high fat and moisture readily absorb microwaves and are cooked or baked. The application of microwave processing for both home and institutional meal preparation has increased because of its speed and convenience (Nelson et al., 1985). Yoshida and Kajimoto, 1988) reported that microwave heating was effective for inactivation of the trypsin inhibitor in whole soybeans, and also for making full-fat soyflour with high vitamin E from raw beans (Yoshida and Kajimoto, 1989). However, little has been reported about how microwave heating affects antioxidative components and quality of the oil.

Authors are affiliated with the Food and Nutritional Science Laboratory, Dept. of Nutrition, Kobe-Gakuin Univ., Arise, Ikawadanicho, Nishi-ku, Kobe 651-21, Japan. Direct inquiries to Dr. H. Yoshida.

Our objectives were to develop a procedure for rapid and simple roasting of sesame seeds using a home microwave oven, and to evaluate composition and quality characteristics of such oils.

MATERIALS & METHODS

Sesame seeds

Commercial sesame seeds [*Sesamum indicum L.* var. "white strain"] harvested at Tokushima Prefecture, Japan, 1992 were selected for uniformity based on seed weight (i.e., 2.8 to 3.2 mg). The seeds were cleaned and divided into groups for storage in stainless steel containers at 4°C until further experimentation.

Antioxidative components

Vitamin E homologues (α, β, γ, and δ) were purchased from Eisai Co. (Tokyo, Japan). All tocopherols were of the d-form (*RRR*-), and the purity of each tocopherol was >98.5% by high-performance liquid chromatography (HPLC). Sesamol (reagent grade) was purchased from Sigma (St. Louis, MO). Sesamin and sesamolin were purified from sesame oil according to the method of Soliman et al. (1985). The purity of each compound was ≈ 98.0% as determined by HPLC.

Microwave heating

A modified domestic size Sharp microwave oven (Model R-5, 550), capable of generating 0.5 kW power at 2,450 MHz, was used. Whole sesame seeds were placed as a single layer in a Pyrex petri dish (8.0 cm diam) and then heated in the microwave oven for 2.0, 4.0, 6.0, 8.0, 12.0, 16.0, 20.0, 25.0, or 30.0 min after covering the dish. Internal temperatures of the oven during microwave treatments were recorded automatically along with heating time. These were 60, 120, 160, 170, 190, 210, 220, 230, and 250°C, respectively. Each petri dish contained ca. 5.83g (2,000 seeds) sesame, and 5 dishes were treated once at each of the different exposures to provide sample material for analysis and testing. After heating, the seeds were allowed to cool to ambient temperature before lipid extraction.

Moisture content of samples exposed to microwave heating were expressed as apparent moisture and this was calculated by dividing the weight of moisture loss after each exposure-time by the weight of the sample before heating.

Lipid extraction

The sesame seeds (1,000 seeds) treated with microwaves were ground with 50 mL chloroform-methanol (1:1 v/v) at 0°C under ice in a Waring Blendor, and lipids were extracted three times with 150 mL chloroform-methanol (once at 1:1 v/v and two more times at 2:1 v/v) in the Blendor. These solvents contained 100 mg of butylated hydroxytoluene/L, which was added to inhibit oxidative degradation of lipids and antioxidative components during extraction. The combined extracts were dried in a rotary vacuum evaporator at 35°C, and residues were dissolved in chloroform-methanol (2:1 v/v). The solution was washed with water as described (Folch et al., 1957), and dried over anhydrous Na_2SO_4. Solvents were removed from filtrates with a rotary evaporatory under reduced pressure at <35°C to obtain total lipids. The extracted lipids were redissolved in chloroform-methanol (2:1 v/v) and stored in a 25 mL brown glass volumetric flask under nitrogen in the dark at −25°C until required for further analysis. Lipids were also extracted from raw seeds by the procedure described.

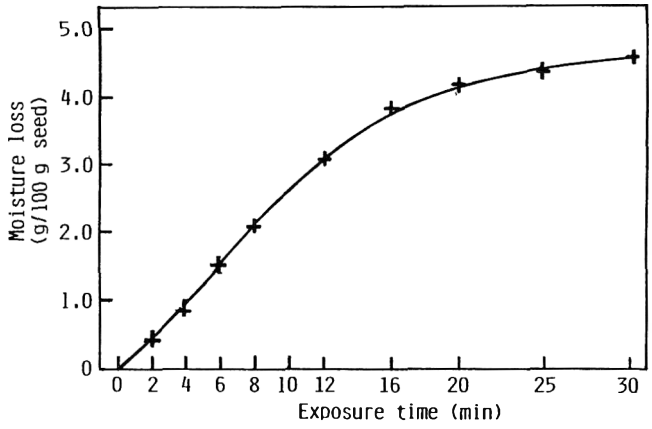

Fig. 1.—Relationship between moisture loss of sesame seeds and exposure-time during microwave heating (at frequency 2,450 MHz). Each data point is the average of 3 replicates; standard errors were within the size of symbols.

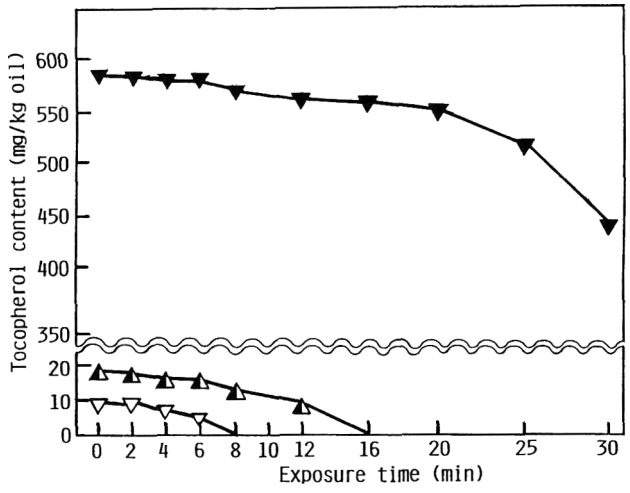

Fig. 2.—Changes in individual tocopherol contents of sesame seeds during microwave heating (at frequency of 2,450 MHz). ▽, α-tocopherol; ▼, γ-tocopherol; ▲, δ-tocopherol. Data points are means of 3 measurements from 3 replicates; standard errors were within size of symbols.

Tocopherols

A 0.2-mL portion of sample, before and after microwave heating, was placed in a 5-mL brown volumetric flask and diluted with mobile phase (for HPLC as described below) after evaporation of the mixture solvents under nitrogen gas. The chromatographic system consisted of a normal-bonded phase Shim-pack CLC-SIL (M) column (5 μm, 250 cm × 4.6 mm i.d.; Shimadzu) protected by a 1-cm guard column (Shim-pack G-SIL). The mobile phase was a mixture of n-hexane: 1,4-dioxane:ethanol (490:10:1 v/v/v) at 2.0 mL/min. Sample (4 μL) was injected with a fully loaded 20-μL loop. The tocopherols were monitored with a fluorescence spectrophotometer (Shimadzu RF-535, Shimadzu Instruments Inc., Kyoto, Japan) set an excitation wavelength 295 nm and emission wavelength 320 nm, quantitated by comparison with peak areas of internal standard (2,2,5,7,8-pentamethyl-6-hydroxy chroman, Eisai Co. Tokyo, Japan). Other HPLC conditions were as reported (Yoshida and Kajimoto, 1989).

Sesamol, sesamin and sesamolin

Sesamol, sesamin and sesamolin in lipids extracted from sesame seeds were determined by a Shimadzu LC-6A HPLC equipped with Shim-pack CLC-ODS (M) column (5 μm, 250 cm × 4.6 mm i.d.; Shimadzu) protected by a 1-cm guard column (Shim-pack G-ODS), a Shimadzu SPD-6A UV-detector and a Shimadzu Chromatopac C-R6B recording data processor, according to the method of Kikugawa et al. (1983). The mobile phase was a mixture of methanol-deionized water (75:25 v/v) at 0.8 mL/min. A 0.1-mL portion of sample, before and after microwave heating, was placed in a 5-mL brown volumetric flask and diluted with mobile phase for HPLC. Sample (4-8 μL) was injected using the method described. The amount of each compound was calculated from peak areas of standards observed at 300 nm.

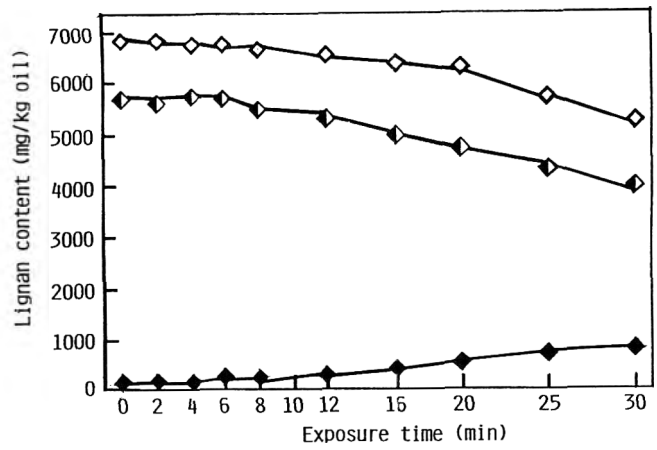

Fig. 3.—Changes in individual lignin contents of sesame seeds during microwave heating (at frequency of 2,450 MHz). ♦, sesamol; ◈, sesamolin; ◇, sesamin. Data points represent means of 3 measurements from 3 replicates; standard errors were within size of symbols.

Lipids

Quantitative changes in free fatty acids and peroxides were determined by the AOCS (1990) methods. Standard methods (JOCS, 1981) were used for determination of carbonyl value. IUPAC (1987) methods was adopted for determination of the p-anisidine value 2-Thiobarbituric acid (TBA) test of Tarladgis et al. (1960) was modified as previously described (Yoshida et al., 1990), and expressed as TBA value: mg malonaldehyde equivalent/kg oil. The fatty acid methyl esters were prepared from the extracted lipids by boron trifluoride-catalyzed transesterification (Morrison and Smith, 1964). A Shimadzu Model 14A-gas chromatograph (GC) equipped with a flame ionization detector and glass column (2m × 3 mm i.d.) packed with 10% DEGSS-X supported on acid-washed Gaschrom Q (100-120 mesh) and connected with an integrator (Shimadzu C-R4A) was used. Other GC conditions were as reported (Yoshida and Kajimoto, 1981).

Lipid class

Total lipid extractions were fractionated on a silicic acid column by a modification of a previous method (Rouser et al., 1967). The silicic acid (Unisil 100-200 mesh, Clarkson Co., In., Williamport, PA) was activated at 120°C overnight and again for 1 hr immediately before the column was prepared. All column chromatographic conditions were as described (Yoshida and Kajimoto, 1977). After the column was washed with 300 mL chloroform, a measured quantity (250 mg) of total lipids was added with 5 mL chloroform. By successive elution with 200 mL chloroform (fraction 1), 700 mL acetone (fraction 2) and further 200 mL methanol (fraction 3), total lipids were separated into three fractions. Total concentrations of separated lipid fractions were determined gravimetrically. Further, triglycerides were isolated from fraction 1 by thin-layer chromatography (TLC) as described (Yoshida and Kajimoto, 1981). Fatty acid composition of each fraction (1-3 and triglycerides) was determined.

Statistical analysis

Statistical evaluation of the results was carried out by the Student's t-test or by one-way analysis of variance (Snedecor and Cochram, 1967).

RESULTS & DISCUSSION

PROXIMATE ANALYSES (in duplicate) using AOAC (1980) procedures showed composition of the seeds before microwave

Table 1—Characteristics of oils from sesame seeds during microwave heating at frequency of 2,450 MHz[a]

Exposure time (min)	Acid	Peroxide	Carbonyl	Anisidine	TBA
0	1.00[b]	1.02[b]	1.29[b]	0.52[b]	0.65[b]
4	1.03[b]	1.15[b]	1.40[b]	0.78[b]	0.72[b]
6	1.04[b]	1.16[b]	1.78[b]	1.02[b]	0.83[b]
8	1.07[b]	2.39[c]	1.89[b]	1.35[b,c]	0.97[b,c]
12	1.11[b,c]	2.25[c]	1.98[b]	2.03[c]	1.03[b,c]
16	1.12[b,c]	3.23[c,d]	2.04[b,c]	2.08[c]	1.26[c,d]
20	1.13[b,c]	3.86[d]	2.16[b,c]	2.23[c,d]	1.63[d]
25	1.21[d]	4.60[e]	2.35[c]	3.61[d,e]	1.75[d]
30	1.42[e]	8.51[f]	2.66[c]	3.96[e]	2.53[e]

[a] Each value is an average of 3 determinations.
[b-f] Values in the same column with different superscript letters are significantly different from those at min 0 ($P < 0.05$).

Table 2—Changes in lipid contents of sesame seeds during microwave heating at frequency of 2,450 MHz[a]

Exposure time (min)	Total lipids	Nonpolar lipids	Glyco-lipids	Phospho-lipids
0	1396.8[b]	1345.1[b]	4.1[b]	47.6[b]
4	1407.4[b]	1355.1[b]	6.0[b,c]	46.3[b]
6	1418.5[b]	1367.7[b,c]	9.8[c,d]	41.0[b,c]
8	1439.3[b,c]	1395.9[b,c]	12.1[d]	31.3[c,d]
12	1433.6[b,c]	1394.9[b,c]	12.8[d]	26.6[d,e]
16	1445.5[b,c]	1406.9[b,c]	14.2[d,e]	24.4[e,f]
20	1462.4[b,c]	1424.6[c,d]	15.0[e,f]	22.8[f,g]
25	1500.5[c,d]	1462.6[c,d]	18.9[f]	19.0[g]
30	1520.2[d]	1476.8[d]	36.7[g]	6.7[h]

[a] Each value is an average of 3 determinations and expressed as mg lipid per 1,000 seeds.
[b-h] Values in the same column with different superscript letters are significantly different from those at min 0 ($P < 0.05$).

heating was: moisture-6.7%, protein-19.5%, fat-54.8%. The rates of moisture loss in sesame seeds exposed to microwave heating (frequency 2,450 MHz) for different times were compared (Fig. 1). Energy absorption during microwave treatment is attributable to two principal processes-elevation of seed temperature and vaporization of water. Although some vaporized water condensed and was reabsorbed by seeds as they cooled, the net moisture loss was substantial. The loss may reflect total volatile substances, but it was considered to be mostly moisture. A linear relationship occurred between moisture loss and exposure-time in the first 16 min of heating: 1.5% after 6 min, 3.2% after 12 min, and 3.8% after 16 min. At 30 min heating, the loss was 4.6% of the original weight, and a burnt odor was apparent. Therefore, under such conditions, optimum microwave treatment time to prepare roasted sesame seeds or oil was probably around 16-20 min. Oomah and Mazza (1992) reported that the microwave oven drying method could be used as a quick method for moisture determination in oilseeds such as canola, flax and mustard. However, they did not study effects of microwave heating on oxidative deterioration of oils.

To clarify the effects of microwave heating on antioxidative components, tocopherol homologues in sesame seeds were analyzed. Before heating (Fig. 2), γ-tocopherol was predominant (576 mg/kg oil), and δ- and α-tocopherols were minor components (8 and 18 mg/kg oil, respectively), but no β-tocopherol was detectable. The results were similar to those reported from several vegetable oils (Speek et al., 1985). Thus, sesame oil does not contain larger amounts of tocopherols than do other vegetable oils (Kanematsu et al., 1983), despite the known strong resistance of sesame oil to oxidation (Table 1). As microwave heating progressed, tocopherol concentrations gradually decreased at a similar rate in the seeds (Fig. 2). The loss for γ-tocopherol was 15 mg (3%) at 12 min of heating and 38 mg (6.5%) at 20 min. The rate of tocopherol loss became more pronounced ($P<0.05$) with microwave exposure up until 30 min. However, > 80% of the original level of γ-tocopherol remained after 30 min heating. On the other hand, δ-tocopherol was not detected at 16 min heating, nor α-tocopherol after 8 min.

Effects of microwave treatment on lignan contents in seeds were determined, as well as their changing patterns (Fig. 3).The dominant components were sesamin (6,824 mg/kg oil) and sesamolin (5,642 mg/kg oil), with much smaller amounts of sesamol (54 mg/kg oil) in the seeds before microwave heating. A distinct difference in amounts was observed between major and minor components during microwave treatments. Sesamin and sesamolin gradually decreased by 5.0% within 12 min and 15.0% in 25 min. However, as much as 80% of both components originally present in sesame seeds remained at 30 min. Conversely, sesamol (as most minor components) gradually increased by a range from twofold at 16 min to tenfold in 30 min. The results were not necessarily in agreement with observations reported by other investigators (Fukuda et al., 1986; Yen, 1990). Fukuda et al. reported that the degradation rate of sesamolin was greater than that of sesamin because sesamolin was decomposed to sesamol during thermal processing. These investigations were carried out under conductive heating conditions (frying: 180°C for 1–2 hr; high isothermal temperature: 180-260°C for 30 min). When sesame seeds were exposed to microwaves, the seeds were heated under non-conductive heating as the result of molecular excitation (Stein, 1972). After 30 min heating, therefore, the loss of γ-tocopherol and sesamolin in sesame seeds may be considerably less than when heated by other methods. The conversion of sesamolin to sesamol was not much greater (Fig. 3) than that under actual cooking conditions such as frying or roasting.

Little is known about how tocopherols or lignans in sesame seeds affect oxidative stability of oils after heating in a microwave oven. Chemical indices of sesame seed oils before and after microwave treatments were compared (Table 1). No apparent differences ($P>0.05$) occurred in most properties such as peroxide value, carbonyl value and anisidine value, of sesame oils until 8 min heating. Thereafter, the peroxide value gradually increased with longer heating, and became 4.60 and 8.51 meq/kg oil, at 25 and 30 min of heating, respectively. In general, the peroxide values do not represent the absolute state of oxidation of an oil because hydroperoxides are unstable on heating. This results in rapid transformation to secondary products (Gray, 1978). However, the indicators for secondary oxidation products were very low during microwave heating (Table 1). After 30 min heating, the carbonyl value was 2.66, anisidine 3.96 and TBA 2.53. These chemical indices were consideraly lower than those reported for soybeans (Yoshida and Kajimoto, 1989). Probably, the differences reflect seed size and initial contents of antioxidative components. Unexpectedly, the acid value was already high in the oils before microwave heating. However, there were no significantly different ($P>0.05$) values until 20 min heating. Although all acidic components were titrated by the acid value method, we assumed that only fatty acids were measured. Fukuda (1990) reported that roasted sesame oils contained more free fatty acids and fat-soluble substances, but were comparatively higher in oxidative stability, than other purified vegetable oils. Results indicated that as much as 80% of γ-tocopherol and sesamin or sesamolin still remained after 30 min heating (Fig. 2 and 3), and these antioxidative components are effective via synergistic action. Fukuda et al. (1986) also investigated the possibility of synergistic actions between γ-tocopherol and sesamol using a model system in linoleic acid, and reported that addition of 0.005% sesamol enhanced antioxidative action of γ-tocopherol at all concentrations. It was especially strong at 0.04%. Contents of γ-tocopherol and sesamol were 0.06% and 0.005% before heating, and 0.04% and 0.5% after 30 min heating (Fig. 2 and 3). Therefore, we reasonably assumed that the antioxidative stability of the oil was mainly due to the synergistic action.

Amounts of lipid components changed (Table 2) after microwave treatments. The nonpolar lipids were eluted in fraction 1, the glycolipids containing browning substances in

Table 3—Changes in fatty acid composition of individual lipids in sesame seeds during microwave heating at frequency of 2,450 MHz[a]

Lipid class	Exposure time (min)	Fatty acid (wt%)							
		14:0	16:0	16:1	18:0	18:1	18:2	18:3	20:0
Total lipids	0	Tr	8.4[b]	0.2[b]	4.5[b]	36.7[c]	49.4[d]	0.5[b]	0.3[d]
	12	Tr	8.8[b]	0.2[b]	4.3[b]	37.6[c]	48.3[c,d]	0.5[b]	0.3[d]
	25	Tr	9.2[b,c]	0.2[b]	4.1[b]	37.7[c]	47.9[c]	0.5[b]	0.2[e]
	30	Tr	9.3[c]	0.2[b]	4.2[b]	37.9[c]	47.8[c]	0.3[b]	0.3[d]
Triglycerides	0	Tr	8.7[b]	0.3[b]	4.6[b]	41.5[d]	43.3[b]	0.7[c]	0.8[b]
	12	Tr	8.6[b]	0.3[b]	4.5[b]	41.9[d]	43.4[b]	0.7[c]	0.6[b]
	25	Tr	9.1[b,c]	0.4[b]	4.8[b]	42.5[d]	42.1[b,c]	0.6[c]	0.5[c]
	30	Tr	9.5[c]	0.5[c]	5.0[c]	42.6[d]	41.4[c]	0.5[b]	0.5[c]
Phospholipids	0	1.3[b]	14.5[d]	0.9[d]	4.5[b]	29.8[b]	47.5[c]	0.8[c]	0.7[b]
	12	1.2[b]	14.4[d]	0.6[c,d]	4.2[b]	32.9[b,c]	45.6[b,c]	0.6[c]	0.5[c]
	25	1.3[b]	15.0[e]	0.4[b]	4.1[b]	33.5[b,c]	44.8[b]	0.4[c]	0.5[c]
	30	2.1[c]	17.5[f]	0.5[c]	4.6[b]	30.4[b]	43.8[b]	0.5[b]	0.6[b]

[a] Each value is an average of 3 determinations. Tr, trace amount (less than 0.1 wt%).
[b-f] Values in the same column with different superscript letters are significantly different from those at min 0 ($P < 0.05$).

fraction 2, and phospholipids in fraction 3. A longer microwave process resulted in greater amounts of total lipids extracted from the seeds. Collins and Beaty (1980) indicated that heat caused some protein denaturation which could have improved lipid extractability. Probably, their lipid extraction was performed after heat treatments rather than before heat treatments. Nonpolar lipids, the major component (96.3–97.5%), showed a significant increase after 20 min heating. Glycolipid components increased slowly in the first 25 min of heating and rapidly thereafter, and attained > ninefold increases after 30 min heating. On the other hand, phospholipid components gradually decreased and at 30 min were less than 14% of the amount before microwave heating. When soybeans were heated in a microwave oven, Hafez et al. observed an increase of browning substances (1985) and also a decrease of phospholipids (1989). The browning substances are generally very polar due to active radicals (Kawakishi et al., 1983). The increase in browning substances may be attributed to the increase of glycolipids. We expected such increases because lipid extraction was carried out after heat treatments.

Profiles of the fatty acid compositions of major lipid classes in the sesame oils before and after microwave heating were compared (Table 3). The oils had a low ratio of saturated-to-unsaturated fatty acids and contained high levels of unsaturated fatty acids (ca. 85%). A small but significant difference ($P<0.05$) occurred in fatty acid compositions between total lipids and triglycerides and between total lipids and phospholipids of sesame oil before microwave heating. However, fatty acid compositions in the first 8 min heating were omitted from Table 3 because they were essentially the same as before heating. Triglycerides comprised above 98.0% of nonpolar lipids in the sesame seeds (Table 2) and no differences occurred in fatty acid compositions between nonpolar lipids (data not shown) and triglycerides. Therefore, phospholipids were minor components of the seeds (Table 2) and their fatty acid compositions were not reflected by total lipids or triglycerides. Significant differences ($P<0.05$) occurred in fatty acid compositions of phospholipids after 12 min heating.

Results suggested that the oxidative stability of the oil in the sesame seeds exposed to microwave heating was comparatively higher than that of soybeans previously described (Yoshida and Kajimoto, 1989). That was despite the longer heating period and smaller sample size. Contrary to reports of Fukuda et al. (1986) and Yen (1990), no significant decreases ($P>0.05$) occurred in antioxidative components in sesame seeds, or in the quality characteristics of the oil when the seeds were heated 16–20 min in an oven. Thus, the oxidative stability of the oil was probably mainly due to the synergistic action of γ-tocopherol and lignans. The optimum microwave treatment was around 20 min for preparation of sesame oil with better quality under these experimental conditions. Microwave ovens can be used as a quick method for preparing sesame oil with good quality when compared with other heating means.

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Fracture Intensity Distributions during Compression of Puffed Corn Meal Extrudates: Method for Quantifying Fracturability

A.H. BARRETT, S. ROSENBERG, and E.W. ROSS

ABSTRACT

The distribution of fracture intensities occurring during compression of puffed corn meal extrudates was described using an exponential function, and parameters from that analysis were used as fracturability indices. Since "jagged" or oscillating stress-strain functions are typical for porous and brittle materials, fracture intensities were determined by measuring the abrupt, sequential reductions in stress produced during compression. Both distribution exponent and cumulative fracture stress correlated strongly with fracturability measured by other techniques, including fractal and Fourier analysis of stress-strain functions. Distribution parameters also indicated textural differences due to process parameters (structural modification through addition of different levels of sucrose) and storage conditions (equilibration at various relative humidities).

Key Words: corn meal, extrudates, fracturability, compression

INTRODUCTION

FRACTURABILITY is an important textural property of many widely consumed foods. "Crunchy" products, which are often porous and brittle, constitute a large category of breakfast cereals and snack foods. Many of these products are formed by high-temperature, short-time extrusion, which effectively puffs the material into a cellular and open structure. Texture in these products arises from an incremental and progressive fracturing of cell wall components in response to deformation. During mastication such fracturing gives rise to specific sensory perceptions such as crunchiness.

Fracturability is a complex mechanism often requiring sophisticated instrumentation and mathematical techniques to evaluate. Previously, texture in extrudates has been evaluated by force-deformation analysis and determination of elastic properties such as modulus (Smith, 1992; Halek et al., 1989; Launey and Lisch, 1985) or single-parameter failure properties such as breaking stress (Lai et al., 1989; Faubion and Hoseney, 1982). More recently, fracturability has been described by analysis of extended strain compression functions, in which repeated fracturing results in oscillating stress levels—giving rise to "jagged" stress-strain relationships. This property was described in terms of Fourier and fractal analysis by Barrett et al. (1992) and Rhode et al. (1993), and used in Symmetrized Dot Patterns (SDP) plots by Peleg and Normand (1992). In those studies Fourier analysis provided power spectra that indicated frequencies of fracturing, and fractal analysis provided a numerical measurement of the overall roughness of the stress-strain function; SDP plots were used to visually determine differences in the failure patterns of foods. Fracturability parameters from fractal and Fourier analysis, respectively fractal dimension and average intensity within specific frequency ranges of the power spectrum, correlated with extrudate structure and sensory texture (Barrett et al., 1994). One of the additives these authors employed to vary cell structure was sucrose, which has been reported to alter extrudate expansion and/or mechanical properties (Hsieh et al., 1990; Ryu et al., 1993; Sopade and Le Grys, 1991).

Authors Barrett and Rosenberg are with the Advanced Foods Branch, Sustainability Directorate, U.S. Army Natick RD&E Center, Natick, MA 01760-5018, Author Ross is with the Dept. of Mathematical Sciences, Worcester Polytechnic Institute, Worcester, MA.

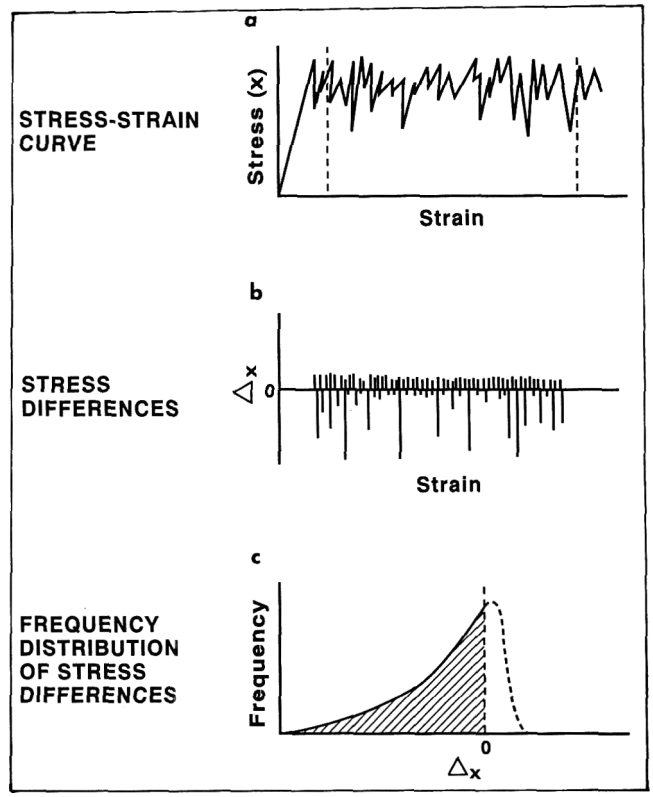

Fig. 1.—Procedural sequence for determining fracture intensity (stress difference) distributions. X refers to stress.

Our objective was to describe quantiatively the distribution of fracture intensities that occurs during compression of extrudates and to use parameters from those distributions as fracturability indices. Such a methodology employs mathematical procedures that are readily available in basic spreadsheet programs. Extrudate structures and mechanical properties were varied through addition of different levels of sucrose; textural changes in the samples were also produced by storage at elevated humidity Parameters from fracture intensity distributions were tested for correlation with fracturability parameters from fractal and Fourier analysis.

MATERIALS & METHODS

SIX BATCHES OF CORN MEAL (Lincoln Grain Co.) extrudates with sucrose contents of 0, 2, 4, 6, 8, and 10% were produced on a Werner and Pfleiderer ZSK-30 twin screw extruder. For each batch, a feed rate of 27 kg/hr, a moisture content of 15%, a six-zone temperature profile of 38-38-116-116-138-138°C and a 4 mm die were used. Extrudates were freeze dried after extrusion. Samples for high-humidity tests were equilibrated over saturated solutions of KCO_3 (43% RH) and NaCl (75% RH) for 48 hr.

Compression

Extrudates were sliced into 12 mm thick discs and compressed to 50% strain on a Texture Technologies TX2 texture press interfaced with a Zenith 286 computer. Before compression, three caliper measurements were taken of the diameter of each specimen and averaged. A compression rate of 0.2 mm/s and force-distance data acquisition

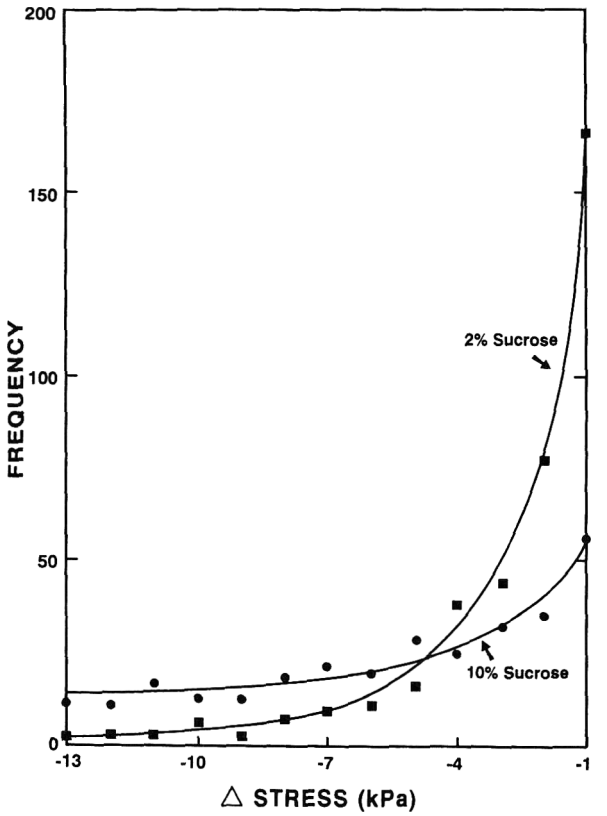

Fig. 2.—Fracture intensity distributions (6 replicates combined) for samples containing 2% sucrose and 10% sucrose.

Table 1—Fracture intensity distribution parameters and extrudate expansion

Sucrose content	Exponential distribution parameters and fit			Extrudate expansion ratio
	b (Exponent)	C (Coefficient)	r^2	
0	0.352	10.5	0.87	30.8
2	0.490	15.5	0.90	28.9
4	0.307	10.8	0.95	28.9
6	0.363	11.6	0.86	26.8
8	0.209	7.0	0.86	24.5
10	0.093	3.0	0.78	16.8

rate of 12.5 pts/sec were used. The middle 2/3 of each compression curve (8–42% strain region) was saved for analysis. Samples were compressed at room temperature (25°C).

Cumulative fracture stress measurement

Force-distance data files were imported into a Minitab™ statistical program, converted to stress-strain values and analyzed in the procedural sequence shown in Fig. 1. Incremental changes in stress were determined by differences in the data according to the formula:

$$\Delta_{xi} = x_i - x_{i-1}$$

In the difference data file negative Δ_{xi} values indicate reductions in stress, or fractures, whereas positive values are incremental increases in stress due to compression (Fig. 1b). A frequency distribution of fracture intensities (negative Δ_{xi} values) was constructed from the difference data, (Fig. 1c) and numerically summed to determine the cumulative fracture stress. Six replicates of each sample were compressed and the summed fracture intensities averaged.

Fracture intensity distribution analysis

The six frequency distribution replicates for each sample were combined and then truncated by removing the largest 5% of the stress reductions (since the distributions were long-tailed). This distribution was divided by 6 to yield an "average" distribution per compression and fitted to the exponential form

$$y = \text{frequency} = Ce^{(b\Delta_x)}$$

where C and b are parameters of the distribution. The exponent, b, indicates the relative preponderance of low intensity vs high intensity fractures; the coefficient, C, indicates the number of fractures below a minimum observable non-zero stress reduction.

The distribution tail (high intensity fracture range) was investigated by the extreme value procedure proposed by Gumbel (1958). This method tested whether the largest fracture intensities were consistent with the main body of the exponential distribution or arose from an entirely different distribution (suggesting a different mechanism for low and high fracture intensities). The following relationships were used: if U is an exponential random variable with density function be^{bU}, and $L_U(n)$ is the largest value of U in a sample of size n from that variable, then

$$\overline{L_u(n)} = \text{mean of } L_u(n) = \frac{1}{b}\sum_{k=1}^{n}\frac{1}{k}$$

$$\sigma^2_{L_u(n)} = \text{variance of } L_u(n) = \frac{1}{b^2}\sum_{k=1}^{n}\frac{1}{k^2}$$

if $n>>1$, then

$$\overline{L_u(n)} \approx (\ln(n) + 0.577)$$

$$\sigma^2_{L_u(n)} \approx \pi^2/(6b^2)$$

if the most extreme value satisfies the inequalities

$$\overline{L_u(n)} - 2\sigma_{L_u(n)} \leq L_u(n) \leq \overline{L_u(n)} + 2\sigma_{L_u(n)}$$

then it can be concluded (with ≈95% confidence) that the extreme value does not conflict with the exponential model, i.e., the mechanism governing very large fractures is not inherently different from that governing moderate ones. The three largest stress reductions in the combined, untruncated distributions were also averaged to assess the relative contribution of high intensity fractures to cumulative fracturability.

Fractal and Fourier analysis

The fractal dimensions of the stress-strain functions were calculated using the Blanket Algorithm method described by Barrett et al. (1992), Normand and Peleg (1988), Peleg et al. (1984) and software written by Mark Normand (University of Massachusetts). Fractal dimension was determined for each of the six compression curve replicates and averaged.

Power spectra of the stress-strain functions were obtained using a Fast Fourier Transform algorithm from Systat. Parameters were obtained from the power spectra according to the procedure described by Barrett et al. (1994) and Rhode et al. (1993); spectra (obtained from the sum of the squares of the sine and cosine terms of the FFT) were averaged within specific frequency ranges and these values were averaged across replicates. The two frequency ranges used were 0.44–0.78 and 0.83–1.07 sec^{-1}, respectively, corresponding to the 2nd and 3rd sixteenth sections of the power spectrum. Means were averaged across the six replicates.

RESULTS

REPRESENTATIVE FRACTURE INTENSITY distributions for the 2% and 10% sucrose samples are shown (Fig. 2), illustrating how the number of fractures increased as fracture intensity decreased. However, the distributions were long-tailed, and showed the existence of a small number of fractures of very high magnitude. A substantial difference exists between the two plots since the distribution for the 10% sucrose samples was flatter and extended much further towards high magnitude fractures (plot does not show extremes of this distribution). By contrast, the bulk of the 2% sucrose distribution was in the low fracture magnitude range. While sucrose level influenced this difference in fracturability, the mechanism was possibly largely structural since high sucrose levels reduced expansion

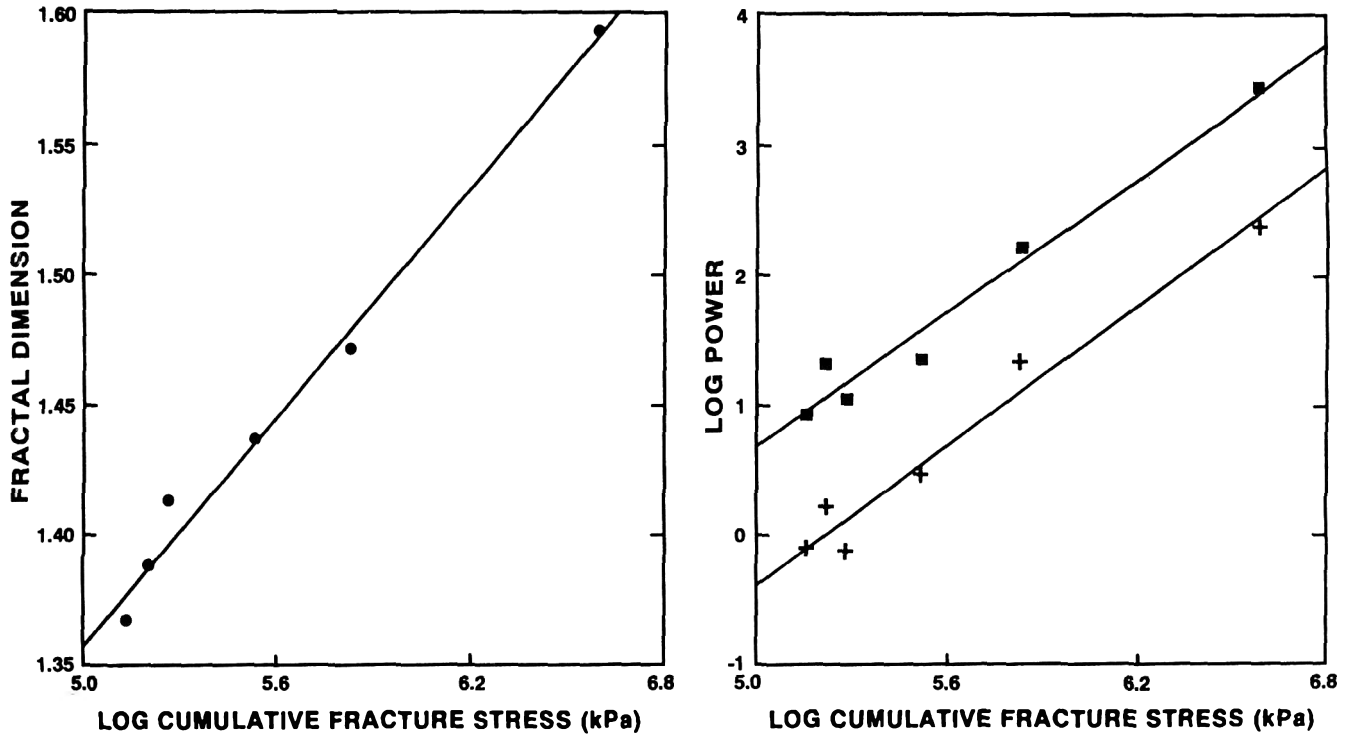

Fig. 3.—Relationship between log cumulative fracture stress and (a) fractal dimension, (b) log average power. ■ 1st frequency range; + 2nd frequency range.

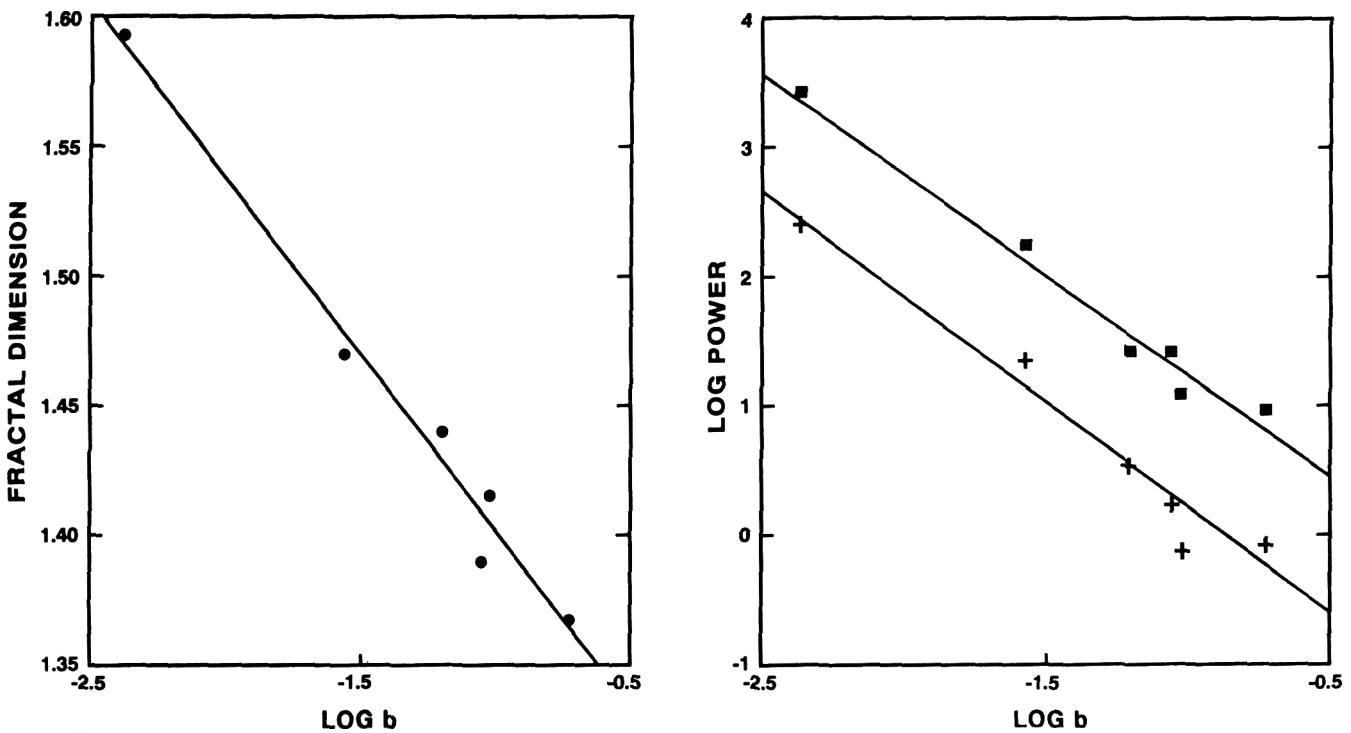

Fig. 4.—Relationship between log b and (a) fractal dimension, (b) log average power. ■ 1st frequency range; + 2nd frequency range.

(defined by extrudate cross sectional area/die cross sectional area, Table 1). Furthermore, cell sizes in the 6 sample lots appeared visually different.

The log of cumulative fracture stress is plotted against fractal dimension (which has theoretical limits of 1 and 2 for images) (Fig. 3a) and log average power in the two frequency ranges (Fig. 3b). Cumulative fracture stress correlated closely with either fractal dimension or average power, and correlation coefficients for those relationships were respectively 0.98, 0.95 (first frequency range) and 0.96 (2nd frequency range).

Parameters from the fitted exponential distributions were compared (Table 1). Regression coefficients were in all cases between 0.86 and 0.95 except for the 10% sucrose sample, which had more scatter in the distribution ($r^2=0.78$). The model exponent, b, is plotted against fractal dimension (semi-log plot) and average power (log-log plot, Fig. 4). Regression

Table 2—Extreme values of fracture intensity distributions and conformance to exponential model

Sucrose content (%)	n	Largest fracture intensity, kPa (in 6 replicates)	Average of 3 largest fracture intensities, kPa (in 6 replicates)	$\overline{L_u(n)}+2\sigma_{Lu(n)}$
0	353	24.5*	18.8	25.5
2	368	27.5	22.8	18.4
4	329	27.9*	25.3	29.2
6	377	21.2*	17.8	24.9
8	413	45.6	35.5	43.8
10	430	62.3*	62.1	99.2

* Within extreme value limit.

Table 3—Effect of relative humidity on cumulative fracturability

Sucrose content (%)	Cumulative fracturability (kPa) Freeze dried samples	Equilibrated samples 43% RH	75% RH
0	184	32	9.6
2	173	34	22
4	252	64	32
6	194	58	19
8	342	68	26
10	723	168	16

coefficients for these relationships were respectively 0.98, 0.97 (first frequency range) and 0.95 (2nd frequency range).

Analysis of the largest stress reductions by extreme value theory showed that these fracture intensities in general conformed to the exponential model. Parameters of this analysis (Table 2) showed the largest fracture intensity, in the six replicates combined, was well within the extreme value limit of $L_u(n) + 2\sigma_{Lu(n)}$ in four cases (0,4,6, and 10% sucrose) and very close in a fifth (8% sucrose). For the remaining sample, the three largest fracture intensities were higher than the predicted value. However, those fractures occurred in only two of the sample replicates, and the remaining four were well within the extreme value limit for this distribution. The averages of the three largest fractures were also compared (Table 2). These values correlated generally with cumulative fracturability and were approximately equal to 10% of the cumulative fracturability.

Loss in fracturability due to moisture sorption was reflected in the cumulative fracturability measurements (Table 3). Reductions in cumulative fracture stress between 71 and 83% for samples equilibrated at 43% RH (yielding wet-basis moisture contents between 8.8 and 10.2%) and between 87 and 98% for those equilibrated at 75% RH (yielding wet-basis moisture contents between 10.9 and 11.8%). Distributions of fracture intensities were not fitted for these samples due to extreme losses in fracturability produced by equilibration. Furthermore, reductions in stress during compression of equilibrated samples were primarily of very low magnitude (> 80% smaller than 2 kPa for 43% RH samples; > 95% smaller than 2 kPa for 75% RH samples).

DISCUSSION

FRACTURABILITY IN BRITTLE, puffed extrudates results from a multitude of individual fractures of varying magnitude. Fracture intensities form a distribution with a relatively greater number of low intensity fractures and proportionally fewer high intensity fractures. This distribution could be reasonably well described by an exponential function. A small number of very large fractures cause these distributions to be considerably long tailed. However, the highest intensity fractures can be shown to be consistent with the exponential distribution by extreme value theory. That the distributions showed a range of fracture intensities was somewhat expected, given that the extrudate structures were nonuniform and also had wide distributions of cell sizes. Extrudate cell size distribution was described by right-skewed functions such as the log Normal and the Rosin-Rammler by Barrett and Peleg (1992); both functions accommodated the preponderance of smaller-sized cells observed from image analysis of the samples.

Fracturability parameters from fracture intensity distributions were related to those obtained through previously published fracturability-assessment techniques. Distribution exponents and cumulative fracturability correlated strongly with either the fractal dimension or power spectrum averages derived from stress-strain functions. Such a correlation was reasonable, given that each technique is based upon analysis of the oscillating behavior or "jagged" quality of the original function. An analogous correlation between fractal dimension and power spectrum averages was reported by Rhode et al. (1993). Furthermore, both fracturability parameters were related to structural characteristics and sensory texture by Barrett et al. (1994).

Quantification of fracturability by distribution analysis provides an alternative method to describe the texture of porous and brittle foods that can, furthermore, be applied using conventional statistical software. Parameters from this distribution can be used to quantify fracturability and to differentiate samples with different processing or storage histories. While use of an exponential function to describe fracture intensity distributions may be practically limited to reasonably brittle structures, cumulative fracture stress can be used to evaluate a wide range of samples that includes both brittle and nearly-plastic products.

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Kinetics of Acid Hydrolysis of Defatted Peanut Flour

RUDEEPAN WATTANAPAT, TOMMY NAKAYAMA, LARRY R. BEUCHAT, and R. DIXON PHILLIPS

ABSTRACT

The kinetics of hydrolysis of defatted peanut flour as affected by treatment at 100, 110 and 120°C in 3N and 5N HCl were determined. Rates of hydrolysis of protein and destruction of reducing sugars were rapid at 120°C and in 5N HCl. About 90% of total amino acids were released after 4 hr. The overall rate of reaction followed second-order kinetics as monitored by the generation of free amino acids over time. This relationship was reliable for all acid and temperature treatment conditions when the extent of hydrolysis was less than 80%. The rate of hydrolysis of peanut protein was about 2.2 times faster for every 10°C increase. Hydrolysis in 3N HCl was about 2.3 times faster than in 3N HCl at the same temperature. The activation energy was ≈24 kcal mol^{-1}. The rate of liberation of individual amino acids with respect to temperature and HCl concentration, however, varied.

Key Words: kinetics, acid hydrolysis, peanut flour

INTRODUCTION

PEANUT is grown primarily for oil extraction, yielding peanut press cake as a by-product. Defatted press cake may be processed to peanut flour which contains 44-63% protein, 0.7-10% fat (Milner, 1962), minerals, B vitamins (thiamin and niacin), and carbohydrates. Because of its relatively high protein content, peanut flour is a potential ingredient for formulating protein-fortified food products. A problem sometimes associated with utilization of peanut press cake or flour is its contamination with aflatoxins. Contaminated products are unfit for use as food or feed and are diverted to fertilizer or discarded. Williams and Dutton (1988) proposed that contaminated peanut meal could be used for production of hydrolyzed vegetable protein (HVP) because acid hydrolysis would totally destroy aflatoxins without leaving mutagenic residues.

Hydrolyzed vegetable protein, widely utilized as a condiment or flavor enhancer in soups, sauces, processed meat, and snack products, can be processed by enzymatic or acid hydrolysis. Acid hydrolysis is preferable in industrial production because it costs less and complete hydrolysis can be achieved in a much shorter time (Prendergast, 1974). Acid-hydrolyzed products also have good flavor because of high free amino acid and sodium chloride contents. Yield and quality of HVP vary according to composition of raw materials and hydrolysis conditions. During preparation of HVP, proteins are dissociated to peptides and free amino acids, while carbohydrates are converted to sugars which are further degraded or react with amino acids to form compounds that contribute to flavor development. The flavor of hydrolyzed protein is also enhanced by peptides and free amino acids (Hall, 1946; Pham and del Rosario, 1983).

Factors affecting the rate and extent of acid hydrolysis of protein substrates have been reviewed (Hill, 1965; Roach and Gehrke, 1970; Davies and Thomas, 1973; Blackburn, 1978; Gehrke et al., 1985; Hunt, 1985; White and Hart, 1992). Emphasis has been directed mainly toward improving analytical techniques, since acid hydrolysis is used to prepare samples for amino acid analysis. Factors studied have included concentration and purity of acid, hydrolysis time and temperature, and the presence of non-protein substances.

Studies to investigate the optimum conditions for production of protein hydrolysates for food use were conducted by Pham and del Rosario (1983) on coconut and soybean meal, and Jaswal (1989, 1990) on shrimp and crab processing waste. However, information on conditions influencing hydrolysis of peanut flour is limited. A study of the kinetics of acid hydrolysis of peanut flour would provide useful information for establishing production conditions for HVP. Our objective was to determine the effects of temperature and acid concentration on the kinetics of hydrolysis of defatted peanut flour and to define the kinetic parameters for this process.

The authors are affiliated with the Center for Food Safety & Quality Enhancement, Dept. of Food Science & Technology, Univ. of Georgia, Griffin, GA 30223-1797.

MATERIALS & METHODS

Preparation of defatted peanut flour

Peanuts (cv. Florunner, medium, 1991 crop) were purchased from McCleskey Mills, Smithville, GA and stored at 7°C and 60% relative humidity until used. Peanuts were adjusted to room temperature (≈23°C) and blanched for 5 min in a rotary electric roaster (model 37, Preedit Electric Roaster Co., Eric, PA) at initial (175°C) and final (150°C) temperatures. Cooled peanuts were then passed through a roller type blancher (model EX, Ashton Food Machinery Co., Inc., Newark, NJ) to remove testae. Defective kernels were visually detected and discarded.

Sound kernels (500g) were partially defatted by pressing three times using a Carver hydraulic laboratory press (model M, Fred S. Carver Inc., Menomonee Falls, WI). The first and second presses were done at 4,500 kg for 5 min and the third press was done at 4,500 kg for 3 min. Peanut press cake was ground through a 2-mm screen in a Thomas-Wiley Laboratory Mill (model 4, Arthur H. Thomas, Co., Philadelphia, PA).

Ground press cake (300g) was extracted with 9000 mL analytical grade hexane in 2-L beakers using a triple-stage batch extraction with a contact time of 15 min for each stage. Extraction was counter current. Hexane from the first stage was discarded. Hexane from the second and third stages of previous batches were used for the first and second stages of the following batch, respectively. Fresh hexane was used in the third stage. During each extraction, the press cake-hexane mixture was agitated at 75 rpm on a rotary shaker and occasionally stirred with a glass rod to facilitate oil extraction. After preliminary desolventizing under a fume hood, the defatted press cake was spread on a wire screen lined with muslin cloth, covered with muslin cloth, and desolventized with saturated steam at atmospheric pressure for 20 min. The meal was dried in a forced-air oven (Blue M, Blue M Electric Company, Blue Island, IL) at 60°C for 4 hr to a final moisture ≈7% and then ground through a 1-mm screen in a Thomas-Wiley Laboratory Mill. The peanut flour was hermetically sealed in a plastic bag and stored at 1°C until used.

Proximate composition of defatted peanut flour

The protein content of defatted peanut flour was determined using the macro Kjeldahl method and a conversion factor of 5.46 (AOAC, 1984). Moisture and ash contents were also determined using the AOAC (1984) procedure. The oil content of moisture-free samples was determined using a Goldfisch extractor (model 3500, Laboratory Construction Co., Kansas City, MO). Carbohydrate content was calculated by difference.

Acid hydrolysis of defatted peanut flour

This was done at 100, 110, and 120°C using 3N and 5N HCl at each temperature. Hydrolysis times varied from 0 to 36 hr unless oth-

Table 1—Amino acid composition of products studied (% of total by wt)

Amino acid	Defatted peanut flour	Peanut meal[a]
Aspartic acid	11.79	8.83–15.43
Threonine[b]	2.53	2.01–2.73
Serine	4.75	4.74–6.04
Glutamic acid	19.99	17.20–22.46
Proline	4.31	4.28–6.36
Glycine	6.82	5.33–7.83
Alanine	4.17	3.41–4.16
Half cystine[b]	ND	1.97–3.31
Valine[b]	3.88	2.39–3.99
Methionine[b]	0.58	0.71–1.21
Isoleucine[b]	3.48	1.64–3.33
Leucine[b]	6.62	5.60–6.79
Tyrosine	4.35	3.36–3.96
Phenylalanine	5.34	4.56–5.54
Lysine[b]	3.83	2.88–4.45
Histidine	2.57	1.94–3.17
Ammonia	2.24	1.26–2.60
Arginine	12.76	10.31–17.17

[a] From Young and Waller (1973).
[b] Essential amino acid.

erwise indicated. Two replicates of each treatment were done. Duplicate samples were analyzed at each hydrolysis time.

Flour was removed from storage and adjusted to room temperature. Five milliliters of 3N or 5N HCl were mixed with 1.25g of peanut flour in a screw cap test tube (16 × 125 mm). Tubes were tightly closed with teflon-lined caps and placed in an oil bath (Thermomix 1480, B. Braun, Germany) in which hydrolysis temperatures were controlled (±0.1°C). Once the hydrolysis temperature was attained, one tube of sample from each treatment was removed after time intervals ranging from 0 to 36 hr, and immediately cooled in an ice bath to stop hydrolysis. The hydrolysate was diluted to 100 mL with deionized water and vacuum filtered through Whatman No. 1 paper. The filtrate was stored at −18°C until analyzed. Standard deviations of mean values reported for reducing sugars, ninhydrin-positive substances and amino acids were in the range of ± 2-6%.

Reducing sugars

The amount of reducing sugars in filtrates was determined by the dinitrophenol method described by Ross (1975), with some modifications. Filtrate (0.15 ml) was pipetted into a 16 × 125 mm test tube and diluted with 0.85 mL deionized water. Deionized water (2 mL) and 3.0 mL of dinitrophenol reagent were added and the contents were mixed thoroughly. The tube was covered with a glass marble and placed in a boiling water bath for 6 min. The tube was then cooled in an ice bath and the absorbance of the reaction mixture was read at 520 nm. A solution of 0.06% glucose was used to prepare a standard curve.

Ninhydrin assay

Ninhydrin-positive substances expressed as L-leucine equivalents in the hydrolysate were determined by ninhydrin assay (Friedman et al. 1984) except that 0.250 mL of filtrate was used instead of a solid sample. A solution of 1.5×10^{-3}M L-leucine was used to prepare a standard curve.

Amino acid analysis

Peanut flour (0.02 g) was weighed into a screw-cap test tube; 10 mL of 6N HCl containing 0.5% phenol was added. The tube was deaerated by subjecting to three cycles of alternate evacuation and argon purging, sealed tightly under argon, and placed in an oil bath 110°C for 24 hr. The tube was then cooled to room temperature and contents adjusted to pH 2.1 with 2N NaOH, diluted with 0.27N sodium diluent (pH 2.2) (Pickering Laboratories, CA), and filtered through a 0.2-μm teflon filter (type FG, Millipore Corp., Bedford, MA). Fifty microliters of filtrate were analyzed for amino acid content using a high performance liquid chromatograph (HPLC, Waters Associates, Milford, MA).

The hydrolysate was deproteinized by diluting 4 to 5 mL of filtrate to 50 mL with 3% sulfosalicyclic acid, mixing thoroughly, and centrifuging at 12,000 × *g* for 10 min (4°C). The supernatant fluid was adjusted to pH 2.1 with 0.27N NaOH and diluted with 0.27N sodium diluent (pH 2.2). The sample was filtered through a Teflon membrane (0.2 μm) and 50 μL were injected into the HPLC.

A Waters system HPLC equipped with a Waters System Interface Module, Model 510 pumps, post column reaction pump, TCM temperature control unit, and Model 440 absorbance detector (Waters Associates) was used for amino acid analysis. Operation control, data collection, and chromatogram integration were done using Baseline 810 software (Millipore Corporation, CA).

Amino acids were eluted at 0.3 mL min^{-1} with a linear gradient of 0.2N (pH 3.28) and 1.0 N (pH 7.40) sodium buffer (Pickering Laboratories, CA) on a cation-exchange column (Pickering Laboratories, Mountain View, CA). The column was maintained at 50°C. Eluted amino acids were subjected to post column reaction with ninhydrin (Trione, Pickering Laboratories, Mountain View, CA) at 120°C (0.2 mL min^{-1}). Absorbance was monitored at 436 and 546 nm. A protein hydrolysate standard containing 1.25 μM mL^{-1} L-cystine and 2.5 μM mL^{-1} of each of 16 other amino acids (except tryptophan) in 0.1N HCl (Amino Acid Standard H, Pierce, Rockford, IL) was diluted with 0.27N sodium diluent (pH 2.2) to obtain 0.05 μM mL^{-1} of each amino acid (0.025 μM ml^{-1} L-cystine), and used to quantify the amino acid content in test samples.

Determination of kinetic parameters

Rate constant. We assumed that acid is a catalyst and water is present in excess so that its change in concentration with time would be negligible. Thus, the rate constant depends on the concentration of unhydrolyzed protein and partially hydrolyzed protein in the hydrolysate mixture. The concentration of unhydrolyzed protein (mol L^{-1}) can be represented by the difference between the concentration of total amino acids when hydrolysis is complete (total amino acids in 1.25 g peanut flour/total volume of hydrolysate) and total amino acids released in the hydrolysate mixture at time t. The rate of reaction is expressed as

$$-dA/dt = k(A)^n \quad (1)$$

where n = order of reaction.

Integration of the differential gives

$$\ln A = -kt + \text{constant, for first order} \quad (2)$$

and

$$1/A = kt + \text{constant, for second order.} \quad (3)$$

The rate constant was taken from the slope of appropriate reaction curve that was obtained by linear regression analysis.

Activation energy. The effect of temperature on reaction rate was determined according to Arrhenius equation:

$$k = Ae^{-E_a/RT} \quad (4)$$

where A = pre-exponential or frequency factor, E_a = activation energy (cal/mol), R = gas constant (cal/K mol), and T = absolute temperature.

Transformation to the logarithmic form gives

$$\log k = -E_a/2.303RT + \log A. \quad (5)$$

Activation energy was calculated from the slope of the Arrhenius plot that was obtained by linear regression analysis.

RESULTS & DISCUSSION

THE AMINO ACID COMPOSITION of defatted peanut flour (51.2% protein, 3.4% fat, 5.0% ash and 40.4% carbohydrate, dry weight basis) containing 7.4% moisture (Table 1) was similar to that reported by Young and Waller (1973). The defatted flour contained 30.6% essential amino acids (not including tryptophan) and 67.2% nonessential amino acids. Lysine, isoleucine, valine, threonine, and the sulfur amino acids were limiting in nutritional value. This limitation should be considered if used for nutritional purposes. However, the limitation is not crucial for production of HVP, because HVP is mostly used for a different purpose and only small quantitites are added to foods. On the other hand, the high content of non-essential amino acids, especially dicarboxylic amino acids (31.8%), in peanut flour is beneficial for HVP production, since they contribute a better flavor than do some essential amino acids (Hall, 1946).

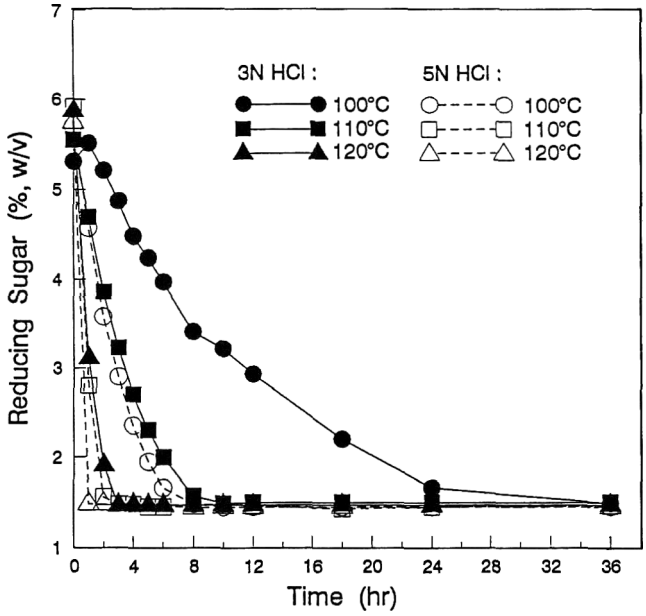

Fig. 1—Effect of hydrolysis temperature and HCl concentration on reducing sugar content in defatted peanut flour hydrolysate.

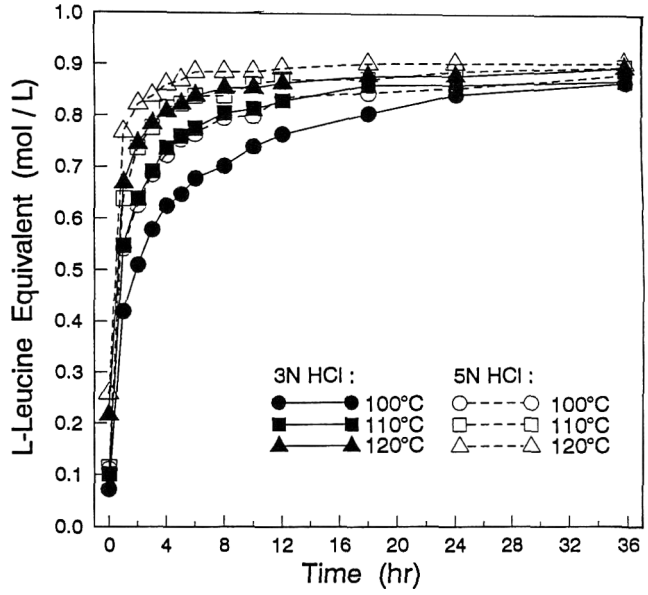

Fig. 2—Effect of temperature and HCl concentration on yield of ninhydrin-positive substances in defatted peanut flour hydrolysate.

Reducing sugars

Reducing sugars in acid hydrolysates result from cleavage of glycosidic linkages of oligosaccharides or polysaccharides and serve as precursors of many flavor components in HVP. The reducing sugar content decreased (Fig. 1) as the hydrolysis time increased. The rate of change in reducing sugars greatly increased with increased temperature and acid concentration. At 120°C, the use of 5N HCl depleted reducing sugars so rapidly that only negligible amounts remained after 1 hr. The glycosidic linkages of carbohydrates are readily cleaved in acid environments, especially at high temperatures (Whistler and Daniel, 1985). Decreases in reducing sugar content during the course of hydrolysis implied that the sugars may have undergone other reactions. Under the strong acid conditions of the reaction mixture, dehydration of monosaccharides or reaction of sugars with proteins, amino acids, or their degradation products undoubtedly occurred. Dehydration of pentoses yields 2-furaldehyde as the main product while hexoses yield 5-hydroxymethyl-2-furaldehyde (HMF). These primary dehydration products can be degraded to formic and levulinic acids, specific flavor components of HVP, from acid hydrolyis. Reaction with proteins, amino acids, or degradation products through nonenzymatic browning reactions yields compounds which impart the dark color and flavor to the product.

Ninhydrin-positive substances

Ninhydrin-positive substances (NPS) are compounds that form a complex with ninhydrin, yielding chromaphores known as Ruhemann's purple (Rosenthal, 1985). These compounds included primary and secondary amines, amino alcohols, ammonia, and ammonium salts as well as amino acids. The ninhydrin assay was used in a preliminary study to monitor the approximate extent of hydrolysis of peanut protein for facilitating selection of samples for the kinetic study. As expected, the NPS content in the hydrolysate increased with time of hydrolysis. The rate increased with an increase in temperature and acid concentration (Fig. 2). Hydrolysis was rapid during the first few hours of treatment. The highest yield of NPS was obtained after about 12 hr at 120°C using 5N HCl, but 16 hr or more was required for less harsh conditions.

Amino acid profile

Based on the ninhydrin assay for free amino groups, samples after hydrolysis for 0, 1, 2, 4, 8, and 12 hr were selected for determination of amino acid profiles. This range encompassed the greatest changes in hydrolysis rate. The yield of free amino acids was calculated based on amino acid content using the formula:

$$\% \text{ Yield} = \frac{\text{Amino acids released in hydrolysate (mmol)}}{\text{Amino acids in defatted peanut flour (mmol)}} \times 100$$

Yields of total free amino acids during 12 hr of hydrolysis (Fig. 3) showed curves similar to those for NPS. About 90% of the total amino acids were released after 4 hr at 120°C when 5N HCl was used, but 12 hr or more were required under less severe conditions. Some yield curves for total free amino acids were similar under different conditions. Thus several combinations of acid concentrations and temperatures could result in similar yields for a given hydrolysis time. Extended treatment after complete hydrolysis caused destruction of amino acids (Pham and del Rosario, 1983; Jaswal, 1989, 1990) and thus decreased the yield. The formation of decomposition products such as ammonia also imparts an undesirable flavor to the final product (Pham and del Rosario, 1983).

The rate of liberation of amino acids with respect to temperature, acid concentration, and time varied from one amino acid to another, probably resulting from differences in peptide bond cleavage, stability of individual amino acids, and presence of non-protein substances. Extensive destruction of asparagine, glutamine, tryptophan, and cystine occurred, as expected. Asparagine and glutamine were probably converted to aspartic and glutamic acid, respectively (Hill, 1965; Gehrke et al., 1985), with concomitant release of ammonia during early stages of hydrolysis.

Tryptophan is generally destroyed during acid hydrolysis. Large losses have been reported with the formation of humin (Olcott and Fraenkel-Conrat, 1947). Low yields of cystine and cysteine have often been encountered, especially when carbohydrates were present (Hil, 1965). Aspartic acid and glycine were rapidly liberated and quite stable during hydrolysis. Isoleucine and valine were liberated at a much slower rate than other amino acids, especially at low temperatures and acid concentrations. Peptide bonds involving isoleucine and valine residues may be difficult to acid hydrolyze due to steric hindrance

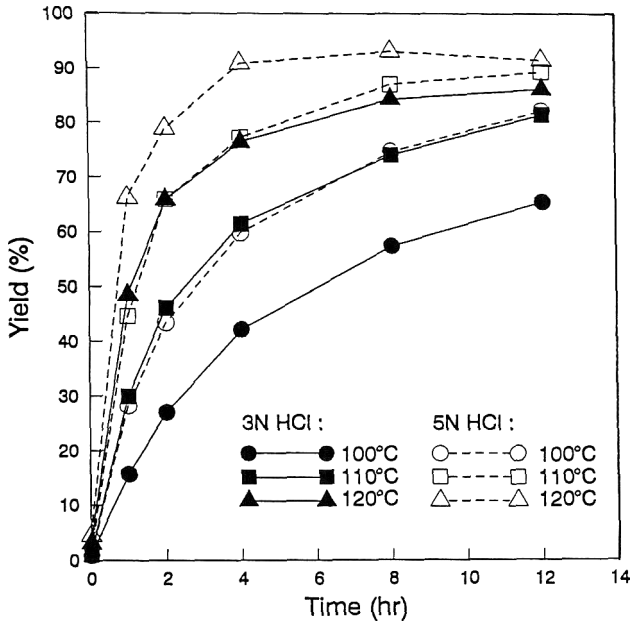

Fig. 3—Yield of total amino acids during 12 hr of hydrolysis of defatted peanut flour at various temperatures and HCl acid concentrations.

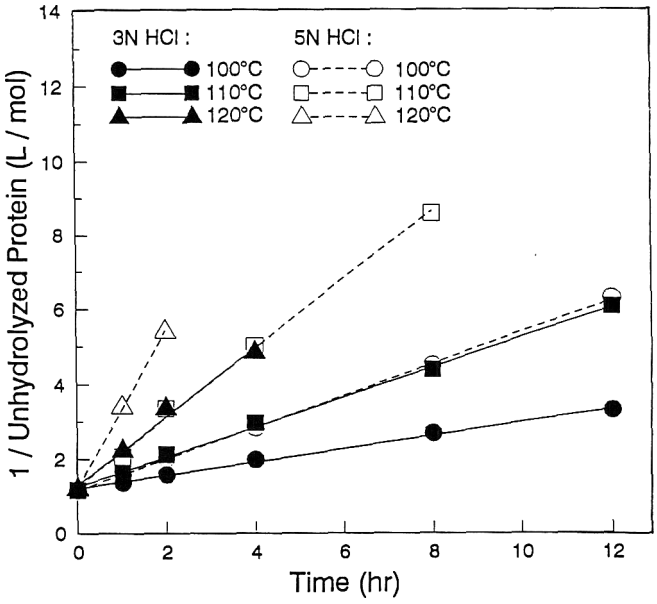

Fig. 4—Second-order reaction plot of acid hydrolysis of defatted peanut flour protein at various temperatures and acid concentrations.

by bulky side chains (Hill, 1965; Roach and Gehrke, 1970; Gehrke et al., 1985). Tyrosine yield was low, under all conditions imposed in our study, probably because of halogenation of the phenolic ring from traces of chlorine and bromine in the HCl, coupled with oxygen in the hydrolysis medium (Hunt, 1985). A considerable decrease in methionine yield was also observed during the first few hours. Differences in individual amino acids can have a pronounced effect on aroma and flavor quality of HVP.

Kinetics of hydrolysis

The linear relationship of the reciprocal of unhydrolyzed protein and time (Fig. 4) suggested a second-order reaction with respect to concentration of unhydrolyzed protein. For defatted peanut flour hydrolyzed at higher temperatures and acid concentrations, the rates of reaction were so rapid that only a few data points were useful. Since this relationship is reliable when the extent of hydrolysis is <80%, only data from analysis of samples that were <80% hydrolyzed are shown (Fig. 4). Deviation from second-order kinetics when hydrolysis is >80% might be attributed to changes in amino acids by other reactions and accumulation of dipeptides which are quite difficult to hydrolyze.

Second-order kinetics were not expected for acid hydrolysis of peptide bonds. The order of reaction with respect to concentration of both unhydrolyzed protien and H^+ was also determined, but results did not indicate H^+ was a reactant. According to several studies (Synge, 1945; Whitaker and Deatherage, 1955; Harris et al., 1956), acid hydrolysis of dipeptides in a large excess of acid is first-order with respect to dipeptide concentration. In lower concentrations of acid, hydrolysis is first-order with respect to dipeptide concentration. In lower concentrations of acid, hydrolysis is first-order with respect to both dipeptide and H^+ concentration (Lawrence and Moore, 1951; Martin, 1955). However, second-order reactions have been reported for acid hydrolysis of gelatin, silk fibroin (Greenberg and Burk, 1927), and casein (Nasset and Greenberg, 1929).

The difference in order of reaction of proteins compared to dipeptides was probably due to the complexity of the proteins. Proteins consist of many different amino acids. Some amino acids, e.g., arginine, histidine, and lysine, with charged side chains might serve as a source of protons to react with peptide bonds which are near (in space). Moreover, in a system containing large amounts of protein, amino acid released during hydrolysis, because of their charged amino group, could be catalysts of the reaction. The second-order rate constants from the slope of linear regressions lines were summarized (Table 2). The rate of hydrolysis of peanut protein was about 2.2 times faster for every 10°C increase. Hydrolysis in 5N HCl was about 2.3 times faster than in 3N HCl at the same temperature.

Table 2—Second-order rate constants of acid hydrolysis of defatted peanut flour protein under various hydrolysis conditions

Temp (°C)	HCl conc (N)	Rate constant (L mol hr^{-1})	Constant (L mol^{-1})	R^2
100	3	1.782×10^{-1}	1.2055	0.9964
	5	4.222×10^{-1}	1.1641	0.9998
110	3	3.993×10^{-1}	1.2557	0.9981
	5	9.224×10^{-1}	1.2679	0.9973
120	3	9.128×10^{-1}	1.3025	0.9897
	5	20.967×10^{-1}	1.2322	0.9994

The effects of temperature on rate constants of hydrolysis in 3N and 5N HCl were compared by Arrhenius plots (Fig. 5). Calculated activation energies were summarized (Table 3). These data indicate that the catalytic effect of HCl was proportional to its concentration. Increasing it from 3N to 5N appeared to increase the frequency factor father than altering the activation energy. Possibly, in stronger acid solutions more hydrogen ions were readily available to react with peptide bonds, thus promoting formation of activated complexes and peptide bond cleavage.

Although the true mechanism of reaction could not be clearly explained from reaction orders and kinetics, the data are useful from a practical standpoint. Data in Tables 2 and 3 can be used to predict the course of hydrolysis of defatted peanut flour protein at various temperatures and HCl concentrations using similar ratios of rat material and acids. These data also make possible the estimation of time required to effect a given degree of hydrolysis (provided it is <80%).

CONCLUSIONS

HYDROLYSIS TEMPERATURE and acid concentration are important process variables that can be used to control the rate of

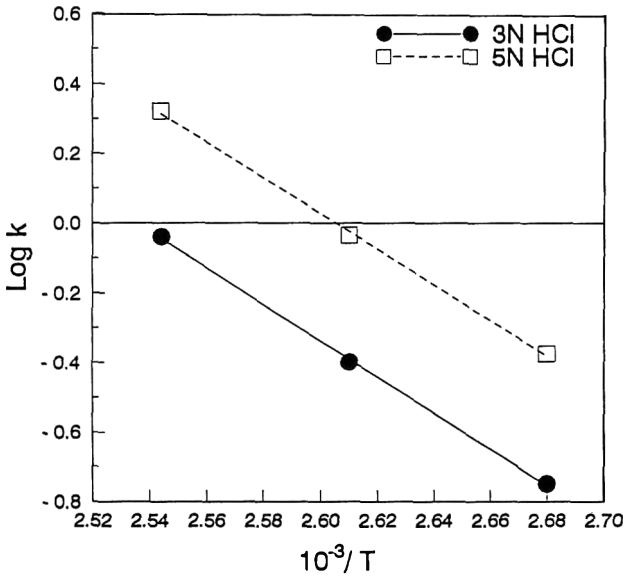

Fig. 5—Arrhenius plots for acid hydrolysis of defatted peanut flour protein in 3N and 5N HCl.

Table 3—Activation energy of acid hydrolysis of defatted peanut flour protein in 3N and 5N HCl

HCl conc (N)	Activation energy (kcal mol^{-1})	Frequency factor (L mol hr^{-1})	R^2
3	23.86	1.67×10^{13}	0.9994
5	23.41	2.13×10^{13}	0.9990

hydrolysis and free amino acid yield in production of HVP from defatted peanut flour. Various combinations of process variables may result in the same yield within the same time. However, product quality may be different due to variations in inidividual amino acid content. Based on a high yield of total free amino acids and minimum processed times, a high temperature of 120°C and an acid concentration of 5N would be preferable for industrial production of HVP. Studies on sensory qualities of HVP made from defatted peanut flour are needed for development of high quality hydrolysates.

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Mathematical Model for Formation Rate and Collapse of Foams from Enzyme Modified Wheat Flours

N. BOMBARA, A.M.R. PILOSOF, and M.C. AÑÓN

ABSTRACT

A zero-order equation described the expansion rate of foams. Lower viscosity and yield values of doughs allowed an increase in rate of expansion of foams as the degree of hydrolysis increased. Foam volume was increased by enzyme hydrolysis of wheat proteins from 63 mL for untreated wheat flour to 93 mL for 36.7% DH flour. Time course of collapse was fitted by a second-order equation suggesting that its rate was determined by coalescence between gas bubbles. The rate of collapse was enhanced by protein hydrolysis. This indicates that a high degree of gluten structure was required for maximum stability.

Key Words: mathematical model, foam expansion, foam collapse, wheat flour.

INTRODUCTION

WHEN WHEAT FLOUR DOUGH is fermented by yeast or is chemically leavened by bicarbonate or carbonate with acids, carbon dioxide bubbles produced are entrapped to produce an expanded foam-like structure. The ability of dough to retain a fine porous structure is attributed to gluten which has high surface activity. The high stability of dough foams arises from the mechanical properties of the adsorbed gluten film, such as surface viscosity and surface rigidity. A high degree of hydrogen bonding between the large number of glutamine residues, and the extensive intermolecular disulphide bonding in glutenin enhance the cohesiveness between polypeptides in the protein film (Cheftel et al., 1989; Kinsella, 1981; Mita et al., 1977).

The functional properties of wheat proteins may be improved by using specific proteases. Addition of proteases to dough is used commercially in production of bread, baked goods, crackers and waffles. It improves the handling properties of dough, the elasticity and texture of the gluten and increases loaf volumes substantially (Lyoons, 1982).

Our objective was to evaluate the effects of enzymic modification of wheat flour proteins on the foaming capacity and stability of dough through a rapid batter expansion test developed by Khan and Elahi (1980) which had shown high correlations with bread volume. This method was essentially the dough expansion test with the modification that yeast was replaced by baking powder. The chemical leavening was a mixture of sodium bicarbonate and glucono-delta-lactone which, when hydrolyzed, lowers pH (McCarthy et al., 1991), promoting carbon dioxide production without formation of salts which would affect rheology (Hoseney, 1991). In addition, modification of the batter expansion test allowed measurement of the rate of foam formation and collapse, so we could develop a mathematical model.

Authors Bombara and Pilosof are with Depto. de Industrias, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Ciudad Universitaria, 1428 – Buenos Aires, República Argentina. Author Añón is with Centro de Investigación y Desarrollo en Criotecnología de Alimentos – CIDCA, 47 y 116, 1900 La Plata, República Argentina. Address inquiries to Dr. A.M.R. Pilosof.

MATERIALS & METHODS

Materials

Wheat flour, additive free, (*Triticum aestivum*) was obtained from Molinos Río de la Plata (Argentina). Proximate composition was: protein content ($N \times 5.7$), 0.095 g/g; moisture (dry basis), 0.135 g/g; and ash, 0.006 g/g. A neutral protease enzyme from *Aspergillus oryzae* was obtained from Biocon Cono Sur L.T.D. (Argentina). The proteolytic activity was 400,000 HUT/g. (One HU is the amount of enzyme which, while acting on hemoglobin substrate, will produce 1g tyrosine in 1 min at pH 5 and 50°C). The pH optimum for the protease was 5 to 7 and the optimum temperature 55°C.

Methods

Protein and degree of hydrolysis (DH). Nitrogen was determined by micro-Kjeldahl method, using a Kjeltec Auto Analyzer 1030 (Tecator, Sweden). Protein was calculated as $N \times 5.7$. For DH the TCA soluble protein was measured according to Bombara et al. (1992). DH was calculated as the ratio of TCA soluble peptides to total initial protein × 100.

Preparation of enzyme modified wheat flour. The hydrolysis of wheat flour (28% w/w) was carried out in a batch laboratory reactor at 54°C. Protein concentration was 28 mg/g and pH of the reaction was not controlled to more closely approximate commercial conditions. The pH varied from 5.9 before hydrolysis to 6.2 at the end of 24 hr hydrolysis (Bombara et al., 1992). In order to obtain enzyme treated samples of different degrees of hydrolysis, wheat flour was hydrolysed for 1 hr with different enzyme (E) to substrate(s) ratios. E/S ranged between 0.25/100 and 3/100. Residual enzyme activity was stopped by lowering pH to 2.3 with (1 mol/L) HCl solution and stirring at 10°C for 30 min. The original pH of the mixture (5.9) was restored by neutralizing with dilute NaOH solution. Enzyme modified samples were freeze-dried.

Foaming. A modification of the rapid batter expansion method of Khan and Elahi (1980) was adopted in order to closely follow expansion and collapse of foams. Wheat flour (50g) and baking powder (10g) composed by a mixture of sodium bicarbonate and glucono-delta-lactone 1:2.25 rates were mixed by sieving. Water (10 mL) at 15–20°C was added to wheat flour (10g) in a 100 mL graduated cylinder and stirred with a glass rod. The cylinder was kept in a water bath at 40°C and the foam expansion was recorded over time until maximum expansion, and after collapse began, until total collapse occurred. About 60 min was required for the entire test.

Soluble protein and flow properties. Amount of protein solubilized in each enzyme treatment was determined according to Bombara et al. (1992).

Flow properties were determined on the resulting dough by mixing 50g wheat flours and 50 mL water at 20°C. A Brookfield Helipath stand with T-spindle was used to characterize the time-dependent flow properties of each system by recording stress or viscosity decay at a constant shear rate (0.3 rpm). The time (t) dependency of viscosity (η) was expressed by the Umstätter equation:

$$\eta = \eta_e + (\eta_i - \eta_e)\exp(-t/\lambda_T) \quad (1)$$

where η_e is the equilibrium viscosity, η_i is the initial viscosity, and λ_T is the thixotropic relaxation time. A stress relaxation method (Toth and Hamm, 1968) was used to estimate yield stress. This was defined as the minimum torque registered by the viscometer with T-spindle F when the rotor was stopped after rotating 3 sec at 0.3 rpm.

RESULTS & DISCUSSION

Experimental foam profiles

The formation and collapse of foams from protease modified wheat flours with different degrees of hydrolysis were com-

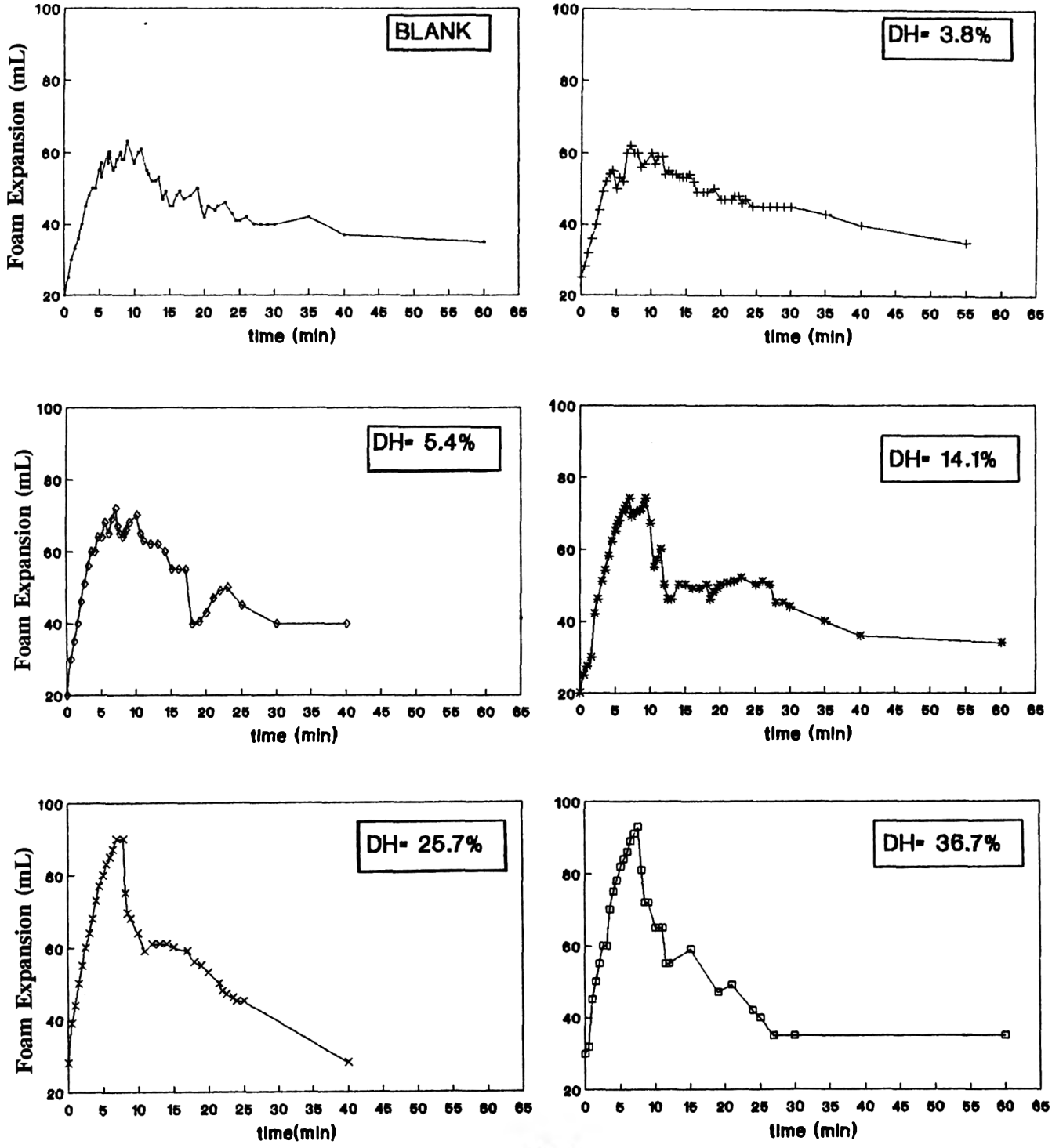

Fig. 1—Formation and collapse of foams from protease modified wheat flours with different degrees of hydrolysis.

Table 1—Rate constants of expansion (K_0) and collapse (K_2) of foams from enzyme modified wheat flours

Degree of hydrolysis (%)	$K_0 \pm \sigma_{K_0}$ (mL/min)	$(K_2 \pm \sigma_{K_2}) \cdot 10^5$ (mL/min)$^{-1}$
0	4.1 ± 0.4	23.6 ± 1.7
3.8	4.8 ± 0.5	25.7 ± 8.2
5.4	6.4 ± 0.5	28.1 ± 4.8
14.1	7.8 ± 0.3	31.3 ± 2.1
25.7	8.4 ± 0.4	57.7 ± 3.0
36.7	8.5 ± 0.5	55.1 ± 3.5

Table 2—Soluble protein and flow parameters of enzyme modified wheat flours

DH (%)	Soluble protein (%)	Initial viscosity (0.3 rpm) (cps 10^{-3})	Yield value (dyne · cm)	λ_T (min)
0	7.1	1357.2	36.0	2.7
3.8	11.6	1177.8	20.0	5.3
5.4	13.4	811.2	10.0	—
14.1	25.5	152.1	4.5	—
25.7	35.3	120.1	3.2	—
36.7	53.0	60.8	10.0	—

pared (Fig. 1). Maximum foam expansion was reached between 6.5 and 9 min and increased as DH increased (Fig. 1). It ranged from 63 mL for the original wheat flour to 93 mL for the 36.7% DH flour. A linear relationship between maximum foam expansion and DH was found ($R = 0.942$, $P < 0.01$). For untreated flour and those of low DH (3.8 – 14.1%)

Table 3—Regression models that relates functional properties of enzyme modified wheat flours and foam parameters

Foam parameters	Variable description	R	Regression coefficient	Std error of coefficient
V	soluble protein	0.934 ($P < 0.01$)	0.73	0.14
K_0	initial viscosity	0.991 ($P < 0.01$)	−0.0032	0.0002
K_0	yield value	0.889 ($P < 0.05$)	−0.12	0.03
K_2	soluble protein	0.906 ($P < 0.01$)	7.96×10^{-6}	1.86×10^{-6}

maximum expansion was maintained for several minutes (3.5 − 2 min); however, for flours with 25.7% and 36.7% DH, the foam decreased suddenly after maximum expansion was reached. The time for total collapse ranged from 50 min for untreated flours to 22 min for the more hydrolysed flours.

Equations for fitting foam expansion and collapse

A zero-order equation:

$$v - v_0 = K_0 t \qquad (2)$$

where v is volume of foam at time t, v_0 the initial volume, and K_0 the specific rate constant of foam expansion, fitted accurately (R : 0.926 − 0.984; $P < 0.01$) the increasing of foam volume over time up to maximum expansion.

Time course of collapse was better fitted (R : 0.910 − 0.981; $P < 0.01$) by a second-order equation:

$$\frac{1}{v} - \frac{1}{V} = K_2 t \qquad (3)$$

where V is maximum foam expansion volume and K_2 is the specific rate constant of collapse. Table 1 summarizes K_0 and K_2 values and standard deviations. Both K_0 and K_2 increased as DH increased. Thus protein hydrolysis enhanced the rate of expansion of foams but concomitantly increased the rate of collapse.

Foam parameters and functional properties

Soluble protein increased as the degree of hydrolysis increased (Table 2) ranging from 7.1% for untreated flours to 53.0% for 36.7% DH flour. Viscosity and yield values also decreased by effects of protein hydrolysis. However major changes occurred up to 14.1% DH. Thixotropy of the untreated flour was lost at very low DH, as shown by the λ_T values. Multiple linear regression analysis of foam parameters (V, K_0 and K_2) with soluble protein, viscosity and yield value as indepenent variables, showed that only simple linear relationships were significant. The most significant relationships between V, K_0 and K_2 and the measured functional properties were soluble protein and initial viscosity (Table 3).

Discussion

A zero-order equation described the expansion rate of foams, in accordance with the releasing of carbon dioxide from chemical leavening. A decreased resistance to flow according to lower values of viscosity and yield values of doughs resulted in an increase in the rate of expansion of foams as DH increased.

This was consistent with previous reports on effects of enzymic hydrolysis of proteins on foaming power (Puski, 1975; Gunther, 1979; Kabirullak and Wills, 1981; Boyce et al., 1986; Bernardi et al., 1991). In general, partial hydrolysis of proteins enhances properties which favor foam formation such as solubility, rate of diffusion and molecular flexibility, to form an interfacial film around nascent gas bubbles.

Several empirical equations have been fitted to drainage profiles on protein-stabilized foams. Few attempts have been made to analyze quantitatively the time course of collapse. Several kinetic processes might determine the over-all rate of collapse of wheat flour foams where drainage is prevented by high viscosity. These include: (a) rupture of films of adsorbed protein at air-foam interfaces; (b) rupture of aqueous lamellae separating bubbles; (c) increases in bubble size by coalescence (disproportionation); (d) rate of transport of larger gas bubbles through the foam to the air-foam interface. A zero-order rate would indicate that coalescence at the interface between air and foam was the rate-determining step. A first-order rate would indicate that rupture of aqueous lamallae separating the bubbles was the lowest step. That collapse here followed a second-order rate law suggested that its rate would be determined by coalescence between gas bubbles.

Foaming power was enhanced by protein hydrolysis, but foam stability, through the measured rate constant of collapse, was decreased. This indicated that partially hydrolyzed protein was needed to increase foam expansion but some larger protein components are needed to stabilize the foam. The inverse relationship between soluble protein and rate of collapse supports the concept that a high degree of gluten structure is required for maximum stability. Mita et al. (1978) and Popineau et al. (1988) showed that gluten foams were very stable. This was attributed to the much greater degree of hydrogen bonding in gluten foams between the large number of glutamine residues which enhance cohesiveness between polypeptides. Between gluten proteins, glutenin had much superior stabilizing properties compared with gliadin or gluten itself (Mita et al., 1978; Popineau, 1988). This may be related to the high degree of polymerisation through disulphide bonds. Reduction in polymer size by enzymic hydrolysis and modification of polymer structure would affect physico-chemical properties related to foam stability. These include surface viscosity, cohesiveness, elasticity, and the extent of surface adsorption.

CONCLUSIONS

THE RAPID BATTER EXPANSION TEST allowed us to evaluate the effects of enzymic modification of wheat flour proteins on foaming properties. By increasing the degree of hydrolysis, foam power was enhanced but stability of foams was decreased. A limited hydrolysis allowing improvement of foaming capacity while maintaining foam stability to a great extent, would be optimal for most applications.

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Microbial Sour Doughs Influence Acidification Properties and Breadmaking Potential of Wheat Dough

CONCEPCIÓN COLLAR ESTEVE, CARMEN BENEDITO DE BARBER, and MARÍA ANTONIA MARTÍNEZ-ANAYA

ABSTRACT

Breads from sour doughs were started with *Lactobacillus plantarum* (L-73, or B-39) or *Lactobacillus brevis* (L-62)-, and incorporated at 10, 17.5, or 25%. Lactic and acetic acid production depended on bacterial starter and yeast. Started doughs had longer mixing time, stability, more softening with time; lower resistance to extension; shorter fermentation, less stability, and greater oven rise. Breads had greater volume, lower density, softer crumb and higher slice height. Interactive effects between inoculum and microbial composition of starter were reflected in sensory scores. Adequate sour dough inoculum was lower for heterofermentative strains (10%, L-62) than for homofermentative strains (17.5%, B-39; 25%, L-73), due to deleterious effects on rheological properties.

Key Words: wheat, sour-dough, lactic acid bacteria, yeast, bread

INTRODUCTION

SOUR DOUGH is a complicated biological system, in terms of microbial composition of starters (Kline and Sugihara, 1971; Sugihara et al., 1970, 1971; Spicher and Stephan, 1987; Seibel and Brümmer, 1991; Brümmer and Lorenz, 1991). There are interactive effects among types of sour dough processes (Barber et al., 1991; Kline et al., 1970; Spicher and Stephan, 1987), conditions (Stear, 1990; Ng, 1972; Salovaara and Valjakka, 1987; Brümmer and Fisher, 1990; Martínez Anaya et al., 1992), and ingredients (Brümmer and Lorenz, 1991; Kline et al., 1970; Stear, 1990) on breadmaking performance of sour dough microflora. Sour dough in breadmaking improves dough properties, leads to a more cohesive crumb with uniform structure, and enhances the flavor of wheat (Spicher and Stephan, 1987; Brümmer et al., 1984; Salovaara and Spicher, 1987; Richard-Molard and Drapron, 1978) and rye (Spicher and Stephan, 1987; Seibel and Brümmer, 1991) products. In addition, bread shelf life is extended by delaying mold growth (Seibel and Brümmer, 1991; Salovaara and Valjakka, 1987; Salovaara and Spicher, 1987).

Effects of sour dough in rye products have been widely investigated (Spicher and Stephan, 1987; Seibel and Brümmer, 1991), but application to wheat products is limited due to the different functions of sour dough in rye and wheat bakery products. In wheat bread, sour dough is partly a rising agent (Galli and Ottogalli, 1973) to improve bread volume (Seibel and Brümmer, 1991; Salovaara and Spicher, 1987), and partly for dough acidification (Brümmer et al., 1984; Brümmer, 19889a, 1989b) to improve aroma (Brümmer and Lorenz, 1991). Dual functions of wheat sour doughs have been pointed out (Kline et al., 1970; Ng, 1972) and closely related to starting microflora (Sugihara et al., 1970, 1971; Kline and Sugihara, 1971). Studies have been conducted to determine how sour dough acidification influenced wheat bread quality (Brümmer and Fisher, 1990; Brümmer and Lorenz, 1991). These led to recommendations for processing conditions for sour dough (1% ash content and high absorption flour, high dough temperature and 7.5–10% of sour dough inoculum) and acidification degree (pH 3.5–4.0) to provide acceptable bread aroma and flavor. Excessive acidification makes it difficult to produce bread with high specific volume, and the pungent bread aroma is not accepted by all consumers (Salovaara and Spicher, 1987).

Yeast and bacterial population from sour dough and percentage of sour dough addition to bread dough are probably important factors affecting dough plasticity and overall wheat bread quality. Extended knowledge on wheat sour dough effects by systematic studies would provide the opportunity to expand the range of wheat bread types available. Our objective was to contribute to this systematic study by investigating acidification and breadmaking quality of white wheat flour doughs prepared with different proportions of bacterial sour dough starters with and without yeast.

The authors are affiliated with Laboratorio de Cereales, Instituto de Agroquímica y Tecnología de Alimentos (CSIC), Jaime Roig, 11.46010 Valencia (Spain). Address inquiries to Dr. Collar Esteve.

Table 1—Starters and flours used for sour and bread dough preparation

Starter composition		
Lactobacilli	**Strain**	**CFU/mL**
Lactobacillus plantarum	L-73	4.0×10^9
Lactobacillus plantarum	B-39	3.0×10^9
Lactobacillus brevis	L-62	2.0×10^{10}
Yeast	**CFU/g**	
Instant Active Dried Yeast (IADY)	3.0×10^{10}	

Flour characteristics		
	flour 1 (L-73,B-39)	**flour 2 (L-62)**
Energy of deformation ($\times10^3$ ergs)	181.6	156.6
Curve configuration ratio	0.85	0.51
Protein (%, dry basis)	12.40	11.70
Ash (%, dry basis)	0.54	0.52
Moisture (%)	13.59	13.38
Hagberg Index (s)	356	426

Sour dough conditions	
Dough yield (g dough/100 g flour)	200
Fermentation time	20 h
Fermentation temperature	30°C
Bacterial inoculum	10^7 bacteria/g flour
Yeast inoculum (+)	10^6 cells/g flour

Dough formula	
Sour dough (%, flour basis)	0, 10, 17.5, 25
Flour (g)	100
Ascorbic acid (ppm)	50
Malt flour (%)	0.18
Water (mL)	63.8 (flour 1), 59.0 (flour 2)
Fat (%)	1
Sugar (%)	1
Salt (%)	1.5
IADY (%)	2

MATERIALS & METHODS

Microbial propagation and performance of doughs and breads

Composition of microbial starter, analytical data on flours, and conditions for sour dough preparation and dough formula were summarized (Table 1). Freeze-dried cultures of *Lactobacillus plantarum* L-73 and *Lactobacillus brevis* L-62 (CHR Hansens Laboratorium. Denmark A/S), and *Lactobacillus plantarum* B-39 (Cereals Laboratory collection) strains were propagated following the procedure of G.B. Hansen

Table 2—Acidification properties of fermented sour doughs, doughs, and breads started with sour dough bacterial starters

Starter	Yeast (+/−)	Percent of sour dough	pH			Total Titratable Acidity (mLNaOH 0.1 N/5 g flour, 14% mb, 10g bread, as is)			DL-LACTIC ACID (g/100g flour, as is, 100g bread, dry basis)		ACETIC ACID (g/100g flour, as is, 100g bread, dry basis)	
			Sour dough	Bread dough	Bread	Sour dough	Bread dough	Bread	Sour dough	Bread	Sour dough	Bread
L-73 (flour 1)	−	10	4.03	5.27	5.26	6.88	3.30	3.08	0.3491	0.1525	0.1360	0.0231
		17.5		4.92	5.01		3.94	3.63		0.2403		0.0247
		25		4.59	4.72		5.50	4.34		0.3338		0.0238
	+	10	4.34	5.39	5.55	4.77	3.06	2.80	0.2571	0.0663	0.1143	0.0229
		17.5		5.24	5.50		3.29	2.87		0.1396		0.0223
		25		4.81	5.05		3.95	3.65		0.257[illegible]		0.0230
B-39 (flour 1)	−	10	4.16	5.43	5.54	6.39	3.11	2.97	0.2894	0.0808	0.1636	0.0225
		17.5		4.91	5.07		3.97	3.58		0.1842		0.0220
		25		4.61	4.72		4.39	4.73		0.3299		0.0206
	+	10	4.13	5.39	5.50	6.22	3.22	3.13	0.4130	0.0900	0.1347	0.0166
		17.5		4.99	5.18		3.78	3.71		0.2059		0.0169
		25		4.80	5.01		4.14	4.25		0.2554		0.0164
L-62 (flour 2)	−	10	3.67	4.91	5.03	9.89	3.63	3.45	1.2436	0.2441	0.1046	0.0120
		17.5		4.53	4.76		4.31	4.23		0.3754		0.0296
		25		4.30	4.49		5.05	5.11		0.6477		0.0101
	+	10	3.75	4.91	5.06	9.98	3.95	3.55	1.1626	0.2613	0.1739	0.0120
		17.5		4.70	4.71		4.25	4.42		0.5979		0.0222
		25		4.37	4.49		5.33	5.19		0.6941		0.0244
control 1	−	0	−	5.49	5.70	−	2.69	2.65		0.0046	−	0.0240
control 2	−	0	−	5.46	5.74	−	3.23	2.61		0.0069	−	0.0209

Table 3—Farinograph measurements on doughs started with sour dough bacterial starters

Starter	Yeast (+/−)	Percentage of sour dough added	Farinograph					
			Development time (min)	Maximum consistency (BU*)	Stability (min)	Degree of softening 10" (BU*)	Degree of softening 20" (BU*)	Water absorption (%)
L-73 (flour 1)	−	10	4.0	450	7.0	40	105	
		17.5	4.0	430	5.5	45	110	
		25	3.5	490	3.5	125	190	
	+	10	1.5	460	9.5	30	85	
		17.5	4.5	445	7.0	30	100	
		25	6.0	440	4.5	75	145	
B-39 (flour 1)	−	10	4.0	510	9.0	30	90	
		17.5	4.5	510	3.5	120	200	
		25	4.0	500	2.5	130	190	
	+	10	4.0	490	9.0	20	90	
		17.5	5.5	450	5.5	60	120	
		25	3.5	480	4.5	110	180	
L-62 (flour 2)	−	10	2.5	520	2.5	190	225	
		17.5	3.0	550	4.0	140	200	
		25	5.0	535	3.5	50	140	
	+	10	3.5	490	2.5	250	270	
		17.5	3.5	500	5.0	130	190	
		25	4.5	510	7.5	55	140	
flour 1			2.0	500	11.0	30	70	60.14
flour 2			5.5	500	17.0	10	40	55.60

(*) BU: Brabender Units

(CHR Hansens Laboratorium. Denmark A/S). Each culture (0.5g) was inoculated in MRS broth (200 mL) at 30°C over night (16–20 hr). Aliquots (10 mL) of full grown MRS broth were pipetted into sterile tubes and centrifuged at 8000 × *g* for 8 min. Bacteria were dispersed in 5 mL of freshly prepared freezing media (MRS broth:glycerol, 5:1 ratio) on a Vortex mixer. Aliquots (2.5 mL) of bacteria suspension were pipetted into cryo tubes and immediately frozen at −40°C. Thawing of frozen cultures (32°C, 3 min) and washing (2 × 5 mL 5% NaCl) were carried out before sour dough inoculation (10^7 bacteria/g flour). Instant active dried yeast (IADY) (Danish Distillers, Denmark) was incorporated in sour doughs (10^6 yeast cells/g flour). IADY characteristics included: dry matter: 94.5%; living cells: 3.0×10^{10} cells/g (malt agar), 4.0×10^{10} cells/g (microscopic counts); bacteria: 1.9×10^4 cfu/g (plate counts).

Bacterial wheat sour doughs with and without added yeast (18 total) were prepared and added in three levels to bread doughs (Table 1). These were mixed at 26–27°C; after dough resting (20 min), scaling (830g), hand rolling and intermediate proofing (10 min), doughs were panned, proofed (60 min, 32°C, 75–80% RH) and baked (230°C, 35 min) following the German Standard method "Kastengebächversuch."

Table 4—Extensigraph, maturograph and oven rise recorder properties of doughs started with sour dough bacterial starters

Starter	Yeast (+/−)	Percentage of sour dough	Extensigram			Maturogram				Oven rise record		
			Maximum resistance (BU*)	Extensibility (mm)	Energy (cm^2)	Fermentation time (min)	Stability (min)	Elasticity (BU*)	Dough level (BU*)	Initial vol (BU*)	Final vol (BU*)	Final oven rise (BU*)
L-73 (flour 1)	−	10	240	120	35.5	82	23	150	275	300	538	63
		17.5	225	105	28.1	47	8	175	283	340	680	120
		25	215	94	23.1	44	11	240	600	395	835	210
	+	10	240	113	31.1	58	10	218	530	388	653	10
		17.5	210	107	24.5	50	10	195	380	383	675	95
		25	185	102	21.3	53	11	195	375	320	725	200
B-39 (flour 1)	−	10	257	116	36.3	78	15	155	298	315	550	60
		17.5	205	114	30.5	77	28	140	255	340	550	73
		25	210	108	27.5	58	13	145	265	325	625	105
	+	10	287	99	34.4	67	8	200	515	378	593	30
		17.5	230	97	23.0	54	8	240	570	400	790	130
		25	185	78	16.8	46	8	180	360	400	620	30
L-62 (flour 2)	−	10	200	123	31.0	92	24	125	240	315	458	40
		17.5	233	115	33.3	72	12	145	265	220	545	150
		25	260	116	36.3	69	6	160	350	410	520	10
	+	10	208	95	21.9	70	18	140	273	325	540	70
		17.5	235	113	32.2	74	12	130	350	360	570	75
		25	285	94	30.9	51	8	210	590	395	640	45
control 1	−	0	265	107	30.5	64	8	200	503	375	610	30
control 2	−	0	380	98	46.8	56	9	215	645	388	560	3

(*) BU: Brabender Units

Table 5—Physico-chemical characteristics of bread started with sour dough bacterial starters

Starter	Yeast (+/−)	Percentage of sour dough	Bread characteristics					
			Weight (g)	Volume (mL)	Density (g/ml)	Texture (g)	Height (cm)	Moisture (%)
L-73 (flour 1)	−	10	738.5	1920	0.39	3360	10.2	43.78
		17.5	733.4	2660	0.28	1725	12.7	43.64
		25	721.1	3080	0.23	1640	13.6	43.70
	+	10	725.6	3970	0.18	2600	12.3	43.30
		17.5	720.9	3860	0.19	2460	12.2	44.94
		25	733.9	3960	0.19	2580	12.1	44.52
B-39 (flour 1)	−	10	745.5	1990	0.37	5270	9.9	34.94
		17.5	740.4	2390	0.31	3715	10.9	43.46
		25	739.0	2430	0.30	3790	11.2	43.26
	+	10	739.7	2550	0.29	2725	11.3	43.22
		17.5	723.0	2990	0.24	1106	12.7	43.64
		25	732.6	2810	0.26	1850	11.8	42.63
L-62 (flour 2)	−	10	745.9	2490	0.30	4400	10.2	41.28
		17.5	745.7	2260	0.33	5330	9.8	41.22
		25	738.8	2060	0.36	9720	8.6	39.95
	+	10	732.6	2780	0.26	2550	12.0	41.46
		17.5	749.7	2040	0.37	5606	10.6	42.11
		25	745.0	2160	0.35	5904	9.4	41.43
control 1			737.6	2370	0.31	3975	11.1	43.18
control 2			732.8	2550	0.29	4500	11.4	44.42

Unsoured loaves (flours 1 and 2) were made using the same basic formula. Breads were allowed to cool (2 h) before packaging (polypropylene bags) and storage (24 hr, room temperature ≈23°C).

Technological evaluation

Thirty-five properties were measured in sour doughs, doughs and breads. Acidification properties were pH, total titratable acidity (TTA) (Arbeitsgemeinschaft Getreideforschung, 1978), and lactic and acetic acid production (Boehringer Mannheim, 1980). Rheological and fermentative dough properties were determined by farinograph, extensigraph, maturograph, and oven rise recorder measurements, following Brabender instructions (Duisburg, Germany). Bread quality properties included physico-chemical characteristics – weight, volume (seed displacement), density, crumb texture (maximum deformation strength, Instron press), slice height and moisture content (AACC, 1975). Evaluation of sensory properties – color, grain, structure, elasticity, smell and taste (intensity, typical, alcoholic or sour character) and acceptability, were based on the German test "Weizenbackversuch" (Arbeitsgemeinschaft Getreideforschung, 1978).

Statistical treatment

Statistical treatment of data (significant interactions, significant differences between means) was performed in a Microvax computer (Dig-

Table 6—Sensory attributes of breads started with sour dough bacterial starters

Starter	Yeast (+/−)	Percent of sour dough	Sensory attributes of bread: Grain	Crumb structure	Crumb elasticity	Smell	Taste	Eatability	Color
		10	rough	even enough	unsatis-factory	slight, odd, bland	slight, odd, bland	gummy	greyish
	−	17.5	somewhat rough	even enough	satisfactory	slight, charac-teristic	strong, charac-teristic	somewhat gummy	greyish
L-73		25	smooth	even enough	soft and elastic	strong, charac-teristic	strong, charac-teristic	satisfactory	white
(flour 1)		10	somewhat rough	even enough	soft and elastic	strong, charac., alcoholic	slight, charac., sour	satisfactory	white
	+	17.5	somewhat rough	even enough	soft and elastic	strong, charac., alcoholic	slight, charac., sour	satisfactory	white
		25	smooth	even	soft and elastic	strong, charac., alcoholic	strong, charac-teristic	satisfactory	white
		10	rough	even enough	satisfactory	slight, odd, bland	slight, odd, bland	somewhat gummy	some greyish
	−	17.5	rough	uneven	satisfactory	satis-factory, charac.	satis-factory, charac.	satis-factory	some greyish
B-39		25	rough	even enough	soft and elastic	satis-factory, odd	satis-factory, odd	satis-factory	some greyish
(flour 1)		10	rough	even enough	satisfactory	slight, odd, bland	slight, odd, bland	gummy	some greyish
	+	17.5	smooth	uneven	soft and elastic	strong, charac., alcoholic	satis-factory, charac.	satis-factory	white
		25	somewhat rough	even enough	satisfactory	strong, charac., alcoholic	satis-factory, charac.	somewhat gummy	some greyish
		10	rough	even enough	unsatis-factory	slight, charac., alcoholic	strong, charac-teristic	gummy	white
	−	17.5	rough	even enough	unsatis-factory	satis-factory, charac., sour	slight, charac., bland	gummy	white
L-62		25	rough	even enough	unsatis-factory	satis-factory, odd	strong, odd	gummy	greyish
(flour 2)		10	smooth	even enough	soft and elastic	strong, charac-teristic	satis-factory, charac.	satis-factory	white
	+	17.5	somewhat rough	even enough	satisfactory	slight, charac., bland	satis-factory, odd	gummy	some greyish
		25	rough	even enough	unsatis-factory	strong, odd, sour	strong, odd, sour	gummy	greyish
control 1	−	0	somewhat rough	even	satisfactory	satisf., charac., alcoholic	satisf., charac., alcoholic	somewhat gummy	white
control 2	−	0	somewhat rough	even enough	satisfactory	satisf., charac., alcoholic	satisf., charac., alcoholic	somewhat gummy	white

ital) by applying the analysis of variance and the Tukey's test (BioMeDical Package programmes).

RESULTS & DISCUSSION

Acidification properties

Data on acidification (pH, total titratable acidity and lactic and acetic acid production) of fermented sour and bread doughs and breads were compared (Table 2). Interactive effects of bacterial starter and yeast were observed in lactic and acetic acid production, as reported for rye products (Spicher et al., 1981, 1982). As expected, unsoured control doughs and breads had the lowest acidification properties, in good agreement with published results (Kline et al., 1970; Richard-Molard and Drapron, 1978). Heterofermentative L-62 resulted in lower pH, higher total titratable acidity and organic acid production (mainly lactic acid) of doughs and breads than homofermentative L-73 and B-39 in agreement with reports for rye

doughs and breads (Spicher and Stephan, 1987). With some exceptions, the presence of yeast in the microbial composition of the starter (comparison between bacterial started samples with and without yeast in sour dough) reduced acidification of doughs and breads (Brümmer and Lorenz, 1991). This was especially reflected in total titratable acidity values and lactic and acetic acid production of L-73 samples.

Sour doughs containing yeast and starter with homofermentative lactobacilli had lower acetic acid production (16 to 18%) and variable lactic acid production (26% −L-73, +43% −B-39) than sour doughs without yeast (Brümmer, 1989b). For heterofermentative L-62 doughs, the presence of yeast increased acetic acid content by 66%. Organic acid levels in breads were also affected by microbial composition of the starter. Breads containing sour doughs with yeast and started with homofermentative strains, had lower lactic acid (L-73, B-39), and similar (L-73) or lower (B-39) acetic acid. Sour doughs with yeast increased in both organic acids at the higher percentage of addition.

The percentage of sour dough starter addition was the most important factor affecting acidity (Table 2). Increasing percentage of sour dough led to lower pH and higher total titratable acidity in doughs and breads. Doughs with yeast had lower degrees of acidification than no yeast doughs except L-62 samples. Greater lactic acid levels in breads were noticed when the percentage of sour dough addition increased. No noticeable differences in acetic acid were observed probably due to volatilization during baking. Consequently, the fermentation quotient (molar ratio between lactic and acetic acid) was higher for breads containing higher percentages of sour dough (17.5, 25%).

Rheological and fermentative dough properties

Interactive effects between microbial starter composition and percentage of sour dough addition affect dough machinability (development time, dough consistency, stability and degree of softening) and functionality (extensibility, resistance to extension, stability and elasticity). With some exceptions, intermediate sour dough (17.5%) resulted in softer doughs with longest development times when homofermentative bacterial starters with yeast were used (L-73, B-39) (Table 3). With heterofermentative L-62, increments in sour dough addition regularly increased development time, whereas no significant changes in dough consistency were noted. Dough stability and degree of softening of different doughs, closely depended on the presence of yeast and the sour dough amount. Increasing the percentage of sour dough, led to a decrease in dough stability as pointed out before (Galal et al., 1978) due to souring action, and to an increase in degree of softening in samples started with homofermentative L-73 and B-39. The effect was greater in absence of yeast.

Unsoured doughs showed higher resistance to extension, similar extensibility and similar or higher energy levels than inoculated doughs (Table 4). As the amount of added sour dough increased, maximum resistance to extension and energy decreased in samples started with L-73 and B-39. However, heterofermentative L-62 started doughs showed the opposite pattern. The presence of yeast in the microbial composition of the sour dough starter led to lower energy values (Table 4). The addition of increasing percentages of sour dough shortened fermentation time (L-73, B-39, L-62), decreased dough level and stability (L-62) and improved dough elasticity (L-62). In general, the presence of yeast did not clearly influence maturogram and oven rise recorder values, with a few exceptions. Control doughs showed lower stability, higher dough level and similar elasticity to started doughs (Table 4).

Bread characteristics

Physico-chemical characteristics. Breads made with no-yeast sour doughs had less weight, volume and yield (Table 5) as sour dough inoculum level rose. Bread volume and slice height values were related to the starting microorganisms. A positive effect of the sour dough inoculum was observed in bread volume and slice height of no-yeast samples except those started with L-62. In L-73 and B-39 samples containing yeast, bread volume and slice height were greater than those of no-yeast and uninoculated samples. Crumb firmness was in general inversely related to bread volume. Note that L-62 gave harder bread crumb for similar bread volumes, mainly when no-yeast bacterial sour doughs were used. Bread moisture values ranged from 41 to 45%, levels beyond the Spanish requirements for pan bread (38%).

Sensory attributes. Estimations of sensory attributes of breads (Table 6) were difficult to quantitate for separate effects of processing conditions due to lower accuracy and less precision. Nevertheless, overall comments on the most noticeable characteristics were included. Crumb grain improved as percent sour dough increased, for L-73 samples. For B-39 and L-62 breads, the effect was only relevant in presence of yeast. Crumb structure was, in general, opened and uniform, and similar to that of control breads. The more elastic and softer crumbs corresponded to breads with higher volume and smoother grain. Stronger flavor was reported in breads containing sour dough with yeast. Smell and taste were usually characteristic (alcoholic), and only in some cases, an unpleasant flavor was detected. Stronger or satisfactory and characteristic pleasant flavor corresponded to started samples with TTA values from 2.8 to 4.34 (Table 2, 6). These were broader acidity intervals than are recommended for wheat breads (Brümmer and Fisher, 1990). Atypical flavor was generally related to low percentage of sour dough when homofermentative starters were used, and to high percentage of sour dough addition in heterofermentative samples. Concerning acceptability, samples started with L-73 and B-39 had better scores than those started with L-62.

CONCLUSIONS

THE IMPROVING effects of microbial wheat sour doughs on breadmaking performance were closely dependent on the microbial composition of starters (yeast and lactic acid bacteria species and strains) and on sour dough inoculum level. *Lactobacillus brevis* L-62 behaved differently from *Lactobacillus plantarum* L-73 and B-39 as sour dough starter. L-62 gave more acidic doughs and breads, and resulted in breads with lower volume, harder texture, unsatisfactory crumb grain, elasticity and acceptability, and atypical flavor than L-73 and B-39. Adequate levels of sour dough with yeast to be added for improvement of bread quality was lower for heterofermentative strains (10%, L-62) than for homofermentative strains (17.5%, B-39; 25%, L-73), due to deleterious effects of excessive acidification on some rheological properties.

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Physicochemical Properties of Field Pea, Pinto and Navy Bean Starches

ELZBIETA GUJSKA, WANDA D.-REINHARD, and KHALIL KHAN

ABSTRACT

Legume starches were compared for physicochemical properties that may explain differences in functional properties. Field pea starch had higher amylose, greater swelling power and solubility, and lower pasting temperatures than pinto and navy bean starches. Scanning electron microscopy (SEM) showed that field pea starch had larger, more irregularly shaped granules and more broken large granules than pinto or navy starches. The most starch damage was observed for field pea. Pinto and navy bean starches had greater resistance to swelling at 60°C than field pea indicating a more strongly bonded micellar network. Higher cold paste viscosity was observed for navy bean and field pea.

Key Words: beans, peas, starches, micrographs, swelling power

INTRODUCTION

INTEREST HAS GROWN in the utilization of flours or factions from different types of legumes. Previous studies of Gujska and Khan (1990, 1991) and Zabik et al. (1983) showed high starch fractions from navy, pinto, garbanzo and flour from black beans could be extruded to products with good expansion. The expanded volumes of cereals and starch products probably relates to extrusion processing conditions and raw material composition and quality. Expansion ratio has been related to starch-amylose content (Chinnaswamy and Hanna, 1988), protein content (Faubion and Hoseney, 1982a; Peri et al., 1983) lipid content (Mercier et al. 1980; Faubion and Hoseney, 1982b) and starch content (Linko et al., 1981). High temperature was applied in our laboratory to whole ground seed and dehulled flour from pinto, navy and field pea. Differences were noted in some physical and functional properties of snacks. The most puffed extruded product was obtained from field pea flour. Our objective was to measure some properties of legumes starches that may help explain their functional properties during high temperature extrusion-cooking process.

MATERIALS & METHODS

Materials, starch isolation and granule size

Seeds from Pinto and Navy Beans, and Field Pea were broken into chunks in an Allis-Chalmers mill, and then milled on a Hammer Mill using screen No. 64/2.

Starch was isolated by the procedure of Schoch and Maywald (1968) with slight modification. The milled flours were suspended in water, passed through No. 70 screen and most hulls removed. The suspension was centrifuged at 4000 rpm for 10 min. The supernatant was discarded and the precipitate was re-suspended in excess of 0.2% sodium hydroxide. After centrifugation the starch was washed two times with water (neutralized to pH=7) and then three times each with 30, 50 and 70% ethanol. The supernatant was discarded after each washing. The starch was dried in an air oven (25°C) and passed through a 100-mesh sieve.

Author Khan is with the Dept. of Cereal Science & Food Technology, North Dakota State Univ., Fargo, ND 58105. Author Gujska's present address: Dept. of Human Nutrition, Univ. of Agriculture & Technology, Olsztyn, Poland. Author D.-Reinhard's present address: Dept. of Technical Microbiology, Univ. of Agriculture, Warsaw, Poland. Address inquiries to Dr. K. Khan.

The size and shape of each legume starch was studied using a Nikon AFM microscope. The range of granule size was determined by measuring the length and width of 50 granules and correcting for magnification levels. 0.1% iodine in potassium iodide was used for staining. Starch samples were mounted on aluminum stubs and sputter coated with gold-palladium. Samples were viewed and photographed on JEOL JSM 35 Scanning Electron Microscope at an accelerating voltage of 15 kV.

Swelling power, solubility, amylographs and analyses

Swelling power and solubility were determined according to Leach et al. (1959).

The Brabender viscograph was used to develop viscosity curves of isolated starches. Slurry was prepared by blending 40g (dry basis) starch in 450mL distilled water (Medcalf and Gilles, 1965).

Amylose was determined according to the procedure described by Williams et al. (1970). Damaged starch content was assessed by the method of Farrand (1964).

Protein, starch, ash and fat were determined according to AACC Approved Methods (1983), No. 46-10; 76-11; 08-01; and 30-25, respectively. Fiber was determined by the method of Prosky et al. (1984) with slight modification by Sigma Chemical Company (1985).

RESULTS & DISCUSSION

CHEMICAL ANALYSIS SHOWED flours of pinto, navy and field pea contained comparable amounts of protein: 24.5 ± 0.16%, 25.4 ± 0.14% and 26.0 ± 0.14% dry basis, respectively, and starch: 40.0 ± 0.24%, 41.1 ± 0.19% and 43.2 ± 0.26%, respectively. Field pea had highest fat but lowest fiber compared to pinto and navy beans (fat: 4.73±0.11, 1.22±0.08, 1.32±0.09 and fiber: 22.7±0.26, 32.0±0.30, 30.1±0.28%, for field pea, pinto and navy beans, respectively). The starches of pinto, navy and field pea were also compared (Table 1). Results indicated pure starches were obtained by the isolation methods. The amylose content of field pea starch was higher than navy and pinto bean. Proportions agreed closely with those reported by Hoover and Sosulski (1985, 1986). The highest degree of starch damage, measured by percentage of granules susceptible to hydrolysis by α-amylose, was found in field pea. Mechanical damage to dry granules was probably caused by milling (Vose, 1977; Naivikul and D'Appolonia, 1978) and could influence some properties measured.

Granule size and microscopic appearance

Starch granule sizes were compared (Table 2). Highest variations in size distribution were found among granules of pinto bean for both measured mean lengths and widths. These results were in close agreement with ranges reported by Naivikul and D'Appolonia (1979) for navy and pinto bean starches. Different ranges were reported by Hoover and Sosulski (1985) for navy bean starch granules (22–49 in length and 22–40 in width). Field pea granules tended to be larger than those of pinto and navy beans. Moss (1961) reported that small granules were less likely to be damaged during milling than large ones. Also through starch damage analysis and scanning electron microscopy, Vose (1977) reported that smaller corn-starch granules appeared more resistant to damage by attrition than did field pea and wheat starches. Researchers have suggested

Table 1—Composition of starch and damaged starch

Starch source	Protein (%)*	Fat (%)	Ash (%)	Amylose starch	Damaged (%)
Pinto Bean	0.08±0.17	0.12±0.027	0.24±0.011	32.2±0.13	3.0
Navy Bean	0.12±0.015	0.09±0.019	0.20±0.014	32.1±0.12	5.0
Field Pea	0.10±0.011	0.23±0.021	0.29±0.021	34.2±0.32	8.0

* On dry basis.

Table 2—Size of legume starches

Starch source	Mean granule length (μm)	Length range (μm)	Mean granule width (μm)	Width range (μm)
Pinto Bean	21.9	14—40	19.0	8—36
Navy Bean	20.9	12—32	19.7	9—32
Field Pea	22.9	18—35	20.6	15—30

a direct correlation between granule size and amylose content (Bertoft et al., 1993; Duffus and Murdoch, 1979; Kulp, 1973). Also Meredith (1981) hypothesized that the larger granules had greater proportions of amylose.

When viewed with the scanning electron microscope (Fig. 1) field pea starch granules appeared different from those of pinto and navy bean. Starch granules of pinto and navy bean were more oval, smooth and regularly shaped compared to those of field pea. Granules from field pea ranged from large irregular shapes to small ovals or spherical granules. These results were similar to those of Bertoft et al. (1993). Field pea starch granules were not smooth but had fissures on their surfaces. SEM showed also the presence of broken large granules incurred by milling process especially in field pea starch. More smaller type granules appeared in pinto and navy beans than in field pea.

Swelling power and solubility

The swelling power and solubility (Fig. 2 and 3) from field pea were higher than from pinto and navy beans at all tested temperatures. Rapid increases in swelling and solubility occurred for pinto and navy beans above 70°C but for field pea it occurred above 60°C. According to Stone and Lorenz (1984), the strength and character of the micellar network within the granule is the major factor controlling swelling behavior of starch. Thus, a highly associated starch with extensive, strongly-bonded micellar structure should be relatively resistant to swelling. In our study pinto and navy starches had greater resistance to swelling at 60°C compared to field pea starch thus indicating a more strongly-bonded micellar network. The swelling profiles for all starches were similar to those reported by Hoover and Sosulski (1985, 1986) but different values were obtained at higher temperatures (lower values for pinto and navy beans and higher for field pea) in comparison to those reported by Hoover and Sosulski (1985, 1986).

As a direct result of granule swelling, there was a parallel increase in starch solubility (Fig. 3). Higher increase in solubility for field pea starch was probably mainly a result of higher granule swelling permitting the exudation of amylose (Schoch, 1965). No negative correlations occurred between amylose content and swelling power as reported by Stone and Lorenz (1984) for amaranth starch. Influence on swelling power could result from different percentages of starch damage caused by milling and isolation. Mechanically deformed and damaged granules absorb more water than undamaged. Dengate (1984) pointed out that differences in swelling behavior appeared to be caused by differences in lipid and amylose content as well as granule organization.

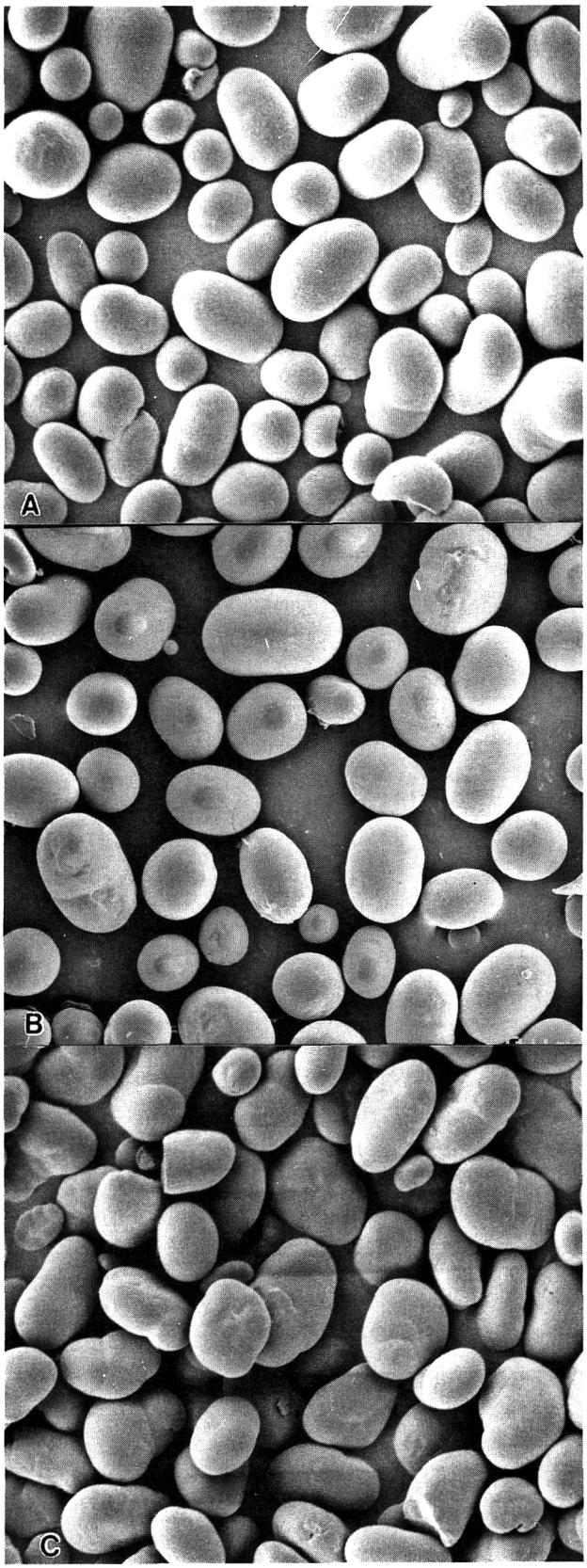

Fig. 1—SEM-micrographs of starches isolated from pinto bean (a), navy bean (ɔ) and field pea (c). (540 ×)

Pasting properties

When pasting properties were compared (Table 3, Fig. 4) Brabender viscosity patterns were characterized by absence of a peak viscosity and a slow viscosity increase during holding and cooling periods. Among the starches, field pea had the lowest pasting temperature followed by pinto and navy beans. Our results confirmed those of Hoover and Sosulski (1985)

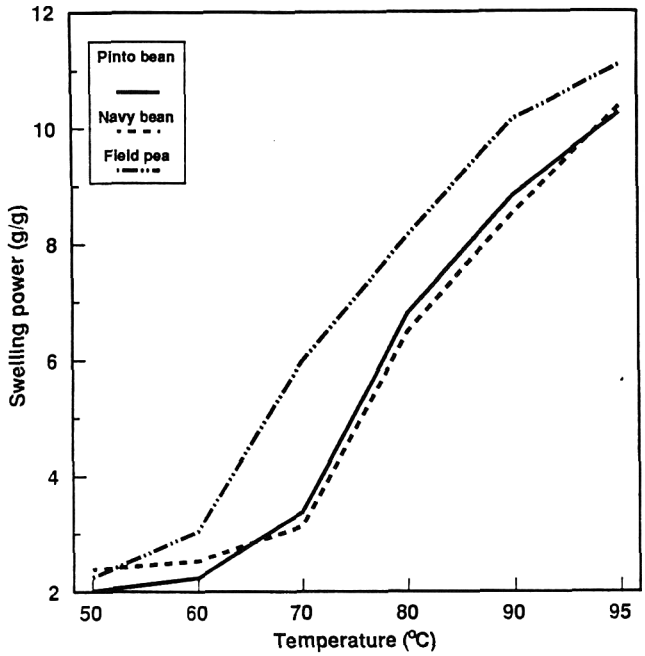

Fig. 2—Swelling patterns of starches.

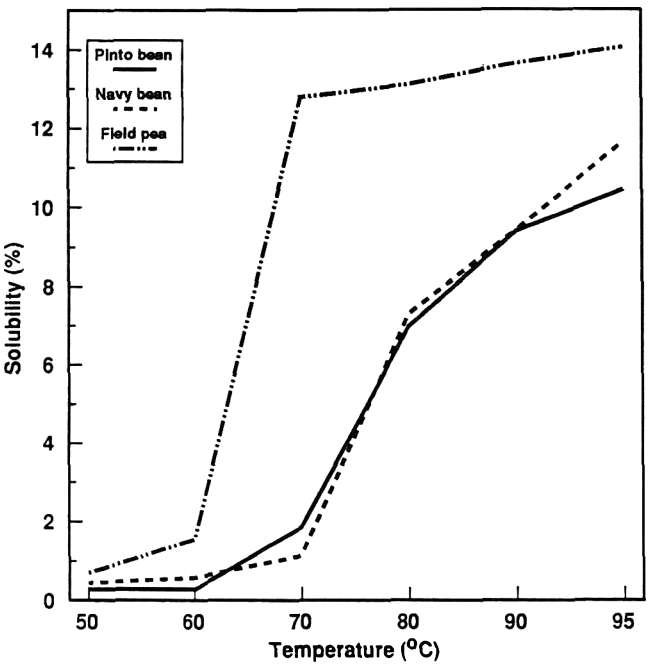

Fig. 3—Solubility patterns of starches.

Table 3—Viscographic characteristics of starches

Starch	Initial pasting temp[a] (°C)	Viscosity at 95°C (BU)	Viscosity after 15 min (BU)	Viscosity at 50°C (BU)	Set-back[b] (BU)
Pinto Bean	79.0	190	320	580	260
Navy Bean	83.5	400	540	820	280
Field Pea	73.0	370	460	800	340

[a] Initial pasting temperature is the temperature at which an increase of 10 BU (Brabender Units) in viscosity is reached. No peak viscosity occurred in any sample.
[b] Set-back denotes the viscosity difference at 50°C and that after 15 min at 95°C.

who pointed out that initial pasting viscosity appeared to reflect, in part, differences in amylose content in starch. Higher amylose content lowers initial pasting temperature. The viscosity at 95°C was much higher for navy bean and field pea than for pinto bean. During the holding period at that temperature, all samples showed gradual increases in viscosity up to end of cooling time. Higher cold paste viscosity was observed for navy and field pea compared to pinto bean. Ghiasi et al. (1982) suggested that increase in viscosity with temperature was caused by removal of water from the exuded amylose by the granules as they swell. However, the relation cannot be absolute. From our results the two starches from navy and pinto beans had similar swelling powers but different viscosity patterns. According to Schoch and Maywald (1968) two different starches may have similar swelling powers but quite different viscosity patterns due to factors such as size and shape of granules, type and degree of crystallinity within granules, ionic charge, presence of fat and protein, and perhaps molecular size and degree of branching.

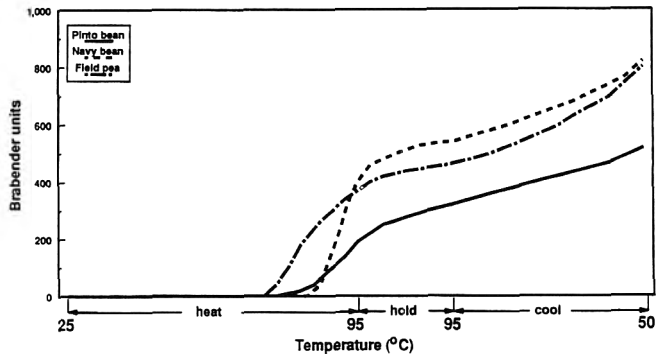

Fig. 4—Amylograph curves of starches.

CONCLUSION

FIELD PEA STARCH GRANULES were larger in size than those of pinto and navy beans, which probably led to the observed highest degree of starch damage despite similar procedures for milling and isolation for all samples. Differences in some properties between field pea and pinto or navy bean starches appear closely related to granule size differences. Some properties of field pea starch may be advantageous for extrusion processing such as higher amylose content and greater swelling power.

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Reversible Molecular Rearrangement of Slightly Acid-treated Starches

KOJI TAKAHASHI, MAKOTO HATTORI, and KEIZO WADA

ABSTRACT

The reersible association and dissociation of starch chain populations obtained from acid-treated starches (ATSs) was investigated. Potato starch, both nonglutinous and glutinous rice starches and sago starch were suspended in 15% sulfuric acid until 1% hydroysis occured. These ATSs had relative molecular weight ranges of 25,00–29,000 daltons. In water, about half the molecules were reconstituted into large aggregates of a few million daltons. This change in molecular size depended on presence of potassium chloride. It was not observed in debranched samples. The aggregates showed a clear endothermic peak on a DSC curve. Thus, the reversibly rearranging starch chain populations were not linear, but were branched and had some type of ordered structure.

Key Words: potato, rice starch, carbohydrates, structure

INTRODUCTION

STARCH CHAINS IN NATIVE GRANULES are regularly aligned in micellar structures; in particular, A and B chains of amylopectin molecules associate both intra- and intermolecularly (Yamaguchi et al., 1979). In general, such crystalline structures are dissociated during heating (gelatinization). Once thermally dissociated, upon cooling, however, they would partially reassociate (retrograde) to reconstitute micelle structures resistance to acids and enzymes (matsukura et al., 1983). The reversible association and dissociation of starch chains as influenced by changes in their environment has not been reported.

Aqueous acids preferentially hydrolyze the amorphous region of starch granules (Kainuma and French, 1971), and the water-solubility of starch can be increased by acid treatment. French (1984) reported that an extensively acid-treated starch, such as Nägeli amylodextrin, was readily soluble in hot water and formed a true solution. However, since Nägeli amylodextrin is a very degraded starch consisting of low molecular weight chains of DP 25 and DP 12-14 (Watanabe and French, 1980), it is not suitable for investigating the reversible reassembly of native starch chains.

Our objective was to slightly treat various starches with sulfuric acid under mild conditions in order to obtain soluble chain populations similar to those of native starch. We also elucidated their properties and proved the soluble starch chain populations with branched structures fractionated by size-exclusion chromatography could anderge rearrangement to a structure with a melting endotherm, depending on ionic strength.

MATERIALS & METHODS

Starch

Potato starch (Hokuren Research Institute, Japan), nonglutinous and glutinous rice starches (Shimada, Japan), and sago starch (Sarawaku Economic Cooperation, Malaysia) were used after being washed repeatedly with distilled water at 4°C prior to air-drying (moisture, 17–20%, dry basis).

Authors Takahashi and Hattori are with the Dept. of Applied Biological Science, Faculty of Agriculture, Tokyo Univ. of Agriculture & Technology, 3-5-8 Saiwai-cho, Fuchu-City, Tokyo 183, JAPAN. Author Wada's present address: East Asia University.

Acid-treated starch (ATS)

Each of the four kinds of starch (10 G) was immersed in 2 L of 15% (w/w) sulfuric acid at 20°C to give a 1% hydrolysis rate (Kainuma and French, 1971). The degree of hydrolysis was calculated from the following, (0.9 × dissolved saccharides) × 100/(starch weight), where dissolved saccharides was determined by phenol-sulfuric acid method (Dubois et al., 1956). After sulfuric acid had been thoroughly removed by filtering through a G-4 glass filter and washing with distilled water, each ATS was obtained by air-drying. For X-ray diffractometry and DSC, ATS was used without drying.

Enzymes

Sweet potato β-amylase (EC 3.2.1.2; Sigma, Type 1-B) was purified by affinity chromatography in a cyclodextrin-Sepharose 6B column (Vretblad, 1974). *Pseudomonas* isoamylase (EC 3.2.1.68) was obtained from Seikagaku Kogyo Co. (Tokyo, Japan).

Fractionation of ATS

ATS dissolved in distilled water by heating to 80°C for 20 min was applied to a column of Toyopearl HW-65S (Tosoh, Japan; column size, 2.6 cm i.d. × 100 cm) and eluted with distilled water as a mobile phase at flow rate 60 mL/hr at 4°C. The peak-1 component (P-1) eluted at the void volume was collected and recovered by lyophilization. P-1 was redissolved in a 0.01M borate buffer at pH 7.0 containing 0.1 M potassium chloride, and rechromatographed in the column with the same buffer as the mobile phase at flow rate 60 mL/hr. Fractions containing the major chains of P-1 with relative molecular weight (Mr) of 2-5 × 10^5 were dialyzed against distilled water and subsequently lyophilized.

Preparation of debranched P-1

A solution of P-1 (500 mg) dissolved in 4 mL of 1M sodium hydroxide at 40°C with stirring for 2 hr was diluted with 30 mL of distilled water prior to adjusting pH to 6 with 0.5M hydrochloric acid. After adding 40 mL of 0.06M acetate buffer, pH 3.5, the P-1 solution was incubated with 26 IU of isoamylase at 40°C for 24 hr. Debranched P-1 was obtained by dialysis against distilled water and lyophilization.

Preparation of β-limit dextrin of P-1

A solution of P-1 (500 mg) dissolved in 4 mL of 1M sodium hydroxide at 40°C with stirring for 2 hr was diluted with 30 mL distilled water and neutralized with 0.5 M acetic acid. After adding 40 mL of 0.2M acetate buffer, pH 4.8, containing 1 mM reduced glutathione, P-1 was incubated with 300 IU of purified β-amylase at 37°C for 24 hr. The maltose produced was determined by the Somogyi (1952)-Nelson (1944) method. β-limit dextrin was obtained by dialyzing against water with subsequent lyophilization.

Chromatographic analysis

Size-exclusion chromatographic (SEC) analysis of starch chains was performed on columns of Toyopearl HW-65S and HW-55S (Tosoh, Japan; column sizes, 1 ID × 60 cm and 0.9 ID × 50 cm, respectively) calibrated with dextran (Pharmacia). SEC for the starch chain aggregates in an associated state was conducted on samples that had been dissolved in a 0.01 M borate buffer, pH 7.0, at 80°C with stirring for 20 min. The solutions were then applied to the Toyopearl columns equilibrated with the same buffer. A flow rate of 12 mL/hr and 20°C were employed. The eluted fractions (0.2 mL/tube) were monitored by

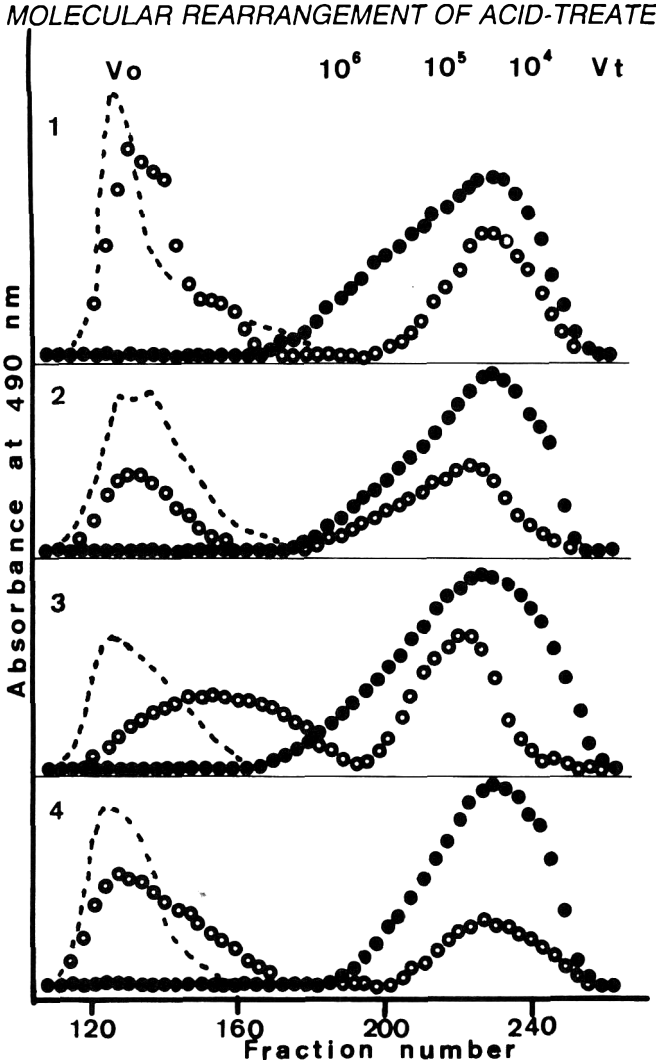

Fig. 1—SEC patterns for several acid-treated starches. Sample, 1% hydrolyzed starch (1, potato starch; 2, sago starch; 3, non-glutinous rice starch; 4, glutinous rice starch); column, Toyopearl HW-65S (1 ID × 60 cm); flow rate, 12 mL/hr; fraction, 0.2 mL/tube; detection, absorbance at 490 nm after coloring by phenol-sulfuric acid; mobile phase: ●—● 0.01M borate buffer at pH 7.0 containing 0.1M KCl: ○—○ 0.01M borate buffer at pH 7.0; temp., 20°C. --- native starch.

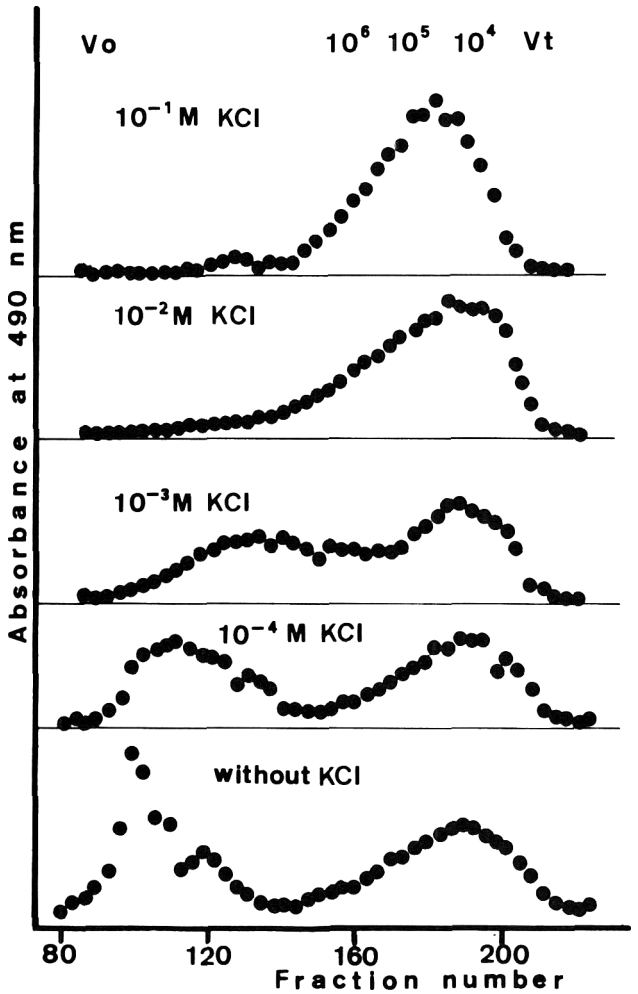

Fig. 2—Changes in the SEC pattern for P-1 of acid-treated potato starch a mobile phase with varying KCl concentration. P-1, the component eluted at the void volume of the Toyopearl HW-65S column, using water as the mobile phase. Column size, 1 × 54 cm. The other conditions are the same as Fig. 1.

the phenol-sulfuric acid (Dubois et al., 1956). SEC for the monomeric starch chains in a dissociated state was similarly performed, except for using a 0.01M borate buffer, pH 7.0, containing 0.1M potassium chloride as sample buffer and mobile phase.

Differential scanning calorimetry (DSC)

After adding water to a sample [acid-treated potato starch (potato ATS) or its components] in a 70 μL silver cell (35 μL of water/15 cm on a dry basis), DSC was conducted using a DSC apparatus (Seiko SSC-5020 DSC 100, Japan) as described by Takahashi et al. (1988). After the first run to 120°C, the heating furnace was rapidly cooled with liquid nitrogen and held at 0°C for 30 min prior to the second run. The cell was opened after DSC and the sample was dissolved in water at 120°C for 20 min. Total saccharide concentration was measured by the phenol-sulfuric acid method (Dubois et al., 1956). The transition temperature and enthalpy (ΔHg) were determined from the DSC curve.

Other determinations

The wavelength of the absorbance maximum (λmax) of a potato ATS-iodine complex was determined by the method of Bailey and Whelan (1961). The intrinsic viscosity of potato ATS was evaluated with an Ostwald viscometer at 30 ° 0.02°C. A sample solution was obtained by dissolving potato ATS in an 85% dimethylsulfoxide solution at 40°C with stirring for 2 hr under N_2 gas, centrifuging at 18,000 rpm and twice filtering the supernatant through a G-4 glass filter. The X-ray diffraction pattern of water-sorbed potato ATS (54.8% moisture, dry basis) was recorded with a diffractometer (JEOL LRX-12, Japan) operated at 40 kV and 100 mA. Crystallinity was estimated by the total peak areas measured from a straight line joining troughs.

RESULTS & DISCUSSION

Slightly acid-treated potato starch

Potato starch (PS) was immersed in 15% sulfuric acid at 20°C for 15 days to give 1% hydrolysis. Potato ATS recovered was designated as slightly acid-hydrolyzed starch as compared with Nägeli amylodextrin with about 25% hydrolysis (Kainuma and French, 1971). Potato ATS (1% hydrolysis) showed a lower λ max (585 nm) than that of native potato starch (PS; 614 nm), indicating that average chain lengths, calculated according to Bailey and Whelan (1961), were DP 58 and DP 128 for potato ATS and native PS, respectively. The intrinsic viscosity values for potato ATS and native PS were 0.1 and 3.7 g/100 mL, respectively. Thus, the slight acid-treatment considerably hydrolyzed the starch chains at acid-labile positions including the amorphous regions of starch granules.

Native PS was almost excluded from the Toyopearl HW-65S analysis (exclusion limit of a few million daltons), while potato ATS indicated the disappearance of macromolecules of a few million daltons and the appearance of a distributed peak with Mr 25,000 daltons (Fig. 1). This demonstrated remarkable cleavage of the glucosidic linkage as already described. Jackson et al. (1992) have shown that Nägeli amylodextrins pre-

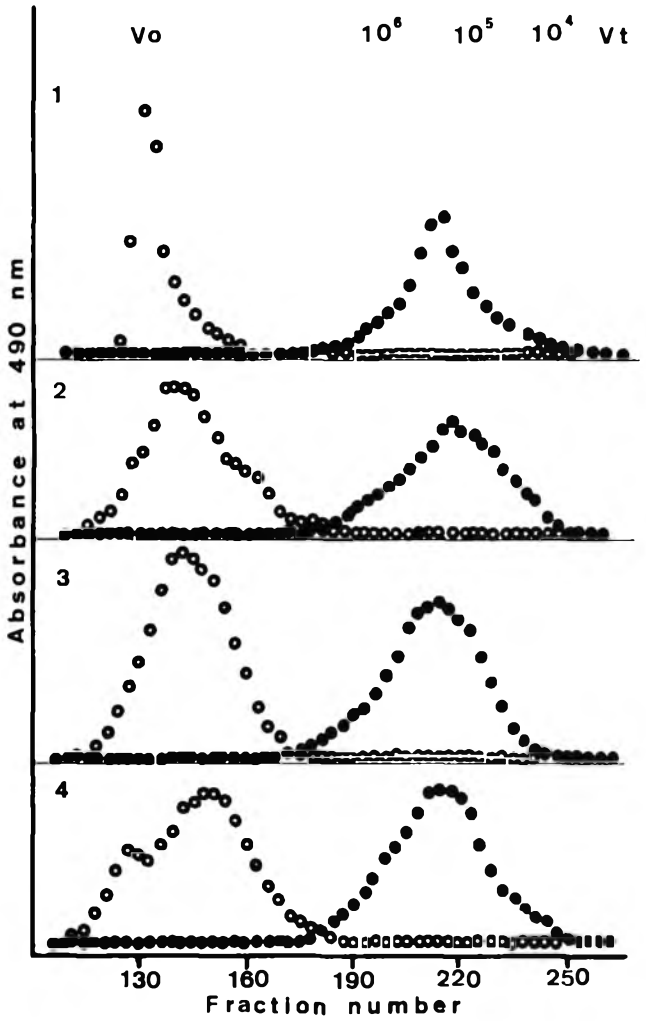

Fig. 3—SEC patterns of the major chain populations (1-4) of P-1s. 1, potato starch; 2, sago starch; 3, nonglutinous rice starch; 4, glutinous rice starch. The other conditions and symbols are the same as those shown in Fig. 1.

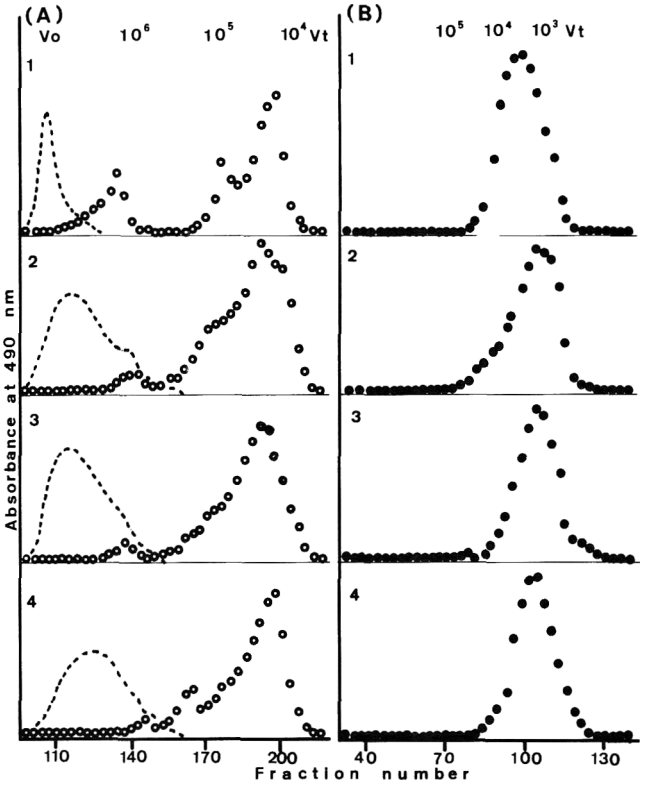

Fig. 4—SEC patterns of the major debranched chain populations. 1, potato starch; 2, sago starch; 3, nonglutinous rice starch; 4, glutinous rice starch. [A], Toyopearl HW-65S column (1 ID × 50cm); [B], Toyopearl HW-55S column (0.9 ID × 50 cm). The other conditions and symbols are the same as those shown in Fig. 1. ---- undebranched chains.

pared from corn starch gradually lost the macromolecules during an acid-treatment. However, our data cannot be directly compared, because they did not report degree of hydrolysis or product characteristics.

PS and potato ATS gave characteristic B type x-ray diffraction patterns. The crystallinity of potato ATS estimated by X-ray diffractometry was 1.2 times that of native PS, and was a little higher than that of 5% hydrolysis sample reported by Komiya and Nara (1986). Such increases indicate that the chain end region resulting from acid cleavage could be regularly rearranged as suggested by Kainuma and French (1971).

Potato ATS had a higher gelatinization temperature and enthalpy (74.9°C and 19.4 mJ/mg, respectively) than the values for native PS (62.9°C, 17.2 mJ/mg), corresponding to the increase in crystallinity. Komiya and Nara (1986) reported no essential change in enthalpy by an acid treatment. However, in their study, air-dried granules were used. Since potato ATS can easily be cracked at the hilum by air-drying (data not shown) and the cracking probably would cause decreases in crystallinity, gelatinization temperature and enthalpy of granules. Thus, we considered that the determination by Komiya and Nara could have been affected by air-drying damage.

Reversible molecular rearangement of ATS

SEC for potato ATS, which was performed by using a 0.01M borate buffer, pH 7.0, as the mobile phase, demonstrated that about half of the starch chain population was eluted at the void volume, as was the case with native PS (Fig. 1). Since potato ATS had no chains of this size, we suggest that the large aggregates could be reconstituted from the chains with MR 25,000 in the buffer solution without potassium chloride. A similar SEC pattern was obtained when water was used as mobile phase (data not shown).

SEC was, therefore, conducted by using a mobile phase with varying potassium chloride content. As the concentration of potassium chloride increased, the large aggregates that were eluted were reduced in size and completely disappeared at 0.1M (Fig. 2). Thus, in the absence of potassium chloride, about half of the chain populations apparantly associated with each other and formed large aggregates. In the presence of potassium chloride (0.1M), the aggregate dissociated to monomeric chains, indicating that reversible molecular reassembly of the starch chains could occur. Since sodium chloride and potassium chloride destabilize the starch micelle structure (Wada et al., 1979; Takahashi et al., 1980), the chloride anion might affect the structure of the starch chain in potato ATS in an aqueous solution.

SEC patterns for sago, non-glutinous rice and glutinous rice ATSs in a mobile phase with potassium chloride were compared. They showed these ATSs had no large aggregates and were also composed of chains with MR 25,000–29,000 (Fig. 1, 2-4). However, every one of those ATSs could form large aggregates in the absence of potassium chloride.

Molecular rearrangement of the major chain population of P-1

P-1s, the large aggregates appearing in the void volume, were obtained from four kinds of ATSs by SEC. These P-1s had a molecular weight (Mr) range of $1 \times 10^6 - 3{,}200$, with a peak of Mr 1.6×10^6 (Fig. 3). The major chain population ($2 - 5 \times 10^5$) of P-1 was recovered for further examination

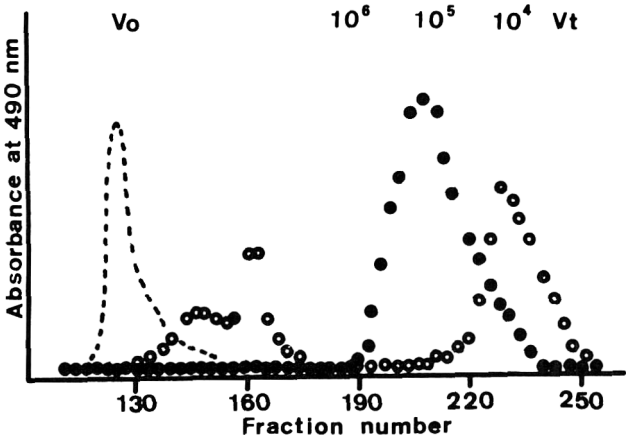

Fig. 5—SEC pattern of β-limit dextrin for the major chain populations of potato starch. Conditions and symbols are given in Fig. 1. ---- without β-amylolysis.

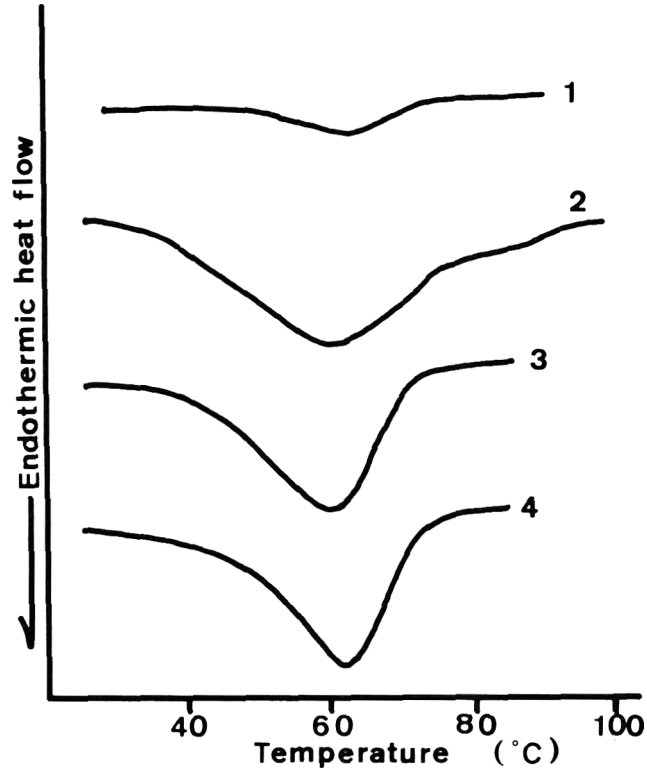

Fig. 6—DSC curves of acid-treated potato starch and its components from the second run. 1, native starch; 2, acid-treated starch; 3, P-1; 4, major chain populations. DSC, Seiko SSC-5020 DSC 100; heating rate, 2 K/min; sample cell, Ag; atmosphere, He at 40 mL/min; reference, water.

Table 1—Transition temperature and enthalpy values* for acid-treated potato starch and components

	Transition temp (°C)			
	T_o	T_p	T_c	ΔH (mJ/mg)
Gelatinized starch	46.8	62.8	72.9	0.6
Acid-treated starch	37.1	58.9	76.4	3.7
P-1	39.1	59.9	70.5	2.2
Major chain population	43.8	60.9	71.8	1.5

* Mean value from duplicate experiments. T_o, onset temp.; T_p, peak temp.; T_c, conclusion temp; ΔH, enthalpy.

of reversible molecular rearrangement. The major chain populations of potato, sago, non-glutinous rice and glutinous rice ATS P-1s showed Mr values of 2.8×10^5, 2.8×10^5, 3.2×10^5 and 3.5×10^5 on SEC patterns in the dissociated state, respectively (Fig. 3). SEC analysis for the associated state proved that the major chain populations could entirely reconstitute the large aggregates. That is, complete molecular reassembly resulted as compared with that of ATS.

Molecular rearrangement of debranched chains of β-limit dextrin

Four kinds of major chain populations were debranched with isoamylase. SEC for the products indicated that the chains were composed of unit chains with about Mr 2,000–2,400, except for about Mr 5,500 for potato P-1 (Fig. 4-B). The relatively large Mr value for potato P-1 may be due to incomplete isoamylase digestion. This was strongly supported by the fact that the average chain length of potato amylopectin was 22.9 (Hizukuri et al., 1983). These debranched chains could not reconstitute the large aggregates like the undebranched samples could (Fig. 4-A). Therefore, this strongly suggests a branched structure must be essential for molecular rearrangement.

The β-amylolysis limits for potato P-1 and its major chains were 45% and 40%, respectively, much lower than 63% for native potato starch (Watanabe et al., 1982). This result also suggested that the reversibly associable chain had a considerably branched structure like that of amylopectin. β-limit dextrin of the major chain population showed Mr of about 100,000 in the dissociated state (Fig. 5). However, about half of the β-limit dextrin could reconstitute large aggregate with a slightly smaller size compared with the major chain populations before β-amylolysis. This also suggested that the branched structure was important for reversible molecular rearrangemen.

Thermal transition behavior of aggregates

DSC curves from previously melted potato ATS, P-1, and its major chain population (second run) showed a clear endothermic peak in a temperature range from about 37°C to 76°C (Fig. 6). This corresponded to the remelting temperature region of the ordered structure reconstituted during retrogradation of native PS (Nakazawa et al. 1985). Since no endothermic peak was observed in 1.0 M guanidine hydrochloride (data not shown), the reconstituted ordered structure was probably mainly reconstituted by hydrogen bonding. Native PS gave only a weak endothermic peak because the retrogradation time was too short (30 min). The major chain populations had a sharper transition due to greater homogeneity in molecular weight distribution when compared with the others (Table 1), while enthalpy values were independent of molecular distribution.

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Volume Expansion during Hot Air Puffing of a Fat-free Starch-Based Snack

H.S. GURAYA and R.T. TOLEDO

ABSTRACT

Puffed volume and puffing uniformity of a fat-free snack were investigated. Strips of drum-dried sweet potato-tapioca pastes were puffed 15 sec at 200°C in a fluidized bed. Puffed product specific volume (SV) was related to starch gelatinization, soluble solids content, initial moisture and method of half-product preparation. SV was maximum (490% increase) and most uniform (3.94% coef. of variation) when 70% moisture paste was drum-dried at 100°C and 1 rpm to produce the half-product. Starch was 93% gelatinized in this process. Soluble solids reduced puffing by reducing starch gelatinization. Half-product produced by sheeting dough containing pregelatinized starch had puffed volumes which significantly correlated with starch gelatinization.

Key Words: starch, drum-drying, tapioca, sweet potato, puff-drying

INTRODUCTION

SNACK PRODUCTS CONSTITUTE one of the fastest growing segment of the food industry. Hazera's (1983) data showed that fat in the U.S. diet was distributed 60% from meats and vegetables and 40% from salad oils, cooking oils, frying and baking fats, butter and margarine. Frying and baking fats was consumed at 11 pounds per capita, or 15% of total dietary fat. Crispy snack products generally contain about 20–40% fat. Sliced frozen potatoes and potato products mostly fried, are produced at ca. 2.3 million metric tons/yr in the U.S. (Anonymous, 1987). An intermediate dried material, produced by shaping and drying pregelatinized moistened starches, which is later puffed into a crispy snack food is called a half-product. Frying transforms it into a low density crisp product. Half products are produced in different shapes and texture using cooking extruders equipped with a cooled die. An original half-product (arare) was produced in the Orient from rice. The arare process (used in Taiwan) involves rolling pregelatinized rice dough into sheets, stamping out esired shapes and hot air drying. Such dry half-products may be stored for later use, or puffed immediately by frying or baking.

Swientak (1987) described a process for extruded half-products formulated with modified starches plus wheat flour, corn flour, potato solids and salt. The 10–12% moisture half product puffed in hot air at 300°C for 10–20 sec. Clausi (1969) described puffing of 21% moisture case-hardened pellets for breakfast cereals in high velocity hot air at 400°C for 8–35 sec. Half-product puffed by frying, baking and microwaving has been produced by extruding corn reduction flour (Wilkinson and Short, 1989). Other extrusion processes for half-product manufacturing which utilized partially or completely gelatinized mixtures of raw materials have been reported (Bretch, 1972; Ruegg and Slovak, 1989; Popel, 1974).

Drum drying may also be used for making a half-product. Although processing cost for expanded snack products may be lower with extrusion than with drum drying, cost may be offset by better nutritional and textural quality of the drum dried product. Significantly higher biological value and nitrogen protein utilization resulted from drum drying flour flakes compared to extrusion (Hakansson et al., 1987). In addition, losses of essential amino acids were significantly higher in extrusion processed corn-soy-milk and mixtures of corn-soy-lactose-hydrolyzed whey (Aguilera and Kosikowski, 1978) than when they were drum dried. Half-products must have a nonporous structure to expand on puffing; therefore, on extrusion, a cooled die is usec to prevent expansion. The subsequent drying step would require substantial moisture reduction; therefore, there would be no major energy savings in this process over drum drying.

Puffing occurs on flash evaporation of water due to product exposure to high temperature and/or sudden drop in pressure. For starchy half-products, the most important change during such processing is starch gelatinization which is time, temperature and moisture dependent (Lund, 1984). Expansion during extrusion was directly related to degree of gelatinization (DG) of starch. Case et al. (1992) reported that maximum puffing occurred when DG ≥ 75% for wheat starch, wheat flour and corn meal while corn starch required higher DG. Bhattacharya and Hanna (1987) reported that more expansion occurred at higher extrusion temperatures where pressure and DG of starch were high.

Sugars particularly dissacharides, have raised starch gelatinization temperatures (Spies and Hoseney, 1982). Sugars increase temperatures of gelatinization by limiting water availability to the starch granule, lowering water activity, forming bridges between starch chains, or by counteracting the plasticizing action of water. The specific mechanism remains unclear since the effects have not been modeled independently.

For fat-free crispy snack foods, commercial formulations and processes are limited. Fruit and vegetable based formulations could provide essential dietary micronutrients and create attractive colors, but such options have hardly been explored. We selected sweet potato as a primary contributor of color and flavor and tapioca flour for its blandness and crispiness effects, as a base formulation for a crisp snack product. A major impediment to vegetable based fat-free crisp snacks is uniformity of puffing and adequate flavor development. Our major objective was to evaluate the effects of sugar, method of half-product preparation and starch DG on half-product puffed volume.

MATERIALS & METHODS

Preparation of dry sweet potato-tapioca half-product

Jumbo (ca. 12 cm diam) sweet potatoes (cv. Red Jewel) were obtained from our local producer. Following washing, hand peeling to completely remove blemished flesh and slicing (ca. 1 cm thick), cooking was done in atmospheric steam for 20 min. followed by cooling 1 hr at ambient. Cocled slices at 76.5% moisture were pureed in a Waring blender and packed in Ziploc® (Dow Chemical Co.) bags to prevent further loss of moisture while storing at 4°C until use.

When paste moisture was < 70%, dry sweet potato powder was added. The powder was made by dehydrating raw sweet potato dices. Washed and peeled raw sweet potatoes were diced into 1 cm cubes using a portable dicer, and dried 1 hr at 80°C in a tunnel drier followed by grinding in a Wiley mill through 0.5 mm screen. Flour with 3.5% moisture was stored at room temperature (≈23°C) in Ziploc® bags until used. Enough tapioca flour for the study (9.9 kg) was purchased at one

Authors Guraya and Toledo are affiliated with the Dept. of Food Science & Technology, The University of Georgia, Athens, GA 30602. Address inquiries to Dr. R. Toledo.

Table 1—Effect of speed (S) and surface treatment (T) of drum dryer and initial moisture (M) of paste and degree of starch gelatinization on Specific Volume (SV) increase of puffed half product

No.	Treatment[a]	Mean SV(%)[b]	COV (%)[c]	DG (%)[d]
1	1:100:70	533a	3.94	86
1	1:100:60	488ab	27.3	76
3	6:100:70	399bc	42.06	67
4	11:110:60	393bcd	5.41	57
5	6:110:70	391bcd	6.11	92
6	1:120:60	369bcde	31.45	71
7	1:110:80	364cdef	16.41	74
8	6:120:60	363cdef	19.01	79
9	11:110:70	350cdefg	13.10	68
10	11:100:60	348cdefgh	45.93	56
11	1:100:80	325cdefghi	8.15	57
12	6:100:60	297cdefghij	30.59	60
13	6:110:70	273defghijk	22.01	92
14	11:110:80	269defghijk	31.45	74
15	6:100:80	261efghijk	26.08	86
16	6:110:80	248efghijk	22.54	95
17	1:110:70	244fghijk	5.45	86
18	1:110:60	233ghijk	18.57	71
19	11:120:80	225hijk	41.07	76
20	11:100:80	216ijk	37.38	69
21	11:120:60	213ijk	27.39	65
22	6:110:60	211ijk	20.23	77
23	11:100:70	202ijk	32.49	63
24	6:120:60	195jk	23.54	79
25	11:120:70	183jk	11.19	89
26	1:120:70	177jk	19.65	86
27	1:120:80	154k	35.57	85

[a] Treatments are drum speed (rpm) : temperature (°C) : initial moisture (%).
[b] Means followed by same letter are not significantly different at $p<0.05$
[c] Coefficient of variation.
[d] Degree of gelatinization.

time from a local grocery and thoroughly mixed to ensure homogeneity. Moisture content (vacuum oven) was 10.33%.

Each batch consisted of paste with different initial moisture level made by mixing sweet potato puree containing 100g sweet potato solids, 100g dry tapioca flour and water. Dry sweet potato flour was added as part of the sweet potato solids instead of water to prepare products of moisture < 70%. The flour was blended with the cooked sweet potato puree and heated 20 min in flowing atmospheric steam to inactivate indigenous amylases.

An atmospheric double drum dryer was used, consisting of counter-rotating 21.64 cm diam, 29.0 cm long drums with end plates. Drum surface temperture was set using a controlled temperature steam supply and measured with an infrared pyrometer (Model RAYR2CFSC1, Omega). When temperature stabilized, drum clearance was set at 0.279 mm, drum speed (rpm) was set, and paste was applied.

All paste for each batch was applied to the drums at one time. It was not possible to completely dehydrate a thick layer of paste on the drum in one rotation; therefore, paste was deposited from the pool in the nip between the two drums as a thin layer on the drum surface with each rotation of the drum. The high moisture undeposited paste continued to receive heat from the heated drums gelatinizing the raw tapioca starch. the thin layer deposited on the drum lost moisture as the drum completed its revolution and received a fresh thin coating of wet paste as the cycle was repeated. Thus the sheet was formed by successive deposition of thin layers of paste. This continued until wet paste was no longer visible on the drum surface. The doctor blades were lowered one at a time on each drum to remove the sheet of dried material. While still warm and pliable, the sheets were cut into 1×1 cm strips, dried 2 hr at 80°C in a tunnel drier, and stored in plastic Ziploc® bags at ambient temperature until puffed. This half-product was stable against rancidity development at least 1 yr at ambient temperature (≈23°C).

Treatment variables were: initial moisture content (60, 70, 80%), drum speed (1, 6, and 11 rpm), and surface temperature (100, 110, and 120°C). Starch in the sweet potato was 100% gelatinized. Raw tapioca starch was mixed with the cooked sweet potato puree, and DG of starch in the dried half-product varied with treatment variables. DG was determined using the procedure of Guraya and Toledo (1992).

Puffing and determination of specific volume

Per cent increase in specific volume (SV) was used as an index of puffing. Half-product was equilibrated at least 7 days in an environmental chamber at 54% RH prior to the test. Water activity was measured (Beckman Hygroline with NovaSina sensor) to ensure a_w was 0.54 before puffing 15 sec at 200°C in a HTFB dryer (Kim and Toledo, 1990). Puffed samples were immediately stored in a desiccator over $CaCl_2$. A pycnometer (Fisher Scientific Co.) was used to measure volume by mercury displacement.

Preparation of half-product by making sheets from dough

Dough containing a cooked mixture of sweet potato puree and dried sweet potato flour and specified proportions of raw and gelatinized tapioca (1:1 total sweet potato solids:tapioca solids) was prepared with 60% moisture. After thorough mixing and equilibrating 48 hr under refrigeration, the ball of dough was flattened by hand, positioned between two layers of 2 mil polyethylene film and rolled into a sheet by passing between (0.533 mm clearance) the two rolls of an unheated double drum dryer. The sheet of dough was transferred to a tray, the top film peeled off, and the sheet was scored with a sharp knife into squares. The sheet was then dried in a tunnel drier at 55°C until it was firm but pliable. The bottom film was then removed, the rectangular pieces separated and drying was continued for another 5 hr. The half-product was equilibrated 2 days at 60% RH. Half-products containing 0, 61, 74, 87 and 100% total starch DG were prepared. Powder containing 100% gelatinized tapioca was made by boiling for 20 min a suspension of 20% raw tapioca powder in water. After transferring to a pan to form about a 2 mm thick layer, the gelatinized paste was dried in a tunnel dryer at 55°C. The dry starch film was shredded by hand and ground in a Wiley mill through a 0.5 mm screen.

Effect of soluble solids on puffing

Sweet potato exhibits wide variability in soluble solids contents depending upon cultivar, curing and storage conditions (Pischa, 1985). To simulate the effects of variations in soluble solids on starch DG and half-product puffing, half product containing added sugar was prepared by drum drying. Half-products containing 100% gelatinized sweet potato and tapioca starch and 0, 5, or 10% (dry weight basis) of a 1:1 mixture of dry sucrose and maltose were also prepared using the dough sheeting procedure described.

Effect of moisture content of half product on puffing

Half-product equilibrated to a_w of 0.54 or vacuum oven dried to constant weight were puffed in HTFB 15 sec at 200°C and evaluated for specific volume.

RESULTS & DISCUSSION

Degree of starch gelatinization and puffing of drum drier prepared half-product

Expansion during puffing of half-product prepared by drum drying depended on factors other than starch DG. No correlation occurred between values of DG in the half-product and SV increase (Table 1) on puffing. The samples had DG from 78 to 98% of total starch and SV increased on puffing from 150 to 533%. Product which exhibited maximum and most uniform puffing was produced at 70% initial moisture and the lowest speed and temperature (Treatment 1, Table 1). The slow and uniform deposition of gelatinized paste during sheet formation seems to have an important influence on the puffing characteristics of the half-product. At lower speeds raw starch in the paste gelatinized when heated by the drums before deposition. The deposited thin layer was dry upon completion of a drum rotation and before another layer of paste was deposited in the next rotation. At higher drum speeds starch in the paste deposited early on the drums had low starch DG and dried quickly therefore little additional gelatinization occurred on the drum surface. The undeposited paste on the other hand was not heated sufficiently to gelatinize because of the short exposure time to heat at high rotation speeds. The deposited layers would have varying starch DG with each layer which could explain the high coefficient of variation in SV of puffed product prepared at high drum speeds (Table 1). Blends with initial moisture content of ≤ 70% had the consistency of dough

while at higher moisture content they were more pasty. Homogeneity of puffing is important for a snack product so therefore coefficient of variation of SV should be minimized. The ANOVA (Table 2) showed that initial moisture, temperature and speed were significant factors affecting SV of puffed drum dryer prepared half-product. Interactions were also significant.

Degree of gelatinization and puffing of half-product prepared by rolling sheets from dough

Volume increase on puffing of half product was proportional to starch DG. Results of triplicate tests showed that increase in specific volume was >1%, 29%, 56%, 70% and 121% when starch DG in the half product was 0, 62.5%, 75%, 87.5% and 100%, respectively. Since all sheets were prepared the same, and no heat was applied making the sheets, only the starch DG varied in those products. The increase in SV was linear with starch DG ($r^2 = 0.96$).

Soluble solids, starch DG and puffing

Sucrose in sweet potatoes may increase during storage from 2.11% to 4.72% of dry solids, in 4 mo. Maltose formed by amylase induced breakdown of starch during cooking decreased as sweet potato roots were stored longer (Walter and Hoover, 1983). Total sugars (glucose, fructose, sucrose and maltose) in baked sweet potato (cv. Jewel) have been reported at 14.02% (Picha, 1985). Soluble solids of sweet potatoes affected starch DG and SV increase on puffing of drum dryer prepared half-product. The sweet potatoes were the same cultivar (Jewel) but the batch containing 16.4% soluble solids had 84% starch DG and 230% SV increase on puffing compared to 92.5% and 498% for the batch containing 11% soluble solids. When the paste soluble solids was adjusted from 11.0% to 16.4% by sucrose addition prior to drum drying, starch DG was 80% and SV increase on puffing was 186%. However, when the half-product was prepared with 92.5% starch DG using the dough sheeting procedure, SV increase on puffing was 70% and did not change with sucrose addition. Thus, soluble solids affected starch DG during exposure to heat in the drum dryer and ultimately affected half-product puffing. On visual examination, the drum drier processed half-product appeared translucent and had a shiny tightly packed surface while that prepared by dough sheeting had a slightly powdery surface, appeared more opaque and porous. The half product with reduced porosity appeared to expand more on puffing.

Half-product moisture and puffing

Puffing appeared to occur as a result of flash vaporization of water when the half product was exposed to high temperature. SV increases were 311% and 97% when half product moistures were initially 6.06% and 0.01%, respectively. Moisture content dropped from 6.06 to 5.89% on puffing, indicating that only a small amount of moisture release was responsible for the increase in volume.

CONCLUSIONS

HALF-PRODUCT MADE BY DRUM DRYING sweet potato-tapioca mix puffed uniformly in HTFB dryer at 200°C for 15 sec.

Table 2—ANOVA for percent increase in specific volume

Source	DF	SS
Moisture (M)	2	89726.25**
Linear (M)	1	696222.8**
Temperature (T)	2	148748.5**
Linear (T)	1	1326381**
Speed (S)	2	39592.72*
Linear (S)	1	355377.4**
M×T	4	88258.06**
T×S	4	173843.4**
M×S	4	48040.62*
M×T×S	8	160321.2**
Error	54	300700.6
Total	80	1042231

* Significant at $p<0.05$
** Significant $p<0.01$

Specific volume increase on puffing was related primarily to degree of starch gelatinization. Restricted transmission of water vapor while exposed to the HTFB ultimately determined the increase in specific volume. Puffing related highly to half-product method of preparation and not only composition. When prepared by drum drying, half-product with highest puffing resulted from paste with low soluble solids, initial moisture of 70% drum temperature 100°C and slow drum rotation. A half-product moisture content of about 6% was needed to expand the product fully in a HTFB.

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Potato Starch Paste Behavior as Related to Some Physical/ Chemical Properties

DENNIS P. WIESENBORN, PAUL H. ORR, HOWARD H. CASPER, and BETH K. TACKE

ABSTRACT

Potato starch with anomalous stable (constant with time) paste viscosity has been proposed as a natural replacement for cross-linked starch, but its behavior and the influencing factors are not well understood. Starch from 44 samples of potato tubers representing 34 genotypes was analyzed for phosphorus, mean granule diameter, amylose, calcium, magnesium, sodium, and potassium. Except for granule diameter, sodium, and potassium, each of those properties correlated significantly with one or more of five paste characteristics determined using the Brabender Visco-amylograph. Three samples had a stable viscosity in 1 yr of the study, but their behavior was not associated with unusual values of the other measured properties. The stable viscosity is probably a heritable trait influenced by cultural and climatic factors.

Key Words: potato, starch, paste, viscosity

INTRODUCTION

POTATO STARCH is used to thicken foods such as soups and sauces. Unmodified potato starch has an exceptionally high cooked viscosity per dry weight of starch, partly attributed to its high content of starch phosphate esters. Gelatinized starch granules, however, are readily disrupted by shear during conveying and mixing operations, resulting in greatly reduced viscosity. A typical method of characterizing starch paste viscosity is the Brabender Visco-amylograph test, in which viscosity is plotted versus time during a standard cycle of heating with continuous stirring. Unmodified potato starch yields a distinct peak viscosity on the amylograph plot (Fig. 1). Initially, the starch granules swell, resulting in a rise in viscosity; however, stirring causes swollen granules to disintegrate, leading to subsequent decrease in viscosity (de Willigen, 1976a). Paste viscosity that is relatively constant with time, (a "stable" paste viscosity) can be achieved by chemical modification in which constituent molecules are covalently cross-linked.

Chemical modification of starch is often needed to tailor functional characteristics to desired applications. Modifications are performed to achieve, under conditions of high temperature, high shear, freeze-thaw cycles, or acid pH, a desired texture (McCormick, 1985). One alternative to chemical modification is to identify sources of native starch with desired functionality (Duxbury, 1989). This may be accomplished by screening crop germplasm collections, and by breeding and selecting desired genotypes or applying recombinant DNA transfer techniques. The resultant ingredient may appeal to consumers as being more "natural." Also, this approach might identify starch with unique characteristics for possible new uses and markets.

McComber et al. (1988) studied paste characteristics of starch from four potato genotypes. One red-skinned genotype, (Red) Pontiac, yielded an atypical stable paste viscosity. This suggested that starch from such tubers might be a natural replacement for synthetically cross-linked starch (McComber and Osman, 1991).

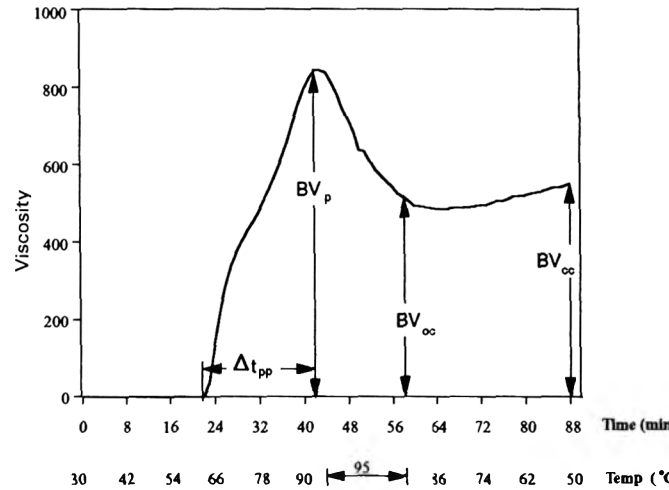

Fig. 1—Typical amylograph test results for potato starch. Viscosity is in Brabender Units (BU). Δt_{pp} denotes the peak-paste time, or time elapsed from initiation of pasting to peak viscosity. BV_p, BV_{oc}, and BV_{cc} denote viscosity at the peak, at the onset of cooling, and at the completion of cooling, respectively.

The stable paste viscosity of the Red Pontiac starch might have resulted from soluble impurities, or degradation of the starch during processing, such as exposure to excessive temperatures during drying (Meiss et al., 1944). A critical level of divalent cations (e.g., magnesium or calcium) during the amylograph test has sharply suppressed paste viscosity, probably by ionically cross-linking starch phosphate esters (de Willigen, 1964). Heat-and-moisture tretment and annealing have led to amylograph plots similar to that reported for Red Pontiac (Stute, 1992).

McComber et al. (1988) washed starch thoroughly with distilled water and dried it at low temperature (McComber, personal communication, 1990). All such starch samples were processed under similar conditions, but only Red Pontiac yielded the atypical amylograph plot. Nevertheless, possibly the results had been influenced by ionic effects. Starch extracted from potatoes with distilled water may have enough magnesium to influence paste characteristics (de Willigen, 1964).

Paste characteristics might also be affected by the amylose: amylopectin ratio, starch phosphate esters, granule diameter distribution, or other physical/chemical properties. For example, a positive correlation was found between starch phosphate and maximum paste viscosity (Veselovsky, 1940). To some extent, such characteristics vary with genotype and cultural practices (Leszczynski, 1989; de Willigen, 1976a; Barichello and Yada, 1991).

Our objective was to study selected factors which might relate to the novel paste characteristics of Red Pontiac starch, and the reproducibility and uniqueness of such paste charac-

Author Wiesenborn is affiliated with the Agricultural Engineer& Fooding Dept. and Cereal Science Dept., North Dakota State Univ., Fargo, ND 58105-5626. Author Orr is with the USDA Agricultural Research Service Potato Research Laboratory, East Grand Forks, MN 56721. Authors Casper and Tacke are with the Veterinary Science and Microbiology Dept., North Dakota State Univ., Fargo, ND 58105.

teristics. Starch from samples of several genotypes of potato tubers was extracted, characterized and compared.

MATERIALS & METHODS

Source of tubers

Almost all potato tubers (*Solanum tuberosum L*) were grown under dryland conditions (no irrigation) in the Red River Valley of Minnesota and North Dakota, primarily at Red River Valley Potato Research Farm, Grand Forks, ND in the 1989 and 1990 growing seasons. Exceptions were Triumph and Lemhi tubers obtained from the 1989 growing season at Beltsville, MD. One lot of Red Pontiac tubers grown in 1990 was used to prepare 4 samples of starch identified as Red Pontiac (90)[a], (90)[b], (90)[c], and (90)[d]. Tubers were stored 4 to 32 wk at ~4°C until processed for starch.

Starch extraction, purification, and ion exchange

Water for extraction was tap water, except distilled and deionized water where noted. Tubers (2.5 kg total wt) were rinsed in water to remove dirt, peeled and cut into 2–3 cm cubes. They were macerated in 400g lots at low speed (Blendor 7010 Model 31BL91, Waring, New Hartford, CT) in 400 mL water for 45 sec, then combined with like macerates. Each mixture was allowed to stand 20 min, then the liquid and remaining suspended solids were decanted away from the sediment and macerated for an additional 45 sec. This remacerated material was added back to the sediment, and allowed to stand at room temperature (~23°C) for 40 min. The liquid was decanted from sediment and discarded.

The sediment was suspended in 500 mL water and passed through a U.S. No. 20 sieve. The material retained by the sieve was washed on the sieve with 200–300 mL water, resuspended in 400 mL water, macerated for 45 s, then again passed through a U.S. No. 20 sieve. Solids retained on the sieve were rinsed on the sieve with 1–2 L water then discarded. Starch in the filtrate and rinse water was allowed to settle 30-45 min, and the liquid decanted off and discarded. The starch was then resuspended in 1L water and passed through a U.S. No. 60 sieve. Retained solids were rinsed on the sieve with 1–2 L water and discarded. Starch in the filtrate and wash water was allowed to settle 30-45 min and liquid decanted. Starch was resuspended in 500 mL water and settling and decanting steps repeated once without sieving.

Except where noted, the starch was converted to sodium form using a modification of the procedure described by de Willigen (1964). The starch was twice suspended in 0.1N HCl (2 mL/g wet weight of starch) for 1 hr and filtered under vacuum. The starch was immediately rinsed 3 times with deionized water (4 mL/g wet wt of starch), and the suspension adjusted to pH 7.8 with 0.05N NaOH during 30–60 min.

The suspension was vacuum filtered, and the starch dried to constant weight in a convection oven at 35–40°C for 1–3 days then ground with a mortar and pestle to pass through a U.S. No. 60 sieve. The ground starch was spread 1 cm deep on a tray open to ambient temperature (23–24°C) and humidity (25–60% RH) for 2–3 days prior to transfer to air-tight storage containers. Samples were stored at 4°C.

Moisture, amylose, phosphorus and cation analysis

Moisture content was determined in triplicate using a modified form of AACC Method 44-40 (AACC, 1983). Samples (2g) were weighed in aluminum pans before and after drying at 95°C and 710–740 mm Hg vacuum for 12 hr or more. Samples were removed from the oven and immediately placed in a dessicator and weighed to the nearest 0.1 mg, within 20 min.

Amylose content was determined in triplicate using the colorimetric method and formula of Williams et al. (1970).

Phosphorus was determined in duplicate using Method B-47 (Corn Industries Foundation, 1985), with samples ≤0.50g of starch each.

Calcium, magnesium, potassium and sodium contents were determined by atomic absorption spectrophotometry (Model 603, Perkin Elmer with air/acetylene flame) at 422, 286, 766, and 590 nm, respectively. Briefly, starch (2.5g) in a 100 mL beaker was ashed overnight in a muffle furnace at 475°C. The ash was refluxed in 5 mL 3N HCl containing 1% (w/v) lanthanum for 1 hr. The solution was brought to 10 mL with 1% lanthanum and serially diluted twice. Standards were prepared by diluting 1000 ppm standards (Ricca Chemical Co., Arlington, TX). All dilutions and standards were in 3N HCl containing 1% (w/v) lanthanum. This method was validated by triplicate analysis of one starch sample (potato genotype 13653).

Table 1—Analysis of potato starch (Samples were in the sodium form except where noted)

Genotype (Year grown)	Phosphorus (ppm)	Mean granule diam (microns)	Amylose (%)
Agassiz (89)	830 ± 2	49.9 ± 1.3	27.8 ± 0.7
Atlantic (89)	950 ± 4	50.3 ± 1.2	27.3 ± 0.3
Bintji (90)	745 ± 1	42.9 ± 1.8	25.9 ± 0.1
Dark Red Norland (89)	860 ± 1	42.7 ± 0.9	27.4 ± 0.1
Erik (89)	815 ± 4	44.4 ± 1.9	27.4 ± 0.5
Gemchip (89)	765 ± 1	46.4 ± 0.5	26.6 ± 0.2
Katahdin (89)	895 ± 5	49.4 ± 0.7	25.8 ± 0.2
Katahdin (90)	850 ± 1	49.3 ± 5.1	26.0 ± 0.1
Kennebec (89)	659 ± 1	41.9 ± 3.1	23.9 ± 0.1
Krantz (89)	804 ± 8	42.9 ± 1.4	24.3 ± 0.2
Lemhi (89)	691 ± 5	53.8 ± 4.5	29.0 ± 0.1
Monona (89)	773 ± 4	43.4 ± 0.9	31.0 ± 0.5
Norchip (90)	738 ± 4	53.1 ± 1.7	27.4 ± 0.1
Norgold Russet (89)	609 ± 1	46.3 ± 2.4	23.6 ± 1.4
Red LaSoda (89)	955 ± 8	43.6 ± 2.4	24.8 ± 0.8
Red LaSoda (90)	915 ± 2	46.9 ± 0.7	23.5 ± 0.1
Red Norland (90)	797 ± 11	44.7 ± 3.1	24.5 ± 0.1
Red Pontiac (89)	831 ± 4	53.5 ± 0.5	26.4 ± 0.0
Red Pontiac (90)[a]	672 ± 6	49.5 ± 3.3	29.2 ± 0.1
Red Pontiac (90)[b]	677 ± 1	n.a.[c]	27.9 ± 0.1
Reddale (89)	1031 ± 2	43.3 ± 0.2	23.5 ± 0.3
Reddale (90)	848 ± 1	48.3 ± 2.5	30.0 ± 0.1
Redsen (90)	738 ± 2	48.2 ± 3.9	26.5 ± 0.1
Russet Burbank (89)	765 ± 1	52.3 ± 0.6	23.4 ± 0.6
Russet Burbank (90)	644 ± 2	53.3 ± 0.2	26.1 ± 0.1
Russet Norkota (89)	794 ± 4	49.6 ± 1.4	27.3 ± 0.4
Russet Norkota (90)	693 ± 7	47.8 ± 2.5	25.8 ± 0.1
Saginaw Gold (89)	745 ± 2	49.7 ± 1.0	26.6 ± 0.5
Spartan Pearl (89)	813 ± 2	42.1 ± 2.1	26.2 ± 0.1
Tolaas (89)	943 ± 4	38.7 ± 2.3	25.0 ± 0.5
Triumph (89)	781 ± 3	n.a.[c]	26.3 ± 0.2
Triumph (90)	744 ± 6	39.9 ± 2.4	26.0 ± 0.1
MS718-15 (89)	884 ± 2	45.1 ± 1.3	27.0 ± 0.1
ND 860-2 (89)	662 ± 4	42.1 ± 1.5	24.1 ± 0.2
ND 1538-1Russ (89)	960 ± 2	46.6 ± 1.7	29.7 ± 0.1
ND2008-2 (89)	934 ± 3	42.3 ± 0.7	29.2 ± 0.1
12567 (89)	849 ± 2	43.5 ± 1.6	23.5 ± 0.2
12823 (89)	837 ± 2	47.3 ± 3.5	28.9 ± 2
13035 (89)	848 ± 6	46.3 ± 0.7	28.8 ± 0.6
13420 (89)	918 ± 1	42.5 ± 0.2	26.9 ± 0.2
13653 (89)	849 ± 2	44.6 ± 1.2	25.6 ± 0.1
mean	803	46.4	26.5
S.D.	105	4.0	2.0
Nonsodium starches			
La Belle (89)	609 ± 1	40.8 ± 1.1	26.2 ± 0.1
Red Pontiac (90)[c]	722 ± 2	51.4 ± 2.8	28.7 ± 0.1
Red Pontiac (90)[d]	669 ± 2	n.a.[c]	27.7 ± 0.1

[a-d] Separate preparations of starch from a common lot of Red Pontiac tubers grown in 1990.

[c] n.a. sample was not analyzed.

Mean ± SD of duplicate phosphorus or triplicate diameter and amylose samples.

Starch granule diameter

Granule diameter distribution was determined in triplicate by laser light scattering (Microtrac Particle Size Analyzer Model 7991-01, Leeds & Northrup, Largo, FL). Analysis was performed by recirculating a suspension of 0.60g starch in 200 mL isopropanol, with retention time 2.0 min. Mean diameter based on volume distribution is reported.

Starch pasting characteristics

The paste characteristics of potato starch samples were determined within 8 wk of extraction using a Brabender Visco-Amylograph Type VA-1B with a 700 cmg cartridge. Suspensions of 3.25% starch (dry wt basis) in distilled water were heated from 30° to 95°C at 1.5°C/min, held at 95°C 15 min, then cooled to 50°C at 1.5°C/min (McComber et al., 1988). Paste temperature was defined as that temperature at which increase in viscosity was first detected. Peak-paste time was defined as that time elapsed from initiation of pasting to detection of peak paste viscosity (Δt_{pp}, Fig. 1). Paste stability ratio was defined as the ratio of viscosity at onset of cooling (BV_∞) to peak viscosity prior to cooling (BV_p). Paste setback ratio was defined as the ratio of viscosity at completion of cooling (BV_α) to viscosity at onset of cooling (BV_∞).

RESULTS & DISCUSSION

Composition and mean granule diameter

Relatively wide variation was observed in phosphorus contents (S.D./mean = 0.13 for all sodium starch samples, Table

Table 2—Atomic absorption analysis of potato starch for select cations

Genotype (Year grown)	Calcium (ppm)	Magnesium (ppm)	Potassium (ppm)	Sodium (ppm)
Agassiz (89)	4	<1	<1	583
Atlantic (89)	<1	<1	<1	565
Bintji (90)	19	5	4	747
Dark Red Norland (89)	21	<1	2	788
Erik (89)	2	<1	<1	431
Gemchip (89)	<1	<1	1	584
Katahdin (89)	31	19	48	434
Katahdin (90)	28	9	2	808
Kennebec (89)	10	<1	<1	422
Krantz (89)	11	<1	<1	607
Lemhi (89)	20	6	3	656
Monona (89)	<1	<1	2	579
Norchip (90)	15	5	5	747
Norgold Russet (89)	<1	<1	<1	393
Red LaSoda (89)	4	<1	7	848
Red LaSoda (90)	143	9	10	1059
Red Norland (90)	20	7	4	774
Red Pontiac (89)	17	3	9	498
Red Pontiac (90)[a]	18	5	2	650
Red Pontiac (90)[b]	2	2	24	449
Reddale (89)	<1	<1	4	600
Reddale (90)	34	10	3	888
Redsen (90)	25	8	3	697
Russet Burbank (89)	<1	<1	3	355
Russet Burbank (90)	4	2	<1	592
Russet Norkota (89)	7	<1	4	418
Russet Norkota (90)	17	5	2	659
Saginaw Gold (89)	<1	<1	3	506
Spartan Pearl (89)	2	<1	1	274
Tolaas (89)	5	1	2	583
Triumph (89)	2	<1	16	607
Triumph (90)	32	7	4	809
MS716-15 (89)	7	<1	1	673
ND 860-2 (89)	<1	<1	<1	470
ND 1538-1Russ (89)	3	<1	2	697
ND 2008-2 (89)	2	<1	2	717
12567 (89)	8	3	10	653
12823 (89)	16	1	<1	588
13035 (89)	<1	<1	2	554
13420 (89)	4	2	2	665
13653 (89)	16	4	1	629
Validation with 13653				
trial 1	15.8	4.1	1.3	745
2	16.1	4.0	1.1	756
3	13.7	3.5	1.0	697
mean ± S.D.	15.2 ± 1.3	3.9 ± 0.3	1.1 ± 0.1	733 ± 31
Nonsodium starches				
La Belle (89)	207	114	204	44
Red Pontiac (90)[c]	6	30	704	23
Red Pontiac (90)[d]	12	31	249	15

[a-d] Separate preparations of starch from a common lot of Red Pontiac tubers grown in 1990.

Table 3—Effect of starch extraction procedure on pasting characteristics of Red Pontiac Starch (1989 season)

Water	Ion Exchange	Paste Temp (°C)	Viscosity (Brabender Units)[a] Peak	43.3 min	58.3 min	88.3 min
Tap	yes	63.5	840	840	510	550
Distilled	yes	64.5	840	820	620	670
Distilled	no	64.5	835	800	620	690

[a] 43.3 min corresponds to the attainment of a constant temperature of 95°C; 58.3 min corresponds to the onset of cooling; 88.3 min corresponds to the completion of cooling.

1). Highest phosphorus contents were found in the red-skinned genotypes Red LaSoda and Reddale, but other red-skinned genotypes did not have unusually high values. Veselovsky (1940) reported a similar range (≈600–1000 ppm) with exception of Katahdin (1150 ppm) and South American Andean genotypes of *S. andigenum* (up to 1240 ppm). High phosphorus content is desired in potato starch to attain high paste viscosity (Veselovsky, 1940). Leszczynski (1989) and de Willigen (1976a) indicated phosphorus content could be increased by application of phosphates during cultivation.

Starch from Russet Burbank and Norchip genotypes had some of the largest mean granule diameters (Table 1). Both are leading genotypes in the processed potato industry where starch can be recovered as a byproduct. A large starch granule is desirable for reasons such as ease of separation and luster. Large grains are desired for use in both textile and paper industries (de Willigen, 1976b). Laser light scattering to characterize granule diameter is not strictly accurate, because it is based on the assumption that granules are spherical. In fact, potato starch granules tend to be slightly oblong; nevertheless, laser light scattering provides a relatively rapid indication of comparative granule diameter distributions between samples.

The proportion of amylose to amylopectin is a relatively constant characteristic of starch from a given crop. Thus, the somewhat narrow range in amylose content we observed (S.D./mean = 0.075 for all sodium starch samples in Table 1) was somewhat as expected. Other investigators reported amylose content for mature potato tubers of 16–24% (Leszczynski, 1989) and 19.1–25.3% (Barichello and Yada, 1991). Although our results were higher, they were reproducible and should at least reflect the relative amylose content among genotypes.

Potato starch naturally contains metal cations bound to starch phosphate esters by ionic forces. Calcium, magnesium, and other multivalent cations apparently cross-link phosphate esters on adjacent amylopectin chains by ionic forces (de Willigen, 1976a). Paste characteristics of the starch should resemble those of covalently cross-linked starch. Ionic cross-linking in potato starch is not generally advantageous to food processors, since in formulated foods, divalent cations could be displaced by non-linking monovalent sodium or hydrogen ions. To minimize the potential for ionic cross-linking, starch samples were converted to sodium form by ion-exchange. Analysis by atomic absorption showed that the ion-exchange procedure usually eliminated all but relatively low levels of calcium and magnesium (Table 2). One notable exception was a Red LaSoda sample.

The cation content of starch extracted into distilled water is predominantly potassium (de Willigen, 1976a). In starch extracted into tap water, some of the potassium is displaced by calcium, magnesium and other cations (Table 2). Starch extracted into distilled water and not subjected to ion-exchange, which were Red Pontiac (90)[c] and (90)[d], contained high levels of potassium, intermediate levels of magnesium, and relatively little calcium and sodium. The inconsistent potassium content of Red Pontiac (90)[c] vs (90)[d], and also in Red Pontiac (90)[a] vs (90)[b], may reflect variability in samples and preparation methods and errors in potassium analysis. Starch extracted into tap water and which did not undergo ion exchange (La Belle) had the highest calcium and magnesium. Moisture content ranged from 7–15%, because of the wide range of relative humidities during starch drying. No significant effect of moisture content on paste characteristics was observed.

Paste characterization

In a preliminary investigation, the effect of tap water extraction and ion-exchange procedures on paste characteristics was studied (Table 3). Starch extracted into tap water and which underwent the ion-exchange procedure had a slightly lower paste temperature and slightly higher peak viscosity. During cooling, however, viscosity was lower resulting in lower paste stability ratio. For starch extracted into distilled water, the ion-exchange procedure did not result in any change in amylogram properties.

A wide range in paste behavior was seen from results of amylograph tests (Table 4). Viscosities of selected samples were reproducible to within <8%, except when the interval between replicates was >8 wk. The paste characteristics of potato starch gradually change with aging (de Willigen, 1976a; Leszczynski, 1989).

Of particular interest were paste stability ratios (Table 4). The stability ratio ranged from 0.38 to 1.00. A high ratio usually indicated the viscosity attained a relatively stable value (constant with respect to time). By definition, unity is the high-

Table 4—Paste characteristics of potato starch by Brabender Visco-analyzer

Genotype (Year grown)	Paste temp (°C)	Peak-paste time (min)	Peak visc. (B.U.)	Stability ratio	Set-back ratio
Agassiz (89)	65.5	13.5	1285	0.47	1.02
Atlantic (89)	65.3	15.5	1045	0.52	n.a.[c]
Bintji (90)	63.6	18.7	1000	0.52	1.09
Dark Red Norland (89)	66.3	16.1	1290	0.56	1.00
Erick (89)	65.1	17.9	910	0.59	1.08
Gemchip (89)	66.3	13.8	1020	0.49	1.09
Katahdin (89)	64.5	19.7	935	0.60	1.10
Katahdin (90)	64.8	18.1	1000	0.60	1.08
Kennebec (89)	61.5	19.0	940	0.48	1.02
Krantz (89)	62.5	15.0	1040	0.43	1.07
Lemhi (89)	63.6	21.2	925	0.66	1.10
Monona (89)	67.0	17.3	1100	0.63	1.06
Norchip (90)	63.0	21.3	1050	0.62	1.04
Norgold Russet (89)	64.3	18.8	905	0.61	1.08
Red LaSoda (89)	62.0	16.7	1140	0.46	1.01
Red LaSoda (90)	65.9	21.4	1110	0.78	n.a.[c]
Red Norland (90)	65.1	31.4	630	0.99	1.23
Red Pontiac (89)	63.6	20.6	840	0.60	1.09
Red Pontiac (90)[a]	66.0	27.1	770	0.96	1.18
Red Pontiac (90)[b]	65.5	29.0	745	0.95	1.16
Reddale (89)	65.0	12.7	1120	0.47	1.10
Reddale (90)	64.0	20.1	960	0.63	1.13
Redsen (90)	68.0	21.2	930	0.77	1.05
Russet Burbank (89)	64.5	19.0	975	0.57	1.10
Russet Burbank (90)	62.8	22.8	920	0.64	1.09
Russet Norkota (89)	67.0	20.7	895	0.74	1.11
Russet Norkota (90)	65.3	20.1	870	0.71	1.10
Saginaw Gold (89)	66.2	10.9	1170	0.41	1.08
Spartan Pearl (89)	66.5	15.7	1030	0.57	1.06
Tolaas (89)	63.7	10.2	1300	0.38	1.07
Triumph (89)	66.4	20.8	1005	0.63	1.01
Triumph (90)	67.2	33.5	755	1.00	1.16
MS716-15 (89)	65.5	11.3	1260	0.45	1.05
ND860-2 (89)	64.0	20.3	960	0.60	1.03
ND1538-1Russ (89)	63.6	16.2	1335	0.53	1.05
ND2008-2 (89)	66.0	14.3	1205	0.60	1.04
12567 (89)	63.5	16.3	1230	0.44	0.99
12823 (89)	67.0	20.7	980	0.74	1.01
13035 (89)	65.3	8.1	1320	0.38	1.06
13420 (89)	64.5	11.7	1270	0.43	1.04
13653 (89)	63.5	15.7	1160	0.51	1.04
Nonsodium starches					
LaBelle (89)	65.0	25.0	570	0.90	1.15
Red Pontiac (90)[c]	65.5	22.0	905	0.71	1.07
Red Pontiac (90)[d]	65.5	25.7	735	0.83	1.13

[a-d] Separate preparations of starch from a common lot of Red Pontiac tubers grown in 1990.

[c] n.a. not available.

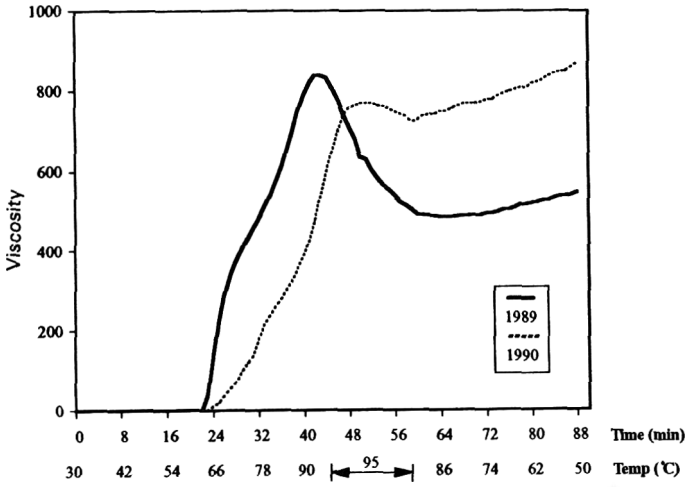

Fig. 2—Amylograph test results for 3.25% Red Pontiac starch in water. Starch was from tubers grown at the same location in different years, and was converted to the sodium form using an ion-exchange procedure.

Table 5—Correlation matrix between selected chemical/physical characteristics and paste properties of 41 samples of potato starch (sodium form)

	Paste temp	Peak-paste time	Peak viscosity	Stability ratio	Setback ratio[a]
Phosphorus	0.02	−0.47**	0.61**	−0.38*	−0.26
Mean granule diameter[a]	−0.02	0.17	−0.24	0.17	0.20
Amylose	0.35*	−0.03	0.12	0.15	0.05
Calcium + magnesium	0.09	0.34*	−0.16	0.38*	0.35*
(Ca + Mg)/P[b]	0 09	0.40*	−0.23	0.43**	0.39*

[a] Correlations for setback ratio and mean granule diameter based on 39 samples each.

[b] (Ca + Mg)/P represents the ratio of total calcium+ magnesium to phosphorus.

* and ** significant at P<0.05 and P<0.01 levels of probability, respectively.

est possible ratio, and is obtained only when a maximum or peak viscosity is not observed before onset of cooling. Reproducible, high ratios for Red Pontiac (90)[a] and (90)[b] (Table 4) confirmed novel paste characteristic for that genotype as reported by McComber et al. (1988). Two other genotypes, Triumph and Red Norland, had very high ratios in 1990. The stability ratios of the other 37 sodium starch samples were distinctly lower. Triumph is a parent of Red Pontiac, and Red Pontiac is both a maternal grandparent and paternal great grandparent of Red Norland. Thus, the high stability ratio may be related to a heritable trait.

Neither stability ratios for Red Pontiac nor Triumph were unusually high in samples grown in 1989 (Table 4) nor in samples grown in 1991 and 1992 (data not shown). Amylographs for starch from Red Pontiac grown in 1989 and 1990, (90)[a] in Table 4, were compared (Fig. 2). Probably cultural practices or climatic conditions influenced paste characteristics. The 1989 and 1990 growing seasons (June to August) were similar with respect to mean temperature, but the 1990 season had about 50% more precipitation (J. Enz, personal communication, 1993). The plants were not irrigated, thus precipitation might have been a factor. However, data from 6 yrs, including the 2 yr (1984 and 1985) of the McComber et al. (1988) study, did not show correlations between Red Pontiac paste stability and total precipitation or mean temperature.

Starch from Red Pontiac, Red Norland, and Triumph which exhibited high stability ratios, contained the expected low concentrations of calcium and magnesium. Furthermore, comparing Red Pontiac (90)[a] and (90)[b] (converted to sodium form) with Red Pontiac (90)[c] and (90)[d] (unconverted), the stability ratio was higher in the sodium form, though total calcium plus magnesium was lower. Thus, in those samples, the high stability ratios probably did not result from calcium or magnesium cross-links. We assumed there were not significant concentrations of other multivalent cations, since the ion exchange procedure usually removed most calcium and magnesium. Other cations are generally not present in tubers or tap water at comparable concentrations. The content of amylose and phosphorus and the mean granule diameter were also not unusual compared to other genotypes.

Correlation of results

Phosphorus, granule diameter, amylose, and total calcium and magnesium were compared with paste characteristics using multiple linear regression (Table 5). Samples containing <1 ppm calcium or magnesium were assumed to have 1 ppm for calculating correlation coefficients. They were not high, which was somewhat expected based on the complex nature of starch and its pastes. However, some of the measured characteristics were significantly related to paste characteristics (Table 5). Paste temperature correlated best with amylose content ($P \leq 0.05$). The most pronounced correlations were found between phosphorus content and several quantities from the amylograph analysis: peak viscosity, $P \leq 0.01$; peak-paste time,

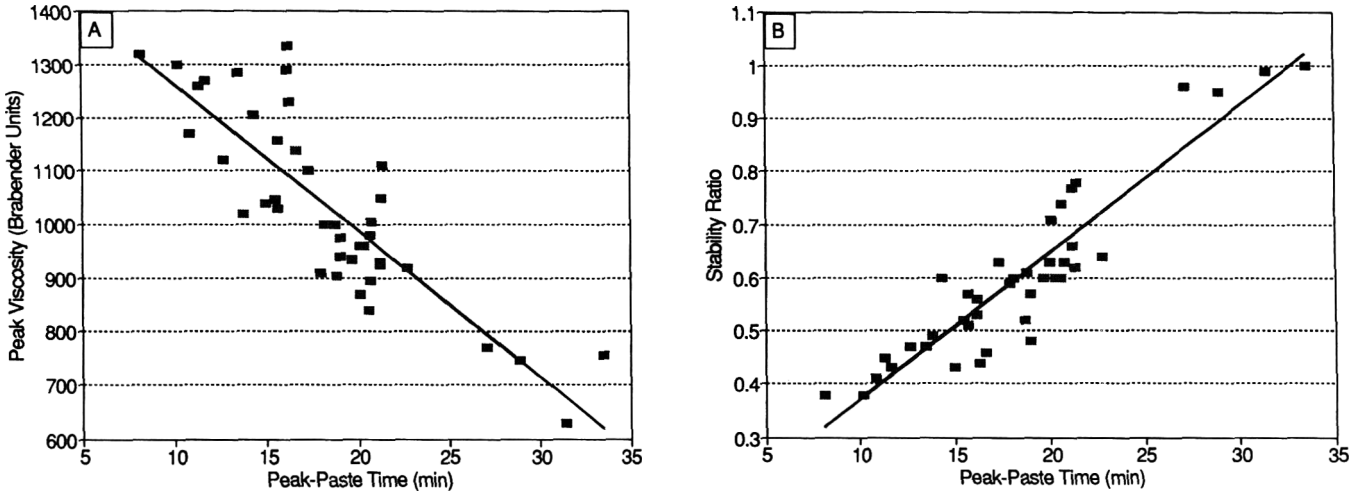

Fig. 3—Scatter plots relating selected amylograph results for 41 samples of potato starch (sodium form). Correlation coefficients for plots were (A) −0.85 and (B) 0.92.

P ≤0.01; and stability ratio, P ≤0.02. The correlation of phosphorus with peak viscosity has been reported (Veselovsky, 1940). The correlation between mean granule diameter and paste characteristics was not significant. Nor was the correlation with sodium and potassium.

Despite efforts to eliminate calcium and magnesium from the samples, correlations between total calcium and magnesium and certain paste characteristics were significant (P ≤0.05). The ratio of total calcium and magnesium to phosphorus might indicate the proportion of phosphorus groups bound to divalent cations. Thus proportion correlated better with stability and setback ratios than the other quantities (Table 5).

Although values ranged widely for the amylograph tests, the various results were correlated with one another. Peak-paste time varied inversely to peak viscosity (Fig. 3A), and demonstrated a strong positive correlation with stability ratio (Figure 3B). A low peak-paste time likely means that the granules had relatively low resistance to swelling. In that case, they would be expected to swell rapidly, and become susceptible to concurrent shear-induced disintegration. A high peak-paste time may be associated with granules which swell more gradually, and thus are not as susceptible to mechanical damage. This trend, observed with unmodified, freshly extracted potato starch from different genotypes appears similar to trends reported on progressive effects of thermal treatment, cross-linking, and aging of starch.

CONCLUSIONS

ATYPICALLY STABLE PASTE VISCOSITY (high paste stability ratio) was found only in starch from Red Pontiac and in two related genotypes. Thus stable paste viscosity may be a heritable trait. Amylograph plots for those three genotypes resembled those of commercial cross-linked starch, and they did not appear to result from ionic cross-linking of starch phosphate groups. These results, however, were found only in 1 of 4 years of this study, and thus the characteristic may depend on cultural and climatic factors. Unless such factors can be identified and controlled, it may not be feasible to use Red Pontiac starch as a natural replacement for cross-linked starch. Despite high variability in pasting characteristics, significant correlations occurred among several of those characteristics, with paste stability.

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Mention of trade names is for identification only and does not constitute an endorsement to the exclusion of other products that may be suitable.

Phenolics in Aqueous Potato Peel Extract: Extraction, Identification and Degradation

D. RODRIGUEZ DE SOTILLO, M. HADLEY, and E.T. HOLM

ABSTRACT

Phenolics were extracted from potato peel waste using water or methanol. Phenolic acids in the extracts were quantified by HPLC. The greatest amounts of phenolic acids resulted when potato homogenate was refluxed with water for 30 min, yielding a total concentration of 48 mg/100g. Four phenolic acids (chlorogenic, gallic, protocatechuic, and caffeic) were characterized as major components. Aqueous extracts were stored 20 days and after 7 days at 25°C exposed to light, chlorogenic acid had degraded to caffeic acid.

Key Words: potato peel waste, phenolic acids, chlorogenic acid, caffeic acid

INTRODUCTION

PHENOLIC COMPOUNDS, widely distributed within plants (Wong, 1973; Gross, 1981), are commonly isolated, using aqueous or organic solvents. High performance liquid chromatography (HPLC) is reliable and reproducible and its application for quantification and identification of various plant phenolic acids has been reported (Walter and Purcell, 1979; Dreher and Holm, 1983; Hartley, 1987). Potato peel contains many phenolic compounds, some in free form and some bound. The largest portion consists of chlorogenic acid (CGA), a derivative of caffeic acid (CFA) and quinic acid (QNA) (Lisinska and Leszczynski, 1987). Identification and quantification of CGA and other phenolic acids in most potato cultivars by HPLC have been reported (Lyon and Barker, 1984; Malmberg and Theander, 1985; Kumar et al., 1991; Ramamurthy et al., 1992).

Our objectives were to study the extraction of phenolics from potato peel waste using methanol (MeOH) or water and the stability of such liquid extract after storage at 4, 25, and 37°C.

MATERIALS & METHODS

Reagents

Chlorogenic acid, meta-coumaric acid (m-CMA), para-coumaric acid (p-CMA), ferulic acid (FRA), para-hydroxy benzoic acid (p-BAC), protocatechuic acid (PCA), gallic acid (GAC), vanillic acid (VNA), and CFA were purchased from Sigma Chemical Company (St. Louis, MO). Methanol (MeOH) was purchased from Fisher Chemical Company (Pittsburgh, PA) and glacial acetic acid (HAc) was purchased from Baxter Scientific Products (Minneapolis, MN).

Potato sample preparation

Potato peel waste was obtained from Simplot Food Division. A 2 kg aliquot of the peel waste was autoclaved for 10 min at 121°C and ≈1kg/cm². After determining that autoclaving did not affect the phenolics (HPLC profiles and concentration for samples not autoclaved were the same) autoclaving was done to prevent fermentation. Aliquots (500g) from each autoclaved sample were mixed with 100 mL water and homogenized in a Dual-range Osterizer™ Blender (Fisher Scientific Co., Pittsburgh, PA). Homogenized slurries were combined and aliquots were kept frozen at −12°C until analyzed.

The authors are with the Dept. of Food & Nutrition, North Dakota State Univ., Fargo, ND 58105.

Isolation of phenolic compounds from potato peel waste

Phenolic compounds were extracted from potato peel waste using MeOH at 4°C, aqueous extraction at 25°C, or aqueous extraction at 100°C. Three samples were thawed and 5g aliquots were homogenized for 4 min in a Dual-range Osterizer™ Blender with 29 mL cold MeOH (4°C). The resulting slurry was centrifuged using an IEC Centra-8R Centrifuge (International Equipment Co., Needham Heights, MA.) at 3000 × *g* for 10 min at 5°C. The supernatant liquid was filtered through Whatman™ No. 4 paper, and the filtrate was collected for quantitative analysis. The residue was extracted one more time, except the residue was not homogenized, but mixed for 4 min using a Vortex-Genie 2™ Mixer (Fisher Scientific Co., Pittsburgh, PA.). Supernatant liquid after centrifugation from the second extraction was collected for quantitative analysis and the residue was discarded.

This same method was followed using water at 25°C as solvent. In an attempt to increase the yield of extracted material, water-extracted slurries were reflexed 30 min using a suitable size of flat bottomed Pyrex™ boiling flask attached to a Pyrex™ Reflex Condenser (Fisher Scientific Co., Pittsburgh, PA.) cooled with tap water. A Corning™ Combination Stirrer Hotplate (Fisher Scientific Co., Pittsburgh, PA.) was used as heat source and the slurry stirred with a Teflon-coated magnetic stir bar. After cooling to room temperature (≈23°C) the slurry was centrifuged. The residue was re-extracted in 29 mL water at 25°C by mixing for 4 min with a Vortex-Genie 2™ Mixer and then refluxing for 30 min.

HPLC

A series of solutions containing from 2 to 80 μg/mL of CGA, PCA, GAC, p-BAC, m-CMA, p-CMA, o-CMA, CFA, and VNA were injected and peak area responses were used to prepare standard curves. The HPLC instrument included a 501 HPLC pump from Waters Millipore (Mildford, MA), an injector (Rheodyne Co., Catati, CA), a guard column (3 mm i.d. × 22 mm) containing C18/Corasil, a 3.9 mm × 30 cm μBondapak™C_{18} column, a 440 absorbance detector with 313 nm filter, and a Data Module from Waters Millipore (Mildford, MA). The mobile phase Water:MeOH:HAc (64:35:1 v/v/v), was delivered isocratically at 1 mL/min, and the effluent monitored at 313 nm. A 10 μL aliquot of peel extract was analyzed by HPLC. Where possible, major peaks were identified by co-migration with phenolic acid standards.

Stability of liquid extract

Three 5-g samples of potato peel waste were extracted using the water reflux method of and a 10 μL aliquot of filtrate was analyzed for phenolic acids. The remainder of the filtrate was divided into three 7-mL aliquots. The first aliquot was stored under refrigeration (4°C), the second at room temperature (25°C), and the third incubated in a Blue M Stable-Therm 108 oven (Fisher Scientific Co., Pittsburgh, PA) at 37°C for 20 days. Periodically over 20 days, a 10 μL aliquot from each tube was analyzed for concentration of phenolic acids by HPLC.

Statistical analysis

Data were analyzed by the general linear model (GLM) program for analysis of variance and regression estimation (SAS Institute, Inc., 1985).

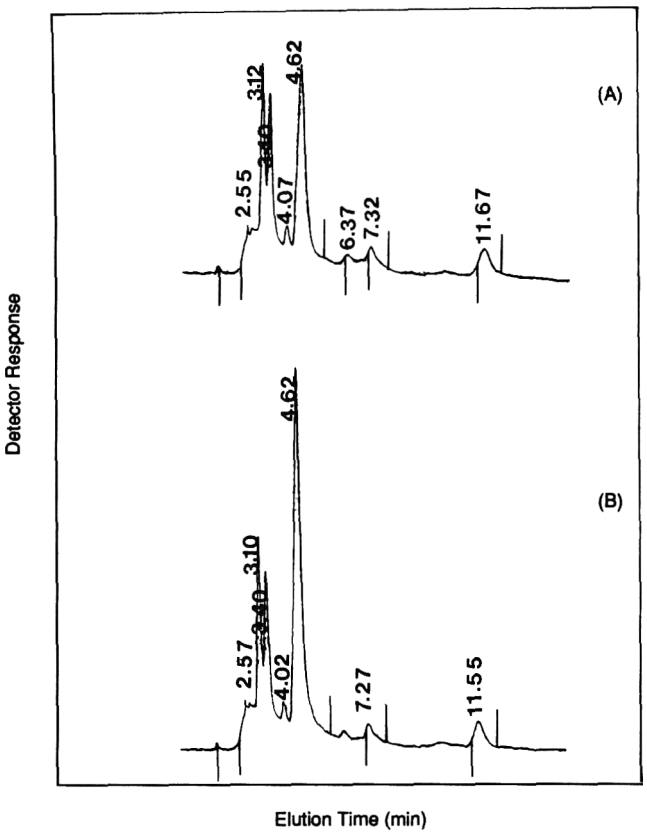

Fig. 1.—Identification of CGA (retention time 4.62 min) in potato peel waste extract. HPLC chromatograms of: (A) an aliquot of potato peel waste; (B) potato peel waste augmented with pure CGA.

Table 1—Phenolic acids extracted from potato peel waste using water or methanol

Solvent and Method	Phenolic acids (mg/100g)[a]			
	CGA[b]	GAC[b]	PCA[b]	CFA[b]
Methanol 4°C	24.06 ± 0.85	12.66 ± 1.88	2.44 ± 1.06	2.49 ± 1.59
Water 25°C	20.45 ± 1.20	8.24 ± 0.96	0.78 ± 0.34	2.68 ± 0.38
Water 100°C refluxed	29.91 ± 2.26	12.96 ± 1.23	3.77 ± 2.64	1.36 ± 0.22

[a] Means ± standard deviation of eight replicates expressed on wet matter basis.
[b] CGA=chlorogenic acid, GAC=gallic acid, PCA=protocatechuic acid, CFA=caffeic acid.

RESULTS & DISCUSSION

Selection of best solvent and extraction method

The total concentration of phenolic acids obtained (Table 1) using MeOH at 4°C was 41.65 mg/100g and water at 25°C was 32.15 mg/100g. MeOH at 4°C was a more efficient extractor of phenolic acids than water at 25°C. We attempted to reduce cost and improve efficiency of extraction using water because MeOH is toxic. The temperature for extraction was increased by refluxing homogenates for 30 min, resulting in a total yield of 48.00 mg/100g. The water reflux method gave the greatest amounts of CGA, GAC, and PCA, but gave the lowest amount of CFA in the extracts. CFA may be unstable at 100°C and decomposed during reflux to compounds that did not absorb at 313 nm, the wavelength used for analysis. After examination of phenolic recoveries by the methods used, MeOH extractions were discontinued.

Identification of phenolic acids

The major phenolic acids in the potato peel extract were identified as CGA, GAC, PCA, and CFA after the first and second extractions. Lyon and Barker (1984), Malmberg and Theander (1985), Lisinska and Leszczynski (1987), Kumar et al. (1991), and Ramamurthy et al. (1992) all reported the same phenolic acids, or CGA as a major constituent, in the peels and/or flesh of potato tuber cultivars. The HPLC chromatograms A and B (Fig. 1) are profiles of an aliquot of a second extraction from potato peel waste and the same extract to which pure CGA had been added, respectively, for identification of CGA, retention time 4.62 min.

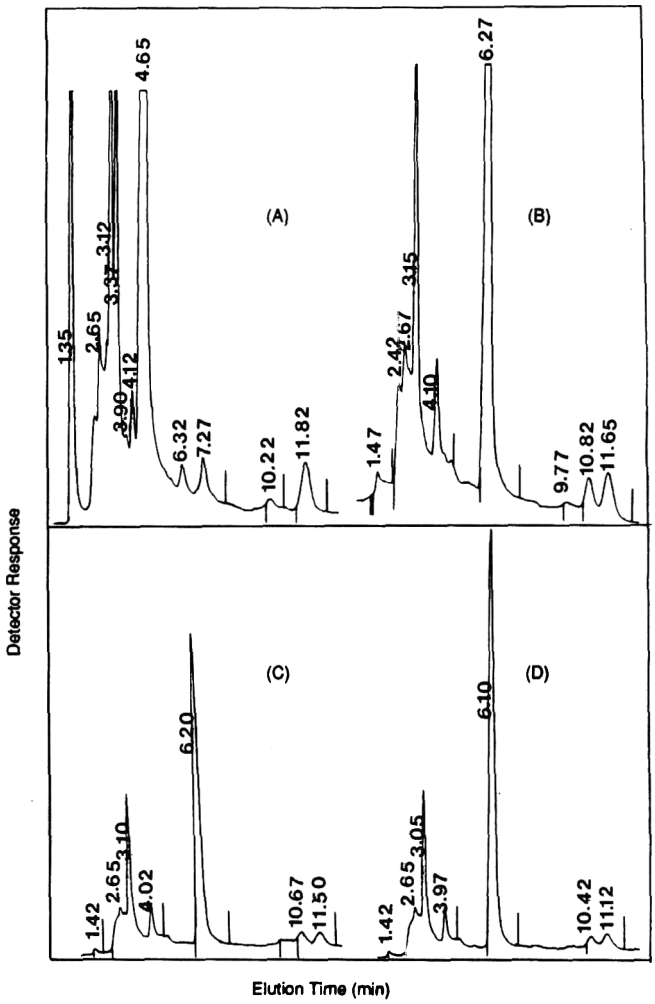

Fig. 2.—Stability of the components of liquid extract from potato peel waste. HPLC chromatograms of: (A) aliquot of extract on day 0; (B) aliquot after 7 days at 25°C; (C) diluted aliquot after 7 days at 25°C, (D) diluted aliquot of extract augmented with pure CFA after 7 days at 25°C.

Stability of components

Samples stored at 4°C and those stored at 37°C did not show major changes in concentrations of extract components; samples stored at 25°C did. Samples held at 4°C and 37°C were stored in the dark, while those at 25°C were kept on the bench and were exposed to light. Concentrations on day 0 of analysis were CGA 0.96 μmol/g extract and CFA 0.04 μmol/g extract. After 7 days at 25°C, there was no CGA, and the concentration of CFA had increased to 0.64 μmol/g extract. The loss of CGA may have been due to conditions of storage. Some components including CGA may be sensitive to light. The increase in concentration for CFA, apparently contributed by degradation of CGA, was only 0.60 μmol/g. This suggested that the remaining portion (0.36 μmol/g) may have degraded into another compound or compounds. Profiles of the fresh extract and those stored after 7 days at 25°C were compared (Fig. 2). No

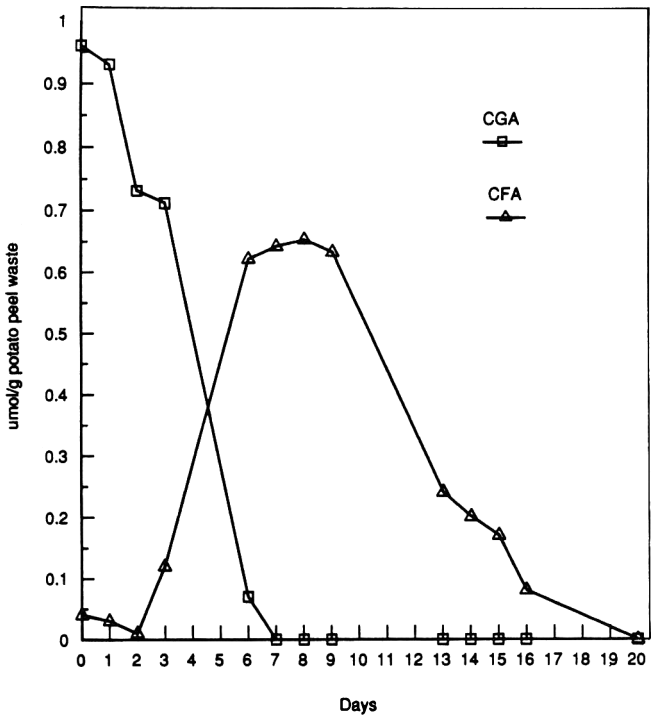

Fig. 3.—Changes of CGA and CFA in a liquid extract from potato peel waste stored at 25°C.

changes were seen in the chromatograms after 7 days storage at 4°C or 37°C.

Peaks that eluted on day 0 of the study (Fig. 2A) showed chlorogenic acid was the major component, with retention time 4.65 min; CFA was a minor component, with retention time 6.32 min. The HPLC profile of the same extract after 7 days at 25°C (Fig 2B) showed chlorogenic acid (4.65 min) was not detected, and the area of the peak at 6.32 min had increased more than 13-fold.

After 7 days storage, the extract was diluted in water and analyzed by HPLC (Fig. 2C). An aliquot of the diluted extract was augmented with authentic CFA and analyzed by HPLC. Adding CFA increased the area of the peak at 6.20 min. by 2-fold (Fig. 2D), indicating that it was probably CFA. CFA may have increased due to the degradation of CGA. Chlorogenic acid is composed of CFA and QNA. QNA was not observed as it does not absorb at 313 nm.

Differences in areas of the peaks corresponding to CGA and CFA were noticeable after the first day of storage at 25°C. A plot (Fig. 3) of concentration changes of CGA and CFA in potato peel extract with storage time showed a decrease in CGA and an increase in CFA concentration until day 7. Between days 7 and 9 of storage the concentration of CFA seemed to be stable; after day 9, CFA concentration began to decrease until, after 20 days storage, CFA was not detected. This suggested that CFA may have decomposed into a compound that was not detected at 313 nm.

CONCLUSION

PHENOLIC ACIDS can be readily isolated from potato peel waste, using a simple and economic extraction procedure. Degradation of CGA into CFA in the extract suggested that water extraction, storage temperature, and exposure to light, promoted chemical reactions and consequent hydrolysis of the phenolic acids.

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Melting Characteristics and Hardness of Milkfat Blend Sucrose Polyesters

M.A. DRAKE, T.T. BOUTTE, F.L. YOUNCE, D.A. CLEARY, and B.G. SWANSON

ABSTRACT

Sucrose polyesters (SPE) are noncaloric fat substitutes prepared from fatty acid methyl esters (FAME) and sucrose. Our objectives were to determine and compare the effects of supplementing FAME from hydrolyzed milkfat with 25% FAME derived from beef tallow, coconut oil, or myristic acid on melting characteristics, hardness, and fatty acid profiles of SPE. DSC thermograms revealed different melting profiles among the SPE and anhydrous milkfat. Milkfat:tallow, milkfat:coconut, and milkfat SPE differed ($p<0.05$) in hardness from milkfat at 12.5, 22, and 27.5°C. Fatty acid compositions of the SPE were also different. Adjustments of melting characteristics, hardness, and fatty acid substituents of SPE are essential when substituting milkfat SPE for selected natural fats in foods.

Key Words: milkfat, sucrose polyesters, fat substitute, cheese, melting characteristics

INTRODUCTION

SUCROSE POLYESTERS (SPE) ARE LIPOPHILIC, nonabsorbable, noncaloric, potential fat substitutes in foods (Mattson and Nolen, 1972). SPE are hexa-, hepta-, and octaesters of fatty acids esterified to the hydroxyl groups of sucrose (Mattson et al., 1971). SPE have the potential of replacing undesirable fats in foods without sacrificing the flavor and textural properties that fats contribute to foods. Both liquid and solid SPE with a wide range of melting points can be synthesized by varying the fatty acid methyl esters (FAME) used in synthesis (Mattson et al., 1971). FAME of naturally occurring fats and oils, as well as methyl esters of purified fatty acids have been effective reactants. A large stockpile of milkfat exists in the U.S. (USDA, 1991). A potential market for milkfat would emerge if hydrolyzed milkfat fatty acids were used to synthesize SPE with selected melting points and hardness suitable for food products.

Differential scanning calorimetry (DSC) is used to study the phase behavior of milkfat and other fats (Norris et al., 1971; Timms, 1980; Hale and Schroeder, 1981). Cone penetrometry is a rheological technique used to assay hardness of fats (Haighton, 1959; Tanaka et al., 1971; DeMan, 1976). Our objectives were to study the melting characteristics, hardness, and fatty acid profiles of SPE prepared from milkfat or milkfat fatty acids supplemented with 25% fatty acids of other fats or oils.

MATERIALS & METHODS

ANHYDROUS MILKFAT was purchased from Darigold, Inc. (Spokane, WA). Beef tallow was obtained from IBP, Inc. (Wallula, WA). Coconut oil, 99% pure methyl myristate, sucrose, and potassium carbonate were purchased from Sigma Chemical Co. (St. Louis, MO). Spectrophotometric grade cyclohexane, used as a calibration standard in the DSC experiments, was purchased from Aldrich Chemical Co. (Milwaukee, WI).

The authors are affiliated with the Dept. of Food Science & Human Nutrition, College of Agriculture & Home Economics Research Center, Washington State Univ., Pullman, WA 99164-6376. Address inquiries to Dr. B.G. Swanson.

Anhydrous milkfat, beef tallow, and coconut oil FAME were prepared according to Akoh and Swanson (1988) with modifications. Hexane was used as a solvent in place of pentane or diethyl ether, and 1N KOH was used in place of 1N NaOH. FAME of milkfat or 75% milkfat FAME and 25% FAME of tallow, myristate, or coconut oil were blended prior to initiating SPE synthesis according to McCoy et al. (1989). These reaction mixtures generated milkfat SPE and 75:25 milkfat:tallow, milkfat:myristate, and milkfat:coconut SPE, respectively.

SPE were purified by washing with 10 volumes of hot water followed by 2 two-volume washes with hot ethanol (50°C). Further purification of the SPE was accomplished by short-path distillation (UIC, Joliet, IL) with an oil bath temperature of 160°C and a propylene glycol bath temperature of 10°C. To remove excess dark color, SPE were mixed with 0.05% (w/w) clay at 100°C while stirring on a rotoevaporator for 2-3 hr. The clay was then removed by filtration.

Purity of the SPE was determined by ascertaining the amount of FAME left in the SPE after purification. Purity of the SPE was determined using a Lee Scientific Series 600 supercritical fluid chromatograph (SFC) (Dionex, Salt Lake City, UT). Thirty mg of each SPE were dissolved separately in 1 mL hexane. The sample was injected, using a split injection time of 0.1 sec, onto an SB-cyanopropyl-50 column (50 µm I.D., 0.25 µm film) (Dionex, Salt Lake City, UT). Density of the CO_2 mobile phase was set at 0.13 g/mL and held for 10 min and then increased to 0.61 g/mL at 0.1 g/mL/min using a 100 µm flow rate frit restrictor. Oven temperature was 150°C and FID 400°C. The resulting FAME peak area was compared to a standard curve generated under the same conditions using methyl myristate to determine the amount of FAME present. The calculated FAME weight was divided by the total weight of SPE to determine the percentage (w/w) of FAME present.

Differential scanning calorimetry (DSC) was performed on a Perkin-Elmer DSC7 interfaced with a Perkin-Elmer Delta series computer (Norwalk, CT) to ascertain the melting characteristics of the fats. The melting onset temperature, defined as the first deviation from the baseline, was determined by visual inspection of curves. While the DSC is accurate to ± 0.5°C, due to possible human error in determining melting onset temperatures, SPE were not considered significantly different unless there was more than a ± 5°C difference in average melting onset temperature. Four replicates of each fat were used to generate curves. About 9.0 mg was used for each run. The curves of each fat were reproducible and similar with the determined melting onset and finish temperatures within ± 2°C. Cyclohexane served as calibration standard. The SPE and the calibration standard were analyzed using liquid nitrogen as a heat sink with a slow helium gas purge to improve thermal conductivity between the platinum DSC sample pans and heat sink. The SPE were heat-cycled *in situ* prior to collecting DSC data by raising the temperature from 20°C to 50°C at 200°C/min, holding at 50°C for 1 min, cooling to −40°C at −5°C/min, and finally holding at −40°C for 20 min prior to beginning data collection. The data collection was from −40°C to 50°C with the temperature increasing 5°C/min.

Hardness of the SPE at 12.5, 22, and 27.5°C was analyzed with an Instron (Canton, MA) servohydraulic universal testing machine with a 90.8 kg load cell and a cone penetrometer probe with a cone angle of 30°C. SPE were melted and poured into stainless steel cylindrical molds with a volume of 1.73 cm^3 and hardened at 5°C for 4 hr. The SPE were removed from molds and allowed to temper at the assay temperature for 24 hr prior to assay. A penetration depth of 0.5 cm was used and the resulting peak force at a constant speed was converted to Newtons and used as an indicator of fat hardness. An analysis of variance was performed on the four replicates of each fat at each temeprature ($p<0.05$).

To determine fatty acid profiles of the SPE, they were deesterified by saponification. Fatty acid substituents were then methylated and SFC was used to separate and quantify the FAME. A modified method

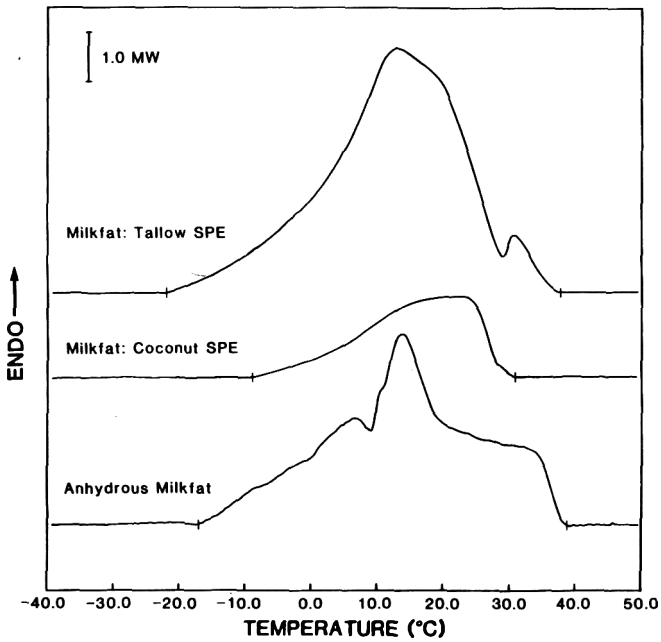

Fig. 1—DSC thermogram of milkfat:tallow SPE, milkfat:coconut oil SPE, and anhydrous milkfat. MW = milliwatts.

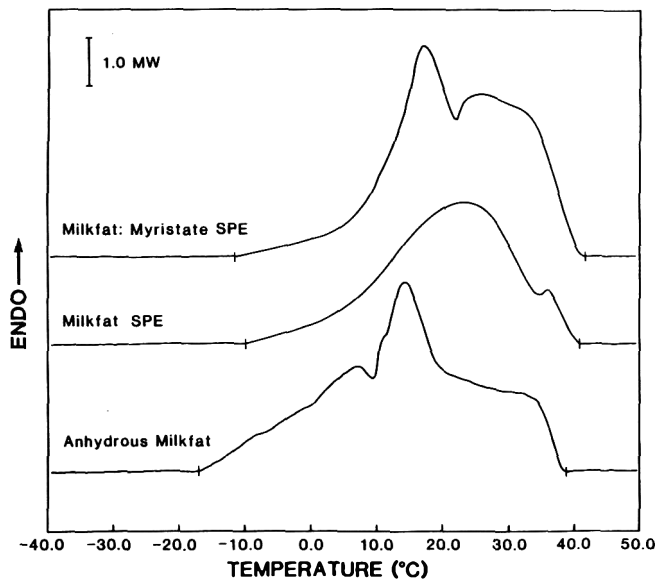

Fig. 2—DSC thermogram of milkfat:myristate SPE, milkfat SPE, and anhydrous milkfat. MW = milliwatts.

of Morrison and Smith (1964) was used for saponification and methylation. About 15 mg of each SPE were transferred to a 20-mL-screw-cap test tube. One mL of 0.5N methanolic KOH was added to the vial, and the cap was screwed on tightly, followed by heating in an 80°C water bath for 6 min. Then, 1 mL of 14% boron trifluoride in methanol was added to the vial and it was held at 80°C for another 6 min. After cooling to room temperature (≈23°C) 4 mL hexane and 5 mL of a saturated NaCl solution were added. Five min later, the hexane layer was transferred to another vial containing 2g sodium sulfate, and the contents were mixed. After brief mixing and standing 5 min, the hexane layer was transferred to a screw-cap vial and stored under nitrogen until SFC analysis. FAME samples were injected, using a split injection time of 0.1 sec, onto an SB-methyl-100 column (50 µm i.d., 0.25 µm film) (Dionex, Salt Lake City, UT). The density of the CO_2 mobile phase was initialized and maintained at 0.2 g/mL for 15 min. Density was increased to 0.48 g/mL at 0.1 g/mL/min using a 50 µm flow rate frit restrictor. An initial oven temperature of 90°C was maintained for 15 min and then raised to 150°C at 4°C/min. The FID temperature was 350°C. Peak retention times were compared with FAME standard retention times to determine fatty acid chain lengths. Each FAME peak area was divided by the sum of the FAME peak areas to determine the percent (w/w) of each FAME in the sample. The fatty acid profile of anhydrous milkfat was obtained from Handbook Number 8 (USDA, 1979).

Table 1—Mean fat hardness in newtons at 12.5, 22, and 27.5°C

Fat/SPE	Temperature (°C) 12.5	22	27.5
Milkfat:myristate SPE	8.80[a]	0.64[c]	0.042[b]
Milkfat SPE	6.09[b]	2.59[a]	0.446[a]
Anhydrous milkfat	2.88[c]	0.42[c]	0.056[b]
Milkfat:coconut oil SPE	2.43[d]	0.90[b]	ND
Milkfat:tallow SPE	2.30[d]	1.05[b]	ND

[a-d] Means within rows followed by different superscripts were significantly different at $p < 0.05$.
ND = not determined.

RESULTS & DISCUSSION

PURITY OF THE SPE was 99% (w/w) as determined by the amount of FAME remaining in them. SPE exhibited smoother, more uniform DSC melting curves then milkfat, although milkfat:tallow SPE, milkfat:myristate SPE, and milkfat SPE exhibited peaks within DSC melting curves similar to milkfat (Fig. 1 and 2). The melting onset temperatures of milkfat:tallow SPE and milkfat:myristate SPE were not different from that of milkfat. Except for milkfat:coconut SPE, the SPE did not differ from milkfat in melting finish temperature.

There was no difference in hardness between milkfat:tallow SPE and milkfat:coconut SPE at 12.5, 22, or 27.5°C (Table 1). No difference occurred in hardness between milkfat:myristate SPE and milkfat at 22 and 27.5°C. All other SPE differed in hardness from each other and from milkfat. The greatest differences in hardness among the SPE were observed at 27.5°C. Unlike the other SPE and native milkfat, milkfat:coconut and milkfat:tallow SPE were soft and did not retain molded shapes. Hardness values of milkfat:coconut and milkfat:tallow SPE were too low for detection by the Instron. Milkfat SPE were more than ten times harder than the milkfat:myristate SPE and milkfat.

Fatty acid profiles of the SPE (Fig. 3) revealed further differences among them and helped explain previous results. The addition of coconut oil containing a large proportion of C:12 fatty acids resulted in SPE that were softer than milkfat at 12.5 and 27.5°C and finished melting at a lower temperature than milkfat. The fatty acid profile of milkfat:coconut SPE revealed that milkfat:coconut SPE contained more laurate (C:12) than the other SPE, which may account for the decreased hardness and melting finish temperature of milkfat:coconut SPE.

Milkfat:tallow SPE were not different in hardness from milkfat:coconut SPE. Fatty acids from tallow, which contains a large amount of saturated C:16 (25%) and C:18 fatty acids (19%), (USDA, 1979) should theoretically increase hardness and would be expected to increase melting onset temperature of milkfat SPE. Milkfat:tallow SPE may not exhibit the expected characteristics due to steric hindrance in esterification of large concentrations of the saturated C:16 and C:18 fatty acids to the sucrose molecule. While the amount of stearate present in milkfat:tallow SPE was slightly greater than those amounts present on other SPE, the palmitate concentration was less than that of milkfat SPE. Milkfat:tallow SPE also contained almost 10% more oleate than the other SPE. The large amount of oleate might account for the hardness similarities between milkfat:tallow SPE and milkfat:coconut SPE. Milkfat:tallow SPE synthesis reactions required longer time and had lower yields than the other SPE synthesis reactions.

Milkfat SPE had a higher melting onset temperature and were harder than milkfat at 12.5, 22, and 27.5°C. The increased hardness and melting onset of milkfat SPE compared to milkfat may have been due to volatilization of C:10 and shorter fatty acids at the reaction temperatures required to synthesize

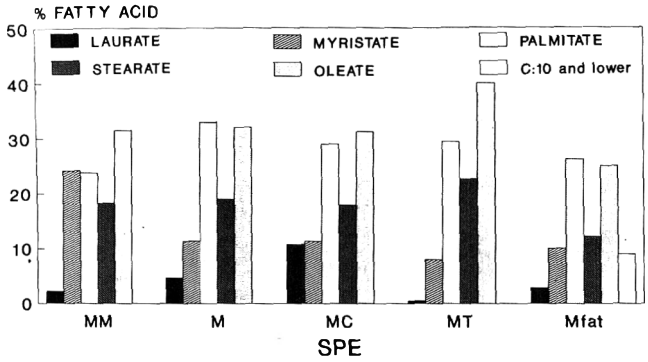

Fig. 3—Fatty acid profiles of SPE and milkfat. MM = Milkfat: myristate SPE; M = Milkfat SPE; Mfat = Anhydrous milkfat; MC = Milkfat:coconut oil SPE; MT = Milkfat:tallow SPE. (Milkfat profile was obtained from USDA, 1979.)

SPE. None of the synthesized SPE contained fatty acids shorter than C:12. The short-chain fatty acids (C:10 and lower) account for ≈ 10% of the fatty acids in milkfat. The other major fatty acids are present in milkfat SPE in about the same ratio, but at higher concentrations. Another reason for the difference in hardness and melting characteristics between milkfat and milkfat SPE may be differences in fatty acid distribution on the glycerol and sucrose molecules, respectively. Such differences may alter functional properties and polymorphic behaviors.

Milkfat:myristate SPE were most similar in hardness to milkfat at the three assay temperatures and exhibited melting curve profiles most similar to milkfat. Milkfat:myristate SPE, similar to the other SPE, do not contain the C:10 or shorter fatty acids present in milkfat. Milkfat:myristate SPE contained a smaller amount of palmitate and a larger amount of myristate than the other SPE. The presence of the larger amount of myristate and smaller amount of palmitate apparently compensated for the lack of C:10 and shorter fatty acids.

Both milkfat SPE and milkfat:myristate SPE exhibited melting finish temperatures similar to milkfat. Milkfat:myristate SPE were most similar to milkfat in hardness and melting onset temperature. Milkfat SPE were harder than milkfat at 12.5, 22, and 27.5°C, and had a melting onset temperature 7°C greater than that of milkfat. The addition of myristic acid methyl esters to hydrolyzed milkfat FAME to synthesize SPE would increase the cost of the SPE. While a milkfat substitute derived solely from milkfat fatty acids would be most desirable, the increased hardness of pure milkfat SPE may result in an unacceptable milkfat substitute. Milkfat SPE or milkfat: myristate SPE are potential ingredients for incorporation as milkfat substitutes in dairy products. The addition of small amounts of FAME from other sources to milkfat FAME would affect fatty acid substituents of the SPE. Other natural fats or oils could be used as supplemental fatty acid sources to produce SPE with predictable hardness and melting behaviors.

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HC values are used by food processors to adjust can fill weights. Regardless of the type of bean, a higher HC allows for lighter fill weights in cans. In our experiments, a 30-min soaking duration gave the optimum desired result.

CONCLUSION

A HIGH TEMPERATURE-short time soaking procedure at 82°C or 93°C for 30 min will provide the shortest rehydration method for dry beans. This time is much faster than the overnight soak used by many processors. No significant differences occurred between hydration coefficients of the treated beans and those of controls. Following soaking temperature of 82°C for 30 min, processing at 121°C for 21 min will provide acceptable canned beans.

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Sucrose Polyester Content in Foods by a Colorimetric Method

M.A. DRAKE, C.W. NAGEL, and B.G. SWANSON

ABSTRACT

Lipids were extracted from the food sample with petroleum ether and sucrose polyesters (SPE) and triglycerides in the extract were hydrolyzed with 1N methanolic KOH for 8 min. Free sucrose was extracted with water, and quantified colorimetrically using a phenol-sulfuric acid test. Results were compared to a standard curve from known quantities of hydrolyzed SPE. Standard curves were linear and reproducible through 90% SPE (r = 0.999).

Key Words: sucrose polyesters, fat substitute, lipids, cheese, colorimetric method

INTRODUCTION

SUCROSE POLYESTERS (SPE) ARE LIPOPHILIC, noncaloric, nonabsorbable fat substitutes synthesized by esterifying 6 to 8 fatty acids onto the hydroxyl groups of sucrose (Mattson et al., 1971). Depending on the type of fatty acids, SPE with a variety of melting effects and functional properties may be synthesized to substitute for natural fats and oils.

Because SPE are lipid-soluble, their quantification in foods requires differentiation between SPE and other lipids. Direct analysis of fat extracts to quantitatively determine SPE has been accomplished using supercritical fluid chromatography (Chester et al., 1985) and high performance gel permeation chromatography (Birch and Crowe, 1976). Hydrolysis of SPE and subsequent benzoylation of the sucrose molecule was used to quantify SPE with high performance liquid chromatography (Wood et al., 1991). Those methods require costly and sometimes complex chromatographic instrumentation. Thin layer chromatography has been used to separate SPE and triglycerides (Birch and Crowe, 1976), but accurate and precise determination of SPE was difficult. Our objectives of this study were to develop a simpler, effective method of quantifying SPE in foods without the need for costly instrumentation or extensive training.

MATERIALS & METHODS

SOYBEAN OIL was purchased in a grocery store (Pullman, WA). Anhydrous milkfat was purchased from Darigold, Inc. (Spokane, WA.). Milkfat SPE were synthesized according to methods of McCoy et al. (1989). SPE were purified according to Drake et al. (1992a). Stock solutions of soybean oil or SPE were prepared in 500 mL hexane at the rate of 1 g oil or SPE/ 10 mL hexane. Mixtures of SPE:soybean oil were prepared in amounts of 10:90, 25:75, 50:50, 75:25, 80:20, 85:15, and 90:10 (v/v). The known SPE:soybean oil solutions were used to prepare standard curves for determining the quantity of SPE in food products. A stock solution of 100% soybean oil (1g oil/10 mL hexane) was used as a turbidity control for generation of the standard curves.

Cheddar-type cheeses with milkfat SPE incorporated as a fat substitute were used to determine the applicability of the assay to food products (Drake et al., 1992b). The cheeses incorporated milkfat SPE as a fat substitute for 10, 25, 50, or 75% of true milkfat. The cheeses were grated, frozen, and lyophilized. Two 5-g replicates of each dried cheese were extracted for 12 hr with petroleum ether using a Soxhlet apparatus. Gravimetric experimentation with standard additions of SPE to lyophilized cheeses demonstrated that fat and SPE were completely extracted in 10 hr. Extracted fat was evaporated to dryness under vacuum using a rotary evaporator. Total fat content of the cheeses was determined by weighing. Each fat extract (2.5g) was dissolved in hexane, transferred to a 25-mL volumetric flask, and made to volume with hexane. Two 10-mL aliquots of each fat extract were hydrolyzed.

Hydrolysis was carried out at room temperature (22 ± 2°C). The SPE/triglyceride mixture (10 mL) was pipetted into a 50-mL beaker containing a spin bar. Ten mL hexane was added for better stirring and the mixture was agitated. A solution (4.5 mL) of 1N methanolic KOH was added followed by stirring for 8 min. The solution was acidified after 8 min with 2.25 mL of aqueous 2N HCl. The contents of the beaker (now containing free sucrose) were transferred to a separatory funnel. The beaker was rinsed twice with 10 mL hexane and twice with 10 mL water, and rinses were added to the separatory funnel. The lower aqueous phase in the separatory funnel was drained into a 50-mL volumetric flask. The hexane phase was extracted two more times with 10 mL water to ensure sucrose was completely extracted. The extract in the 50-mL volumetric flask was made to volume with distilled water. Sucrose solutions were diluted (based on previous experimental trials) to yield 0.2-0.7 absorbance at 490 nm. A sample (10 mL) of an appropriately diluted solution was pipetted into a test tube and the phenol-sulfuric acid test of Dubois et al. (1956) was used to detect and quantify the sucrose. A reagent blank was prepared by running the phenol-sulfuric acid test using 10 mL distilled water in place of the sucrose solution. Absorbance was determined using a model 340 spectrophotometer (Sequoia-Turner Corp., Mountain View, CA).

Two 10-mL aliquots of each standard solution were hydrolyzed and two replicates of each hydrolysate were analyzed for sucrose content. Resulting absorbance values were averaged and used to develop a standard curve. Two aliquots of each hydrolyzed cheese (four replicates/cheese) were also analyzed for sucrose content and resulting absorbance values were averaged and compared to the standard curve to determine the concentrations of SPE. Minor day-to-day variations in color development (0.01– 0.02 absorbance units) were reported by Dubois et al. (1956). A standard curve was developed simultaneously with each cheese assay to minimize effects of such variations.

RESULTS & DISCUSSION

STANDARD CURVES developed on four different days had similar slopes (0.291 ± 0.0045) and were linear (r = 0.999). Standard deviations of absorbance readings were also small (Table 1) (Fig. 1). Results from the cheese analyses (Table 2) showed the method was effective and reliable. Results from the control solution containing no SPE and 100% triglyceride indicated that glycerol from hydrolyzed triglyceride did not interfere with absorbance of sucrose at the dilutions used (absorbance = 0.004) (Table 1). Standard curves were consistent and reproducible through 90% SPE. Increasing the amount of KOH solution and time for hydrolysis did not result in a linear curve through 100% SPE. The increased hydrolysis times and higher concentration of KOH caused the formation of large amounts of soap and made the hydrolysate more difficult to transfer quantitatively. Variance between absorbance readings of the hydrolyzed standard solutions increased and the r value for the standard curve decreased under such conditions. No explanation for the lack of linearity between 90 and 100% (1 g) SPE was apparent. Quantitative analysis of the SPE in cheese indicated the relative error for the calculated SPE concentrations was < 5%.

The sensitivity of the phenol sulfuric acid test is 0.1–0.7g sucrose (Dubois et al., 1956). Sucrose constitutes 15-20% of the molecular weight of SPE depending on the fatty acids, so

The authors are affiliated with the Dept. of Food Science & Human Nutrition, College of Agriculture & Home Economics Research Center, Washington State Univ., Pullman, WA 99164-6376.

Table 1—Standard curve data from four different days

	Day 1			Day 2			Day 3			Day 4		
% SPE	Mean[a] absorbance	StDev	Corrected absorbance	Mean absorbance	StDev	Corrected absorbance	Mean[a] absorbance	StDev	Corrected absorbance	Mean[a] absorbance	StDev	Corrected absorbance
0[b]	0.003	0.002	0.04	0.004	0.003	0.05	0.004	0.003	0.05	0.004	0.003	0.05
10[b]	0.228	0.005	2.85	0.236	0.003	2.95	0.230	0.001	2.88	0.224	0.005	2.80
25[c]	0.279	0.007	6.98	0.289	0.001	7.23	0.282	0.009	7.05	0.269	0.003	6.73
50[d]	0.289	0.003	14.45	0.291	0.006	14.55	0.289	0.012	14.45	0.269	0.004	13.45
75[d]	0.413	0.003	20.85	0.426	0.005	21.30	0.408	0.006	20.40	0.425	0.003	21.25
80[f]	—	—	—	0.478	0.005	23.90	0.460	0.006	23.00	0.455	0.008	22.75
85[d]	—	—	—	0.501	0.003	25.10	0.490	0.005	24.50	0.481	0.004	24.05
90[d]	0.518	0.006	25.90	0.536	0.007	26.80	0.526	0.002	26.30	0.521	0.007	26.50

[a] Mean absorbance values were calculated from four replicates.
[b] Hydrolysate diluted 2/25.
[c] Hydrolysate diluted 1/25.
[d] Hydrolysate diluted 1/50.

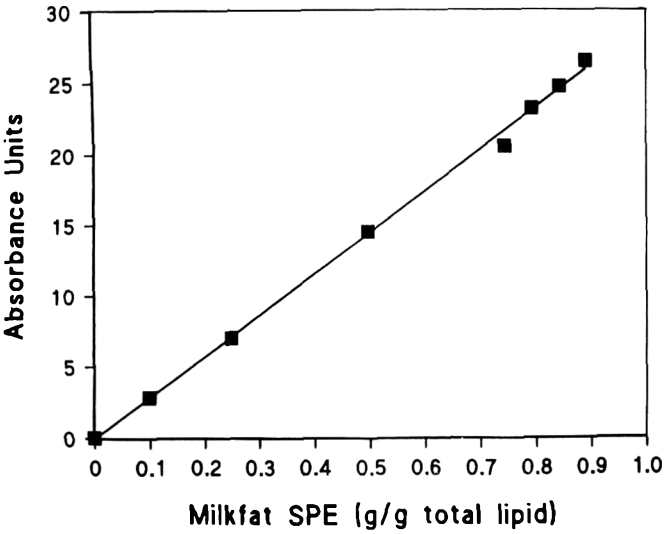

Fig. 1—Standard curve for sucrose polyester determination.

Table 2—Sucrose polyester analysis of cheeses

Calculated % SPE	Mean absorbance	StDev	Corrected absorbance	Confirmed % SPE by analysis	Relative error
10	0.234	0.007	2.93	10.3	3.00%
25	0.286	0.005	7.14	25.0	0.00%
50	0.279	0.008	13.97	48.8	2.40%
75	0.414	0.010	20.68	72.2	3.70%

[a] These values were calculated using the day 3 standard curve which was generated simultaneously with the cheese absorbance values.
[b] Mean cheese absorbance values were calculated from four replicates.

sensitivity of the assay for SPE would be less than that for sucrose. The assay was sensitive to 0.1g SPE. Based on experimental observations, the assay may be sensitive to 0.05g SPE or lower. A hydrolyzed solution of 1.0g triglyceride would need to be diluted to the same degree as a standard solution of hydrolyzed 0.05g SPE and 0.95g triglyceride (probably 3/25 or 4/25, Table 1) prior to the phenol-sulfuric acid test to determine whether glycerol would interfere at those concentrations.

The colorimetric SPE assay requires no costly or complex chromatographic equipment, minimal training, and provides results within 2 days, including time for fat extraction. Since solutions of the same SPE are used to generate the standard curve, the molecular weight of the SPE is not required. A standard curve should be developed each time an analysis is conducted to minimize error due to minor variations in color development. Due to large differences in molecular weights of SPE, standards must be prepared from the SPE substituted in the food. The method is suitable for analyzing concentrations of SPE constituting 10 to 90% of total lipids in foods.

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Antimicrobial Effect of Pressurized Carbon Dioxide on *Listeria monocytogenes*

HO-MU LIN, NINGJUN CAO, and LI-FU CHEN

ABSTRACT

Listeria monocytogenes was inactivated by carbon dioxide at 35 and 45°C under pressures of 70.3 and 210.9 μg/cm². Inactivation rates were sensitive to temperature and pressure. Other factors such as pH, moisture content, and environmental conditions of cell growth also influenced the effectiveness of CO_2 treatment. Bacteria were more difficult to inactivate when they were suspended in the medium with fat or oil, which may have protected the cells from penetration by CO_2. Fat in growth medium where *Listeria monocytogenes* cells were inoculated apparantly increased their resistance. Several methods may be useful for increasing inactivation rates.

Key Words: microbes, inactivation, sterilization, supercritical-CO_2, *Listeria-monocytogenes*

INTRODUCTION

USE OF CARBON DIOXIDE under pressure for sterilization of microorganisms is of great interest (Kamihira et al., 1987; Haas et al., 1989; Kuhne and Knorr, 1990; Wei et al., 1991; Lin et al., 1992, 1993). This method conceptually differs from conventional sterilization in that the primary action of microbial inactivation is likely prompted by extraction of intracellular substances from cells or membranes. A disturbance (or damage in critical areas) of the balance of biologic systems could cause its death without rupture of the cell wall. This concept of cell inactivation could provide alternative techniques for sterilization of bioactive products in food and medicine.

Among the advantages (Lin et al., 1992, 1993), the CO_2 treatment for microbial inactivation is effective not only to airborne and exposed surface bacteria, but CO_2 can also penetrate porous materials to affect microbes inside the food. The treatment can also be applied in conjunction with other sterilization methods when necessary. Past studies have demonstrated the feasibility of this concept of sterilization. Little evidence has been reported on the antimicrobial function of CO_2 in the inactivation process. The exact mechanism of cell inactivation is not clear, and its relationship with inactivation rate not established. Lin and co-workers (1992, 1993) have determined the inactivation rates with a systematic variation in temperature and pressure on common species of microorganisms important in food contamination. Information from those representative species can improve understanding of the lethal effects of CO_2.

As a continuation of those studies, we applied the approach to *Listeria monocytogenes*, which is a small gram-positive organism widely distributed in nature. This pathogen causes listeriosis and some other diseases that are deadly to immuno-compromised humans (Papageorgiou and Marth, 1989; Creager et al., 1990). The diseases can be transmitted through consumption of *Listeria*-contaminated foods or from a wide range of infected animals (Papageorgiou and Marth, 1989). Several characteristics of *L. monocytogenes* as a foodborne bacterium require particular care for processing and storage of foods (Foegeding and Leasor, 1990). It is ubiquitous, moderately heat resistant and psychotrophic and can grow under refrigeration without presence of oxygen. Leasor and Foegeding (Leasor and Foegeding, 1989; Foegeding and Leasor, 1990) have reported on the growth of *L. monocytogenes* in liquid whole egg and its resistance to heat. The results contributed to development of a commercial destruction process for *L. monocytogenes* in liquid egg and egg products (marketed as "Easy Eggs," M.G. Waldbaum Co.). Wei and co-workers (1991) investigated the bacterial effect of CO_2 on *L. monocytogenes* in augmented foods under the experimental pressure of 13.7 MPa for 2 hr at 35°C. The results showed that CO_2 treatment was not particularly effective in killing the augmented *Listeria* in most food systems studied.

Our objective was to use CO_2 as the primary fluid because of its near-ambient critical temperature (31.1°C), which would minimize problems of thermal degradation of delicate biomaterials and natural products. In addition, its phase behavior and other thermophysical properties needed for process development have been well studied. Other advantages of CO_2 in food applications have been documented (Lin et al., 1992, 1993).

The authors are with the Dept. of Food Science, Smith Hall, Purdue Univ., West Lafayette, IN 47907-1160.

MATERIALS & METHODS

Growth of *Listeria monocytogenes*

L. monocytogenes strain Scott A cells were cultivated in a 250 mL flask containing 100 mL of nutrient medium at 27°C for 24 hr. The medium was a solution of 5.5% (w/v) Lactobacillus MRS Broth (DIFCO Laboratories, Detroit, MI) in distilled water. Cell counts of the cultures were generally within the range of 6.5×10^9 to 8.5×10^9 colony forming units (CFU)/mL. The experiments were also extended to the cell samples that were incubated in milk of different levels of fat content in an attempt to assess the effects of fat on inactivation. All the milks (regular, reduced fat and nonfat) were purchased from a local market. *L. monocytogenes* cells required 48 hr of growth time in milk of all types at 27°C to attain cell counts of 10^7–10^8 CFU/mL.

Apparatus

The apparatus was static type that has shown advantages over semiflow extraction apparatus as commonly reported in other studies for cell inactivation (Lin et al., 1992, 1993). This static apparatus was developed to facilitate the penetration of CO_2 into and out of cell that is important to the effectiveness of inactivation (Lin et al., 1992). The major components of the apparatus were a Ruska Pump (Model 2200, Ruska Instrument Corp., Houston, TX) and a pressure vessel (Kuentzel closure reactor with internal volume 6.6 mL, Series KC single ended unit, Autoclave Engineers, Inc., Erie, PA). The pump was used to inject CO_2 into the vessel at the experimental pressure. The vessel served as a sterilizer that was immersed in a thermostated water bath to maintain constant desired temperature. A pressure gauge was installed in the CO_2 inlet of the vessel to measure pressure. The vessel and all other parts exposed to high pressure were of type 316 stainless steel. Carbon dioxide was purchased from Matheson Gas Products with a minimum purity of 99.99%.

Procedure

The experimental procedure was detailed by Lin et al. (1992). Briefly, 1 mL of *L. monocytogenes* was placed in the sterilizer at the beginning of an experiment. The vessel was enclosed and immersed in the thermostated bath at the temperature of interest (35 or 45°C). When the temperature was equilibrated and all tubing connections were

secured, CO_2 was injected from the Ruska pump via a preheated coil into the vessel at the experimental pressure (70.3 or 210.9 kg/cm²). The cells were agitated by a magnetic stirrer. After the cells were exposed to CO_2 for a designated time, the pressure was released. The cell suspension was immediately removed from the vessel and, if needed, diluted in an aqueous solution of 0.1% peptone (pH 6.5) to an appropriate ratio. The number of survival cells was determined by viable counts of plating 0.1 mL of the sample (or diluted sample) on the surface of DIFCO Lactobacilli MRS broth/agar plates. The standard procedure of plate count was described by Banwart (1981). At least six plates were prepared at each experimental condition. Reported results are the averages of multiple samples.

RESULTS & DISCUSSION

TEMPERATURE, PRESSURE AND EXPOSURE TIME are key conditions that control or influences the process of microbial inactivation. The inactivation rates also varied according to the suspending media of treatment and the environmental conditions of cell growth. However, direct determination of the lethal mechanism of CO_2 treatment was extremely difficult. Possible explanations for bactericidal actions were developed based on experimental observations. Several mechanisms have also been postulated in past reports (Daniels et al., 1985; Haas et al., 1989; Wei et al., 1991; Lin et al., 1993).

Some experimental results were compared on inactivation of *L. monocytogenes* that were incubated in nutrient broth at 27°C for 24 hr (Table 1). The extent of inactivation was represented by the ratio of surviving cells after CO_2 for time (N) to the cell count of original cultures before treatment (i.e., at zero exposure time, No). Inactivation rates were apparently sensitive to both temperature and pressure. Results also showed two distinct stages in the inactivation process at a constant temperature. The earlier stage was governed primarily by the penetration of CO_2 into the cells. Once the concentration (or density) of CO_2 within cells was augmented to a critical level, it would extract vital constituents from cells or membranes enough to lethally disturb or alter the balance of biological systems and prompt inactivation. A damage to structure of membranes, biofunction of proteins or balance of enzymatic activities could contribute to microbial destruction without rupture of cell walls. Inactivation rates were slow at the earlier stages and increased sharply afterward, suggesting that the diffusivity of CO_2 into cells was a controlling factor. About 10 min were required, for example, to inactivate one log cycle of *L. monocytogenes* under CO_2 pressure of 70.3 kg/cm², while 8 log cycles of cells were destructed within 20 min at the later stage. The cellular penetration of CO_2 was extremely sensitive to the applied pressure. The exposure times needed for inactivation of the same amount of cells were significantly reduced at higher pressures. At 210.9 kg/cm², CO_2 treatment became effective in 2 min and zero cell counts (i.e., no survival cells in plate of 0.1 mL undiluted sample) occurred in 14 min. The extraction process that transfers vital constituents from the biological systems to cause inactivation can be slow, depending on the species of microorganism and experimental conditions. However, the action appeared to be stimulated (as a result of sudden expansion of CO_2 within the cells) when applied pressure was released.

Pressure dictates both the solubilization rate of CO_2 and its solubility in suspending medium. Higher pressure enhanced solubilization of CO_2 to facilitate its contact with the microbes, a key factor to cellular penetration. The importance of promoting the contact was also demonstrated by inactivation rates being substantially reduced at any experimental conditions without agitation. Furthermore, CO_2 dissolves in aqueous solution to form acid. The solubilization of CO_2 could therefore be related to the pH changes in suspension. Lower pH inhibited bacterial growth (Daniels et al., 1985), and also attenuated microbial resistance to inactivation (Kamihira et al., 1987; Haas et al., 1989; Wei et al., 1991). The mechanism for this antimicrobial action of pH is not known, although several theories have been suggested (Daniels et al., 1985; Haas et al., 1989). Nevertheless, acidification by CO_2 alone probably does not account for its lethal effect, as demonstrated by experimental results from Haas et al. (1989) and Lin et al. (1993). The pH reduction by dissolved CO_2 depends on applied pressure at a constant temperature. Meyssami and co-workers (1992) have reported on pH changes in some aqueous solutions by pressurized CO_2. In pure water, pH changes were sensitive to CO_2 pressures only in the low pressure range (< 70.3 kg/cm²); and are a weak function of temperture at all pressures. The solution was less acidic at higher temperatures, as a result of decreased solubility of CO_2 in water. Such experimental evidence of pH changes with pressure and temperature were not in accordance with cell inactivation. Inactivation rates increased consistently with an increase in temperature or pressure, or both, under all conditions studied, further suggesting that acidification by CO_2 alone was not cause of microbe lethality. An important function of pH in inactivation is likely that the acidic environment helps increase cell permeability to ease cellular penetration of the fluid (Lin et al., 1993). Lin and co-workers (1993) suggested that formation of carbonic acid by dissolved CO_2 in aqueous solution could be response for some of the lethal action. The acid at high concentration would dissociate into bicarbonate and hydrogen ions. When the applied CO_2 pressure was released, bicarbonate would convert to carbonate which precipitates intracellular calcium and other ions causing damage to the cells.

Experiments were extended to 45°C. As compared with 35°C, inactivation rates appearred insensitive to temperature at earlier stages (within 3 min exposure time), where the pressure had more effect. However, the sensitivity was sharply changed as treatment continued. It took about 4 min exposure time to reduce one log cycle of *L. monocytogenes* but only 4 more min were needed to deactivate all living cells at that temperature. Inactivation rates were again a strong function of pressure. Temperature relates to the characteristics of mass transfer of a fluid. For a biological system, it also affects microbial activities. Higher temperatures favor the diffusivitity of CO_2 and could probably relax the cell wall to ease penetration. However, too high a temperature can create problems (Lin et al., 1993), particularly degradation of food quality.

In addition to temperature, pressure and exposure time, the physical and chemical properties of suspending medium can be influential in inactivation. Water content in the suspension, for example, is vital to the antimicrobial action of CO_2. One reason that wet cells are more accessible to CO_2 treatment

Table 1—Reduction in living cells of *Listeria monocytogenes* (N/No) after treatment with CO_2 at various experimental conditions

	N/No*		
Exposure	35°C		45°C
time (min)	70.3 kg/cm²	210.9 kg/cm	70.3 kg/cm²
0	1.0	1.0	1.0
0.67	—	0 35	—
1	0.84	0 14	0.94
2	0.63	2.2×10^{-2}	0.73
3	—	—	0.38
3.5	—	8.4×10^{-4}	—
4	0.45	1.3×10^{-4}	7.8×10^{-2}
5	0.37	—	3.4×10^{-3}
6	—	3.5×10^{-6}	3.8×10^{-7}
6.5	0.28	—	—
8	0.21	6.6×10^{-8}	0
10	6.2×10^{-2}	1.5×10^{-8}	0
12	3.2×10^{-3}	8.4×10^{-9}	—
14	4.2×10^{-4}	0	—
15	—	0	—
17.5	8.8×10^{-6}	—	—
20	2.9×10^{-7}	0	0
25	1.2×10^{-8}	—	—
30	0	0	0
35	0		
40	0		

* No = 6.5×10^{8}–8.5×10^{8} CFU/mL.

Table 2—Effect of fat contents in milk (as growth medium) on inactivation *Listeria monocytogenes* at experimental conditions of 45°C, 1000 psi and 1 hr

Milk	No	N/No
Nonfat	6.5×10^{7}	6.1×10^{-7}
Reduced fat	6.9×10^{7}	1.3×10^{-6}
Regular	1.7×10^{8}	8.8×10^{-2}

Table 3—Effect of growth media on inactivation of *Listeria monocytogenes*

Pressure[a] kg/cm²	In "Protein" (No=3.8×10^{8} CFU/mL) N/No	In "Nonfat" (No=2.3×19^{8} CFU/mL) N/No
210.9	0	8.6×10^{-7}
70.3	1.3×10^{-6}	1.7×10^{-6}
70.3[b]	6.5×10^{-6}	6.5×10^{-6}

[a] At 45°C for 1 hr.

[b] Applied CO_2 pressure was released (and repressurized afterward) once in the midst of process.

probably directly results from swollen cell walls due to presence of water. The expanded walls become more penetrable to CO_2. Water is also essential to hydration of CO_2 to form acid. The acification has an effect on cell permeability. Water activity and pH of suspension can also be important. Environmental conditions of cell growth and age of cells are other factors that may be important with some species of microorganisms. In general, young cells are more susceptible to CO_2 treatment than mature cells. Spores are much more recalcitrant than vegetative cells. Lin and co-workers (1993) also observed that bacterial cells were less resistive to inactivation when they were cultivated at 10°C rather than 27°C. The properties of growth (or culture) medium can affect the characteristics of cell walls and membranes. Since milk is a medium in which *L. monocytogenes* easily adapt, experimental results were obtained for cells that were incubated in different types of milk. Of various essential contents in milk, fat was most significant to microbial resistance to inactivation.

Effect of fat contents

Lactose Reduced Nonfat milk (grade A) from C.F. Burger Creamery Co. (Detroit, MI), reduced fat milk and regular milk (Vitamin D milk) from Dean Foods Co. (Franklin Park, IL) were compared with exposure time to CO_2 of 1 hr. The results showed the N/No values of 1.0×10^{-7} in regular milk and zero in both reduced fat milk and nonfat milk (Table 2). Obviously, the fat content in milk increased the resistance of *L. monocytogenes* to CO_2 treatment. This evidence was consistent with results from the second sites of experiments (Table 3). Regular milk was centrifuged into three layers: "fat," "nonfat" and "protein" layer. "Nonfat" and "protein" layers were collected (after separation from "fat" layer) to serve as growth media. *Listeria monocytogenes* that were incubated in the "nonfat" or "protein" media for 48 hr were subject to CO_2 pressures at 45°C. More than seven log cycles of cells from both media were inactivated in 1 hr. Both "nonfat" and "protein" layers has some amount of saturated fat after separation from the centrifuged milk. The control experiments also showed a decrease of inactivation rates (compared with Table 1), by the presence of fat in the suspension.

All these experimental results point to a conclusion that *L. monocytogenes* were more recalcitrant to CO_2 treatment when grown or suspended in a media that contained a fat or oil. The presence of fat in growth and suspending media probably resists CO_2 penetration into cells by different mechanisms. Fat content in growth medium in which the cells are incubated can have a biological effect on the structure of cell walls and membranes and/or a physical effect on characteristics of porosity. Yeast cells, after incubation in regular milk for at least 48 hr, were scrutinized by scanning electron microscope for peculiar changes in cell walls. Preliminary results showed a distinctive difference in smoothness of outer cell walls compared with those grown in nutrient broth or nonfat milk. Further studies are needed for identification of those filmed materials on cell wall surfaces.

Improvement of inactivation rate

The inactivation process with pressurized CO_2 is governed essentially by penetration of CO_2 into cells and its effectiveness could be further improved by enhancement of the transfer rate of CO_2. This may be accomplished by agitation, which facilitates solubilization of CO_2 through better contact with cells. The inactivation rates were decreased sharply without appropriate agitation. Addition of entrainers (chemical and biochemical agents) to the treatment is another potential means to improve the inactivation rate. A variety of antibiotic agents could be used for this purpose. Useful agents also might include those that could react with cell walls or membranes to increase permeability. Lin and co-workers (1992, 1993) suggested that improvement in inactivation rate could be achieved by repetitious release of applied CO_2 pressure (and repressurization afterward) during the inactivation process. *Listeria monocytogenes* were inactivated substantially faster after a series of pressure releases repressurizations.

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Bioproduction of Perillyl Alcohol and Related Monoterpenes by Isolates of *Bacillus stearothermophilus*

HAE C. CHANG and PATRICK ORIEL

ABSTRACT

Bacillus stearothermophilus BR388 was isolated from orange peel by an enrichment culture using (+)-limonene. The thermophilic isolate exhibited growth between 45 and 68°C., with optimum growth near 55°C. BR388 could grow on limonene as a sole carbon source, but grew and degraded limonene more effectively when supplemented with small amounts of yeast extract. Perillyl alcohol was the major conversion product, with α-terpineol and perillyl aldehyde as minor products. Strains of *B. stearothermophilus* previously isolated from aromatic enrichments also grew on limonene, but had higher sensitivity to limonene toxicity than did BR388.

Key Words: Bacillus stearothermophilus, perillyl alcohol, orange peel, (+)-limonene

INTRODUCTION

THE MONOCYCLIC TERPENOID (+) limonene [(R)1-cyclohexen-1-methyl 4-(1-methylethyl)] has potential as a monoterpene starting material for microbial bioconversion to higher value monoterpenes utilized in flavor and perfume applications (Kieslich et al., 1986). (+) limonene is a common constituent of many essential oils and is the major component in oils from citrus peel waste (Braddock and Cadwallader, 1992). Thus, it is a low cost, widely available terpene, and is used directly several food and other applications (Krasnobajew, 1984). Useful oxidation products of (+) limonene include perillyl alcohol [(R)1-cyclohexene-1-methanol-4-(1-methylethyl)]; perillyl aldehyde [(R)1-cyclohexene-1-carboxaldehyde 4-(1-methylethyl)], and perillic acid [(R)1-cyclohexene-1-carboxylic acid-4-(1-methylethyl)]. These are naturally found in low quantities in citrus, lemon grass, and perilla oils, and are utilized as flavorings and as antimicrobial agents in foods and pharmaceuticals (Feranoli, 1975). Several investigators have examined the possibility of microbial conversion of limonene to other monoterpenes of interest, and have isolated bacteria capable of growth on limonene yielding metabolites such as (+)-α-terpineol, (+)-limonene-1,2-diol, (+)-perillic acid, and (+)-β-isopopenyl pimelic acid (Cadwallader et al., 1989; Dhavlikar and Bhattacharyya, 1966; Kraidman et al., 1969; Krasnabajew, 1984; Bowen, 1975; Rama Devi and Bhattacharyya 1977a,b). Such pioneering studies are high interest but commercialization of such processes has been thwarted by the multiplicity of products produced, and the low product concentrations due to the relative toxicity of limonene to most microorganisms.

Recognizing the rapid growth rates, broad metabolism, and resistance to chemical toxicity demonstrated by some aerobic thermophilic bacteria (Gurujeyalakshmi and Oriel, 1989; Natarajan et al., 1994), we initiated a search for thermophiles capable of (+) limonene conversion to higher value products. Recognizing the ubiquity of *Bacillus* thermophiles (Buchanan and Gibbons, 1974), we reasoned that since (+) limonene is the major constituent of citrus essential oils, (ca. 95% in orange and grapefruit peel oils), the peel of oranges might provide a source for limonene-degrading thermophiles. Our objective was to search for, isolate and characterize *Bacillus stearothermophilus* strains which could utilize (+) limonene as sole carbon sources for growth, and to identify major monoterpene conversion products.

The authors are affiliated with the Dept. of Microbiology, Giltner Hall, Michigan State Univ., East Lansing, MI 48824-1101.

MATERIALS & METHODS

Reagents and microorganism isolation

(+) Limonene and (+) perillic acid were obtained from Aldrich Co. (+) Perillyl alcohol and (+) perillyl aldehyde were purchased from Nippon Terpene Chemical Co., Japan. Thermophiles capable of growth on limonene were isolated by inoculation of small pieces of orange peel into 50 mL of DP salt media (per liter: NH_4Cl, 1.0g; K_2HPO_4, 0.5 g; $MgSO_4 \cdot 7H_2O$, 20mg; pH 7.2) with 1 mL of neat (+) limonene (97%, Aldrich Co.) in a 125 mL screw-cap bottle, and incubation at 55°C with shaking. After 24 hr incubation, 100-200 μL of the culture were plated onto DP salt agar media in Petri dishes containing 100 μL of limonene in a small glass tube attached to the cover, and incubated at 55°C. Cultures demonstrating growth on repeated transfer were retained as putative limonene users.

Toxicity test of limonene

To determine the relative toxicity of limonene, thermophile isolates were cultured in LB broth (Bacto-tryptone, 10g; yeast extract, 5g; NaCl 5g per liter) containing concentrations of (+) limonene between 0–0.2% (v/v). Growth was observed using turbidity.

Growth and biotransformation

Triple-baffled 250 mL nephelo culture flasks with cleanout arm and depressed side arm (Bellco Inc.) were used for thermophile growth and biotransformation studies. Such flasks allowed vapor introduction of (+) limonene into the culture from liquid contained in the side arm. The medium for growth and limonene transformation contained 75 mL of DP salt and vitamin complex (0.4 mg nicotinamide, 0.4 mg of thiamine and 2 μg biotin/L) either alone or supplemented with 0.0125% yeast extract. Cultures were incubated at 55°C. in a gyratory shaking water bath. Relative growth at various temperatures was measured by culture turbidity with time measured at 550 nm in a Gilford single beam spectrophotometer (corrected for absorbance of medium). Maximum specific growth rates at various temperatures were determined from the maximum slope of semi-log plots of turbidity with time.

Extraction of the biotransformation products

To recover biotransformation products, 16–36 hr cultures were centrifuged at 12,800 × *g* for 20 min at 4°C. Following passage through a Millipore 0.45 μm filter, the filtrate was acidified to pH 2.0 and extracted (3 × 0.5 vol) with ether. The ether fraction was evaporated to 25 mL and separated into neutral and acidic fractions by extraction (3 × 0.6 vol) with 5% (w/v) NaOH solution. The ether fraction was concentrated under a stream of nitrogen, neutralized with 5% (v/v) HCl and then analyzed by high performance liquid chromatography (HPLC) and GC-MS using procedures of Cadwallader et al., (1989). The NaOH fraction was acidified to pH 2.0 and re-extracted (3 × 0.3 vol) with ether. The ether fraction was concentrated under a stream of nitrogen and analyzed by HPLC and GC-MS.

Analytical methods

Products were analyzed by HPLC for routine analysis, and analyzed by GC-MS for product identification. The HPLC system used was a

HP 1050 series (Hewlett Packard). Injection volume was 5.5 μL, volumes for draw speed and eject speed were each 200 μL/mL. Separation utilized a 3.9 mm × 15cm NOVA-PAK C18 column (Millipore Co.) equipped with 3.2 × 15 mm RP-18 Brownlee New 7 micron guard column (Anspec Co.). Peaks were detected at 245 nm using a HP1050 series mutiple wavelength detector. Samples were eluted using a 20 min linear gradient from 99.9% water to 99.9% acetonitrile with an initial hold time of 1 min and a second hold time of 5 min and again a 8 min linear gradient from 99.9% acetonitrile 99.9% water.

The GC-MS system was a mass spectrometer HP 5970 coupled with gas chromatograph HP 5890 (Hewlett Packard). The mass selective detector was an MSD HP 5970 (Hewlett Packard). A 0.25 mm i.d. × 30m DB-wax fused silica capillary column (J & W Scientific Co.) was used for separation. Conditions were: 1 μL injection; He carrier gas; injection port and detector port at 240°C; column temperature programmed from 40–240°C at 7°C/min with a 2 min initial hold time.

RESULTS & DISCUSSION

Microorganism isolation and characterization

Two colonies, designated BR 388 and 389 were isolated from limonene enrichment culture using an orange peel inoculum. Both demonstrated growth at 55°C on DP plates utilizing limonene as sole carbon source, producing small creamy colonies in 3–4 days. Both isolates were rods of ≈3–4 μm length, demonstrated variable Gram staining, and produced oval spores under starvation conditions (Fig. 1). These characteristics, and their ability to grow well under aerobic conditions at 65°C were consistent with those of the broadly-defined species *Bacillus stearothermophilus* (Buchanan and Gibbons, 1974), to which we tentatively assigned them. They were differentiated from *Bacillus schegellii*, which can also grow aerobically at 65°C. by shorter cell length, lower optimum growth temperature, and oval rather than spherical spore shapes (see Schenk and Arago, 1979). Two other *Bacillus stearothermophilus* strains BR316 (Nararajan et al., 1994) and BR219 (Gurujeyalakshmi and Oriel, 1989), which were isolated from toluene and phenol enrichments, respectively, were also tested for growth on limonene. Of these, BR316 demonstrated that ability, and was retained for further testing.

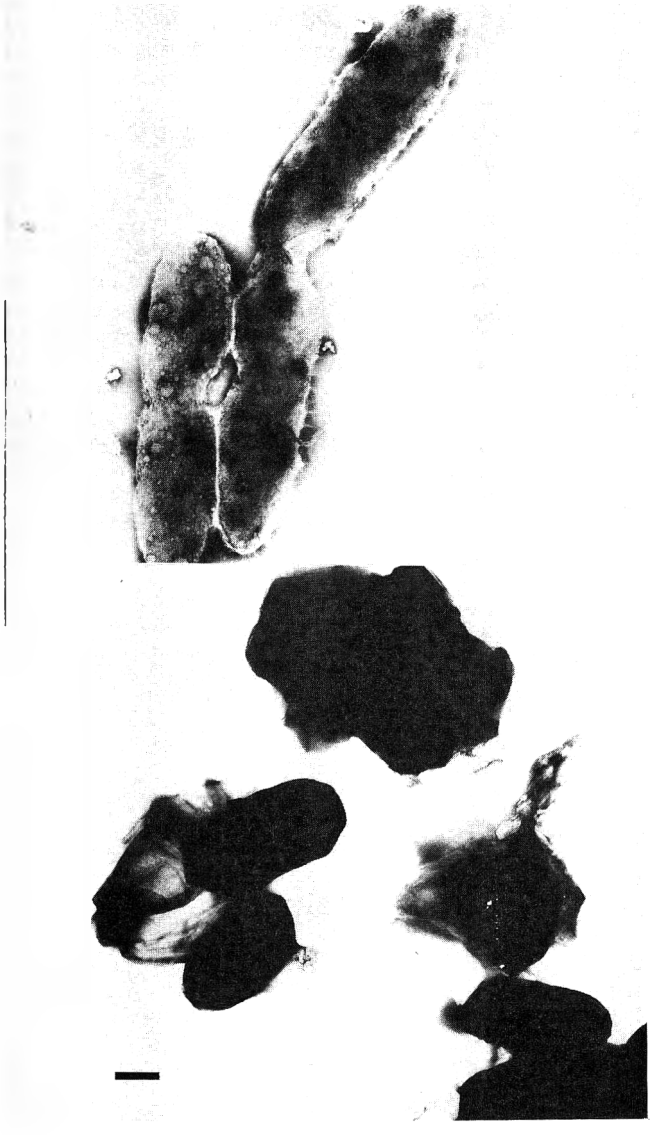

Fig. 1—Electron microscopy of *Bacillus stearothermophilus* BR 388. Top: vegetative cells (26,350X); Bottom: spores (26,350X). Bar is 0.38 μm.

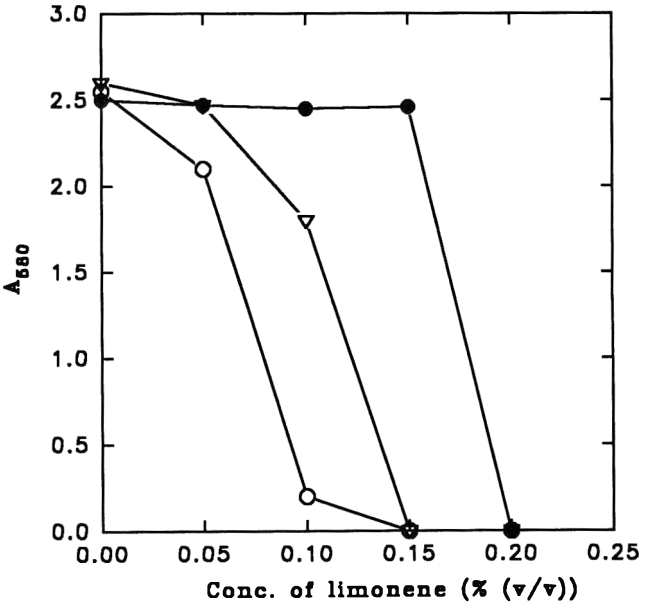

Fig. 2—Growth inhibition by (+) limonene of *Bacillus stearothermophilus* strains BR388 (●——●, BR389 (△——△), and BR316 (○——○).

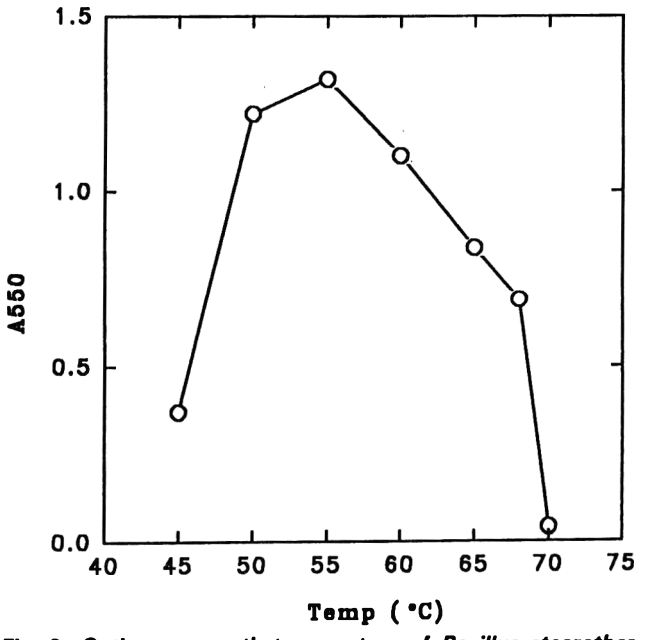

Fig. 3—Optimum growth temperature of *Bacillus stearothermophilus* BR388.

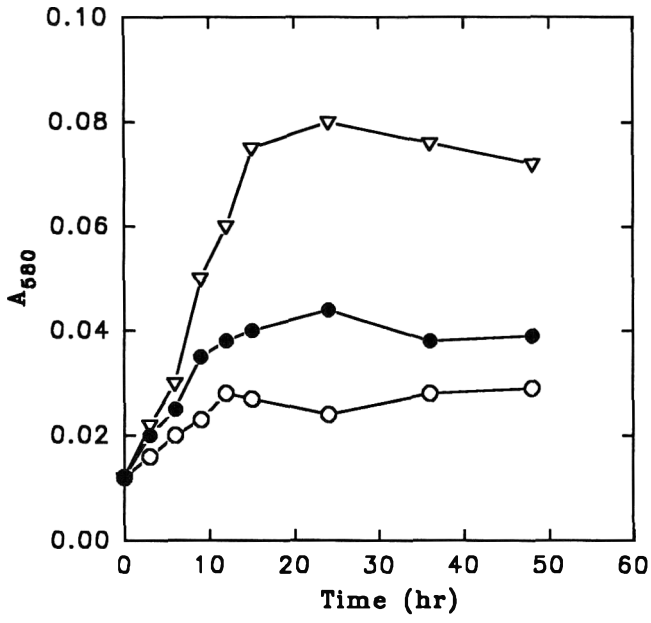

Fig. 4—Growth of *Bacillus stearothermophilus* BR388 in DP minimal broth. h——h w/limonene & w/yeast extract; ●——● w/o limonene and w/yeast extract; o——o w/limonene and w/o yeast extract.

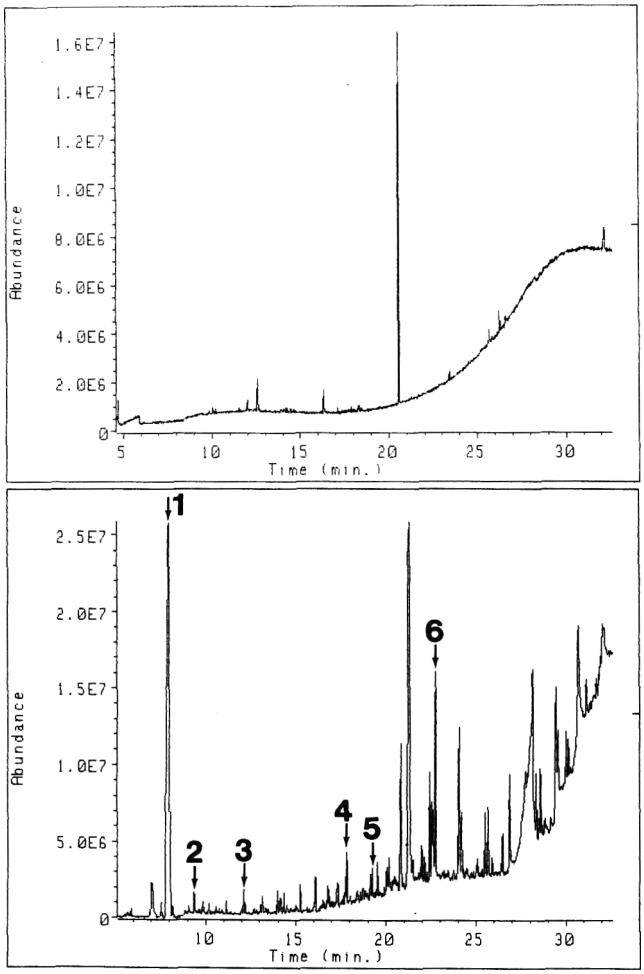

Fig. 5—GC chromatogram of bioconverted products from limonene by *Bacillus stearothermophilus* BR388. Top: bacterial control extract; Bottom: limonene control extract. (1) limonene; (2) 1,8-cineole; (3) bicylic monoterpene; (4) α-terpineol; (5) isomer-perillyl aldehyde; (6) perillyl alcohol.

Limonene tolerance

Limonene is toxic to many microorganisms, even at low concentrations (see, for example Uribe and Pena, 1990). To test the ability of thermophile isolates BR388, BR389, and BR316 to withstand limonene toxicity, we observed their growth in rich medium containing limonene at concentrations between (0.0–0.25% (v/v)) (Fig. 2). Note that the concentration of limonene shown is that added, uncorrected for volatilization. In these studies, BR388 demonstrated higher resistance to limonene toxicity than BR389, which in turn was more resistant than BR316 which was not isolated from a limonene-containing environment. As a result of these tests, BR388 was selected for biotransformation studies.

Optimum growth temperature and BR388 growth in liquid culture

To verify that *Bacillus stearothermophilus* BR388 was an obligate thermophile, growth in LB medium at 45°C to 70°C was measured. As seen (Fig. 3) BR388 grew well from 45 to 68°C. The optimum growth temperature was at 55°C where a maximum specific growth rate of 2.4 hr^{-1} was observed (data not shown).

Growth of *Bacillus stearothermophilus* BR388 in DP minimal medium with limonene was measured (Fig. 4). Although the isolate was able to grow in this medium, biomass levels were modest. To enhance growth, 0.125 g/L yeast extract was added for supplementation. This level of yeast extract supported a low level of growth in the absence of limonene, but significantly enhanced biomass in the presence of limonene.

Limonene bioconversion products

Growth of *Bacillus stearothermophilus* BR388 on DP minimal medium supplemented with 0.0125% yeast extract and limonene vapor resulted in formation of multiple metabolites. Their number and amounts varied with culture time. From the GC-MS chromatograms of the neutral fraction (Fig. 5), perillyl alcohol was identified as a major component, reaching levels of 200 μg/mL of culture. Control cultures with no limonene vapor did not produce this metabolite. Other metabolites present in lower amounts were α-terpineol, perillyl aldehyde, bicyclo[2,2,1]-hepten-2-one, cyclohexanol and 1,8-cineole. Highest yields of perillyl alcohol were obtained from cultures in the exponential phase (data not shown). Perillic acid was not observed in the acidic fraction.

Thus, the broad metabolic capability of *Bacillus stearothermophilus* includes utilization of limonene, resulting in production of monoterpenes of biotechnological interest. Our strategy to control the number and concentration of monoterpene metabolites is to clone the limonene oxygenase genes from the thermophilic isolates and place them under control of exogenous promotors.

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Quantification of Factors Which Influence Nisin's Inhibition of *Clostridium botulinum* 56A in a Model Food System

ANN M. ROGERS and THOMAS J. MONTVILLE

ABSTRACT

We modeled nisin's anticlostridial activity and assessed the antagonistic or potentiating influences of food ingredients. The model systems contained yeast extract, proteose peptone, and glucose; were supplemented with protein (0.075, 0.75, 7.5% w/v), phospholipid (0.075, 0.75, 7.5% w/v), or soluble starch (5, 17.5, 30% w/v); and were adjusted to pH 5.5, 6.0, or 6.5. Samples inoculated with 10^4/mL spores were incubated at 15, 25, or 35°C. Statistical analysis developed an equation ($r^2 = 0.76$) that modeled the response and identified temperature as the most significant ($\alpha \leq 0.001$) variable. Nisin lost effectiveness with increasing temperature. Nisin concentration had significant positive and phospholipid negative, linear effects. Many interactive effects were significant ($\alpha \leq 0.20$). Nisin inhibited *C. botulinum* until its residual level dropped below a threshold, which decreased from 154 IU/mL at 35°C to 12 IU/mL at 15°C.

Key Words: nisin, *Clostridium*, bacteria, spores, growth inhibition, modeling

INTRODUCTION

PRESERVATION OF FOODS involves a calculated and proven balance of product composition, process and package. The addition of bacteriocins to products may effectively prevent growth of pathogenic microorganisms. Nisin, a bacteriocin produced by *Lactococcus lactis* has broad spectrum activity against gram-positive bacteria. Those in the genera *Listeria* (Benkerroum and Sandine, 1988), *Bacillus, Clostridium, Staphylococcus, Streptococcus, Lactobacillus, Micrococcus* (Carminati et al. 1989; Denny et al., 1961; Gould, 1964; Mattick and Hirsch, 1944; O'Brien et al., 1956; Scott and Taylor, 1981a,b; Somers and Taylor, 1981; Spelhaug and Harlander, 1989), and *Mycobacterium* (Gowans et al., 1952) are sensitive to nisin. It also acts against gram-negative bacteria that are treated with EDTA (Stevens et al., 1992) or are injured (Kalchyanand et al., 1992). Nisin activity is reduced in complex media and model food systems containing meat or meat emulsions (Delves-Broughton, 1990).

Nisin is approved for a wide range of applications in many countries. In the U.S., its approval is restricted to certain pasteurized cheese spreads (Federal Register, 1988). Although its efficacy in any food system should be validated through inoculated pack studies, knowledge of how food ingredients influence nisin's efficacy would help identify foods particularly suited or unsuited for nisin addition. For example, phosphotidylcholine is a nisin antagonistic (Henning et al., 1986a), and protein-protein interactions may reduce activity in cooked meat media (Scott and Taylor, 1981a). pH is also a significant factor in the effectiveness of nisin. At pH 6.0, nisin is active at levels above 2,500 IU/mL against spore inocula up to 10^4/mL. Nisin is inactive at pH 7.0 and 8.0 (Scott and Taylor 1981a). At 22,000 IU/mL nisin did not inhibit the growth of *C. botulinum* type A in pork slurries at pH 5.8 (Rayman et al., 1983). Nisin at 4000 to 6000 IU/mL in combination with 120 ppm nitrite was effective in bacon as an antibotulinal agent, but with minimal shelf life extension (Taylor and Somers, 1985) as compared to nitrite alone at 150 ppm. The same nisin levels were not effective when used with 40 ppm nitrite. In chicken frankfurters, nisin (4,000 to 10,000 IU/g) was an effective anticlostridial agent even when nitrite levels were reduced to 40 ppm (Taylor et al., 1985). The concentration of nisin required for anticlostridial action in pasteurized cheese spreads depends on moisture, salt, and sodium phosphate concentrations (Somers and Taylor, 1987). Thus, published results clearly demonstrate that nisin activity decreases in complex media and that food ingredients can increase or decrease its anti-botulinal activity. Quantification of such individual and composite effects in a model food system would elucidate previous studies and help develop rational strategies for nisin applications.

Our objective was to determine the influence of common food ingredients, pH, and temperature on the antibotulinal activity of nisin. Statistical analyses of results from a challenge study in a model system identified those variables significantly associated with nisin effectiveness against *C. botulinum* 56A, a strain which is relatively nisin-insensitive (Montville et al., 1992).

Author Montville is with the Dept. of Food Science, Cook College, New Jersey Agricultural Experiment Station, Rutgers, The State Univ. of New Jersey, New Brunswick, NJ 08903-0231. Author Rogers is with Kraft General Foods, Tarrytown, NY 10591. Address inquiries to Dr. T.J. Montville.

MATERIALS & METHODS

Cultures, maintenance, and spore preparation

Lactobacillus sake ATCC 15521 was obtained from American Type Culture Collection (Rockville, MD), and used as the indicator organism in the nisin assay (Rogers and Montville, 1991). It was maintained on Lactobacilli MRS agar (Difco Laboratories, Detroit, MI) with stock cultures kept at −80°C. *Clostridium botulinum* strain 56A was obtained from Virginia Scott, of the National Food Processors Association (Washington, DC) and stored either as the original spore suspension or as a mixture of glycerol and cooked meat media at −80°C.

C. botulinum strain 56A spores were produced using the biphasic method (Anellis et al., 1972) and counted using a Petroff-Hauser chamber. Confirming plate counts were conducted after heat-activation at 80°C by spread plating on modified Mc Clung agar (Dowell and Hawkins, 1979) and botulinum assay media (Montville, 1981). All culture manipulations were done in an anaerobic chamber with an atmosphere of 5% H_2, 10% CO_2, and 85% N_2. The spore suspension was then diluted and stored in 1.0 mL quantities at −80°C.

Preparation and inoculation of model system

A basal medium consisting of 1.0% w/v glucose (Sigma Chemical), 0.5% w/v peptone (Difco), and 0.5% w/v yeast extract (Difco) and 0.01% w/v resazurin (Sigma) supported growth of and gas production by *C. botulinum* type 56A, at pH values in the range of 5.0 to 7.0, and temperatures from 15°C to 35°C (Rogers, 1991). In preliminary experiments to determine the influence of various food components, egg albumin (10% w/v, Sigma), soy flour (10% w/v, Sta-Pro 3000, Staley Manufacturing Co.), lecithin (10% w/v, Sigma), phosphatidylcholine (10% w/v, Sigma), egg yolk (10% w/v, Difco), carrageenan (0.3% w/v, Sigma), guar gum (0.3% w/v, Sigma), soluble starch (30% w/v, Difco) or xanthan gum (0.1% w/v FMC) were individually added to the basal medium in the presence or absence of 1,000 I.U./mL nisin, the midpoint of the fractional factorial design described below. The pure nisin was a gift from Applied Microbiology, Inc. (New York, NY). The media were adjusted to pH 6.0, and inoculated with *C. bot-*

ulinum 56A spores as described below. The cultures were incubated at 30°C and observed daily for gas produced as the result of clostridial growth.

From the results of the preliminary experiments, protein, phospholipid, and soluble starch were selected for inclusion in the main modeling experiment. The ECHIP statistical software program (ECHIP, Inc., Hockessin, DE) was used to construct a fractional factorial design for a model food system containing three levels of six variables; temperature, pH value, protein, phospholipid, carbohydrate, and nisin. We defined each combination of variables as an "experiment." The ECHIP program identified a fractional factorial design which reduced the number of experiments from 729 to 53. The set of variables for each experiment is given in the first seven columns of Table 1. In addition to the 53 primary experiments, the program designated several experiments to be repeated (these are identified by lettters appended to the experiment number in Table 1) so that the reproducibility and variability of the results could be calculated.

Because the growth rate of *C. botulinum* is influenced by pH and temperature, each experiment was done using media without nisin as positive controls (in triplicate) to determine the time required to detect botulinal growth by gas production in the absence of nisin. Identical experiments were done using media with nisin (in triplicate) to determine how long nisin inhibited *C. botulinum.* The contribution of nisin to the delay in gas production (ΔN, days) was calculated according to the equation ΔN = (days to gas in the presence of nisin) - (days to gas in the absence of nisin). All experimental combinations included duplicate uninoculated tubes of media as sterility controls.

Each combination of food components for each experiment was prepared separately, pH adjusted separately, and inoculated separately. The six experimental variables and their three levels were the addition of protein (chicken egg albumin, 0.075, 0.75, and 7.5% w/v), lipid (lecithin, 0.075, 0.75, and 7.5% w/v), corn starch (5, 17.5, and 30% w/v) and nisin (100, 1,000 and 10,000 IU/ml) to the medium; pH adjustment (using HCl) to 5.5, 6.0, or 6.5; and incubation at 15, 25, or 35°C. The mechanics of media preparation are detailed elsewhere (Rogers, 1991). Briefly, the egg albumin and lecithin were sterilized in the frozen state by gamma irradiation (3.4 Mrad total at a rate of 0.114 kGy/min) using a self-contained Cesium-137 radiation source (Lockheed Corp., Marietta, GA) at the U.S. Department of Agriculture Eastern Regional Research Center in Philadelphia, PA. The nisin was sterilized as a concentrated acidic solution by autoclaving. Appropriate amounts of the basal media and starch were pipetted into Hungate-style test tubes which had septum seals. Inverted Durham vials were added and the tubes were autoclaved for 15 min at 121°C. Appropriate amounts of sterile albumin, lecithin, and nisin were aseptically added to the tubes after autoclaving. The pH values for the model food system were 5.5, 6.0, and 6.5, adjusted aseptically with 0.1N HCl or NaOH after autoclaving. The media were brought into an anaerobic chamber and inoculated when the resazurin in the medium was reduced. If necessary, the media were held on ice to ensure that, once inoculated, the spores were never exposed to a temperature higher than their incubation temperature.

C. botulinum type 56A spores were diluted in sterile distilled water at a concentration of spores 4×10^5/mL and heat-shocked at 80°C for 10 min. The Hungate tubes were each inoculated with 250 μL of the heat-shocked spores to give a final concentration of 10^4 spores/mL, sealed with a septum, placed in a high barrier Kapak pouch (ScotchPak, Inc.) and sealed in the anaerobic chamber. This pouch was placed inside a second pouch and the outside pouch was sealed to insure the integrity of the anaerobic conditions during the prolonged incubation. In addition, resazurin was used to monitor anaerobiosis. Once the pouches were double sealed, the cultures in pouches were incubated at the appropriate temperature in standard incubators which contained recording thermometers.

End point evaluation

Cultures were removed from incubation and refrigerated when gas was observed in the Durham vial. Residual nisin concentrations were determined (Rogers and Montville, 1991), pH values were measured, Δ N values were calculated as described above and botulinal counts on modified McClung agar were done by manual spread plating.

Mouse toxicity testing (Dowell and Hawkins, 1979) was conducted on each of the triplicate cultures in each of the nisin-containing experiments. Supernatants were prepared by centrifugation. Boiled samples were confirmed by anti-toxin neutralization at 37°C for 45 min prior to mouse injection. To reduce the number of animals sacrificed, toxin in putatively positive samples (i.e. those that received no nisin) was determined by the USDA ELISA method (W.S. Lee, personal communication) for the detection of botulinum toxin. This method is based on Dezfulian and Bartlett (1984).

RESULTS & DISCUSSION

PRELIMINARY EXPERIMENTS on the influence of individual food components (Table 2) indicated many factors that determined the design of the main study. In the absence of nisin (control), the time to observe gas in the cultures varied from 2 to 6 days, depending on the other ingredients in the media. Thus, each experiment with nisin required its own control without nisin. In three of the eleven conditions, the results from duplicate tubes were very different. Thus, the time at which gas was observed in the first tube was used to make conservative calculations of ΔN. Using these criteria, albumen, carrageenan, and soluble starch reduced nisin inhibition. Xanthan and soy flour potentiated it. In the basal medium with no addition, gas production was observed coincident with the appearance of turbidity in the culture. This suggested that the presence of gas was an appropriate indicator of growth. Gas production is widely used as an indicator for botulinal growth, although a small percentage of gas-negative samples occasionally have been toxic (Huhtanen et al., 1983, Kautter et al., 1981, Rowley et al., 1983, Townsend et al., 1954, Vahabzadeh et al., 1983). Because gas production may be delayed at low pH and low concentrations of fermentable carbohydrates (Montville, 1983), 1% w/v glucose was included in all experiments and pH 5.5 was the most acidic condition used. Furthermore, all cultures judged negative for growth by absence of gas production were confirmed negative by mouse bioassay (see below).

The results of individual experiments are reported in columns 8 through 14 of Table 1. Because there were two- and three-way interactions in addition to main effects, it is difficult to interpret these results by inspection. Thus, the relative influences of each variable or combination were quantified in Table 3 as the α value and the average ΔN. The α value is the probability that the ΔN for that variable is not significant (with 1 - α equaling the significance for each variable). Thus a low α value indicates a high probability that the variable truly affected the model. Table 3 lists the variables in order of descending significance and excludes those variables where $\alpha > 0.2$, (although all variables were included in the equation generated for the model). The sign of the ΔN indicates the direction of the effect. A negative value for ΔN indicates anticlostridial activity was reduced. As the variable changed from low value to high value, e.g. temperature from 15°C to 35°C, the ΔN decreased. As expected, the ΔN for nisin concentration was positive; increasing nisin levels were more inhibitory. Note that protein, starch, and pH are not listed individually because they did not give significant linear effects when isolated from other interactions. Quadratic effects of protein and phospholipid were significant at $0.05 < \alpha \leq 0.20$.

The residual standard deviation is that standard deviation which remained after the model was fitted for all values. The replicate standard deviation is the standard deviation for the replicates. These two values (Table 3) were similar, indicating that replicates fit as closely to the model as did the individual experiments. The r^2 value for the model was 0.76.

The equation in Table 4 was generated using variables or combinations with $\alpha \leq 0.20$. This equation takes into account two- and three-way interactions as well as main effects. Values for high and low food component concentrations were used in the equation to estimate threshold nisin concentrations. The lines generated using low food component concentrations intercepted the x axis near zero (Fig. 1). This suggested that any nisin added would delay the outgrowth of *C. botulinum.* In contrast, when the equation was solved using high concentrations of food components, the slopes of the lines changed and the x intercepts were at finite nisin concentrations. This sug-

Table 1—Time to growth and gas production (days) of *C. botulinum* 56A in cultures with and without nisin (control) at various combinations of nisin concentration, pH, protein (PRO), carbohydrate (CHO), phospholipid, (PPL) and temperature (T) as dictated by the fractional factorial design; Calculated difference between growth in the presence and absence of nisin (ΔN) is also shown for each experiment

Experiment	Nisin (Log)	pH	PRO (%)	CHO (%)	PPL (%)	T (°C)	Days to growth and gas						ΔN
							Control			With nisin			
1	4	6.5	7.5	5	0.075	15	11	11	11	15	19	19	4
2	4	5.5	0.075	30	0.075	15	32	32	32	32	32	32	0
3	2	6.5	7.5	30	0.075	15	7	7	7	9	9	9	2
4	2	6.5	0.075	5	7.5	15	12	17	17	36	54	63	24
5	4	5.5	7.5	5	7.5	15	11	43	49	48	48	140	37
6	4	6.5	0.075	30	7.5	15	27	27	27	31	46	49	4
7	2	5.5	7.5	30	7.5	15	31	46	123	31	31	123	0
8	4	6.5	0.075	5	0.075	15	8	10	15	48	64	72	40
9	2	5.5	7.5	5	0.075	15	16	20	20	58	58	61	42
10	2	5.5	0.075	30	7.5	15	35	35	35	36	42	42	1
11	4	6.5	7.5	30	7.5	15	11	46	49	58	61	61	47
12	4	5.5	0.075	5	0.075	35	27	52	57	38	59	141	11
13	4	5.5	7.5	5	0.075	35	2	2	2	3	6	8	1
14	2	5.5	7.5	30	0.075	35	2	2	2	2	2	2	0
15	2	5.5	0.075	5	7.5	35	2	4	12	8	8	12	6
16	4	5.5	0.075	30	7.5	35	3	3	5	3	3	5	0
17	4	5.5	7.5	30	7.5	35	2	3	3	3	3	4	1
18	4	6.5	7.5	30	0.075	35	2	2	2	2	2	2	0
19	4	6.5	0.075	5	7.5	35	2	2	2	4	9	14	2
20	4	5.5	7.5	30	0.075	15	10	10	10	48	48	71	38
21	2	6.5	7.5	5	7.5	35	2	2	3	3	4	4	1
22	2	6.5	7.5	5	0.075	15	14	14	14	14	16	16	0
23	2	6.5	7.5	30	7.5	15	43	45	49	49	59	66	6
24	2	5.5	7.5	5	0.075	35	2	2	2	2	2	4	0
25	2	5.5	7.5	30	7.5	35	7	7	7	7	7	7	0
26	4	6.5	7.5	5	0.075	35	2	2	2	2	2	2	0
27	4	6.5	7.5	30	7.5	35	3	3	3	3	3	3	0
28	4	5.5	0.075	5	0.075	15	9	38	41	31	41	41	22
29	4	5.5	0.075	30	7.5	15	49	63	102	40	49	68	0
30	2	5.5	0.075	30	0.075	15	41	48	54	72	72	72	31
31	4	6.5	0.075	30	0.075	15	14	14	14	31	38	43	17
32	2	6.5	7.5	5	7.5	15	7	7	12	8	12	16	1
33	4	6.5	0.075	5	7.5	15	7	8	8	43	64	106	36
34	2	6.5	0.075	30	0.075	15	7	7	7	27	56	62	20
35	2	6.5	7.5	30	0.075	35	2	2	2	2	2	2	0
36	2	5.5	0.075	30	0.075	35	2	5	6	15	19	25	13
37	4	6.5	0.075	30	0.075	35	2	2	2	3	3	3	1
38	4	5.5	0.75	5	7.5	35	12	12	12	23	30	45	11
39	2	6.5	0.075	5	0.075	35	2	2	2	2	2	2	0
40	2	6.5	0.75	17.5	7.5	35	2	2	2	2	2	2	0
41	3	6.0	0.75	30	7.5	35	2	2	2	4	4	4	2
42	3	5.5	7.5	17.5	7.5	35	2	2	3	3	4	4	1
43	4	6.0	7.5	17.5	7.5	35	2	2	4	3	3	4	1
44	2	5.5	0.075	5	0.75	15	25	27	41	25	31	46	0
45	2	5.5	0.075	5	0.075	25	13	13	13	16	33	33	3
46	2	6.5	0.075	30	7.5	35	4	5	5	5	5	5	1
47	2	5.5	7.5	5	7.5	25	5	5	9	5	5	9	0
48	4	5.5	0.075	5	7.5	25	3	3	3	64	73	126	61
49	4	6.5	7.5	5	7.5	25	3	3	3	31	38	41	28
50	4	5.5	0.75	30	0.075	35	2	3	4	30	46	46	28
51	4	6.0	0.75	17.5	0.75	25	3	3	3	9	9	15	6
52	3	6.5	0.075	5	0.75	25	2	2	2	5	5	5	3
53	2	6.5	0.075	5	0.075	15	9	9	14	14	16	16	5
1A	4	6.5	7.5	5	0.075	15	11	11	11	31	31	31	20
2A	4	5.5	0.075	30	0.075	15	45	49	66	46	46	191	1
3A	2	6.5	7.5	30	0.075	15	22	25	25	18	22	25	0
4A	2	6.5	0.075	5	7.5	15	18	25	25	25	31	31	7
5A	4	5.5	0.075	5	0.075	35	4	8	6	64	84	84	60
12B	4	5.5	0.075	5	0.075	35	6	6	8	84	84	84	78
12C	4	5.5	0.075	5	0.075	35	4	4	6	54	84	84	50
12D	4	5.5	0.075	5	0.075	35	4	4	6	64	84	84	60
2B	4	5.5	0.075	30	0.075	15	30	41	41	10	30	84	0
2C	4	5.5	0.075	30	0.075	15	30	40	84	63	84	84	33
2D	4	5.5	0.075	30	0.075	15	10	40	84	40	84	84	30
37A	4	6.5	0.075	30	0.075	35	1	1	1	3	3	5	2
37B	4	6.5	0.075	30	0.075	35	1	1	1	3	4	5	2
37C	4	6.5	0.075	30	0.075	35	1	1	1	3	3	3	2
37D	4	6.5	0.075	30	0.075	35	1	1	1	3	3	4	2
48A	4	5.5	0.075	5	7.5	25	3	4	4	10	18	84	7
48B	4	5.5	0.075	5	7.5	25	3	5	6	8	9	10	5
48C	4	5.5	0.075	5	7.5	25	4	5	6	10	20	84	6
48D	4	5.5	0.075	5	7.5	25	3	5	6	16	33	40	13
4B	2	6.5	0.075	5	7.5	15	8	10	10	10	10	12	2
4C	2	6.5	0.075	5	7.5	15	8	8	12	10	21	40	2
4D	2	6.5	0.075	5	7.5	15	8	8	10	8	10	21	0
52A	3	6.5	0.075	5	0.75	25	3	3	3	4	4	4	1
52B	3	6.5	0.075	5	0.75	25	3	3	3	4	4	4	1
52C	3	6.5	0.075	5	0.75	25	3	3	3	4	4	4	1
52D	3	6.5	0.075	5	0.75	25	3	3	3	4	4	4	1

Table 2—Average time to growth (in days) of *C. botulinum* 56A at pH 6.0 and 35°C in media containing various ingredients with and without 1,000 IU/mL nisin*

Addition to basal medium	Time to growth & gas production (days)	
	No nisin	1,000 IU/mL Nisin
None	2	36, > 60
Albumen 10%	3	3
Soy flour 10%	6	> 60
Lecithin 10%	2	36
Phospatidylcholine 10%	2	36
Egg yolk 10%	2	36
Carrageenan 0.3%	2	5, > 60
Guar 0.3%	2	36
Soluble starch 30%	3	3, 36
Xanthan 0.1%	2	> 60
Xanthan 0.1% pH 5.5	6	> 60

* Identical values were obtained in duplicate cultures except where two values are given.

Table 3—Statistical output from the fractional factorial experiment design* listing those experimental variables that demonstrated $\alpha \leq 0.001$, $\alpha \leq 0.01$, $\alpha \leq 0.05$, and $0.05 < \alpha \leq 0.20$, and the representative ΔN value

α	Variable	ΔN (days)
$\alpha \leq 0.001$	temperature	−15
$\alpha \leq 0.01$	[nisin] [protein] [carbohydrate]	8.8
	[protein] [phospholipid] pH	8.0
	[nisin] [phospholipid] temperature	7.3
$\alpha \leq 0.05$	[nisin]	6.3
	pH [phospholipid] temperature	−7.7
	[phospholipid]	−5.7
	[nisin] [protein]	5.9
	[nisin] [carbohydrate]	−6.4
	[nisin] pH temperature	−6.0
$0.05 < \alpha \leq 0.20$	[phospholipid]²	12.0
	pH [protein] temperature	5.2
	[protein] temperature	−5.0
	[nisin] [protein] [phospholipid]	4.7
	[nisin] temperature	−4.7
	pH [phospholipid]	4.5
	[nisin] pH	4.4
	pH [carbohydrate]	−4.0
	[nisin] pH [carbohydrate]	−4.0
	[protein] [carbohydrate]	3.9
	[protein] [phospholipid]	3.8
	[protein]²	−7.7

* Residual standard deviation = 7.208, replicate standard deviation = 7.752; r^2 = 0.762.

Table 4—Equation describing delay in botulinal growth due to presence of nisin (ΔN, days) as a function of other variables in the model food system

$$\Delta N = -8.71 - 7.10B - 4.09L + 0.16T + 0.096P^2 + 0.73L^2 + 3.10BA - 0.35BP - 0.067BC + 0.26BT + 0.16AC - 0.35AL - 0.17PC - 1.9PL + 0.40PT - 0.054BAC - 0.073BAT + 0.071BPC + 0.06BPL - 0.0094BLT + 0.32APL - 0.074APT + 0.0021ALT$$

where:
N = days of delay in gas production attributed to nisin
B = log target nisin concentration
A = acidity (pH value)
P = protein concentration (%)
C = carbohydrate concentration (%)
L = phospholipid concentration (%)
T = temperature (°C)

gested that a threshold nisin level must be excceded for inhibition to occur. The model predicted threshold residual nisin concentration to be 154 IU/mL at 35°C, 32 IU/mL at 25°C, and 12 IU/mL at 15°C.

We used the equation to generate response surfaces that predicted nisin's efficacy under a variety of conditions. Some of these response surfaces are presented (Fig. 2,3,4). The influence of pH was more pronounced when the food components were at their highest level compared to their lowest level. Similarly the response surfaces showed less nisin concentration

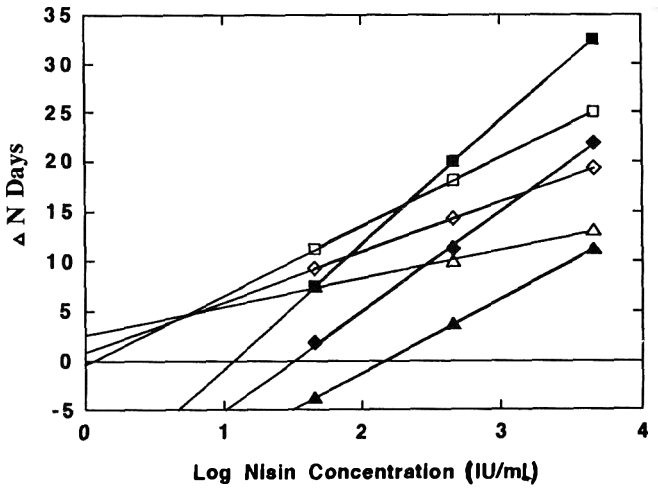

Fig. 1—Calculated delay in botulinal growth (ΔN) in model food system with low levels of added food components at (□) 15, (◇) 25, or (△) 35°C or with high levels of added food components at (■) 15, (◆) 25, or (▲) 35°C.

dependence when levels of other food ingredients were low. In experiments with high concentrations of protein, carbohydrate and lipid, there was a marked effect of nisin concentration. At the high pH, the organism grew out faster in control cultures and thus increased difference between the two (with and without nisin) endpoints. Clearly, at pH 6.5 and high food component concentrations, the ability of nisin to delay botulinal growth was highly dependent on nisin concentration and temperature.

As the phospholipid concentration increased from 0.075% to 7.50% in systems at pH 6.0 where all other food components were at their minimum level, ΔN decreased for all temperatures and levels (Fig. 3). Phospholipid interference with nisin activity was postulated by Henning et al. (1986a) and Somers and Taylor (1987), although concentration dependence was not investigated. High phospholipid concentrations reduced ΔN, as also demonstrated in Table 3. Bell and DeLacy (1986) found that when 100 IU/g nisin was added to meat, residual nisin concentration ranged from 26% in the presence of 3% beef fat to 76% in the presence of 83% beef fat. Other investigators reported that lipids, such as butterfat (Jones, 1974) or phospholipid (Henning, et al., 1986a) interfered with nisin activity. The presence of 2.8 μmol/mL (0.081%) phosphatidylcholine reduced nisin activity by 70% (Henning et al., 1986b). The model system investigated here was the first to evaluate effect of phospholipid, as lecithin, on nisin as a function of lecithin concentration. Further investigation emphasizing the concentration dependence of this and other phospholipids on nisin activity are needed.

Protein concentration influenced nisin activity with phospholipid and carbohydrate at minimum concentrations at pH 6.0 (Fig. 4). There was little difference between 0.075% protein (not shown) and 0.75% protein. However, as protein concentration increased to 7.5%, ΔN decreased at all temperatures and nisin concentrations. The influence of temperature on ΔN was greater at high protein concentrations, demonstrating the protein-nisin interaction at the clostridium optimal growth temperature. This was predicted in Table 3, which gave 95% confidence to the negative quadratic effect of increasing protein.

Nisin's antibotulinal activity has been reported in several food systems. Somers and Taylor (1987) found nisin in cheese spreads at levels of 4,000–16,000 IU/g inhibited *C. botulinum*. These cheese spreads had 54–57% moisture, 2% salt, and pH 6.0. Thus, low moisture and salt interactions could inhibit clostridial growth more than inhibition due to nisin at 30°C. Rayman et al. (1983) demonstrated the ineffectiveness of 12,000 IU/g nisin to inhibit five strains of *C. botulinum* type A in pork

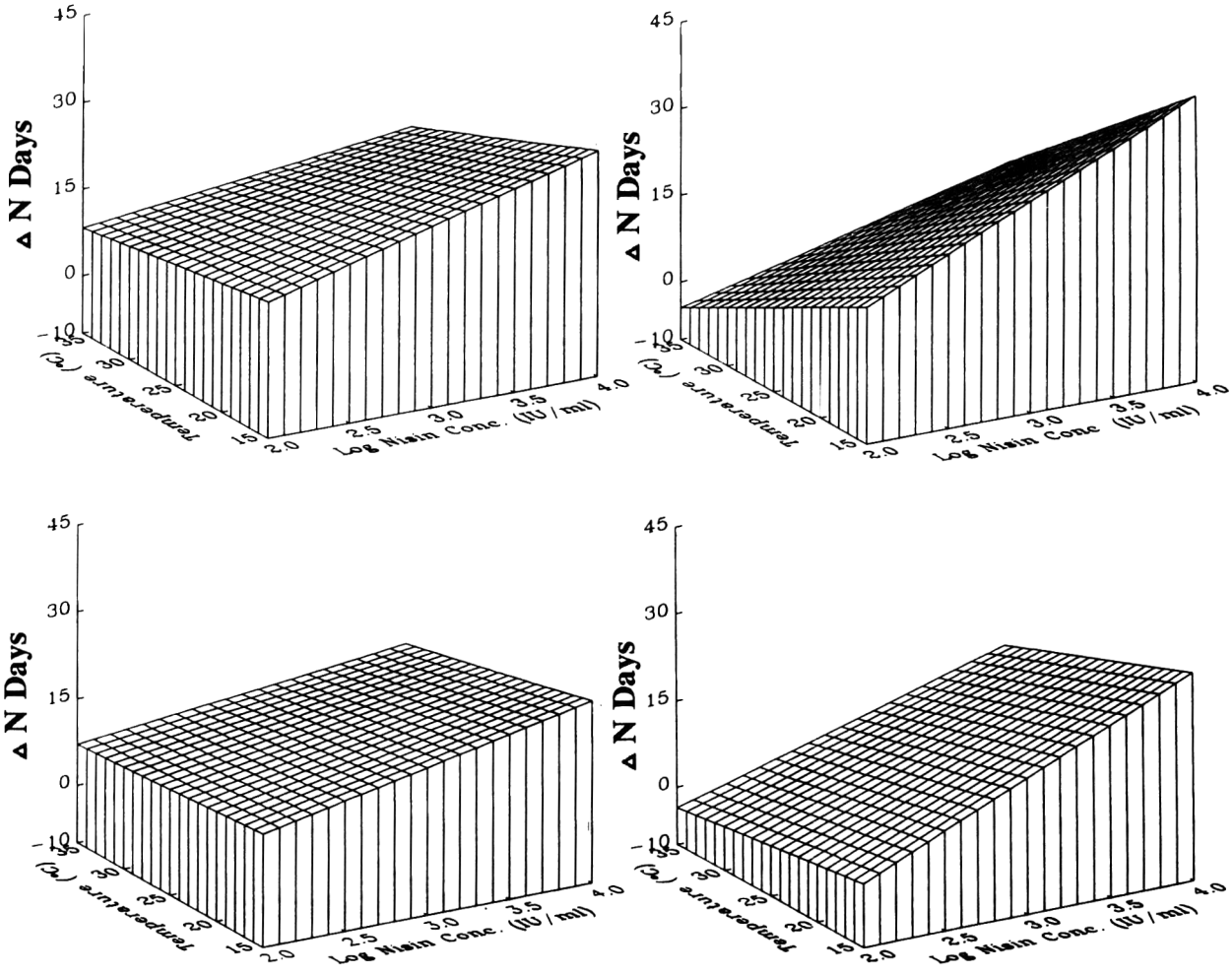

Fig. 2—Influence of temperature and nisin concentration on delay of botulinal growth (ΔN) at pH 6.5 (top) and pH 5.5 (bottom), and when protein, starch, and phospholipid concentrations were fixed at their lowest values (left) or at their highest values (right).

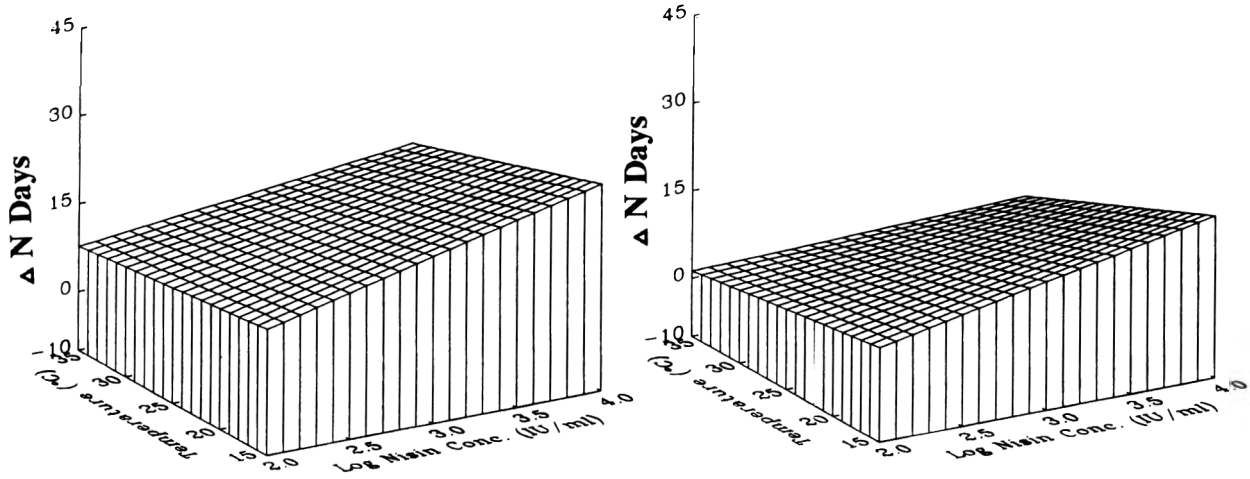

Fig. 3—Influence of temperature and nisin concentration on delay of botulinal growth (ΔN) at phospholipid concentrations of 0.075% (left) and 7.5% (right), both at pH 6.0, when protein and starch concentrations were fixed at their lowest values.

slurries at pH 5.0 and 25°C. Scott and Taylor (1981a and b) reported that 2,000 IU/mL nisin inhibited *C. botulinum* type A in trypticase glucose yeast extract medium, but that 5,000 IU/mL was not inhibitory in cooked meat medium at pH 6.0 and 35°C. No threshold nisin concentrations were estimated in those studies.

Consistency in cell numbers or pH measurements taken at the end of the each experiment was neither observed nor expected. The time between initiation of growth, production and detection of gas, and removal of cultures from incubation differed between experiments and within experiments. Thus those data are not shown here, but were reported by Rogers (1991). All uninoculated samples were negative for toxin in the mouse bioassay. ELISA assays conducted on control (no nisin) inoculated experiments confirmed most positive for toxin. However, experiment 29 and some replicates of experiment 4 were

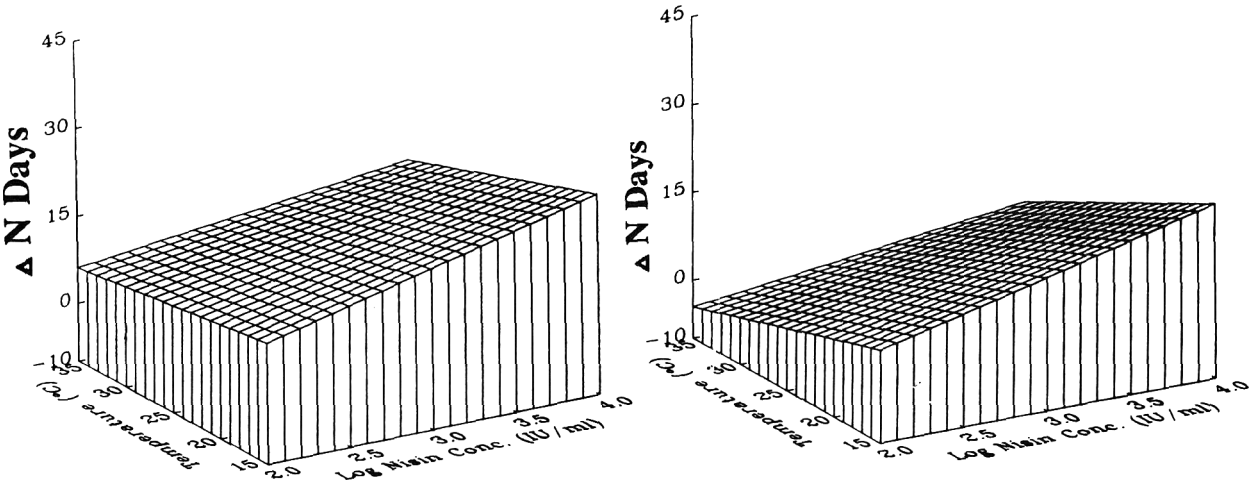

Fig. 4—Influence of temperature and nisin concentration on delay of botulinal growth (ΔN) at protein concentrations of 0.075% (left) and 7.5% (right), both at pH 6.0, when phospholipid and starch concentrations were fixed at their lowest values.

toxin-negative in the absence of nisin but toxin-positive in the same conditions where nisin was present. We considered these results to be false negatives because if toxin was produced in the presence of nisin, it should also have been produced in its absence. We attribute these few false-negatives to the lack of sensitivity in the ELISA method which is about 10-fold less sensitive than the mouse assay (Dezfulian and Bartlett, 1984). We have observed similar results for borderline detectable amounts of toxin in a model gravy system (Crandall and Montville, 1993). Conversely, in experiments 2 (and its replicates), 5 (and its replicates), 16, 20, 28, and 31, toxicity was not detected in the nisin-containing sample. All of these contained the highest nisin concentration. Although growth and gas production may have occurred without toxin production, it is more probable that a small amount of toxin was produced by subsequently degraded during storage. Controls for boiled toxin and antitoxin neutralization revealed no non-specific deaths.

Nisin has been investigated as a possible alternative to many preservatives, including nitrite, and as a backup preservative for temperature abuse of minimally processed unpreserved foods. This study has demonstrated that in conditions of temperature abuse, nisin loses effectiveness proportionately to the increase in temperature. High concentrations of protein and phospholipid may increase the loss of effectiveness.

CONCLUSIONS

NISIN PROVIDED a secondary barrier to botulinal growth, but was active only under suboptimal growth conditions. Under low temperatures and low concentrations of food components, nisin demonstrated greatest antibotulinal activity. Temperature, residual nisin concentration, and specific food components were important in relation to nisin's antibotulinal activity. Interactions of separate components also indicated those such as egg albumen may reduce nisin action. The nisin threshold concentrations required for botulinal inhibition were temperature-dependent.

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Freeze-cracking in Foods as Affected by Physical Properties

N.-K. KIM and Y.-C. HUNG

ABSTRACT

Severe mechanical damage such as cracks can occur in foods during cryogenic freezing. Ten physical properties of 22 different materials were correlated with freeze-cracking incidence. Statistical analyses revealed that several properties correlated significantly with freeze-cracking. This implies that freeze-cracking depends not only on moisture content but on combinations of several properties. A set of equations based on densities and porosities was developed to predict freeze-cracking incidence with an accuracy of 98% in noncracking products and 70% in cracking products during cryogenic freezing. In addition, susceptibility of various food commodities to freeze-cracking was determined.

Key Words: fruits, vegetables, bakery products, cryogenics, freeze cracking

INTRODUCTION

FREEZING IS AN EFFECTIVE METHOD of preservation which retains the quality of foods near their fresh state. Extensive studies on physico-chemical changes of foods during freezing and frozen storage have revealed that freezing rate influences the quality of thawed products and led to the process of 'quick freezing' (Woodroof, 1938, 1965; Morris, 1968; Fennema et al., 1973; Fennema, 1975; Potter, 1978). Quick freezing can be achieved by increasing the temperature gradient between freezing medium and food. Liquefied gases such as nitrogen or carbon dioxide have very low boiling points and can freeze foods faster than most other conventional freezing methods. When the boiling point of the liquefied gas is in the cryogenic temperature range, the process is termed 'cryogenic freezing'. "Cryogenics" is defined as "making icy cold" and is often used as a synonym for 'extremely cold' (Barron, 1985). The National Bureau of Standards (NBS) has defined cryogenic temperatures as 123°K (−150°C). According to that definition, liquid nitrogen (LN_2, −196°C) would be in the cryogenic range but carbon dioxide (−78.5°C) would not.

Advantages of cryogenic over conventional freezing (air blast or contact freezing) have been reported (Woodroof, 1938, 1965; Anon., 1966; Brown, 1967). In general, cryogenic freezing has a faster freezing rate, less moisture loss during freezing, less installation and maintenance costs and less space requirements than mechanical freezing (Anon., 1982; Plassmeier and Frank, 1985; Anon., 1988, 1989). However, exposing food materials to a cryogenic medium will cause internal stress build-up (due to fast freezing rate) in such food materials during freezing (Hoeft et al., 1973; Brennan et al., 1976; Nicholson, 1977). This internal stress may lead to cracking or shattering of frozen material which is critical and irreversible damage.

This research was conducted in the Dept. of Food Science & Technology, Univ. of Georgia Agricultural Experiment Station, Griffin, GA 30223. Author Kim, formerly with the Dept. of Food Science & Technology, Univ. of Georgia, is now with the Research & Development Center, Miwon Co., Ltd., 720 Banghak-dong, Dobong-ku, Seoul 132-020, Korea. Author Hung is with the Center for Food Safety and Quality Enhancement, Dept. of Food Science & Technology, Univ. of Georgia Agricultural Experiment Station, Griffin, GA 30223.

The mechanical damage (cracking or shattering) induced by cryogenic freezing has been explained mostly from volumetric changes associated with the water-ice phase transition. Fennema and Powrie (1964) described volume expansion and amount of empty space in microstructure as the primary factors affecting the degree of mechanical damage on cells during freezing. However, two conflicting presumptions were made, on the cause of mechanical damage (freeze-cracking) at the macroscopic level. Reeve and Brown (1968) suggested that the cracking was probably the result of nonuniform contraction following solidification. The other presumption is that volume expansion of water-ice phase transition causes internal stress development. Sebok et al. (1991) indicated that both contraction and expansion may cause freeze-cracking.

Physical properties of food materials may also affect the susceptibility of freeze-cracking during cryogenic freezing. Porosity (PORO) represents the amount of void space inside a food material. The larger the void space, the greater the chance for internal stress to dissipate instead of accumulate. Density (DEN) is normally proportional to moisture content and inversely proportional to PORO; the greater the DEN, the greater the possibility that stress may build up. Modulus of elasticity (E) is defined as the ratio of stress over strain (Gere and Timoshenko, 1990). Poisson's ratio (NU) is the ratio of lateral strain to axial strain, and it indicates material expansion perpendicular to compression. Generally, materials with greater E and NU are more rigid and more likely to build up stress than those with smaller E and NU values. Visualizing the overall structure of the material during freezing, internal stress can also be affected by size and shape of the material.

Our study was designed to evaluate the physical properties of food materials and assess their importance in the development of freeze-cracking during cryogenic freezing. Specific objectives were to evaluate susceptibility of various foods to freeze-cracking and develop equations for predicting freeze-cracking during cryogenic freezing.

MATERIALS & METHODS

TWENTY FOOD PRODUCTS, pure water enclosed in elastic spheroidal receptacles and cellulose sponges were tested (Table 1). Fruits and vegetables, dairy, bakery and meat products were purchased from a local retailer, sealed in plastic bags and stored at 4°C until used. Cellulose sponges were dried in a forced air oven (Model No. D-2972-Q, Blue M Electric Co., Blue Island, IL) at 105°C (0% MC-Sponge) and then deionized water added to a moisture content of 50% (50% MC-Sponge). Uniform distribution of water in the 50% MC-sponge was achieved as follows: The dry sponge was soaked in deionized water and then compressed so water would be more evenly distributed. Excess water was removed by blotting with tissue paper and moisture content of the wet sponge was readjusted to 50%. All measurements were made in triplicate.

Moisture

The vacuum oven method (AOAC, 1984) was used to determine moisture content (MC) of test samples. All samples were sliced (MAS 5550070, Bosch Appliance Ltd., Great Britain-Hayes) and then placed on aluminum moisture vessels and dried for 12 hr in a vacuum oven (70°C, P_{vac} = 30 mm Hg). MC was calculated based on weight differences before and after drying.

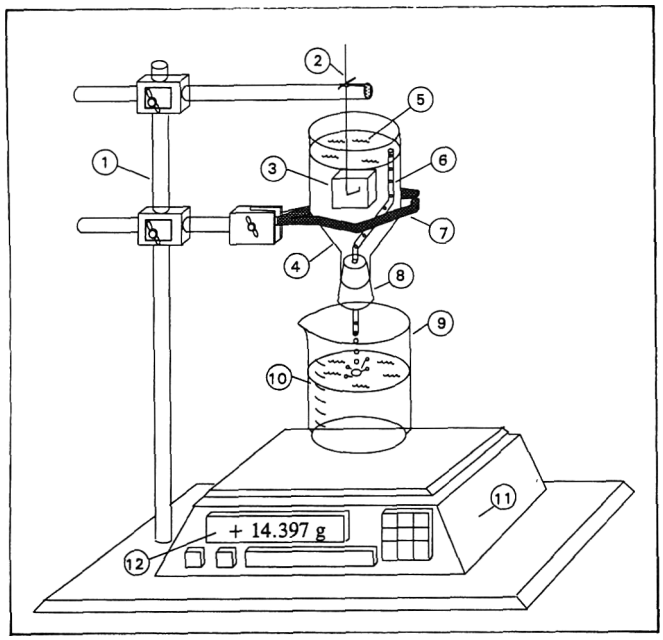

Fig. 1—Density determination device; (1) Stand; (2) Sinker Rod; (3) Sample; (4) Stainless-steel funnel; (5) LDM—ethyleneglycol: water (in volume, 7:3); (6) Glass overflow tube; (7) Clamp; (8) Rubber stopper; (9) Beaker; (10) LDM replaced by sample; (11) Balance; (12) Weight of VDM replaced by sample.

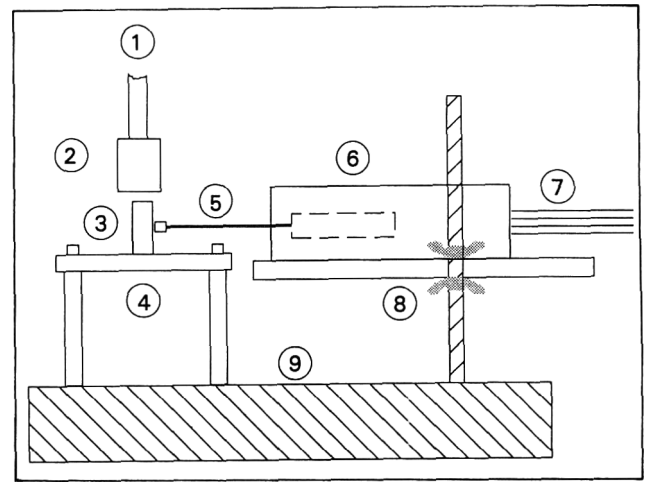

Fig. 2—Uniaxial compression test for determination of elastic modulus and Poisson's ratio: (1) Rod connecting Instron crosshead and compression probe; (2) Compression probe; (3) Sample; (4) Sample stage; (5) LVDT probe; (6) LVDT; (7) LVDT I/O; (8) Adjustable stage for LVDT; (9) Instron base.

Density and porosity

Bulk density was measured at room temperature (≈23°C) and −20°C based on volume displacement. A mixture of ethylene glycol: water (7:3 v/v; freezing point −64.7°C) was used as volume displacement medium (VDM) for both temperatures. A stainless steel funnel with 63.3 mm o.d. and 65.0 mm height was equipped with a glass overflow tube with o.d. 6.0 mm (Fig. 1) for density measurement at room temperature. The funnel was filled until it overflowed. An ≈2 cm^3 sample shaped as a rectangular block was first weighed on a balance in air and then immersed into the VDM by means of a sinker rod. Overflowed VDM was collected in a beaker and weighed ($W_{VDM,\ overflowed}$). The sample was then removed and the VDM on the surface of the sample was removed by blotting with tissue paper. The wet sample was then weighed and the weight of VDM absorbed by the sample ($W_{VDM,\ absorbed}$) was determined. Density of the test sample at room temperature (DENRM) was then calculated:

$$DENRM = \frac{W_s}{V_s} = \frac{W_s \times D_{VDM}}{W_{VDM,\ overflowed} + W_{VDM,\ absorbed}} \quad (1)$$

where W = weight (g); D = density (g/mL); V = volume (mL); subscript, s = sample. Density of frozen samples (−20°C) was determined by using the apparatus in a room maintained at −20°C (DEN20). True density of test samples at room temperature was measured using a helium pycnometer (Model II, Micrometritics, Norcross, GA) (Mohsenin, 1986; Chang, 1988). Porosity (PORO) was calculated as the volume difference between the helium pycnometer and the volume displacement measurements divided by the volume from the displacement measurement; the product was multiplied by 100.

Elastic modulus and Poisson's ratio

Elastic modulus (E) and Poisson's ratio (NU) of test samples were determined with an uniaxial compression test using an Instron Universal Testing Machine (Model 1122, Instron Inc., Canton, MA) at room temperature (≈23°C) and −20°C. At room temperature, samples were cut into cylindrical shape (diameter = 18 mm and height = 36 mm) and placed on the sample stage (Fig. 2). A linear displacement probe connected to a linear voltage differential transducer (LVDT, Model 500HPD, Schaevitz Engineering, Penngauken, NJ) was used to record lateral displacement. The tip of the probe was pulled to contact the lateral side of the sample, at which the null signal was recorded. As the sample was compressed uniaxially, it expanded laterally. Instron load cell capacity was 500 kg, crosshead speed 2 mm/min, and chart speed 25.4 mm/min. Processed signals from strain gage (load cell) of the Instron and LVDT were synchronously plotted on a chart recorder (7132A Recorder, Hewlett-Packard, Rockville, MD). Force-deformation and lateral displacement curves were recorded (Fig. 3).

Axial stress and strain of test samples were obtained from the force-deformation curves. Ratio of stress to strain was calculated at various deformation levels and averaged over the deformation level range before fracture for each individual food material. Elastic modulus at room temperature (ERM) was expressed as the mean value of stress over strain from the calculation. Lateral displacements were determined from curves (Fig. 3) at various deformation levels. Poisson's ratio at room temperature (NURM) was expressed as a mean of ratios calculated by dividing lateral strain by the axial strain. Elastic modulus (E20) and Poisson's ratio (NU20) of frozen samples were determined at −20°C using the procedure described. A cold environmental chamber (Kim, 1993) was attached to the Instron to maintain the temperature of the enclosed test area at −20°C.

Shape, size, and cryogenic freezing

Three shapes (spheroid, cylinder and slab) and two sizes were tested. For each test material size, the volumes of test samples for all shapes were about the same. Axial ratio (AXR), a shape factor, expressed as ratio of shortest dimension to longest dimension in each sample (Mohsenin, 1986) was calculated for all samples. Two different sizes (large or small) of each shape were prepared and represented by sample weight (WT).

Three replicates for each size and shape combination of all test materials were prepared and immersed into liquid nitrogen (LN_2, boiling point = −196°C). Test samples were monitored to detect whether cracking sounds or visible cracks occurred during freezing. Test samples were removed immediately after complete freezing and further evaluated. If a crack was oberved, a "crack" response (RESP) was indicated, otherwise, it was marked "no crack." Samples (338 total) were frozen and evaluated for cracking incidence (learning set).

Development of predictive model

Discriminant analysis is a statistical technique for classifying an item into one of mutually exclusive classes on the basis of certain characteristics (Powers and Ware, 1986). Using that technique, a discriminant function or classification criterion can be developed for classifying an unknown sample into one of the RESP classes. The measured characteristics were physical properties of food materials and the two exclusive classes were "crack" and "no crack" RESP during cryogenic freezing. The Statistical Analysis System (SAS Institute, Inc., 1989) was used for discriminant analysis calculations.

The basic step in development of a predictive model is to decide which of the measured properties should be included in the discriminant funtion. The SAS Stepwise Discriminant Analysis procedure (PROC STEPDISK) was used for screening to select important properties to be included in the predictive model. PROC STEPDISK as-

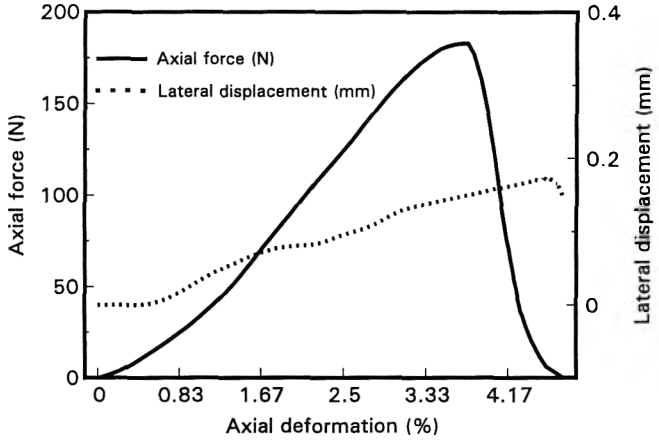

Fig. 3—Typical force-deformation and linear displacement curves of uniaxial compression test.

sumes that the classes are multivariate normal with a common covariance matrix. Both forward stepwise selection and backward stepwise elimination approaches were used. A partial F test was employed to determine whether a variable should be included or eliminated from the function. Stepwise forward selection was repeated until the inclusion of additional variables did not significantly improve the discriminant funtion. Backward elimination was repeated until all variables remaining in the function were judged significant. PROC DISCRIM was then used for developing a classification criterion and for validation of the predictive model. A classification criterion is practically equivalent to the equation for predicting whether a food material would crack or not during the freezing process.

Validation of predictive model

The developed discriminant model was applied to classify an additional 162 food samples (independent set) for validation. These data were collected using 10 other food products (Table 6).

RESULTS & DISCUSSION

THE PROPERTIES OF THE TEST MATERIALS (Table 1) showed moisture content (MC) ranged from 100% (pure water) to 0% (0% MC-Sponge). MC of fresh fruits and vegetables was higher than most other products. In general, DEN's E's and NU's were greater for fresh fruits and vegetables and dairy products than for dry fruits and bakery products. PORO of the bakery products, however, was greater than for most of other products. Most products evaluated had DEN20 very close to DENRM. E20 was much larger than ERM and this indicated that frozen foods were far more rigid than unfrozen foods. For dairy, bakery and meat products, NU20 was greater than NURM. This reflected different behavior of frozen and unfrozen products. Unfrozen products were usually softer than frozen products. The compression stress in unfrozen products dissipates into the structure instead of expanding the material laterally as in frozen products.

Inaccuracy of some PORO results was observed for the low-PORO (high density) products. For PORO calculation, volume difference between liquid displacement and pycnometer methods was divided by liquid-displacement volume and the product was multiplied by 100. In that method, products with low PORO would tend to have smaller volume differences between helium pycnometer and liquid displacement measurements. A small change in liquid displacement measurements would cause calculated PORO to shift to the negative side. For this reason, PORO values with negative signs were considered as PORO near zero.

Susceptibility of food products to freeze-cracking

Effect of sample size and shape on freeze-cracking susceptibility were evaluated using ranges of AXR of 0.5–1.0, 0.07–0.2 and 0.01–0.16 for spheroid, cylinder and slab, respectively. Samples of large size were usualy five- to eight-fold heavier than smaller ones. Margarine and mozzarella cracked regardless of shapes or sizes whereas fresh apple, dry products, bakery products and sponges did not. Thus, immersion of margarine and mozzarella in LN_2 is not recommended. Those samples with no-crack RESP can be frozen in LN_2 without sustaining severe mechanical damage.

Freeze-cracking as affected by physical properties

Other food products showed mixed RESP with different shapes and sizes (Table 2). In these mixed-RESP products, no particular shape resulted in a consistent RESP. The size, however, followed a well-defined pattern in crack RESP. Larger samples had higher tendencies to crack than did smaller samples. For instance, large fresh peach, cheddar and turkey ham samples cracked regardless of shape. Physical properties (Table 1) of test materials were compared with the RESP (Table 2). Materials with higher MC, DEN20, DENRM, E20, ERM, NU20, NURM, WT and lower PORO generally tended to have crack RESP. At 95% confidence level, WT, MC, DEN20, DENRM, E20, NU20, NURM and PORO all had significant influence (Table 3) on freeze-cracking. However, the greatest R-square value was only 0.36 for PORO. This indicated that no single property completely explained the development of freeze-cracking. Thus, several properties must be considered simultaneously to fully understand freeze-cracking phenomena. If freeze-cracking were caused solely by volume expansion of water during phase transition, moisture content should be the most significant variable in the crack RESP. Apple, a high-MC fruit, did not crack for any shape or size combination (Table 1). However, margarine and cheeses with low MC cracked.

One possible explanation is that both initiation of stress development and distribution of developed stress are important. The distribution of initial stress is governed by how the material responds to stress. The stress can be either absorbed (dissipated) into the structure or fully reflected to cause build up of internal stress. As previously mentioned, the greater the DENRM and DEN20, the higher would be the tendency to crack. However, the higher the PORO the less the tendency to crack. This indicates the necessity of using all the important properties for analysis of freeze-cracking.

Correlations among all properties measured were compared (Table 4). The negative sign of correlation coefficients indicates one is inversely correlated with the other. Strong correlations were observed for DEN20, DENRM and PORO. DEN20 and DENRM were near to each other, (also observed in Table 1). DEN was also inversely correlated with PORO (Table 4). DEN, E and NU measured at frozen state also significantly correlated with those measured at unfrozen state.

Development of a predictive model

A stepwise selection procedure applied on the properties and RESP resulted in DEN20, DENRM and PORO selection as potential variables to be included in predictive equations. Backward elimination process resulted in only DEN20 and PORO selection. The classification criterion for distinguishing between the two groups was based on Mahalanobis' D^2, which measures the squared Euclidean distance of each observation to the mean of each treatment group. Since a homogeneity test between variances for the single discriminating response variable indicated possible differences, the squared Euclidean distance was standardized using individual variances (Powers and Ware, 1986). The product with measured set of properties was then assigned to the group for which the squared Euclidian distance was least. The squared Euclidian distances were then used to compute the posterior probabilities for the response groups to which a given product belonged. All materials with

Table 1—Moisture content and physical properties of test materials

Food group	Material	MC[a]	DEN20[b]	DENRM[c]	E20[d]	ERM[e]	NU20[f]	NURM[g]	PORO[h]
Water	Water	100	0.95[i]	1[i]	9842000[i]	—	—	—	0[i]
Fruits & Vegetables	Fresh peach	88.69	0.96	1.01	4350	247	0.4	0.1	7.1
	Potato	79.73	1.06	1.11	22200	299	0.2	0.4	−3.0
	Sweet potato	76.59	1.07	1.04	40100	649	0.6	0.4	5.7
	Fresh apple	86.92	0.78	0.77	5470	229	0.4	0.3	28.0
	Dry peach	28.09	1.32	1.26	40	16	—	—	4.3
	Dry apple	18.84	0.62	0.53	70	21	—	—	59.3
	Dry apricot	22.15	1.39	1.32	260	15	—	—	1.0
Dairy products	Margarine	34.76	1.01	0.98	2500	4.44	0.4	0.01	−3.6
	Mozzarella	46.70	1.10	1.10	16900	3.97	0.6	0.01	−1.3
	Cheddar	38.46	1.08	1.10	21300	5.3	0.5	0.01	−3.9
	Colby	38.86	1.13	1.05	39500	4.31	0.4	0.02	−0.04
Bakery products	Cookie dough	14.98	1.28	1.18	13700	—	0.4	—	10.1
	Angel food	34.99	0.28	0.24	0	0.04	0.01	0	81.9
	White bread	37.83	0.21	0.25	520	0.44	0.01	0	70.9
	Wheat bread	28.31	0.21	0.25	170	1.13	0.0	0	81.3
	French bread	32.03	0.21	0.23	290	0.25	0.02	0	83.1
	Pound cake	29.6	0.41	0.39	20	6.02	0.01	0	68.7
Meat products	Bologna	44.54	1.04	1.03	150	3.98	0.4	0.03	0.8
	Turkey ham	73.87	1.02	1.07	2020	8.28	0.2	0.05	2.5
	Pork sausage	47.12	1.00	0.95	340	0.64	0.05	0	6.3
Sponge	0% MC-Sponge	0	—	—	—	0.11	—	0.08	—
	50% MC-Sponge	50	0.10	0.08	0	1.49	0	0	93.4

[a] Moisture content, wet basis (%)
[b] Density at −20°C (g/mL)
[c] Density at room temperature (g/mL)
[d] Elastic modulus at −20°C ($10^4 \times N/m^2$)
[e] Elastic modulus at room temperature ($10^4 \times N/m^2$)
[f] Poisson's ratio at −20°C (dimensionless)
[g] Poisson's ratio at room temperature (dimensionless)
[h] Porosity (%)
[i] Mohsenin (1986) and Gold (1977)
[j] Presumed value

Table 2—Materials with mixed freeze-cracking response during cryogenic freezing

	No crack		Crack	
Material	Size	Shape	Size	Shape
Fresh peach			Large	all 3
	Small	Cylinder	Small	Spheroid
			Small	Slab
Potato	Large	Slab	Large	Spheroid
	Small	Cylinder	Large	Cylinder
	Small	Slab	Small	Spheroid
Sweet potato	Large	Slab	Large	Spheroid
	Small	Cylinder	Large	Cylinder
	Small	Slab	Small	Spheroid
Cheddar	Small	Spheroid	Large	all 3
			Small	Cylinder
			Small	Slab
Colby	Large	Spheroid	Large	Cylinder
			Large	Slab
	Small	Spheroid	Small	Cylinder
			Small	Slab
Bologna	Large	Spheroid	Large	Slab
	Large	Cylinder	Small	Cylinder
	Small	Spheroid	Small	Slab
Turkey ham	Small	all 3	Large	all 3

Table 3—Regression coefficients of physical properties with response[a]

Variable	R-square	F	PROB>F
AXR[b]	0.0020	0.0482	0.8267
WT[c]	0.1713	56.4510	0.0001
MC[d]	0.0197	5.4960	0.0198
DEN20[e]	0.3401	140.7190	0.0001
DENRM[f]	0.3454	144.0780	0.0001
E20[g]	0.1162	35.8900	0.0001
ERM[h]	0.0095	2.6270	0.1062
NU20[i]	0.2868	109.7800	0.0001
NURM[j]	0.0898	26.9210	0.0001
PORO[k]	0.3570	151.5950	0.0001

[a] Freeze-cracking response (dependent variable)
[b] Axial ratio (dimensionless)
[c] Weight (g)
[d] Moisture content, wet basis (%)
[e] Density at −20°C (g/mL)
[f] Density at room temperature (g/mL)
[g] Elastic modulus at −20°C ($10^4 \times N/m^2$)
[h] Elastic modulus at room temperature ($10^4 \times N/m^2$)
[i] Poisson's ratio at −20°C (dimensionless)
[j] Poisson's ratio at room temperature (dimensionless)
[k] Porosity (%)

a 0.5 or greater probability of belonging to the positive group were classified as crack RESP. The others were classified as no-crack RESP. This procedure was used to develop a discriminant model using the DEN20 and PORO data from all 338 freezing observations. Classification of these data revealed that 111 of 113 "crack" (98.2%) and 141 of 225 "no crack" observations (62.7%) were correctly classified (Table 5).

Validation of the predictive model

The validity of the predictive function was determined by applying the function to an additional data set (independent set). DEN20 and PORO of 10 additional food items were determined. These food samples with various shapes (slab, cylinder or spheroid) and size (large or small) combinations were frozen in LN_2. Results of DEN20, PORO and RESP of these food products (Table 6) showed a similar pattern as in the learning set. Samples with high DEN20 and low PORO had a high tendency of crack RESP. Predicted results on this independent set were compared using the predictive function developed from the learning set (Table 7). The discriminant function predicted that 144 samples would crack and 59.7% of the samples were confirmed as cracked. Also 94% of the predicted "no crack" samples were confirmed as "no crack."

The accuracy of the predictive model for the learning set and the independent set was about the same (Tables 5 and 7). A new predictive function was developed following the same discriminant analysis using all available data (combining the learning set and the independent set). The new predictive function was:

$$\text{PROB}(+) = \frac{DJ_1}{DJ_0 + DJ_1} \quad (2)$$

$$\text{PROB}(-) = \frac{DJ_0}{DJ_0 + DJ_1} \quad (3)$$

$$DJ_0 = \exp(-0.5 \times D_0^2) \quad (4)$$

Table 4—Correlation coefficients of all independent variables

	AXR[a]	WT[b]	MC[c]	DEN20[d]	DENRM[e]	E20[f]	ERM[g]	NU20[h]	NURM[i]	PORO[j]
AXR	1.00									
WT	0.05	1.00								
MC	−0.01	0.26	1.00							
DEN20	0.01	0.50	0.50	1.00						
DENRM	0.01	0.50	0.54	0.99	1.00					
E20	−0.02	0.33	0.31	0.58	0.56	1.00				
ERM	−0.02	0.17	0.72	0.33	0.33	0.64	1.00			
NU20	−0.03	0.42	0.59	0.85	0.85	0.54	0.43	1.00		
NURM	−0.04	0.32	0.53	0.60	0.62	0.52	0.53	0.64	1.00	
PORO	−0.01	−0.50	−0.50	−0.99	−0.99	−0.53	−0.30	−0.83	−0.59	1.00

[a] Axial ratio (dimensionless)
[b] Weight (g)
[c] Moisture content, wet basis (%)
[d] Density at −20°C (g/mL)
[e] Density at room temperature (g/mL)
[f] Elastic modulus at −20°C ($10^4 \times N/m^2$)
[g] Elastic modulus at room temperature ($10^4 \times N/m^2$)
[h] Poisson's ratio at −20°C (dimensionless)
[i] Poisson's ratio at room temperature (dimensionless)
[j] Porosity (%)

Table 5—Grouping of "learning set" responses into crack and no crack categories using a discriminant function

	Predicted crack	Predicted no crack	Confirmed total
Confirmed Crack	111* (56.9)**	2 (1.4)	113
Confirmed No crack	84 (43.1)	141 (98.6)	225
Predicted Total	195 (100.0)	143 (100.0)	338

* Number of classified total.
** Percentage of classified total.

Table 6—Physical properties and freeze-cracking response of "independent set"

Commodity	DEN20	PORO	Response
Red delicious[a]	0.81	24.07	Consistent no crack
Potato[b]	1.04	−2.61	Mixed[c]
Margarine[d]	0.80	2.37	Consistent crack[c]
Butter	1.0	1.97	Consistent crack
Colby jack	1.01	−1.89	Mixed
Montery jack	1.07	−2.14	Mixed
French bread[d]	0.19	73.11	Consistent no crack[c]
Pound cake[d]	0.38	72.20	Consistent no crack[c]
Tylose	0.99	3.67	Consistent crack
Ham	1.08	−0.44	Mixed

[a] Variety of apple in the learning set was Rome beauty.
[b] Harvested year was different from the "learning set".
[c] Same response as the "learning set".
[d] Brands were different from the "learning set".

$$DJ_1 = \exp(-0.5 \times D_1^2) \quad (5)$$

$$D_1^2 = [PORO-1.350\ DEN20-1.015] \times \begin{bmatrix} 0.0283 & 1.677 \\ 1.677 & 171.74 \end{bmatrix} \times \begin{bmatrix} PORO-1.350 \\ DEN20-1.015 \end{bmatrix} - 0.7213 \quad (6)$$

$$D_1^2 = [PORO-47.254\ DEN20-0.596] \times \begin{bmatrix} 0.0126 & 1.116 \\ 1.116 & 104.28 \end{bmatrix} \times \begin{bmatrix} PORO-47.254 \\ DEN20-0.596 \end{bmatrix} + 2.5756 \quad (7)$$

where PROB(+) is the probability that the test material would crack and PROB(−) is the probability that the test material would not crack; D_0^2 is the squared Euclidian distance from the no-crack group mean; D_1^2 is the squared Euclidian distance from the crack group mean; 1.350 and 47.254 are PORO means of crack and no crack groups, respectively; 1.015 and 0.5960 are DEN20 means of crack and no crack groups, respectively; the elements in the 2 × 2 matrices are the variances associated with the no crack and crack groups; 0.7213 and 2.5756 in Eq. (6) and (7) are the natural logs of the variance of crack and no crack groups, respectively. Combined data were classified based on the predictive function (Table 8). The function correctly classified 98.0% (196 out of 200) of the "crack" samples and 72.3% (217 out of 300) of the "no crack" samples. On the prediction side, 70.3% predicted "crack" samples were confirmed cracked, whereas 98.2% of those predicted not to crack did not.

Table 7—Grouping of "independent set" responses into crack and no crack categories based on the discriminant function developed from the learning set

	Predicted crack	Predicted no crack	Confirmed total
Confirmed Crack	86* (59.7)**	1 (0.6)	87
Confirmed No crack	58 (40.3)	17 (94.4)	75
Predicted Total	144 (100.0)	18 (100.0)	162

* Number of classified total.
** Percentage of classified total.

Table 8—Grouping of responses into crack and no crack categories using a discriminant function developed from the combined data sets (learning and independent)

	Predicted crack	Predicted no crack	Confirmed total
Confirmed Crack	196* (70.3)**	4 (1.8)	200
Confirmed No crack	83 (29.7)	217 (98.2)	300
Predicted Total	279 (100.0)	221 (100.0)	500

* Number of classified total.
** Percentage of classified total.

Use of prediction equations

The value of prediction equations developed lies in their ability to accurately predict "crack" and "no crack" response of a selected product during cryogenic freezing process. The information from the prediction equations is the calculated crack or no crack probability. As an example, French bread with determined DEN20 0.19g/mL and PORO 73.11% (Table 6), when used in Eq. (6) and (7) resulted in D_0^2 of 4.8 and D_1^2 63.48. These values were then placed in the right hand side of Eq. (4) and (5) and resulted in DJ_0 of 0.0907 and DJ_1 1.65×10^{-14}, respectively. PROB(+) and PROB(−) were calculated as

$$PROB(+) = \frac{1.65 \times 10^{-14}}{0.0907 + 1.65 \times 10^{-14}} \approx 0$$

$$PROB(-) = \frac{0.0907}{0.0907 + 1.65 \times 10^{-14}} \approx 1$$

This indicates that French bread would not "crack" during cryogenic freezing.

CONCLUSIONS

REGARDLESS OF SHAPE AND SIZE, fresh apple, dried fruits, bakery products, pork sausage, and sponges did not crack whereas water, margarine and mozzarella cracked when immersed in liquid nitrogen. Porosity, densities and Poisson's ratioes at room temperature and at −20°C, size, modulus of elasticity at −20°C and moisture content were significant properties which affect freeze-cracking susceptibility of foods. Equations based on DEN20 and PORO were developed to predict the incidence of freeze-cracking during cryogenic freezing. Products with higher density and lower porosity are generally more susceptible to freeze-cracking than those with lower densities and higher porosities. The prediction equation had an accuracy of 98% in the noncracking products and 70% in products that crack during cryogenic freezing.

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Convective Heat Transfer at Particle-Liquid Interface in Continuous Tube Flow at Elevated Fluid Temperatures

V.M. BALASUBRAMANIAM and S.K. SASTRY

ABSTRACT

Liquid-to-particle convective heat transfer coefficients are useful in developing aseptic food processing systems. They were determined for continuous flow through a holding tube at 115.5°C using liquid crystal and relative velocity methods with sodium carboxymethylcellulose solution to simulate non-Newtonian fluid characteristics. An on-line tube viscometer was used for *in situ* estimation of rheological characteristics. Minimum and maximum values of h_{fp} determined from the liquid crystal method ranged from 986 W/m²°K to 2270 W/m²°K, (Nusselt numbers from 26.4 to 54.6). Values from the relative velocity method ranged from 1143 to 2270 W/m²°K (Nusselt numbers from 33.2 to 63.1) when using the Ranz and Marshall relation, and from 598 to 1456 W/m²°C (Nusselt numbers from 13.6 to 24.1) with a flat-plate correlation. Heat transfer coefficients increased significantly with decreasing carrier medium viscosity and decreasing particle-to-tube diameter ratio and increased with flow rate.

Key Words: heat transfer, continuous flow, relative velocity, aseptic processing convection, particle-liquid

INTRODUCTION

ASEPTICALLY PROCESSED LIQUID FOODS are commercially produced in Europe and in the U.S. Some advantages for this technology include reduced packaging and processing cost, improved product quality, and reduced energy consumption. Interest is increasing in extending aseptic technology to low-acid foods containing particulates (Ohlsson, 1992). Routine commercial processing of particulates has not been adopted partially due to lack of understanding of heat transfer between liquid and particles in continuous flow through heat exchangers and holding tubes (Pflug et al., 1990). Studies have attempted to characterize heat transfer relationships in continuous tube flow (Heppell, 1985; Sastry et al., 1990; Stoforos and Merson, 1991; Balasubramaniam and Sastry, 1993a,b; Mwangi et al., 1993; Zitoun and Sastry, 1993). Most such studies have been conducted below atmospheric boiling point temperatures. Further understanding of heat transfer and associated fluid-particle interactions requires experimental studies at elevated process temperatures. Our objectives were to determine liquid-to-particle convective heat transfer coefficients (h_{fp}) using liquid crystal (LC) and relative velocity (RV) methods (Balasubramaniam and Sastry, 1993) at elevated fluid temperatures, and to characterize factors influencing relative velocity and heat transfer coefficients.

MATERIALS & METHODS

Liquid crystal method

This is a heat transfer technique, wherein the temperature history of a particle moving inside a fluid medium is determined by a liquid crystal thermal sensor, which changes color with temperature. The temperature history of a liquid crystal coated particle was determined by videotaping it during passage through a tube, and subsequently analyzing color changes. The measured color values (described by hue, defined below under Liquid Crystal Calibration) were then converted to temperatures using a color-temperature calibration equation. When a hollow metal transducer particle was used, heat transfer coefficients could be determined using the Newtonian law of heating valid for Biot number <0.1 (Eq. 1).

Author Balasubramaniam, formerly of The Ohio State University, is presently with the University of Georgia Center for Food Safety and Quality Enhancement, Griffin, GA. Author Sastry, to whom correspondence should be addressed is with The Ohio State University, Department of Agricultural Engineering, 590 Woody Hayes Drive, Columbus, OH 43210.

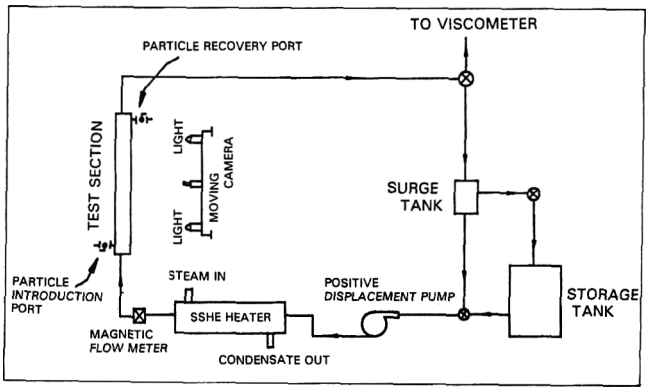

Fig. 1—Experimental setup.

$$\frac{m}{A} C_p \ln\left(\frac{T - T_\infty}{T_i - T_\infty}\right) = -h_{fp} t \quad (1)$$

Relative velocity method

This method is a flow field visualization approach to experimentally determine the relative velocity between fluids and particles by videotaping the motion of small fluid tracers and particles. The videotape was replayed in still mode at selected moments to determine the relative velocity by noting the time elapsed for a selected tracer to pass over a particle. Values of h_{fp} were then back calculated from relative velocity using well known correlations (Ranz and Marshall 1952) given by Eq. (2).

$$Nu = 2.0 + 0.6 Re_{G,S}^{0.5} Pr_G^{0.33} \quad (2)$$

The Ranz and Marshall correlation (Eq. 2) and other similar Nusselt number correlations (Kramers, 1946; Whitaker, 1972) were originally developed for unbounded flow conditions using spherical particles. However, our study involved cubic particles, a situation for which heat transfer correlations were not readily available. To assess the effect of geometry, an available flat plate correlation (Incropera and DeWitt, 1990) (Eq. 3) was also tested for its applicability under such conditions. The values of h_{fp} back calculated from the correlations were then compared against the data obtained using the liquid crystal method.

$$Nu = 0.332 Re_{G,S}^{0.5} Pr_G^{0.33} \quad (3)$$

Since the accuracy of the relative velocity method depends on the correlation, it could not be used as a reliable measure of h_{fp} per se. The primary purpose of the relative velocity experiments was to verify whether data from the heat transfer based liquid crystal method was consistent with values expected from relative velocity data (Balasubramaniam and Sastry, 1993a).

Experimental setup

A scraped surface heat exchanger (Cherry Burrell Thermutator 648 L, Cherry Burrell, Cedar Rapids, IA) was used to heat the fluid (Fig.

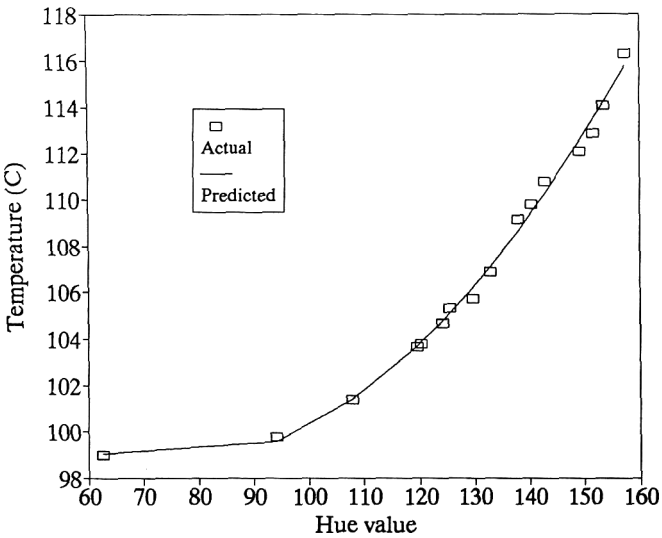

Fig. 2—Color-temperature calibration of liquid crystal.

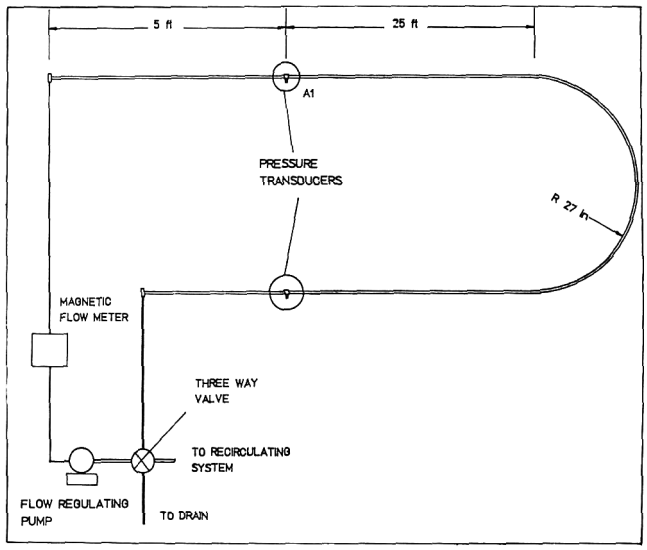

Fig. 3—Tube viscometer setup.

Table 1a—Dimensions and properties of cubic particles used in liquid crystal experiments

Material	Size (m)	Apparent density (kg/m³)	Apparent specific heat (kJ/kg°C)
Brass	0.0177	1065	0.515
Brass	0.0137	1060	0.554

Table 1b—Dimensions and properties of cubic particles used in relative velocity studies

Material	Dimension (m)	Apparent density (kg/m³)
Polystyrene	0.0199	1072
Polystyrene	0.0149	1071
Polystyrene	0.0117	1072

1). A transparent Pyrex section (0.0508m diam and 3.048m long), inclined 2.08 cm/m as required of commercial holding tubes, was used as the test section to videotape the particle and fluid for both methods. A positive displacement pump was used to circulate the carrier fluid. The particle was introduced into the test section by a specially constructed assembly, consisting of two ball valves (Pittsburgh Brass Manufacturing, Irwin, PA) in series. The cold test particle was introduced into the port by opening the outer valve, while keeping the inner valve closed to retain pressure. The outer valve was then closed and the inner valve opened to accomplish introduction without leakage. A similar port downstream was used for particle recovery. Flow rates were measured using a magnetic flow tube and transmitter assembly (Rosemount Inc., Eden Prairie, MN). All experiments were conducted under pressurized conditions, thus a transparent shatter resistant plastic partition was set around the experimental setup (not shown) for safety.

Liquid crystal calibration

The liquid crystal (supplied by Hallcrest, Glenview, IL) was calibrated by videotaping its color changes when coated on the surface of a hollow brass cube (wall thickness 0.00038m) and comparing with data collected simultaneously from a thermocouple (Teflon insulated fine gauge copper-constantan (TT-T-36, OMEGA™, Stamford, CT)) welded to the geometric center of the brass wall. The thermocouple was precalibrated with a quartz thermometer (Model 2804A, Hewlett Packard, Loveland, CO) accurate to 0.0001°C. The calibration was conducted *in situ* using actual test fluids. Care was taken to maintain similar lighting conditions during calibration and experiments. The test fluids (aqueous solutions of sodium carboxymethylcellulose, CMC) were transparent within the range of concentrations we studied. The influence of CMC concentration on observed color was negligible and within error limits of the liquid crystal calibration equation.

The color changes of the liquid crystal were measured using image analysis software (Image-Pro, Media Cybernetics, Silver Spring, MD). Colors from a video image could be separated into red-green-blue (RGB) or hue-intensity-saturation combinations. The hue value (scale from 0 to 255, red ≈ 75 and blue ≈ 220) was selected as the temperature indicator, since it closely correlated with temperature. Hue-temperature data were regressed with corresponding calibration thermocouple readings to obtain the color-temperature relation, valid between 98-115°C (R^2 = 0.99) (Fig. 2 and Eq. 4).

$$T = 112.65 - 0.337H + 0.0025H^2 \quad (4)$$

Experimental conditions

Experiments were conducted at a fluid temperature of 115.5°C, based on the maximum working temperature of the liquid crystal sensor. Aqueous solutions of sodium CMC (CMC, 7HF; Aqualon Company, Wilmington, DE) were used as carrier media. For each method described, experiments were conducted for three concentrations of CMC (0.5%, 0.8%, and 1.0%), three flow rates of 2.52×10^{-4}, 3.79×10^{-4} and 5.05×10^{-4} m³/s and different cube-shaped particle sizes (Table 1a,b).

Transducer particles were introduced into the fluid stream through the upstream ball valves and videotaped as they moved to the downstream end. Later the videotape was replayed and stopped at selected moments to determine color change/relative velocity values as described.

Replications

At least three replications were performed for each of the conditions and methods described. The h_{fp} values from the liquid crystal method were developed from 54 videotaped experimental runs and subsequent analysis of about 750 still frames for color change. For the relative velocity method about 81 experimental runs were videotaped and the values of relative velocity were determined by analyzing about 250 still frames for location (for both particle and tracers) and time.

Rheological characterization

The rheological characterization of carrier fluids at elevated temperatures was performed using an on-line tube viscometer (Fig. 3). The viscometer had a diameter of 0.0127m and the length between pressure taps was 17.39 m. The distance between entrance and the first pressure tap was ≈ 250 diameters so that end effects were effectively eliminated. The pressure drop was measured using a differential pressure transducer (Model 1151DP, Rosemount Inc., Eden Prairie, MN). The design (with one bend as wide as possible to minimize error) was developed based on availability of floor space in the laboratory and capabilities of the pressure transducer. The entire length of the viscometer was insulated to minimize temperature drop across pressure measurement points. A maximum temperature drop of 2.5°C was observed

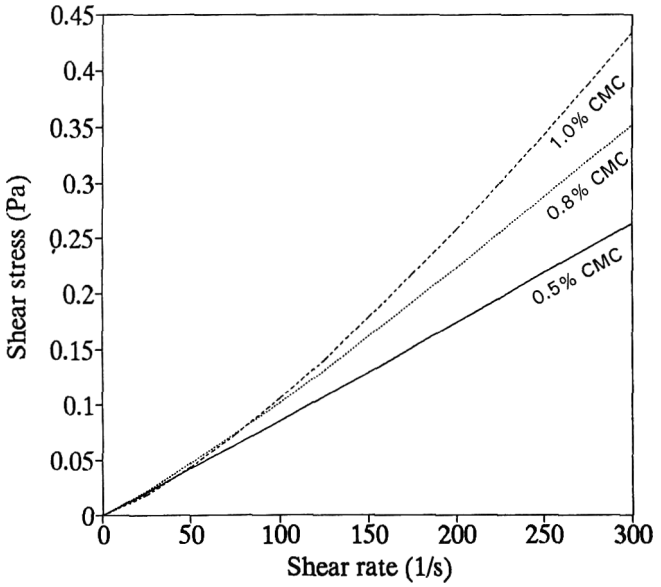

Fig. 4—Sample flow curves for carrier fluids (CMC).

Table 2—Rheological characteristics of carrier fluid (CMC) at 115.5°C for relative velocity (a) and liquid crystal experiments (b)

Conc (%)	Duration of Expt (Hr)	Consistency coefficient (K) Pas^n 10^4			Flow behavior index (n)		
		Initial	Final	Mean	Initial	Final	Mean
		(a) Relative velocity experiments					
0.5	3.5	9.12	7.64	8.38	1.056	1.024	1.040
0.8	4.0	8.52	4.49	6.51	1.140	1.127	1.132
1.0	6.0	6.9	1.86	4.35	1.236	1.284	1.260
		(b) Liquid crystal experiments					
0.5	4.0	8.55	7.83	8.19	1.072	1.054	1.063
0.8	4.0	7.63	6.47	7.05	1.161	1.140	1.155
1.0	4.0	3.05	4.05	3.55	1.27	1.26	1.265

at low flow rates. The maximum Reynolds number measured within the tube viscometer was 1528, ensuring that laminar flow was maintained.

Shear stress and shear rate. We measured time-averaged (over 60 sec) values of pressure drop (ΔP) at steady state for selected ranges of flow rates (5.68×10^{-5} m³/s to 6.31×10^{-5} m³/s). A time average was used since pressure drop readings fluctuated slightly. Shear stress values were calculated by substituting pressure drop values in the following expression:

$$\tau_w = \frac{\Delta PR}{2L} \quad (5)$$

Shear rate values were then calculated using the Rabinowitsch-Mooney equation (Skelland, 1967):

$$\dot{\gamma}_w = \frac{3Q}{\pi R^3} + \tau_w \frac{d(Q/\pi R^3)}{d\tau_w} \quad (6)$$

The derivative term was evaluated by fitting $Q/(\pi R^3)$ vs τ_w with a polynomial ($R^2 \geq 0.98$). The consistency coefficient (K) and flow behavior index (n) for various concentrations of CMC were estimated using a power-law model (R^2 ranged from 0.91 to 0.997):

$$\tau_w = K\dot{\gamma}^n \quad (7)$$

Rheological properties were determined before and after each set of studies to determine whether significant changes had occurred in the carrier fluid (Table 2a,b). Estimated flow curves for different concentrations of carrier fluids were developed (Fig. 4). Power law parameters used in the relative velocity method were those corresponding to the beginning rather than the end of experiments, since the carrier fluid was most viscous at that stage, and h_{fp} calculations would be more conservative.

Results of the tube viscometer were verified at atmospheric pressure and room temperature (≈21°C) by comparison with results obtained from a rotational viscometer (Rheomat 115, Contraves AG, Zurich, Switzerland). Results compared well, with a maximum deviation of 3.5% in the shear stress readings at a given shear rate. Comparisons could not be conducted at high pressures due to lack of a high-pressure rotational viscometer.

Table 3—Means and standard deviations of relative velocity and h_{fp} determined using the relative velocity method and different correlations (R & M refers to Ranz and Marshall correlation, and RV to relative velocity)

CMC conc (%)	Flow rate (m³/s)	Size (m)	RV m/s		h_{fp} W/m²°K	
			Mean	SD	R & M	Flat plate
0.5	0.000252	0.0199	0.092	0.015	1606	856
		0.0149	0.086	0.011	1810	957
		0.0117	0.079	0.013	1973	1059
	0.000379	0.0199	0.113	0.012	1770	948
		0.0149	0.110	0.009	2036	1083
		0.0117	0.106	0.032	2238	1182
	0.000505	0.0199	0.124	0.035	1841	987
		0.0149	0.114	0.026	1996	1060
		0.0117	0.112	0.032	2330	1233
0.8	0.000252	0.0199	0.090	0.016	1583	819
		0.0149	0.086	0.011	1746	922
		0.0117	0.082	0.012	1929	1010
	0.000379	0.0199	0.103	0.018	1633	872
		0.0149	0.102	0.023	1873	993
		0.0117	0.098	0.022	2075	1091
	0.000505	0.0199	0.115	0.022	1718	919
		0.0149	0.110	0.036	1944	1033
		0.0117	0.106	0.009	2166	1143
1.0	0.000252	0.0199	0.063	0.013	1306	689
		0.0149	0.059	0.006	1448	756
		0.0117	0.058	0.009	1627	842
	0.000379	0.0199	0.091	0.012	1523	811
		0.0149	0.089	0.014	1733	915
		0.0117	0.087	0.011	1924	1008
	0.000505	0.0199	0.100	0.013	1582	844
		0.0149	0.097	0.023	1794	949
		0.0117	0.096	0.020	1994	1047

Statistical analyses

Statistical analyses were performed to examine the influence of carrier medium concentration, flow rate, and particle dimension on h_{fp} values using General Linear Model Program (SAS Institute, Inc., 1989). Student's-t test (Mendenhall, 1979) was used to test for significant differences between means.

RESULTS & DISCUSSION

MEANS AND STANDARD DEVIATIONS of h_{fp} as influenced by carrier medium viscosity, flow rate, and particle-to-tube diamter ratio using the relative velocity (Table 3) and the liquid crystal methods (Table 4) were compared. The range of dimensionless numbers was also tabulated (Table 5). The maximum and minimum measured values of h_{fp} (based on individual replicates) for the relative velocity method using the Ranz and Marshall correlation were 2730 W/m²°K and 1143 W/m²°K respectively (Nusselt numbers of 63.1 and 33.2). These compared with 2270 W/m²°K and 986 W/m²°K (Nusselt numbers of 54.6 and 26.4) for the liquid crystal method. Flat plate correlations yielded lowest estimates of h_{fp} values using the relative velocity data for cubic particles (598 W/m²°K to 1456 W/m²°K; corresponding to Nusselt numbers from 13.6 to 24.1).

The data fell partially in the bulk range of h_{fp} values reported by earlier researchers using moving particles at below atmospheric boiling point temperatures (Heppell, 1985; Sastry et al., 1990; Stoforos and Merson, 1991; Balasubramaniam and Sastry, 1993a; Mwangi et al., 1993; Zitoun and Sastry, 1993). The data also confirmed that the rate of heat transfer is a complex function of flow field around the particle, and forced convec-

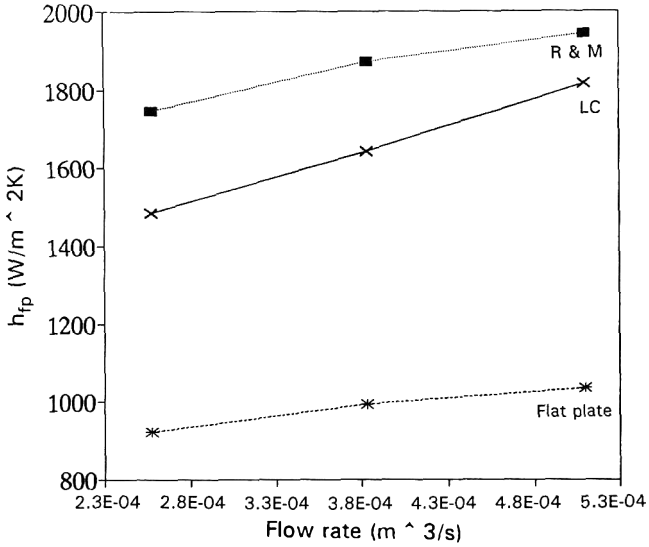

Fig. 5—Effect of flow rate on h_{fp} values (Conc: 0.5% and a = 0.0199m (RV), a = 0.0177m (LC)).

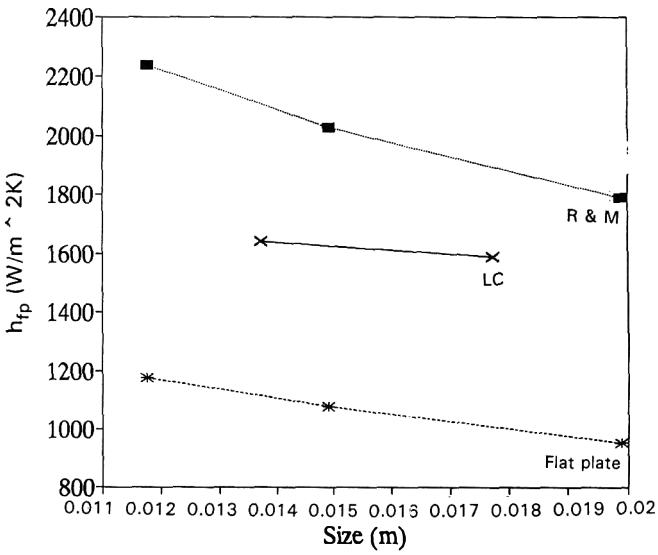

Fig. 6—Effect of particle dimension of hfp values (Conc: 1% and Q = 0.000252 m³/s).

Table 4—Means and standard deviations of h_{fp} determined using the liquid crystal method in continuous tube flow conditions

CMC conc (%)	Flow rate (m³/s)	Size (m)	h_{fp} W/m²°K Mean	SD
0.5	0.000252	0.0177	1662	142
		0.0137	1719	183
	0.000379	0.0177	1844	253
		0.0137	1924	244
	0.000505	0.0177	1888	208
		0.0137	1939	206
0.8	0.000252	0.0177	1521	111
		0.0137	1484	151
	0.000379	0.0177	1521	119
		0.0137	1641	276
	0.000505	0.0177	1653	343
		0.0137	1816	189
1.0	0.000252	0.0177	1256	230
		0.0137	1307	179
	0.000379	0.0177	1381	149
		0.0137	1440	92
	0.000505	0.0177	1438	189
		0.0137	1467	176

Table 5—Range of experimental parameters

Parameter	Maximum	Minimum
Fluid Reynolds no.	16,422	2174
Prandtl no.	11.82	2.46
Biot no.	0.07	0.03

tion is likely the dominant factor (Balasubramaniam and Sastry, 1993a).

Influence of process parameters

Flow rate. The h_{fp} values increased significantly with increasing flow rate ($p < 0.05$) (Fig. 5). An increase in flow rate increased the fluid Reynolds number and the localized relative motion between fluid and particle also increased. The mean values of relative velocity at 5.05×10^{-4} m³/s was 0.11 m/s in comparison to a lower value 0.078 m/s at 2.52×10^{-4} m³/s. The Nusselt number range from the liquid crystal method was 26.4 to 54.6, much higher than the limiting Nusselt number value of 2.0 for a zero relative velocity condition as favored for process evaluation by the FDA (Pflug et al., 1990). The flow rates were representative values commonly employed in the industry.

Particle dimension

The measured relative velocity values increased with particle-to-tube diameter ratio, in agreement with published reports (Sastry et al., 1990; Balasubramaniam and Sastry, 1993a; Mwangi et al., 1993; and Zitoun and Sastry, 1993). However, h_{fp} values decreased with increasing particle-to-tube diameter ratio ($p < 0.05$) (Fig. 6). These findings were not totally contradictory, and arose due to two conflicting effects. For the same relative velocity, h_{fp} decreases with increasing particle dimension, as predicted from the Ranz and Marshall equation. Also, larger particles in tube flow exhibit higher relative velocities due to increased channeling effects. Our results suggest that within the conditions, the increase of relative velocity with particle size was not great enough to compensate for the negative effect of particle dimensions on h_{fp} values. This was in agreement with Zitoun and Sastry (1993).

Particle size effects merit careful consideration since size changes can occur during processing. Burfoot and Self (1988), studying heating times for beef cubes during water cooking observed a change in density and dimensions of particles. They reported mean percentage weight losses of 24.67% and 23.26% for cubes of 0.02m and 0.03m in size (change in particle sizes were not reported). Many of the published heat transfer and residence time studies used simulated food particles of constant shape and size. This was necessary to understand the basic physics of flow and heat transfer mechanisms between fluids and the particles. However, it is also important to conduct further studies with real food particles at ultra high temperatures to understand the effects of changes in particle density and dimensions during cooking, on fluid-particle interactions.

Carrier medium viscosity. The h_{fp} values increased significantly with decreasing carrier medium viscosity ($p < 0.05$) (Fig. 7). When the carrier medium CMC concentration was decreased from 1.0% to 0.5%, the h_{fp} values increased by 556 W/m²°K (based on minimum observed individual replicate values of h_{fp} determined using liquid crystal method). At elevated temperatures, degradation of CMC solution was observed over an extended period (Table 2). Effects were quite apparent for 1.0% CMC solution used in relative velocity experiments, possibly due to longer duration (six hours) of experiments. Heat transfer coefficients based on initial and final viscosity data for different correlations were compared (Fig. 8). The figure il-

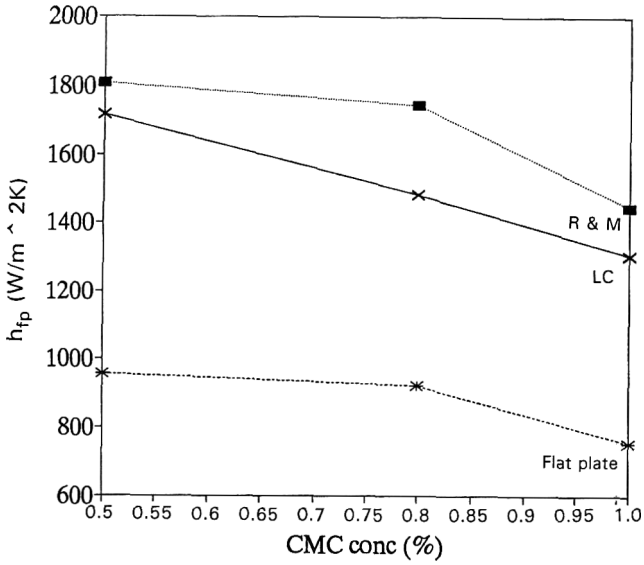

Fig. 7—Effect of carrier medium concentration on h_{fp} values (Q = 0.000252 m³/s and a = 0.0149m (RV), a = 0.0137m (LC)).

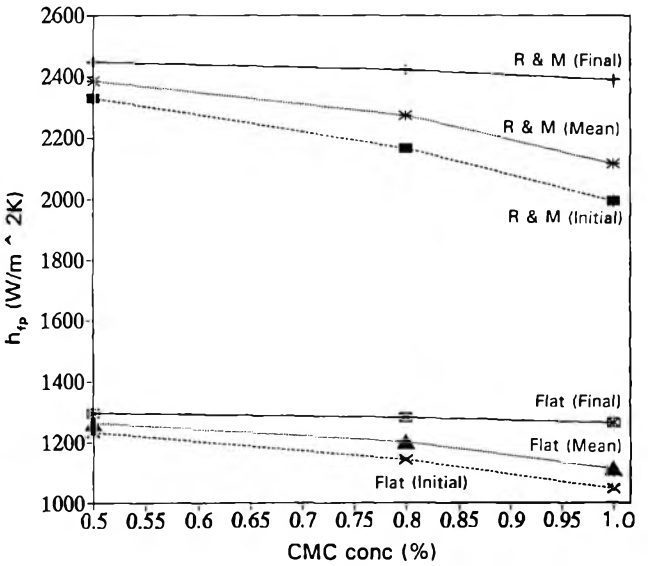

Fig. 8—Effect of variation of rheological parameters during experiments on h_{fp} values determined using relative velocity data and different correlations (Q = 0.000252 m³/s, and a = 0.0149m).

lustrates the possible extent of variation in h_{fp} values due to change in carrier viscosities. The h_{fp} values reported for the relative velocity method for different correlations (Table 3) were determined using rheological parameters that corresponded to a high viscosity carrier (i.e., beginning of the experiments). The corresponding maximum and minimum values of h_{fp} for different carrier fluids and correlations were also compared (Table 6). The liquid crystal method is a heat transfer based experimental technique, the changes in carrier fluid viscosities were reflected in the liquid crystal color changes (and h_{fp} values), and no separate correction was necessary.

Rheological properties. Rheograms were compared (Fig. 9) for 0.8% CMC (at fluid temperatures of 45°C and 115.5°C), water at 115.5°C (Incropera and DeWitt, 1990), and 2.7% starch (at fluid temperatures of 121.1°C and 143.3°C (Dail and Steffe, 1990)). For a given CMC concentration, as fluid temperature increased, the estimated range of viscosity decreased. (Comparing the viscosity of 0.8% CMC at 45°C to that of 115.5°C; similarly viscosity of 2.72% starch solution at 121°C to that of 143°C). The rheograms show that under those shear rate ranges, the carrier fluids (Fig. 4) were slightly less viscous

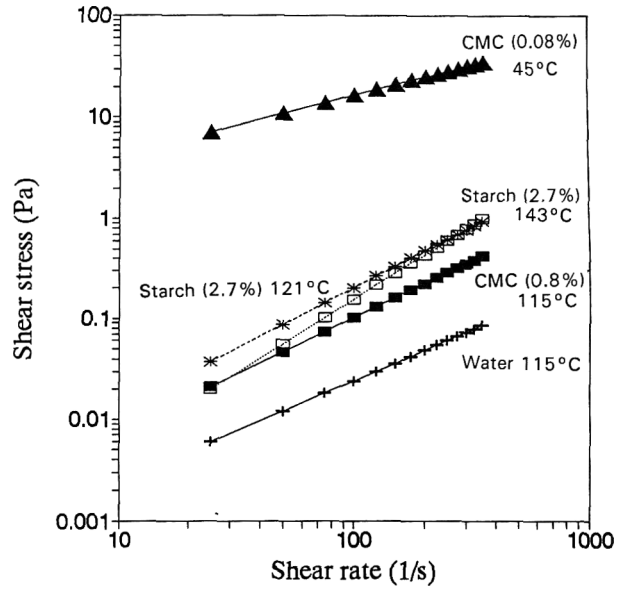

Fig. 9—Rheograms of different carrier fluids at different temperatures.

Table 6—Range of h_{fp} values determined using relative velocity data and different correlations at the beginning and end of the experiments

Experimental condition	h_{fp} (w/m²°C)			
	R&M		Flat plate	
	Maximum	Minimum	Maximum	Minimum
Relative velocity (Beginning[a])	2730	1143	1456	598
Relative velocity (End[b])	2876	1383	1535	729

[a] h_{fp} Values determined based on rheological parameters estimated at the beginning of the experiments.

[b] h_{fp} Values determined based on rheological parameters estimated at the end of the experiments.

than starch solution at 121°C. Also note that 0.8% CMC at 45°C (Balasubramaniam and Sastry, 1993a) is more viscous within the shear range considered in comparison with 2.72% starch solution at 121°C or higher. Based on flow rate and holding tube sizes used in the food industry, most commercial systems would operate in turbulent flow (Dail and Steffe, 1990). Thus the drop in viscosities of carrier fluids at ultra high temperatures could result in increased turbulence and heat transfer between fluids and particles. As an additional point of caution, that some small errors in rheological estimation in our setup were unavoidable, primarily because of the necessity of the bend. Further studies are needed for comparison of rheological data between tube and rotational viscometers at high pressures.

Note that significantly high heat transfer coefficients were measured for carrier fluids (CMC) which exhibited low to moderately dilatant behavior at elevated temperatures (Table 2 and Fig. 4). They were high though CMC exhibited pseudoplastic behavior at below atmospheric boiling point temperature studies (Balasubramaniam and Sastry, 1993a,b). The dilatant effect was not as pronounced as with starch solution (Dail and Steffe, 1990), with a reported flow behavior index of 2.08 (with $K = 1.83 \times 10^{-5}$ Pas^n) for 2.72% starch (based on individual runs) at 132.2°C fluid temperature. This is expected to result in greatly increased viscosities at high shear rates. However, use of flow behavior indices alone to describe rheological characteristics of a fluid is not sufficient. It is necessary to use both K and n values. Both our studies and those of Dail and Steffe (1990) report low values of consistency coefficient (K) at elevated temperatures [present study: K =

Table 7—Range of reported values of relative velocity (RV) and h_{fp} in the literature

Source	Reynolds no.	RV (m/s)	hfp W/m²°K)	Remarks
Chandarana et al. (1988)	5.57–141.84	0.0044–0.011	55.6–89.5	h_{fp} data determined using simulated fluid flow (129°C) over a stationary cubic particle.
Chang and Toledo (1989)	—	0.0–0.0086	239–303	h_{fp} data determined using simulated fluid flow (75°C) over a stationary cubic particle.
Sastry et al. (1990)	7300–43,600	0.0007–0.054	180–1327[a] 688–3005[b]	RV predicted ($V_r = V_f - V_p$) in continuous tube flow between a sphere and fluid (45°C).
Balasubramaniam and Sastry (1993a)	14.5–798	0.02–0.19	401–1684[a] 857–2010[c]	Experimentally measured RV in continuous tube flow between a sphere and fluid (45°C).
Zitoun and Sastry (1993)	21.2–270.0	0.03–0.13	286–1034[d] 268–928[c]	Experimentally measured RV in continuous tube flow between a neutrally buoyant cube and fluid (31°C).
Present study	2,174–10,422	0.05–0.18	1143–2730[a] 986–2270[c] 598–1456[d]	Experimentally measured RV in continuous tube flow between a cube and fluid (115.5°C).

[a] h_{fp} Determined using Ranz and Marshall correlation.
[b] h_{fp} Determined using moving thermocouple method.
[c] h_{fp} Determined using liquid crystal method.
[d] h_{fp} Determined using flat plate correlation.

1.86×10^{-4} to 9.1×10^{-4} Pas^n; Dail and Steffe (1990): K = 1.83×10^{-5} to 4.57×10^{-3} Pas^n] indicating the likelihood of turbulence under the test conditions. Our flow visualization videotapes and significant measured relative velocities (0.05 to 0.18 m/s) between fluids and particles further support this. Hence, one could have high heat transfer coefficients between phases even for a fluid with low to moderate dilatant characteristics, provided it has low apparent viscosity under the conditions of the study. However, different carrier fluids may have different flow patterns and rheological characteristics. Hence, anyone in process development or verification for low acid food, should make independent rheological and heat transfer characterizations for carrier fluids and particulates of interest over the conditions of interest.

Comparison of reported vaues of relative velocity and h_{fp} values

Relative velocities and h_{fp} values reported by several researchers were compared (Table 7). Our experimentally measured relative velocity values were much higher than those values assumed in earlier simulation studies using stationary particles (Chandarana et al., 1988; Chang and Toledo, 1989). The measured values of relative velocity were also higher than those predicted using the difference between average fluid velocity (V_f) and particle velocity (V_p) (Sastry et al., 1990). Although a given particle may move with a velocity near the *average* fluid velocity, the fluid near the particle has a local velocity far higher than its mean velocity due to Bernoulli type channeling effects. (Balasubramaniam and Sastry, 1993a).

Our studies were conducted under a pressurized environment (172.4 to 206.8 kPa [25-35 psi gauge]) and hence it was not possible to control the radial location of the particle. At elevated fluid temperatures, it was also difficult to exactly match the fluid-particle densities. Turbulence and uncontrolled radial location of the particles could have resulted in the relatively wide spread in measured relative velocity data (0.04 m/s to 0.18 m/s).

Limitation of Nusselt number correlations

Studies of Balasubramaniam and Sastry (1993a) using spherical particles in continuous flow through holding tubes, indicated that the Ranz and Marshall correlation yielded the lowest estimates of h_{fp} values in comparison to those of Whitaker (1972), and Kramers (1946). The data were also in agreement with other heat transfer based experimental tecniques (moving thermocouple method and liquid crystal method). However, in our study, cubic particles were used, and the validity of such correlations for non-spherical particles in tube flow conditions is not known. The h_{fp} data obtained from the Ranz and Marshall correlation were compared against the flat plate correlation (Eq. 3, Fig. 5 to 7). The same relative velocity data were used for both correlations. The Ranz and Marshall correlation yielded higher values of h_{fp} than the flat plate correlation. The data from the liquid crystal fell between the two data sets. Since all developed correlations were empirical in nature, using them beyond the applicable conditions could produce erroneous results. The conclusion from our relative velocity data was that the liquid crystal results were consistent with those expected from measured relative velocities.

Visual observations regarding flow field around the particle

Several observations were made from fluid-particle interactions from the relative velocity videotapes of cubic particles (using 1.0% CMC). For visual comparison, the motions of spherical and cylindrical particles were videotaped under similar conditions. The cubic particles oriented themselves parallel to the flow, with bottom faces practically in contact with the tube bottom. No rotation or sliding motion was observed, and only occasional turning (at a 45° angle to the tube axis) was noted. The spheres were in touch with the tube bottom at a single point. Possibly due to the difference in velocity gradients near the particles, the spheres tended to slide along the tube bottom even at low flow rates. The cylinders moved through the tube in a characteristic wavy-discontinuous type of motion. At high flow rates (e.g. 5.05×10^{-4} m^3/s), the cubes and the cylinders exhibited transverse oscillation. The fluid streamlines near the vicinity of the cubes were parallel to the cube surface, and wake formation was observed at high flow rates. These results were for single particles only. For multiple particles, the motion may be different. Our understanding of liquid-solid flow is derived from the study of flows about spheres, flat plates, and similar bodies. Studies on fluid-particles interactions for cubic particles and cylindrical particles (which may be of interest to food engineers) needs further investigation. For example, studies of Mwangi et al. (1993) indicated at 80-200% increase in heat transfer values when the solid fraction was increased to 3.22%. Further studies are necessary to characterize flow interactions and estimates of h_{fp} values for multiple particle suspensions.

NOMENCLATURE

a	Cube size or dimension (m)
A	Surface area (m²)
Bi	Biot number ($h_{fp}/l/k_p$)
C_p	Specific heat (J/kg°C)

d	Equivalent diameter of the cube (m) ($d = (6/\pi)^{(1/3)}a$)
D	Diameter of the holding tube (m)
Fo	Fourier number
h_{fp}	Liquid-to-particle convective heat transfer coefficient (W/m²°K)
H	Hue value of liquid crystal
k_f	Thermal conductivity (W/m°C)
k_p	Thermal conductivity of particle (W/m°C)
K	Consistency coefficient (Pas^n)
l	Wall thickness of transducer particle (m)
L	Length of the tube viscometer in between pressure taps (m)
m	mass (kg)
n	Flow behavior index
Nu	Nusselt number ($Nu = h_{fp}d/k_f$)
P	Pressure (Pa)
Pr_G	Generalized Prandtl number ($Pr_G = [C_pK((3n+1)/n)^n\ 2^{(n-1)}]/[4\ k_f V_f^{(1-n)} D^{(n-1)}]$)
Q	Volumetric flow rate (m^3/s)
R	Radius of the tube (m)
Re_G	Generalized fluid Reynolds number ($Re_{G,F} = [8V_f^{(2-n)}D^n\rho]/[2^n((3n+1)/n)^n]$
$Re_{G,S}$	Generalized slip Reynolds number ($Re_{G,S} = [8V_r^{(2-n)}d^n\rho]/[2^n((3n+1)/n)^n]$
V_f	Velocity of fluid (m/s)
V_r	Relative velocity between fluid and particle (m/s) ($V_r = V_f - V_p$)
V_p	Velocity of particle (m^3)
t	Time (s)
T	Temperature (°C)
T_i	Initial temperature of particle (°C)
T_f	Temperature of fluid (°C)

Greek symbols:

α	Thermal diffusivity (m^2/s)
γ	Shear rate (s^{-1})
Δ	Gradient
τ	Shear stress (Pa)
ρ	Density (kg/m^3)

Abbreviations:

LC	Liquid crystal method
RV	Relative velocity method or Relative velocity

CONCLUSIONS

MAXIMUM AND MINIMUM VALUES of h_{fp} measured by the liquid crystal method ranged from 986 to 2270 W/m²°K respectively over a fluid Reynolds number range of 2,174 to 10,422. The relative velocity ranged from 0.05 m/s to 0.18 m/s. The h_{fp} values increased with increasing flow rate, and decreased with increasing viscosity, and particle-to-tube diameter ratio. The Ranz and Marshall correlation yielded higher estimates of h_{fp} values than the flat plate correlation. The data from the liquid crystal method fell in between the two estimates, and seemed consistent with measured relative velocities. Significant values of relative velocity (and h_{fp}) were determined even when carrier fluids exhibited mildly dilatant behavior, partly due to low fluid apparent viscosity values (and the presence of turbulence) at ultra high temperatures.

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Flavor Modification by Sodium Chloride and Monosodium Glutamate

SARAH E. KEMP and GARY K. BEAUCHAMP

ABSTRACT

Five concentrations of sodium chloride or monosodium glutamate were added to pure taste or flavor substances. The five mixtures were ranked for pure taste or flavor intensity. No true potentiation was found. Suprathreshold sodium chloride or monosodium glutamate additions generally suppressed pure tastes and flavors. The extent of suppression depended upon the pure taste or flavor present. This differential suppression may result in apparent potentiation in more complex mixtures (e.g., three or more components) due to an apparent (relative) increase in intensity of less-suppressed flavor components. Apparent potentiation may also be due to addition of flavor components of sodium chloride and monosodium glutamate.

Key Words: salt, glutamate, threshold, bitter, sweet

INTRODUCTION

NaCl has several gustatory functions in food. It contributes salty taste, sweet taste at low concentrations (Bartoshuk et al., 1978), bitter side tastes in some subjects (Kroeze, 1982), and it modifies taste and flavor. Pangborn (1960) reported that subthreshold concentrations of NaCl reduced caffeine bitterness. Kroeze (1977) reported a significant decrease in absolute thresholds when NaCl and sucrose were combined in different proportions while keeping the total concentration of the two compounds constant at absolute threshold. He later attributed the sweetness potentiating effect to the addition of the sweetness of NaCl (Kroeze, 1982). Low concentrations of NaCl are reported to increase the sweetness of low concentrations of sucrose in aqueous solution (Kamen et al., 1961; Pangborn, 1962; Bartoshuk et al., 1978; De Graaf and Frijters, 1989), in lima bean purée (Pangborn and Trabue, 1964) and in canned tomato juice (Pangborn and Chrisp, 1964). They decrease the sourness of citric acid in aqueous solutions (Kamen et al., 1961).

Higher concentrations are reported to decrease the sweetness of sucrose in aqueous solution (Kamen et al., 1961; Pangborn, 1962; Bartoshuk, 1975; De Graaf and Frijters, 1989), in lima bean purée (Pangborn and Trabue, 1964) and in canned tomato juice (Pangborn and Chrisp, 1964) and the sweetness of low concentrations of glucose and fructose (Moskowitz, 1972). Higher concentrations of NaCl are also reported to decrease the sourness of lactic acid, acetic acid (Hellemann, 1992) and high concentrations of citric acid (Kamen et al., 1961) in aqueous solution. They also increase the sourness of low concentrations of citric acid in aqueous solution (Kamen et al., 1961) and mixtures of lactic acid and acetic acid in rye bread (Hellemann, 1992), but had little effect on sourness of hydrochloric acid in aqueous solutions (Bartoshuk, 1975). The bitterness of quinine hydrochloride (Bartoshuk, 1975; Schifferstein and Frijters, 1992) and quinine sulfate (Kroeze, 1982) were reported to be decreased by higher levels of NaCl in aqueous solution, although Kamen et al. (1961) reported no effects of a range of concentrations of NaCl on bitterness of caffeine.

Author Kemp is with Givaudan-Roure Corp., 1775 Windsor Road, Teaneck, NJ 07666. Author Beauchamp is with Monell Chemical Senses Center, 3500 Market St., Philadelphia, PA 19104.

Gillan (1983) used magnitude estimation of perceived intensity to investigate the effect of mixing NaCl with citral and anethole. Overall suppression occurred, with taste-taste mixture suppression greater than taste-odor mixture suppression. Other effects of NaCl in food systems included an increase in flavor blending, flavor impact and thickness (Yamaguchi and Kimizuka, 1979; Gillette, 1985) and a suppression of off-notes (Gillette, 1985).

In numerous studies on the taste of MSG, no firm concensus has been reached on its sensory attributes. Galvin (1948), Sjöström and Crocker (1948) and Lockhart and Gainer (1950) reported it was salty and sweet whereas Mosel and Kantrowitz (1952), Bartoshuk et al., (1974) and Halpern (1987) found it to elicit sweet, sour, salty and bitter tastes. The predominant taste quality also appears to change with concentration (Galvin, 1948; Halpern, 1987). The taste modification properties of MSG have been studied using a variety of psychophysical (Yamaguchi, 1977; Yamaguchi and Kimizuka, 1979) and electrophysiological (Yoshii, 1987) methods (see Cagan (1977) for a review of earlier literature). Results were confusing and it is difficult to draw firm conclusions although increasingly research results suggest that MSG has a unique taste (see Kawamura and Kare, 1987).

There have also been reports that MSG influenced flavor and odor, although results were confusing. Glutamate is found in free form in almost all food types (Maga, 1983) but appears to affect flavor to differing extents in different food types (Maga, 1987). The gustatory effect of MSG in food systems seems similar to that of NaCl with reports of an increase in flavor blending, flavor impact and flavor intensity and a decrease in off-notes (see Maga, 1983, for a review of earlier literature). Yamaguchi et al. (1971) and Rifkin and Bartoshuk (1980) showed that there was potentiation between MSG and certain 5'-ribonucleotides. Yoshii (1987) suggested that the potentiation of flavor in food was due to such synergistic action.

The objective of this study was to improve understanding of the flavor-modifying effects of NaCl and MSG. Effects on four pure tastes and seven flavors were investigated with five concentrations of NaCl or MSG. Flavor-modifying effects were assessed and a clarification of the terms used to describe these effects was proposed.

MATERIALS & METHODS

Subjects

Subjects were 15 staff of the Monell Chemical Senses Center, aged between 22 and 43, who were screened for their ability to detect, recognize and discriminate tastes and who were trained in the techniques described. They were paid for participation.

Sample preparation and presentation

Bottled spring water was used to make test solutions and for rinsing between test solutions in order to ensure a supply of water of consistent quality with minimum taste. In a pilot study, the brand was selected from commercially available bottled waters, deionized water and distilled water for having the least taste in blind tests.

Test solutions were prepared by making double the concentrations required. When a sample containing two compounds was required, equal volumes of the double-strength solutions were mixed. When a

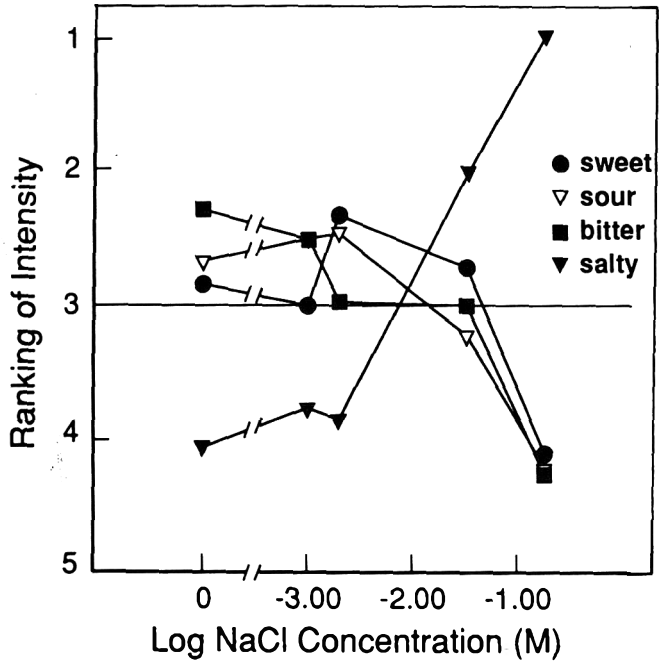

Fig. 1—Effect of NaCl on taste. In four ranking assessments, five concentrations of NaCl were added to a single suprathreshold concentration of the taste exemplars sweet (sucrose), sour (citric acid), bitter (quinine sulfate) and salty (NaCl). Panelists ranked each of the five mixtures for each of the four taste exemplars from most intense to least intense. If equally sweet (or sour or bitter or salty) then the expected ranking for each would be 3; deviation indicates added salt altered the perceived intensity of other taste qualities.

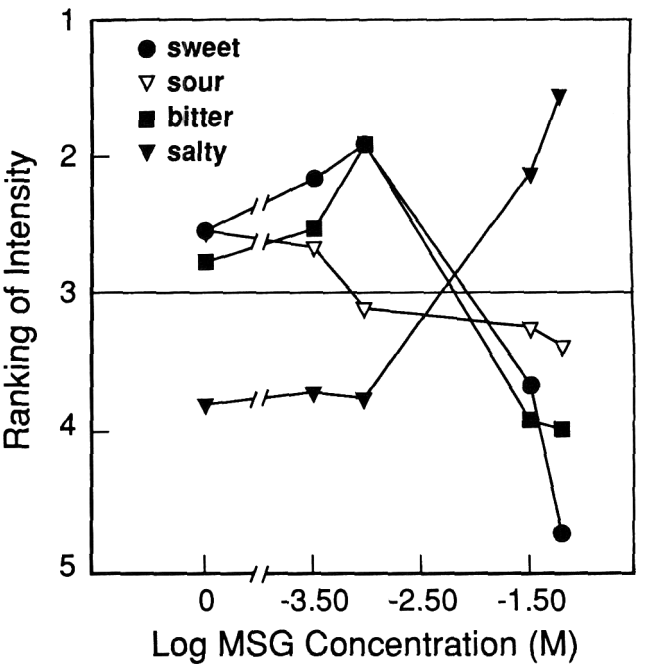

Fig. 2—Effect of MSG on taste. See caption to Fig. 1 for details.

sample containing one compound was required, the double strength solution was mixed with an equal volume of water. Concentrations reported are the final concentrations in the test samples.

Gustatory stimuli were presented as 10-mL aliquots in plastic medicine cups labelled with random, three-figure number codes. Subjects were instructed to sip and expectorate the stimuli, rinsing after each and retasting as often as necessary. Olfactory stimuli were presented as 50 mL aliquots in 250 mL polypropylene sniff bottles labelled with random, three-figure number codes. The bottles and caps were deodorized prior to use by boiling for 2 hr.

Procedures

A ranking procedure was used to assess modification of taste, flavor and flavor with a somatosensory component, by NaCl or MSG at varying concentrations. Subjects were given five samples in each ranking test. Each of the five samples contained the same concentration of pure taste substance, or flavor substance, mixed with one of five different concentrations of NaCl or MSG. Subjects were asked to rank the samples according to the intensity of the pure taste or flavor present (ignoring all other taste(s) or flavor(s) present) using 1 as strongest to 5 as weakest.

The 5 concentrations of NaCl used were 0, 0.001M, 0.00229M, 0.032M, and 0.171M (1%) NaCl, and the five concentrations of MSG used were 0, 0.00032M, 0.000979M, 0.032M and 0.059M (1%). These concentrations were chosen as follows: the lowest was below detection threshold, the second was about at detection threshold, the third was moderately intense and the fourth was at or above the level commonly found in foods. Detection thresholds for MSG and NaCl were obtained previously using the two-alternative forced-choice method of ascending and descending levels with 5 reversals, discarding the first of these reversals, run three times for each of 20 subjects.

Modification of taste, flavor and flavor with a somatosensory component were assessed in separate sessions. Modification by NaCl and MSG were also assessed in separate sessions. All assessments were run in duplicate in separate sessions, giving a total of 12 sessions.

Effect of NaCl and MSG on taste. Subjects assessed four sets of solutions, each set containing a different basic taste substance. The taste substances used were 0.05 M sucrose, 1.25×10^{-3} M citric acid, 2.5×10^{-5} M Q_2SO_4 or 0.025M NaCl. The tastes were all easily detected and identified by panelists and were of moderate strength. The order of stimulus presentation was balanced across sets and across concentrations of NaCl or MSG using a latin square design.

Effect of NaCl and MSG on flavor. Subjects assessed five sets of solutions, each set containing a different flavor substance. The same ranking procedures as above were employed. Instead of pure taste substances, NaCl or MSG were added to medium strength solutions of the following flavor substances; imitation butter flavor (1% v/v), celery extract (1% v/v), lemon extract (1% v/v), mint extract (0.67% v/v), and imitation pistachio flavor (1% v/v) (commercially available flavoring essences from McCormick & Co. and Virginia Dare Extract Co.). Subjects first assessed odor intensities of all five flavor sets from sniff bottles and then orally assessed flavor intensities of the five sets. Thirteen subjects evaluated the solutions containing MSG.

Effect of NaCl and MSG on flavor with a somatosensory component. The same ranking procedures as for flavor assessment were employed. Celery and pistachio flavors were replaced with 0.01% menthol and 0.003% citral (made using 0.6 mL/L of a solution of 0.5 mL of citral in 10 mL of alcohol).

RESULTS & DISCUSSION

Effect of NaCl and MSG on taste

When salt was the additive, overall rankings for sweetness, bitterness, sourness and saltiness of sucrose, Q_2SO_4, citric acid and NaCl, respectively, were significantly different than chance (Friedman test; Ps < 0.05; Fig. 1). The control for the experiment was the ranking of the salt taste of the NaCl solution with NaCl added at a range of concentrations. As expected, subjects ranked the solutions from weakest to strongest as concentration increased, although that was not the result at sub-detection threshold concentrations of NaCl. Perceived intensities of sweetness, bitterness and sourness were ranked as weaker at suprathreshold concentrations of NaCl. The average ranking of sweetness was greatest at threshold concentrations of NaCl. The modal ranking at threshold was 1 for the first replication only.

Addition of MSG produced very similar but not identical results (Fig. 2). Addition of MSG to NaCl, sucrose and Q_2SO_4 significantly (Ps < 0.01) altered the saltiness, sweetness and bitterness, respectively. MSG showed no effect on sourness (Friedman test; $P_s > 0.2$). The ranking of saltiness showed the same-shaped function as ranking of saltiness with added NaCl. MSG suppressed the perceived intensity of sweetness and bitterness at supra-detection threshold concentrations. When threshold concentrations of MSG were added, sweetness and bitterness rankings were not significantly different from those for no added MSG (using the sign test). The modal ranking of threshold concentration in these cases was 1.

Table 1—Probability of chance rankings using the Friedman two-way analysis of variance by ranks for the effect of NaCl and MSG on odor and flavor

	Odor		Flavor	
	Replication 1	Replication 2	Replication 1	Replication 2
		NaCl		
Butter	<0.3	<0.95	<0.8	<0.2
Celery	<0.05*	<0.99	<0.5	<0.95
Lemon	<0.8	<0.05*	<0.5	<0.01*
Mint	<0.3	<0.2	<0.001*	<0.001*
Pistachio	<0.5	<0.7	<0.2	<0.5
		MSG		
Butter	<0.8	<0.7	<0.7	<0.9
Celery	<0.7	<0.5	<0.5	<0.8
Lemon	<0.1	<0.2	<0.05*	<0.02*
Mint	<0.1	<0.7	<0.01*	<0.01*
Pistachio	<0.1	<0.5	<0.5	<0.5

* Significantly different from chance

Little evidence of potentiation of taste was apparent when either NaCl or MSG was added, at various concentrations, to solutions of NaCl, Q_2SO_4 and citric acid. Suppression at suprathreshold concentrations was evident. The lack of potentiation with pure tastes led us to evaluate possible potentiation of flavors.

Effect of NaCl and MSG on flavor

No effects of NaCl on odor were found (Friedman test, Ps > 0.05; Table 1). The only significant effect of NaCl on flavor occurred for mint, which was ranked lower in intensity as NaCl concentration increased (Fig. 3). Effects of NaCl on lemon flavor were marginal ($p < 0.01$ for the second replicate only). Results with MSG were similar (Table 1). No effects of MSG on odor were found. There was a significant effect on mint flavor and lemon flavor only, which indicated suppression at supra-detection threshold concentrations of MSG.

Mint and, to a lesser extent, lemon, had been noted in pilot work to have a greater somatosensory component compared with the other flavors used. The suppressive effect of NaCl and MSG on these flavors might be due, in part to their action on the somatosensory component. To investigate this, menthol, the characteristic flavor and somatosensory component of mint extract, was assessed using the ranking procedure. Also used was citral, at a concentration that gives an olfactory but not somatosensory sensation (Schmidt and Beauchamp, 1989). In order to evaluate reliability of results, mint flavor, imitation butter flavor and lemon extract were repeated.

Effect of NaCl and MSG on flavor with a somatosensory component

The addition of NaCl, again, had no effect (Ps > 0.05) (Table 2) on the odor of the flavors except for lemon. That effect, since suppression was not evident at the highest NaCl concentration, was probably a chance event. The results for oral evaluation of the flavors (Fig. 4) showed the two new flavors, citral and menthol, and mint and lemon were suppressed at higher concentrations of added NaCl ($P_s < 0.05$). Results with MSG were similar to those with NaCl (Table 2). The flavors of citral, lemon, menthol and mint were suppressed at high MSG concentrations. There was no significant effect on butter flavor ($p > 0.1$) although in both replicates there was an indication of suppression at the highest concentration. Results of this experiment were generally consistent with the previous results. There was no flavor potentiation and flavor suppression at higher concentrations was flavor-specific. The two new flavors, citral and menthol, were suppressed at higher NaCl and MSG concentrations.

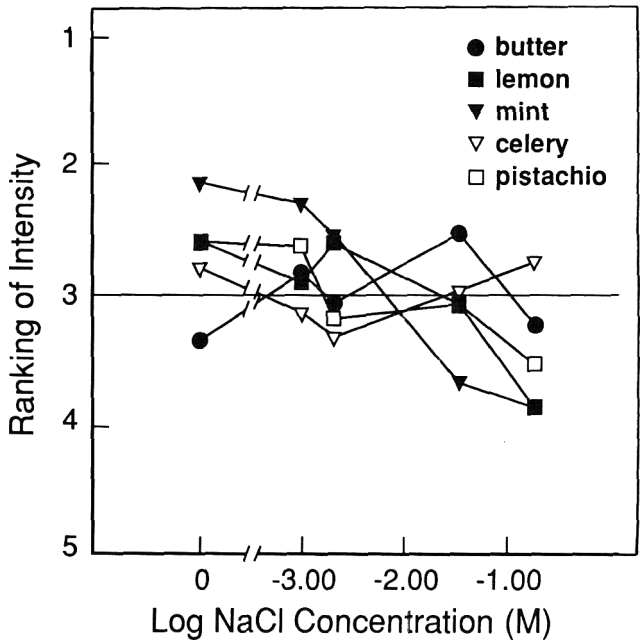

Fig. 3—Effect of NaCl on flavor. In five ranking assesments, five concentrations of NaCl were added to a single suprathreshold concentration of substances eliciting butter, celery, lemon, mint and pistachio flavors. Panelists ranked each mixture for each of the five flavors from most to least intense. If all five mixtures were equal, the expected ranking for each would be 3. Deviation indicates added salt altered the perceived intensity of the flavors.

Table 2—Probability of chance rankings using the Friedman two-way analysis of variance by ranks for the effect of NaCl and MSG on odor and flavor with a somatosensory component

	Odor		Flavor	
	Replication 1	Replication 2	Replication 1	Replication 2
		NaCl		
Butter	<0.5	<0.9	<0.1	<0.9
Citral	<0.05*	<0.7	<0.05*	<0.05*
Lemon	<0.01*	<0.01*	<0.02*	<0.02*
Menthol	<0.8	<0.5	<0.1	<0.02*
Mint	<0.2	<0.7	<0.001*	<0.001*
		MSG		
Butter	<0.5	<0.2	<0.1	<0.1
Citral	<0.8	<0.05*	<0.01*	<0.01*
Lemon	<0.02*	<0.5	<0.02*	<0.01*
Menthol	<0.8	<0.7	<0.001*	<0.01*
Mint	<0.2	<0.3	<0.001*	<0.001*

* Significantly different from chance

General discussion

Sub-detection threshold concentrations of the "flavor potentiators", NaCl and MSG, did not affect taste, odor or flavor. This may be because sub-detection threshold concentrations of NaCl and MSG were too low to produce effects or the methods used were not sensitive enough to detect changes.

At detected threshold concentrations of MSG, sweetness and bitterness were given the lowest rankings (greatest intensity) of any solution in any experiments involving MSG (with exception of saltiness at highest concentration of MSG, discussed below). The apparent potentiation of sweetness and bitterness at threshold concentrations of MSG may have been due to the added sweetness and bitterness that has been reported for MSG (Halpern, 1987) and not due to potentiation per se. At detection threshold concentrations of NaCl, sweetness was perceived as strongest of all solutions in the taste experiment that contained NaCl. Several other reports have indicated NaCl increased sweetness at low concentrations. Probably the low ranking of sweetness was due to addition of the sweet com-

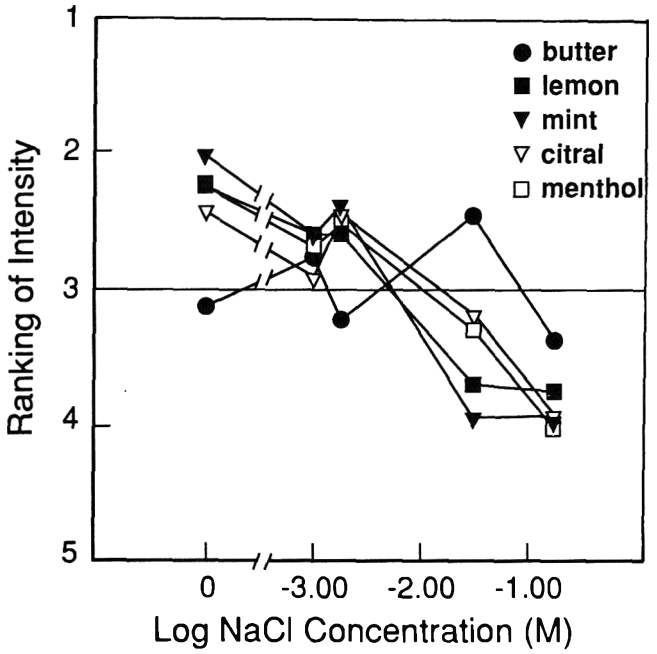

Fig. 4—Effect of NaCl on flavor with a somatosensory component. See caption to Fig. 3 for details.

ponent the taste of NaCl has been reported to impart at low concentrations (Bartoshuk et al., 1978) and not due to potentiation.

At supra-detection threshold concentrations, all significant effects reflected decreases in perceived taste and flavor intensity. This may be due to mixture suppression or masking in agreement with the findings of Yamaguchi (1977) and Yamaguchi and Kimizuka (1979). NaCl appeared to suppress sweetness, sourness and bitterness and MSG appeared to suppress sweetness and bitterness. The action of NaCl in suppressing bitterness (Schifferstein and Frijters, 1992) as concentration increased may in part explain the masking effect reported for NaCl on unpleasant and off tastes (Gillette, 1985).

MSG had no effect on sourness. This may be because there was an equilibrium between four ionic species of glutamate that depends on pH. The species with the greatest effect on taste may not be formed in solution below pH 4 (Fagerson, 1954). MSG increased saltiness as concentration increased, probably due to addition of the salty component of its taste and not due to potentiation.

Supra-detection threshold concentrations of NaCl and MSG acted by suppressing flavor. The magnitude of suppression depended upon flavor type. This differential effect on flavors changed with concentration. Maga (1987) also reported MSG acted with different effectiveness on different flavors. The suppression of menthol provided evidence that prominent somatosensory components are characteristic of suppressed flavors. However, the suppression of citral did not, since at the concentration used citral had little or no somatosensory sensation. Flavors with a somatosensory or chemesthetic (Green et al., 1990; Green and Lawless, 1991) component appeared to be particularly susceptible to suppression. It is unclear why this occurs. NaCl has an irritant effect, although probably not at concentrations we used (Green and Gelhard, 1989). Conceivably this low level of irritation increased overall perception of irritation leading to an apparent decrease in flavor perception (Lawless and Stevens, 1989), due to masking or suppression of the characteristic odor component when sampled retro-nasally.

Generally, effects of NaCl and MSG have been to suppress perceived intensity. This does not appear compatible with the premise that they are flavor potentiators. From our results they were not found to have true potentiating properties. They may, however, have "apparent" flavor potentiating properties due to differential suppression. This is, they may be "flavor modulators." Their perceived suppression of intensities of certain taste and/or other flavor components may result in an increase in perceived (relative) intensity of the remaining (less suppressed) taste and/or other flavor components.

NaCl and MSG are used in foods at supra-detection threshold concentrations. At those levels, they appear to alter relative intensities of tastes and retro-nasally-sampled odors of flavors. They may not modify the perception of all flavors present in the same way. Different combinations of taste, retro-nasally-sampled odor or somatosensory components may be altered for different flavors. This, in turn, could modify the flavor profile of foods, which may increase pleasantness. In this way, NaCl and MSG may act as flavor enhancers, increasing the hedonic value of foods.

Probably NaCl and MSG alter the flavor profile of foods by differential effects on taste and retro-nasally-sampled odors. This appears to increase the palatability of foods, which may have led to the misapprehension that NaCl and MSG potentiate flavor intensity. They may in fact make flavor more palatable or, "enhance" flavor pleasantness. Since our work used only aqueous systems, similar investigations of the effects of NaCl and MSG are needed in real food systems. Potentiating properties NaCl and MSG have may be more apparent in solid foods.

Terminology

It is a widespread belief in the food industry that salt and MSG are not only flavor enhancers (increasing palatability), but are also flavor potentiators (increasing intensity of other flavors). Little published evidence supports this view. The belief may stem from a failure to distinguish the concepts *flavor enhancer* from *flavor potentiator* (Van der Heijden et al., 1983).

Responses to a flavor can be divided into sensory and hedonic components. We suggest that the word "potentiator" be restricted to the sensory component. We define a flavor potentiator as a substance that increases perceived intensity of the flavor of another substance. Consequently, in the simplest case, a flavor potentiator would increase the intensity of a flavor by some mechanism other than simply adding to it. This may be what results from the combination of MSG and 5'-ribonucleotides (Yamaguchi et al., 1971; Rifkin and Bartoshuk, 1980) and some sweeteners (e.g., Van Der Heijden et al., 1983). However it has been reported for no other effect of NaCl or MSG added to any flavor substance, as illustrated in our current experiments.

We reported effectiveness of flavor suppression of MSG and NaCl depended upon the flavor under consideration. For example, MSG at high concentrations suppressed sweetness, bitterness and menthol but not sourness or butteryness. Although this could result in an apparent (relative) potentiation of one flavor component (e.g., an unsuppressed one) compared with another (a suppressed one), this would be due to differential suppression rather than to true potentiation. We propose that a substance giving this effect be termed a *flavor modulator.* Again, with the exception of MSG and 5'-ribonucleotides, we hypothesize that all reported sensory effects of NaCl and MSG have been due to their modulating rather than their potentiating properties.

Finally, we suggest that the use of the term potentiator not be used in reference to hedonic properties. The term "flavor enhancer" should be restricted to hedonic improvement. We define a flavor enhancer as a substance that increases pleasantness of the flavor of another substance. Many foods with added salt or MSG taste better to most people (Yamaguchi, 1987). Whether this is because of effects these substances have in modifying sensory attributes of other flavors and/or effect

of addition of flavors from these substances themselves requires further research.

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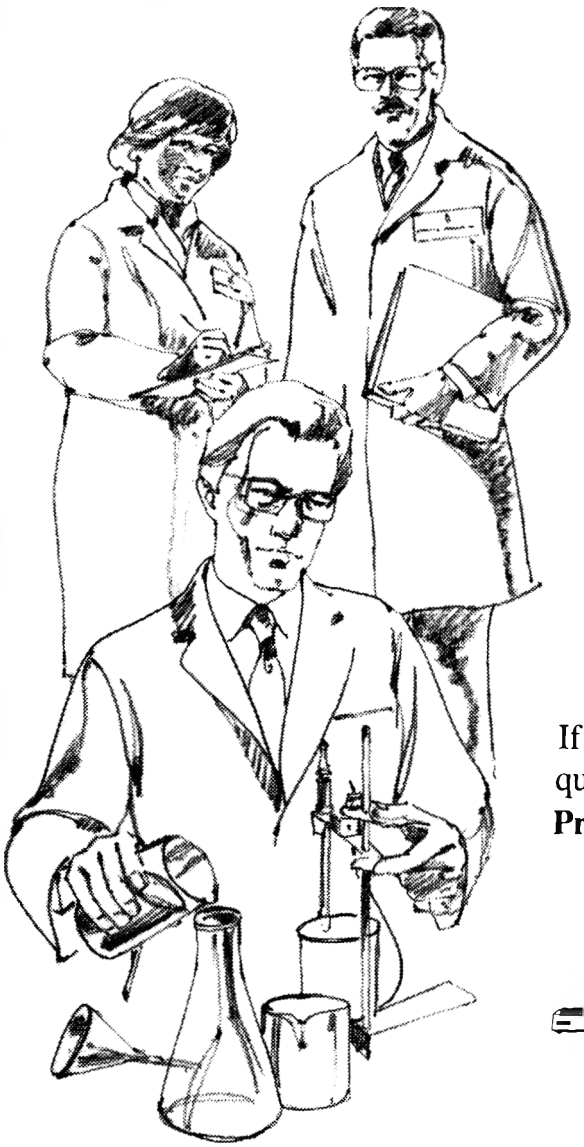

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