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AUTHOR INDEX
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Memo *FROM THE SCIENTIFIC EDITOR*

Quality Improvement of Scientific Papers

PUBLISHED REPORTS are a very important forum for dissemination of scientific knowledge. Even with computer searches and electronic scanners, scientists still face difficult challenges to remain knowledgable about new developments. The volume of papers published is reflected in the large number indexed and abstracted by different services. *Current Contents* (1993a) lists the Tables of Contents of the most important papers in the series of seven general categories. The Agriculture, Biology and Environmental Sciences issue that includes food-related disciplines lists more than 9,600 indices extracted from about 900 journals. The Life Sciences issue which also includes food science-related papers lists more than 262,000 titles from more than 1,250 journals (*Current Contents,* 1993b). Chemical Abstracts Services (1992) provides abstracts and references from 1,000 journals of many scientific disciplines. The International Food Information Service (1992) summarizes reports from over 2,000 sources with a data base of about 415,000 entries and is increasing at about 1,500 references each month. Though these reports are from many disciplines of science and some are duplicated in different abstracts, the number in a given subject area such as food science is large and clearly illustrates how difficult it is to stay abreast of new developments.

AS WITH OTHER scientific journals, we strive to publish only high quality research papers. *Current Contents* uses a Scientific Citation Index (SCI) (*Current Contents,* 1993c) to rank citation rates of each journal in each discipline. According to that index, on average, a 1973 article was cited about 15 times between 1973 and 1988. Today an article in a major subject area may be cited more than 400 times whereas one in a field of lesser activity may be referenced about 100 times. In the SCI survey, JFS was ranked in the top 25% of 66 food science journals. According to a survey of the most frequently cited journals in two volumes of *Chemical Abstracts* (Garfield, 1993) from July 1991 through June 1992, JFS was ranked in the top 15% of 1,000 journals in all disciplines.

WE ARE DOING what we can to make important information on new developments more readily available to scientists. The formula for good scientific papers has several elements. Knowledge generated by research should be original, accurate, and advance food science. Reports should be as brief as possible but complete with precise details and clarity of presentation. Quality, not quantity, should be the goal of investigators. Interpretations of results and conclusions must be supported by adequate strong evidence. The time required to get a paper published is an important concern of scientists, the Journal and the food industry. We will keep this time as short as possible. However, the nature of the research and its significance to food science are key components of the final product. Researchers are principally responsible for the quality of scientific papers and their importance to food science. Originality, design of the study, proper selection of experimental methods, concise interpretations and conclusions are responsibilities of the investigators. Authors can shorten the review process time by checking carefully for format, grammar, clarity and completeness before submitting to the Journal.

RELIABLE REVIEWERS are prerequisites for good scientific papers. They evaluate originality, adequacy of experimental details, accuracy of findings and interpretations, justification of conclusions, importance of the research to food science, and recommend whether publication is warranted. Reviewers' expert comments and critical but constructive suggestions can improve a paper. Good reviewers may be swamped with manuscripts—some may receive as many as four at one time. Occasionally, late reviewers may return an apparent "get it out of the way" type review. Such reviews seem to have been read hurriedly because rather obvious problems have not been indicated. In such cases a third reviewer usually is required, resulting in further delay of the review process. Most reviewers have been authors and can understand concern over delays in publication. Scientific editors contribute to the quality of papers by evaluating the suitability of the study, selecting qualified specialists as reviewers, reconciling differences between authors and reviewers and expediting publication. Through the earnest efforts of researchers, reviewers and scientific editors, we will continue to maintain high standards for acceptance and work toward higher quality scientific papers.

—*Stanley J. Kazeniac,* Associate Editor, *Journal of Food Science*

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Meat Batters Manufactured with Modified Beef Connective Tissue

S.J. EILERT, D.S. BLACKMER, R.W. MANDIGO, and C.R. CALKINS

ABSTRACT

Three fat levels (8%, 16% and 24%) and five modified connective tissue (MCT) levels (0%, 10%, 20%, 30% and 40%) were used in a 3 × 5 complete factorial design to prepare and characterize meat batters. Proximate analysis, batter pH, emulsification temperature increase, raw batter extrusion, batter stability, collagen levels, and processing yield data were collected. Increasing levels of MCT increased batter pH, emulsification temperature increase, batter extrusion values, and collagen levels. Thermal processing yield losses declined with increased MCT level. Addition of MCT may be effective for reducing such losses in low-fat meat batters without major changes in batter stability.

Key words: meat batters, connective-tissue, beef

INTRODUCTION

DURING production of stable, finely comminuted meat products, the maximum amount of high collagen meats should not exceed 15% (Wiley et al., 1979) to 25% (Kramlich, 1971) of formulation weight. Higher levels of collagen (≥ 20% tripe) have correlated with diminished batter stability (Jones, 1982). Meat batters may be stabilized by collagen when the batter is being formed, but protein matrix disruption and fat release from the matrix probably occur upon gelatinization of the collagen (Whiting, 1989). The level of collagen that can be utilized in a processed meat product depends on ionic strength, pH, fat level, comminution method, heat processing conditions, and other factors (Jones, 1984).

The source of collagen, its physical form and the amount of heat stable crosslinks might also influence the amount of collagen that could be incorporated. Satterlee et al. (1973) added hydrolyzed pork and beef skin as a substitute for non-fat dry milk in sausage emulsions. Hydrolyzed pork and beef skins resulted in greater emulsion stability, higher fat and water, and softer texture. As those proteins had been hydrolyzed, any effects due to thermal shrinkage of intact collagen would not be noted. Rao and Henrickson (1983) added 20% food grade hide collagen to 10%, 20% and 30% fat bologna. That source of collagen has a low solubility fraction, which may partially explain why emulsion stability, water activity or expressible juice were not altered in those formulations. Sadowska et al. (1980) found the effects of pig skins on rheology of meat batters was dependent upon level of addition and degree of precooking.

Mechanical desinewing of beef provides another source of collagen, connective tissue. That process is being used on a large scale in major beef packing plants. Currently, that high protein (>20%) material is being rendered at a low transfer cost. In an effort to provide connective tissue a higher value, Quint (1987) added flaked, frozen chuck connective tissue to 15% and 30% fat frankfurters. Such tissue appeared to have less influence on properties of low-fat batters, as no decrease in thermal stability was noted at the lower fat level. Questions remained regarding how much connective tissue could be employed in such low-fat products. The objective of our study was to characterize the properties of meat batters prepared at various fat and modified beef connective tissue levels.

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MATERIALS & METHODS

Connective tissue modification

Beef connective tissue, primarily from hind-shank meat was obtained from a commercial desinewing operation. Fresh connective tissue was shipped to the Loeffel Meat Laboratory, University of Nebraska-Lincoln, where bones and small bone-like material were hand sorted from the connective tissue (amount of removed material, <1%). The connective tissue consisted largely of epimysium, perimysium and tendons. Connective tissue was placed on metal trays in blocks of 10 cm × 30 cm × 30 cm, covered and blast frozen at −26°C overnight. Frozen connective tissue was tempered at 10°C for 3 hr, and ground with a double plate grinder (Wolfking Winkelwolf, Wolfking Danmark, Inc., Blacklick, OH) using a kidney-shaped and a 1.27 cm plate. It was important to grind the connective tissue semi-frozen (−2 to −4°C), as completely thawed connective tissue did not grind cleanly. The semi-frozen, ground connective tissue was placed on metal trays and refrozen overnight at −32°C. Ground, frozen connective tissue was manually broken apart and flaked with a 1.5 mm head on a Model 3600 Comitrol (Urschel Laboratories, Valparaiso, IN). The flaked connective tissue was immediately bagged and placed in a −32°C freezer. The modified beef connective tissue (MCT) was free-flowing and easy to handle during batter preparation.

Product manufacture

Lean beef trimmings were obtained from an "A" maturity steer slaughtered at the Loeffel Meat Laboratory. Pork picnic cushions (IMPS No. 405B) were obtained commercially. These raw materials were stored frozen, tempered, trimmed of some excess fat and ground semi-frozen on a double plate grinder using a kidney-shaped and a 1.27 cm plate. Frozen sow bellies (sows slaughtered at Loeffel Meat Laboratory) were tempered and ground through the same plates in semi-frozen state (−2 to −4°C). Raw materials were held at −3°C for 72 hrs to obtain proximate analysis (AOAC, 1980; Table 1) for formulation. A Least Cost Formulator (Least Cost Formulations, LTD, Virginia Beach, VA) was used to formulate three fat levels (8%, 16% and 24%, finished weight basis) with five MCT levels (0%, 10%, 20%, 30% and 40%, meat block (MB) basis) at each fat level.

Meat ingredients (Table 2), water (4.5 kg/11 kg MB), salt (2.0% MB), seasonings (0.5% MB), dextrose (0.35% MB), cure (156 ppm MB; 6.25% nitrite) and sodium erythorbate (550 ppm MB) were mixed 5 min and passed twice through an emulsifier (Hobart Model MCV 12, Hobart Inc., Troy, OH). The batter was vacuum stuffed (Vemag Model 1000DC Vacuum Stuffer, Robert Reiser and Co., Canton, MA) into 24 mm cellulose casings. Frankfurters were thermally processed to internal temperature 66°C in an Alkar single truck smokehouse (Alkar, Lodi, WI). After 24 hr chilling, franks were peeled (Apollo peeler, Ranger Tool Co., Bartlett, TN) and vacuum packaged (−1.0 Bar; Dixie-Union, Model DV 2000, Robert Reiser and Co., Canton,

Table 1—Proximate analysis (in percent) of raw materials

	Moisture	Protein	Fat
Lean beef	71.41	20.77	7.13
Pork picnic cushions	72.66	19.53	7.88
Pork bellies	40.68	11.00	47.56
Modified beef Connective tissue	53.77	26.66	20.33

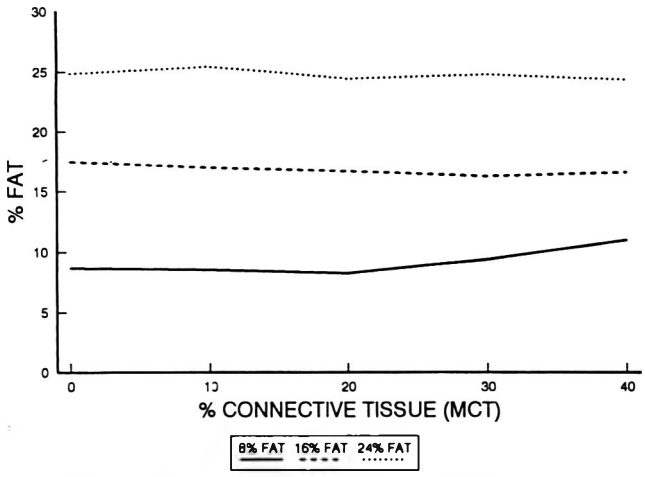

Fig. 1—Fat × modified connective tissue interaction on cooked product fat content (P<0.01, S.E. = 0.46, L.S.D. = 1.32).

MA). Frankfurters were stored under vacuum and fluorescent lighting (194 lux) at 4°C for finished product evaluation, reported elsewhere.

Proximate analysis and pH

Raw batter (50g) and two randomly selected, peeled, thermally processed frankfurters were collected for moisture, fat, protein and ash determinations (AOAC, 1980). On another sample (50g), pH was determined with a hand-held meter (Omega Model PHH-50, Omega Engineering Inc., Stamford, CT) and a spear-tip electrode (Orion Model 8163BN, Orion Research Inc., Boston, MA).

Emulsification temperature rise

The temperature of the batter just before first emulsification and after the second emulsification was determined with an Omega 450 ATT Type T thermocouple (Omega Engineering Inc., Stamford, CT). Differences in the two temperatures were recorded as emulsification temperature increase.

Batter extrusion tests

To characterize rheological properties of the raw batters, 3 different extrusion tests were conducted on an Instron Model 1123 (Instron Corp., Canton, MA). Duplicate, 300 g samples were weighed for each plate, wire bar (Voisey and Larmond, 1971) and back extrusion test (Bourne and Moyer, 1968). Plate and wire bar extrusions were performed using a food extrusion cell (Canners Machinery Ltd, Simco, Ontario, Canada). The extrusion cell was 12.5 cm high, with internal area of 50 cm^2. The wire bar plate contained eight wire bars spaced 0.5 cm apart. The plate extrusion test utilized a plate with 94 0.5 cm holes. Maximum force to extrude the sample in the three tests (kg) and total energy to extrude the sample (area under curve, AUC, in cm^2) was recorded to determine whether connective tissue was becoming integrated into the batter or disrupting batter integrity.

Back extrusion was performed with a cylinder, 10.1 cm internal diameter and 10.0 cm high. The plunger was 9.3 cm diam, providing an annulus of 4.0 mm for the sample to be extruded around the diameter of the cylinder. The sample was packed into cells to eliminate as many air pockets as possible, without overworking the sample. All three tests utilized a 500 kg load cell, a cross-head speed of 100 mm/min and a chart speed of 200 mm/min. The back extrusion test was conducted with a full scale load of 0-50 kg. The plate and wire bar extrusion tests used a full scale load of 0-20 kg.

Table 2—Meat ingredients in each formulation (kg)

Treatment	Lean beef	Pork picnic cushions	Pork bellies	MCT[a]
8% Fat				
—0% MCT	5.13	5.13	0.67	0.00
—10% MCT	4.76	4.76	0.32	1.09
—20% MCT	4.38	4.38	0.00	2.19
—30% MCT	3.83	3.83	0.00	3.28
—40% MCT	3.28	3.28	0.00	4.38
16% Fat				
—0% MCT	3.78	3.78	3.38	0.00
—10% MCT	3.41	3.41	3.03	1.09
—20% MCT	3.30	3.03	2.69	2.19
—30% MCT	2.66	2.66	2.34	3.28
—40% MCT	2.29	2.29	1.98	4.38
24% Fat				
—0% MCT	2.42	2.42	6.12	0.00
—10% MCT	2.05	2.05	5.76	1.09
—20% MCT	1.67	1.67	5.40	2.19
—30% MCT	1.30	1.30	5.03	3.28
—40% MCT	0.93	0.93	4.72	4.38

[a] Mocified beef connective tissue.

Batter stability tests

Triplicate, 34g samples were stuffed into 50 cc polycarbonate tubes for an emulsion stability test following the procedure of Townsend et al. (1968). Three additional tubes/batch were stuffed for smokehouse batter stability test. These tubes were thermally processed in a smokehouse along with frankfurters (rather than in a water bath). Losses of total fluids and solids, gel water, fat and proteinaceous solids were read from graduated centrifuge tubes and expressed as mL/100 g for both tests.

Processing yields

Smokehouse yield was determined by dividing the weight of thermally processed frankfurters by the weight of stuffed, raw batter. Total processing yield was the weight of frankfurters after thermal processing and 24 hr chill (before peeling) divided by the weight of stuffed, raw batter. Chill loss was the difference between thermally processed weight and 24 hr chilled frankfurter weight divided by raw weight. All losses and yields were expressed as percentage.

Table 3—Fat and modified connective tissue main effects on raw batter proximate analysis, pH, temperature rise during emulsification (Temprise) and final emulsion temperature (Finaltemp); final product moisture, protein and ash

Variable	Fat level					Modified connective tissue level						
	8%	16%	24%	L.S.D.[e]	S.E.[f]	0%	10%	20%	30%	40%	L.S.D.	S.E.
Raw batter												
—Lipid[a],%	7.59	14.45	20.42	1.02	0.36	13.57	13.74	13.39	15.13	14.94	1.32	0.46
—Moisture[b], %	75.95	70.34	65.52	0.83	0.29	71.80	71.69	71.01	69.30	69.22	1.07	0.38
—Protein[b], %	15.33	13.91	12.59	0.37	0.13	12.72	13.30	14.24	14.40	15.06	0.48	0.17
—Ash[c], %	1.86	1.81	1.93	0.23	0.08	2.07	1.76	1.97	1.88	1.66	0.30	0.10
—pH[d]	5.90	5.89	5.92	0.03	0.01	5.82	5.86	5.90	5.94	5.99	0.03	0.01
—Temprise[b], °C	3.15	5.30	8.05	0.76	0.27	2.42	3.25	4.41	7.42	10.00	0.98	0.34
—Finaltemp[b], °C	1.00	3.15	5.95	0.72	0.25	0.42	1.25	2.33	5.08	7.75	0.92	0.32
Final product												
—Moisture[a], %	70.86	65.22	59.67	0.84	0.29	65.97	65.12	65.90	65.05	64.21	1.08	0.38
—Protein[b], %	17.84	15.81	13.66	0.36	0.13	14.64	15.58	15.67	16.26	16.68	0.47	0.16
—Ash[c], %	1.94	2.04	1.80	0.28	0.10	1.77	1.89	1.95	2.04	1.98	0.36	0.12

[a] Fat main effect, P <0.01; Connective tissue main effect, P <0.05.
[b] Fat main effect, P <0.01; Connective tissue main effect, P <0.01.
[c] No fat main effect, P >0.05; No connective tissue main effect, P >0.05.
[d] Fat main effect, P <0.05; Connective tissue main effect, P <0.01.
[e] Least significant difference.
[f] Standard error.

Table 4—Fat and modified connective tissue main effects on Instron plate extrusion (Plext) peak force (Peak) and area under the curve (Auc) values, wire bar extrusion (Wbext) Peak and Auc, and back extrusion (Baext) Auc

	Fat level					Modified connective tissue level						
Variable	8%	16%	24%	L.S.D.[d]	S.E.[e]	0%	10%	20%	30%	40%	L.S.D.	S.E.
Plext												
—Peak[a], kg	8.84	9.08	8.85	0.56	0.20	4.41	6.81	9.03	11.18	13.17	0.72	0.25
—AUC[a], cm²	125.85	131.09	123.53	6.69	2.34	65.98	99.96	129.55	157.73	180.92	8.64	3.03
Wbext												
—Peak[b], kg	3.33	3.50	3.26	0.19	0.07	1.94	2.70	3.31	4.19	4.67	0.25	0.09
—Auc[c], cm²	52.18	53.89	49.38	2.44	0.85	31.15	42.79	51.72	63.35	70.08	3.15	1.10
Baext												
—Auc[c], cm²	52.51	50.11	44.93	1.88	0.66	36.11	44.60	50.78	55.52	58.89	2.43	0.85

[a] No fat main effect, P>0.05; Connective tissue main effect, P<0.01.
[b] Fat main effect, P<0.05; Connective tissue main effect, P<0.01.
[c] Fat main effect, P<0.01; Connective tissue main effect, P<0.01.
[d] Least significant difference.
[e] Standard error.

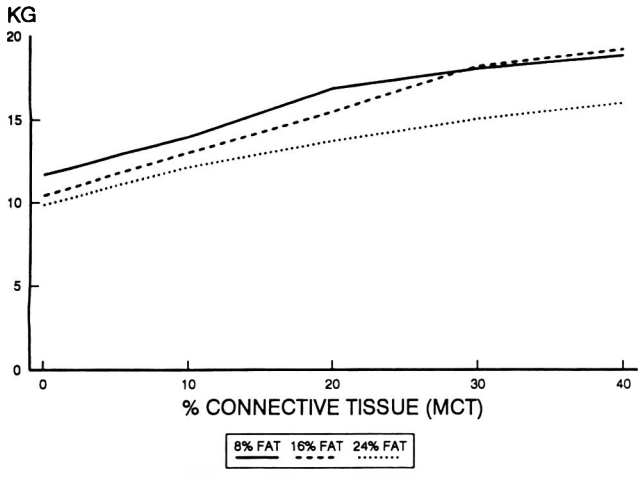

Fig. 2—Fat × modified connective tissue interaction on Instron back extrusion peak force (P<0.05, S.E. = 0.40, L.S.D. = 1.15).

Collagen determination

Raw batter samples were collected for soluble, insoluble and total collagen determination. Soluble and insoluble fractions were separated by the procedure of Hill (1966); spectrophotometric determination of hydroxyproline in the soluble and insoluble fractions was performed (Bergmann and Loxley, 1963). Conversion factors used were soluble (7.52) and insoluble collagen (7.25) (Cross et al., 1973). Collagen values were expressed in mg collagen/g of total sample.

Experimental design and data analysis

Treatments were assigned to a 3 (fat levels, 8%, 16% and 24%, finished weight basis) × 5 (MCT levels, 0%, 10%, 20%, 30% and 40%, meat block basis) complete factorial design (Steel and Torrie, 1980). The experiment was designed as a randomized complete block, replicated four times, with day of production as blocking criterion. Data were analyzed using SAS (SAS Institute Inc., 1985), and means separated using Least Significant Differences (LSD) (Steel and Torrie, 1980). The LSD values were generated at P≤0.05. Main effects and interactions were reported significant at P<0.05 and P<0.01.

RESULTS & DISCUSSION

Proximate analysis

Moisture and protein in raw batter declined with increased fat in the raw batter (P<0.01; Table 3). The lipid content of the raw batter increased at the 30% and 40% MCT levels (P<0.05), with a corresponding decrease in moisture (P<0.01). Protein content of raw batter increased at higher levels of MCT (P<0.01). Similar effects for fat and MCT level were noted on moisture and protein in the final product (Table 3). A fat × MCT interaction (P<0.01) affected the fat content of the final product (Fig. 1). No MCT effect was noted in the 16% and 24% fat treatments, whereas the fat content (of 8% formulations) increased slightly at the 30% and 40% MCT levels. The MCT was 20.3% fat, 53.8% moisture and 26.7%, presenting difficulty in formulating 8% fat frankfurters with 30% and 40% MCT. Overall mean fat contents of final products averaged a little higher than respective target levels. Actual values were 9.15%, 16.78% and 24.75%. Neither fat nor MCT had an effect on raw batter or final product ash values (P>0.05, Table 3). Ash did not change with an increase in MCT, indicating adequate removal of bone.

Raw batter pH

Raw batter pH increased from 5.82 in the 0% formulation to 5.99 in the 40% MCT formulation (Table 3). The pH of the MCT was 6.3, higher than most raw materials. Only a small fraction of myofibrillar protein remained on this connective tissue. One possible explanation for the MCT effect on pH was that myofibrillar protein probably underwent more post-mortem glycolysis than stromal tissue. Fat had a significant effect on pH (P<0.05), but the actual range in pH values was 5.90 to 5.92, of little practical importance.

Emulsification temperature rise

Fat (P<0.01) and MCT (P<0.01) affected emulsification temperature increase. The temperature rise increased from 3.15 to 8.05°C and from 2.42 to 10.00°C for increasing fat and MCT levels, respectively (Table 3). With a lower moisture content, batters with more fat were expected to have a greater temperature rise. Batters with MCT had less moisture and more protein, contributing to the greater temperature rise. Additionally, the resilient nature of the connective tissue contributed to greater resistance through the emulsifier, compensating for the greater amount of frozen material added to these products.

The temperature of the batters from the mixer varied less than 1°C between different fat and MCT levels (Range = −2.00°C to −2.33°C; data not presented). Final comminution temperatures increased with increased fat and connective tissue (P<0.01; Table 3). Ambrosiadis and Wirth (1984) indicated that in production of frankfurter-type sausages with high collagen meats, the final comminution temperature should not exceed 10–12°C when phosphates were not used. All final batter temperatures in our study fell below that range. If the connective tissue had been added in the thawed, unmodified form, the temperature rise would probably have been much higher, causing final comminution temperature to exceed those values.

Ladwig et al. (1989a) found that batters with a high proportion of epimysium and perimysium required less time to reach target chop temperatures than did control batters. That was due to the fibrous nature of the collagen causing frictional heat buildup. In a related study, Ladwig et al. (1989b) stated that the physical action/duration may be more important to batter characteristics than final temperature. In our study, we did not equilibrate batch temperatures by additional chopping or passes through the emul-

Table 5—Fat and modified connective tissue main effects on emulsion stability (ES) gel water (Gel) and proteinaceous solids loss (Prosol); smokehouse stability (SS) total fluid and solid loss (Total), Gel and Prosol losses; smokehouse yield (Smoyld) and 24 hr chill loss (chill)

	Fat level					Modified connective tissue level						
Variable	8%	16%	24%	L.S.D.[e]	S.E.[f]	0%	10%	20%	30%	40%	L.S.D.	S.E.
ES												
—Gel[a], mL/100g	6.88	7.85	9.73	0.91	0.32	6.33	7.59	9.00	9.37	8.47	1.17	0.41
—Prosol[a], mL/100g	0.09	0.12	0.18	0.04	0.01	0.03	0.12	0.16	0.17	0.16	0.05	0.02
SS												
—Total[a], mL/100g	13.92	15.17	16.69	1.20	0.42	12.26	16.05	16.71	16.21	15.08	1.55	0.54
—Gel[b], mL/100g	13.68	14.53	15.22	1.08	0.38	12.06	15.26	15.69	15.11	14.24	1.39	0.49
—Prosol[c], mL/100g	0.19	0.24	0.23	0.08	0.03	0.07	0.23	0.22	0.26	0.31	0.10	0.04
Smoyld[a], (%)	86.31	86.84	88.86	1.27	0.45	85.88	85.21	88.89	87.74	88.96	1.64	0.58
Chill[d], (%)	1.77	1.81	1.52	0.28	0.10	1.61	1.81	1.54	1.74	1.79	0.36	0.12

[a] Fat main effect, P<0.01; Connective tissue main effect, P<0.01.
[b] Fat main effect, P<0.05; Connective tissue main effect, P<0.01.
[c] No fat main effect, P>0.05; Connective tissue main effect, P<0.01.
[d] No fat main effect, P>0.05; No connective tissue main effect, P>0.05.
[e] Least significant difference.
[f] Standard error.

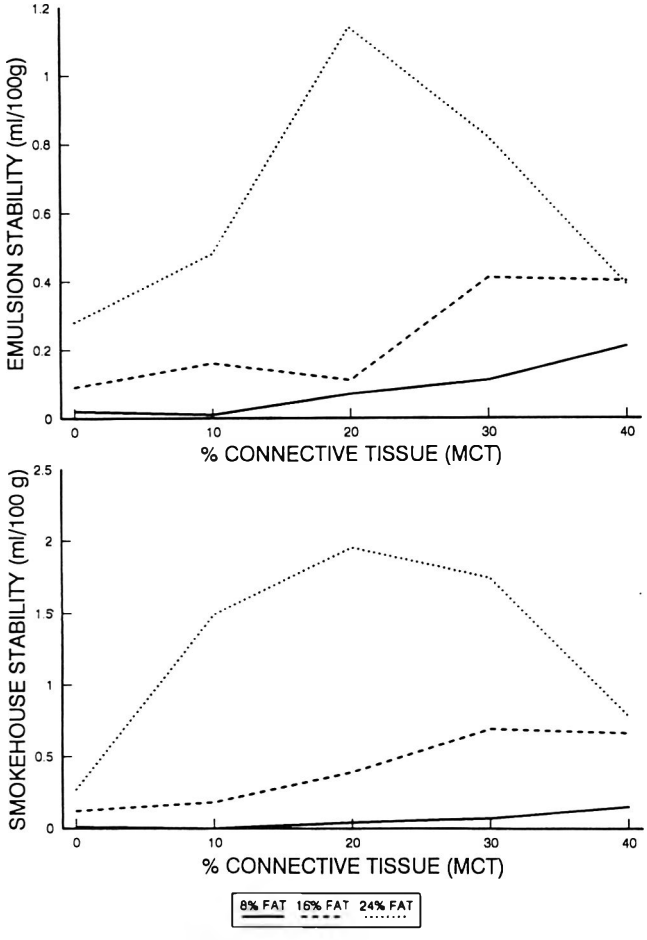

Fig. 3—Fat × modified connective tissue interaction on emulsion stability fat loss (P<0.05, S.E.=0.14, L.S.D.=0.39) and smokehouse batter stability fat loss (P<0.01, S.E.=0.23, L.S.D.=0.67).

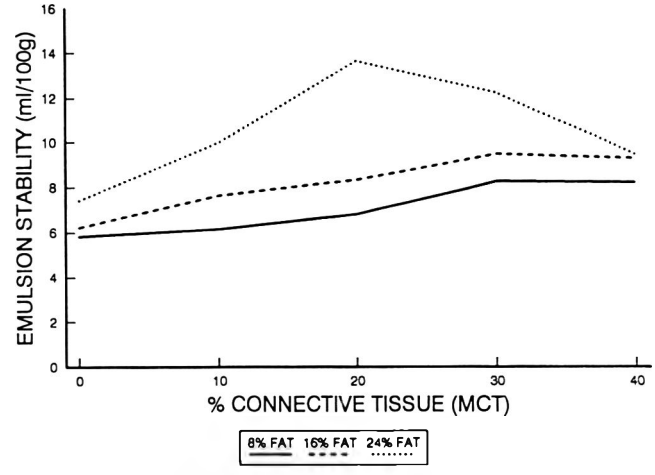

Fig. 4—Fat × modified connective tissue interaction on emulsion stability total fluid and solid loss (P<0.05, S.E.=0.78, L.S.D.=2.24).

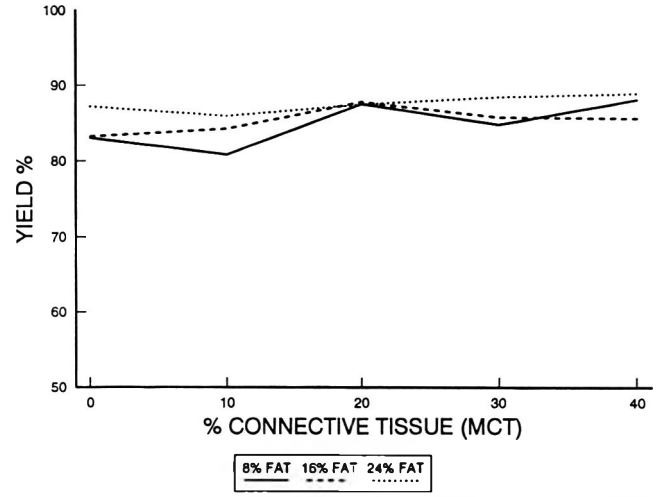

Fig. 5—Fat × modified connective tissue interaction on yield after thermal processing and 24 hr chill (P<0.05, S.E.=0.95, L.S.D.=2.70).

sifier. Batter temperatures were treatment dependent. Any attempt to equilibrate temperatures may have masked or confounded treatment effects from other variables.

Batter extrusion tests

Main effects of fat and MCT on batter extrusion values were compared (Table 4). Fat had no effect (P>0.05) on plate extrusion peak or AUC. For wire bar extrusion peak, AUC and back extrusion AUC, the highest values were noted at 16% fat level, with significantly lower values at 24%. No attempt was made to equilibrate temperatures of these batters before extrusion, as the temperatures were dependent upon treatment. The effects of fat on rheology of meat batters is related to the degree of melting of the fat (Acton et al., 1983). The higher fat batters were higher in temperature after emulsification, which would result in fat being in a more fluid state, decreasing the resistance to flow. Though higher batter temperatures were also noted with greater

Table 6—Fat and modified connective tissue main effects on raw batter collagen values

Variable	Fat level					Modified connective tissue level						
	8%	16%	24%	L.S.D.[c]	S.E.[d]	0%	10%	20%	30%	40%	L.S.D.	S.E.
Raw batter												
—Soluble[a], mg/g	6.79	6.40	5.88	0.30	0.11	2.01	4.12	6.31	8.58	10.77	0.39	0.14
—Insoluble[b], mg/g	25.21	25.37	25.66	0.86	0.30	7.43	16.22	25.46	34.89	43.07	1.11	0.39
—Total[b], mg/g	32.00	31.77	31.55	1.09	0.38	9.44	20.34	31.77	43.47	53.84	1.40	0.49

[a] Fat main effect, $P<0.01$; Connective tissue main effect, $P<0.01$.
[b] No fat main effect, $P>0.05$; Connective tissue main effect, $P<0.0^{*}$.
[c] Least significant difference.
[d] Standard error.

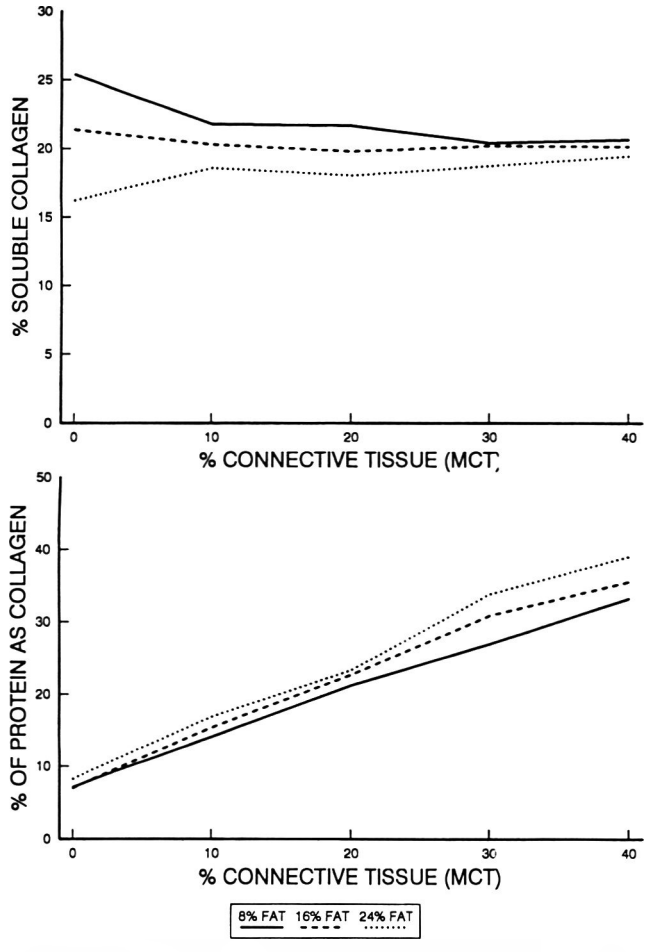

Fig. 6—Fat × modified connective tissue interaction on percentage of total collagen that was soluble collagen ($P<0.01$, S.E. = 0.54, L.S.D. = 1.53) and percentage of total protein that was collagen ($P<0.01$, S.E. = 0.70, L.S.D. = 2.01).

amounts of MCT, all parameters (Table 4) showed sharp increases with an increase in MCT ($P<0.01$). The plate extrusion AUC almost tripled from 0% MCT compared to 40% MCT. Sadowska et al. (1980) reported a decrease in viscosity of meat homogenates when raw pig skin was added. Jones et al. (1982) reported the extrusion yield force of control bologna batters was higher than those containing 0–40% tripe. These experiments with other sources of collagen were contrary to trends noted in our study with MCT. Likely, MCT was being incorporated into the protein matrix of the batter rather than disrupting it, causing an increase in resistance to flow. Scanning electron micrographs of the batters after thermal processing support this hypothesis (Eilert et al., 1992), as frankfurters with higher amounts of MCT showed a more dense protein matrix.

An interaction between fat and MCT affected back extrusion peak force (Fig. 2). Possibly, fat melting in the low fat, high MCT formulations caused extrusion values to approach those of higher fat, high MCT formulations. In general, higher extrusion values were noted with higher levels of MCT and lower fat levels.

Emulsion and smokehouse batter stability

Fat and MCT percentage effects were compared on emulsion add smokehouse batter stability parameters (Table 5). Higher gel-water losses were noted in both tests with increased fat percentage. In addition, emulsion stability proteinaceous solids (PS) and smokehouse batter stability total losses were higher with increased fat ($P<0.01$). Fat level did not affect smokehouse batter stability PS loss ($P>0.05$) but such losses increased with increased MCT. However, values of such losses were very small (<0.4 mL/100g). Emulsion stability gel-water, smokehouse batter stability total and gel losses peaked at 30%, 20% and 20% MCT, respectively. Significant fat × MCT interactions affected emulsion and smokehouse batter stability fat loss (Fig. 3) and emulsion stability total loss (Fig. 4). Loss volumes increased with increased MCT at 8% and 16% fat, although actual increases were small. At 24% fat, losses peaked at 20% MCT.

The general decline in stability due to fat and MCT was expected. Quint (1987) added modified beef chuck connective tissue to frankfurter batters of 15% and 30% and found similar fat and connective tissue interactions. He determined that modified chuck connective tissue had little impact on thermal stability of low-fat frankfurters. Our research confirmed those findings. Apparently, MCT stabilized meat batters when a large proportion of the protein present was collagen. Variations in batter temperature with MCT level may have confounded the data. Perhaps the slightly higher temperatures at higher levels of MCT helped create a collagen:myofibrillar protein matrix that bound water and fat better. This effect may not have been noted if the final emulsification temperatures had been higher (Table 3), which would have caused increased fat coalescence before thermal processing. In low-fat batters, the effect of MCT on stability parameters was minimal.

Processing yields

Main effects of smokehouse yield and chill loss were compared (Table 5). The smokehouse yield at 24% fat was higher ($P<0.01$) than at 16% and 8% fat. Additionally, smokehouse yields of formulations with MCT levels ≥20% were 2–3% higher than those of 0% and 10% MCT. Neither fat nor MCT had effects ($P>0.05$) on chill loss. A fat × MCT interaction ($P<0.05$, Fig. 5) affected total processing which, in general, were higher at higher fat and MCT levels. After thermal processing, frankfurters showed no signs of distortion, fat caps or gelling.

In a detailed study examining factors affecting weight loss during thermal processing of frankfurters, Mittal and Blaisdell (1983) determined that moisture loss was inversely proportional to fat:protein ratio. Fat is hydrophobic, thus moisture diffusion out of a product was less at higher fat levels. This probably explains our fat effect on smokehouse yields. The fat:protein ratio was relatively constant among levels of MCT. When collagen was heated, the fibers shrank and swelled. Ambrosiadis and Wirth (1984) stated that water absorption was associated with this swelling. Jobling (1984) listed several benefits of bone collagen in meat products, including water bind-

ing. Sadowska et al. (1980) reported that the forced drip of meat homogenates decreased in high collagen meats. Bovine hide collagen had enhanced hydration properties upon heating (Ranganayaki et al., 1982). The ability of collagen to bind water during heating may have led to enhanced thermal processing yields with addition of MCT.

Soluble, insoluble and total collagen values

Soluble collagen values decreased ($P < 0.01$) with increased fat level in the raw batter (Table 6). Pork bellies were the primary fat source in these formulations, and contained the least soluble collagen of any of the raw materials (0.54 mg/g pork bellies; 30.40 mg/g MCT; 2.80 mg/g lean beef; 4.08 mg/g lean pork). Fat level did not affect ($P > 0.05$) insoluble or total collagen in the raw batter (Table 6).

Soluble, insoluble and total collagen in the raw batter increased steadily with increased MCT ($P < 0.01$, Table 6). An increase in high collagen meat source does not always cause an increase in soluble collagen values. Jones (1982) added 0–40% tripe to bologna formulations and found soluble collagen levels ranged from 3.2 mg/g in the control to 3.9 mg/g in the 30% tripe formulation. He attributed this to the very low level of soluble collagen in tripe. As noted, the soluble collagen in MCT was much higher than in any other raw materials we used.

The makeup of high collagen meats from a total, soluble and insoluble collagen standpoint differs widely. The soluble, insoluble and total collagen contents of the MCT we used were 30.40, 135.03 and 165.43 mg/g, respectively. Quint (1987) reported a total collagen content of 53.25 mg/g in modified chuck connective tissue. Many characteristics of raw batter and the final product would be affected by true levels of soluble, insoluble and total collagen, not by level of high collagen meat source addition alone.

A fat × MCT interaction affected the percentage of total collagen that was soluble in the raw batter (Fig. 6). With 0% MCT, differences were notable among fat levels, due to the low solubility of pork bellies. When MCT was added, the differences were reduced, and the percentage soluble collagen was almost constant. The fat × MCT interaction (Fig. 6) on total protein that was collagen in the raw batter showed at high levels of MCT, the percentage collagen was greater at the higher fat levels, due to the lower total protein contents. After thermal processing, the collagen values of those batters showed almost identical trends, except that the percent soluble collagen was 3–5% lower (Eilert, 1992). A modified procedure employed (Eilert and Mandigo, 1992) more accurately reflected the level of soluble collagen in the final product, which explained the lower percent soluble collagen values.

Final product analysis

A related study (Eilert et al., 1991) reported the effects of MCT on final product characteristics. In general, products were lighter in color with higher levels of MCT. The addition of MCT resulted in no effect on consumer panel analysis of juiciness of flavor, with limited responses on texture and overall desirability.

CONCLUSIONS

THE ADDITION of MCT to meat batters increased resistance to flow and temperature rise during emulsification. The stability of low fat meat batters with higher levels of MCT decreased slightly, but a tendency for increased stability was noted with higher levels of MCT, especially in high fat formulations. The collagen solubilized and thermal processing yields increased with increased MCT. Adding connective tissue in the frozen, flaked form may help offset some detrimental effects of collagen. Collagen seemed to have useful functional characteristics, such as water and fat binding. Modified beef connective tissue incorporation may be a means to control processing losses and bind water in low-fat products, while adding value to this by-product.

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Iron Distribution in Heated Beef and Chicken Muscles

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ABSTRACT

Distribution of iron in six fractions (water-soluble, water-insoluble, diffusate, hematin, total heme, and ferritin) of beef and chicken muscles heated to 55, 70, 85, and 100°C was determined. Iron content decreased in water-soluble fractions and increased in water-insoluble fractions as temperature increased from 27°C to 100°C. Heme iron decreased more from 55°C to 85°C than from 27°C to 55°C or 85°C to 100°C. The increase in diffusate iron appeared to be less than the decrease in heme iron at each heating temperature. As temperature increased from 27°C to 100°C, hematin iron content increased and extractable ferritin iron content decreased. These findings may help explain rapid development of oxidative rancidity in cooked meat.

Key Words: iron, chicken, beef, heme, lipid oxidation

INTRODUCTION

IRON in muscle is in several different compounds, including low molecular weight molecules, heme compounds such as myoglobin (Mb), hemoglobin (Hb), hematin, and storage complexes of ferritin and hemosiderin (Torrance et al., 1968; Hazell, 1982). Most iron in meat is associated with muscle pigment Mb; a lesser amount is found in blood pigment Hb (Love, 1987). Meat is a rich source of highly available iron, since heme iron has been reported to have a much higher bioavailability than other forms (Monsen et al., 1978; Park et al., 1983). Accurate estimation of total available iron in a meal requires knowledge of the amounts of different forms of iron in food. Iron is the major catalyst for oxidative rancidity in meat (Love, 1987), but the catalytic functions of various forms of iron for lipid oxidation are different. The major catalysts of lipid oxidation have been reported to be heme iron in raw red meats (Younathan and Watts, 1959; Tappel, 1962; Rhee, 1988) and nonheme iron in cooked meats (Sato and Hegarty, 1971; Love and Pearson, 1974; Igene et al., 1979).

Several studies have shown that heat decreased heme iron content and increased nonheme iron content in meat or meat extracts (Igene et al., 1979; Schricker et al., 1982; Chen et al., 1984; Buchowski et al., 1988). The change of distribution of iron in various fractions in meats would affect the bioavailability of iron and the degree and rate of lipid oxidation in cooked meat. However, few studies have reported the effects of heat on amounts of iron in various fractions in meat. Most previous investigations have been based on the assumption that only heme iron is insoluble in acidic conditions. Thus they have focused on determinations of the contents of heme and nonheme iron after solubilization and precipitation with trichloroacetic acid (Igene et al., 1979; Schricker et al., 1982). Further information on the effects of heat on the distribution of different iron fractions in meat could elucidate the functions of various forms of iron in lipid oxidation. This would allow more accurate estimation of the total available iron in meals containing meat. The object of our study was to determine the effect of increased temperatures on the distribution of iron in different fractions of beef and chicken muscles.

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MATERIALS & METHODS

Muscle samples

Longissumus dorsi muscle (≈250g) (pH 5.6) was obtained from each of five beef carcasses of marketweight steers less than 24 mo of age at the Louisiana State University Agricultural Center Meat Laboratory. Beef muscle was hand-trimmed of all visible fat and connective tissue and hand-cut into small pieces of ≈ 1.25 cm^3. Samples of 165g each were selected randomly from the trimmed and cut muscle from each of the five carcasses, combined, mixed, and ground twice through a 4.7-mm stainless steel plate. Ground beef muscle was randomly separated into 10 80-g batches that were vacuum-packaged in vinyl bags and stored at −18°C for <15 days. Thighs from five refrigerated chickens (about 1.8 kg each) were purchased from a local supermarket and prepared in the same way as beef for storage at −18°C. The moisture and crude fat of ground beef and chicken samples were determined by rapid microwave procedures (AVP80 and Automatic Extraction System, CEM Corporation, Matthews, NC).

Method of heating

Frozen vacuum-packaged samples were thawed (27°C) 2 hr. Each 80g thawed sample was mixed with 80 mL deionized water in a Waring Blender at low speed for 30 sec to provide a semi-liquid meat slurry for more uniform heat transfer. Meat slurries were transferred to 250 mL Erlenmeyer flasks with well-fitting stoppers and placed inside 500 mL beakers in a covered steam water bath at 101.6°C (Precision Scientific Co., Chicago, IL). Four flasks with samples were placed in the steam water bath simultaneously. The flasks were randomly assigned for removal from the water bath at internal temperatures of 55, 70, 85, or 100°C. A copper-constantan thermocouple was placed in the center of the sample in each flask through a hole in the stopper. Thermocouples were linked to a Digital Data Acquisition System (MackMac 1240-Touch Display with Thermo VI program, GreenSpring Computers, Menlo Park, CA) to monitor temperature changes. Flasks were cooled to 27°C in ice water after removal from the water bath. The heated samples were then mixed in the blendor at low speed for 30 sec to obtain a homogenous meat slurry. The experiment was replicated twice with two samples from each replication selected at each heating temperature for iron determination. Raw beef or chicken control samples were kept at room temperature (≈27°C).

Total iron

Meat slurries were digested with a mixture of nitric acid and perchloric acid (7:1 by volume) following procedures of Guzmán (1987). Total iron contents were determined on wet ashed samples by atomic absorption spectrophotometry (AAS) (Model 3030B, Perkin Elmer Corp, Norwalk, CT) at a wavelength of 248.3 nm and a slit width of 0.2 nm with an air-acetylene oxidizing flame (Schricker et al., 1982; Guzmán, 1987).

Iron in water-soluble and insoluble fractions

Meat slurry (≈20g) was homogenized in a Waring Blendor at low speed for 2 min and centrifuged at 3000 × *g* for 20 min for beef samples and 40 min for chicken samples. A longer centrifugation time was necessary for chicken extracts to have the same clarity as beef extracts. The top solution was decanted, and residues were re-sus-

Table 1—Iron content[a] in various fractions of heated beef longissimus muscle

Iron type	Internal temperature					
	Control[b]	55°C	70°C	85°C	100°C	S.E.M.[c]
Total Fe	24.56	24.88	24.76	24.55	24.80	0.99
Water soluble Fe	16.93[e]	15.20[e]	8.18[f]	6.61[f]	5.65[f]	0.99
Water insoluble Fe	8.11[e]	9.41[e]	16.45[f]	17.29[f]	18.36[f]	1.29
Diffusate Fe	1.74[e]	1.80[e]	2.16[f]	2.76[g]	2.77[g]	0.09
Fe in hematin	0.24[e]	0.28[ef]	0.28[ef]	0.29[f]	0.35[g]	0.02
Fe in total heme	14.46[e]	14.08[e]	13.08[f]	11.82[g]	11.74[g]	0.14
Fe in ferritin	0.32[e]	0.25[e]	0.13[f]	ND[d]	ND[d]	0.03

[a] (μg Fe/g sample) least-squares means of four measurements.
[b] Controls maintained at 27°C.
[c] SEM = standard error of least-squares means.
[d] ND = not detectable.
[efg] Means in same row bearing common superscript are not different ($p<0.05$).

pended with 10 mL deionized water, centrifuged, and supernatant recovered through three extractions. All supernatants were filtered through No. 3 filter paper (Whatman International Ltd., Maidstone, England), wet-ashed with the mixture of nitric acid and perchloric acid, and the iron content in water-soluble fractions was determined by AAS as described. The meat residues after decanting were also wet-ashed and the iron content of water-insoluble fractions determined by AAS.

Diffusate iron

Aliquots of meat slurry supernatants (10mL), after water solubilization, were dialyzed against 30 mL deionized water at 4°C for 48 hr using 4.5 cm standard dialysis tubing (12,000 daltons molecular weight cutoff; Fisher Scientific, Pittsburgh, PA). After a 48 hr dialysis, the diffusate fraction was wet-ashed with nitric acid and perchloric acid, and the iron content determined by AAS.

Hematin Iron

Meat slurry (≈10g) was homogenized with 15 mL acetone in a Waring Blendor at low speed for 2 min to extract hematin at neutral pH. Homogenates were centrifuged at 3000 × *g* for 20 min in covered centrifuge tubes. The acetone extract was incanted into a 50 mL beaker, acidified to pH <3 with addition of 2.5 mL 1N HCl to intensify the red color of heme, and filtered through No. 3 Whatman filter paper. Hematin content in the acidified acetone extract was determined by measuring the absorbance at 540 nm (Model U-2000 UV/Vis Spectrophotometer, Danbury, CT) (Lewis, 1954; Ladikos and Wedzicha, 1988). Hemin chloride (Sigma chemical Co., St. Louis, MO) was used as a standard. Hematin concentration in samples was calculated from the standard curve of hemin chloride, and the iron content in hematin in the samples was calculated as follows:

$$\text{Iron content } (\mu g/g) = \text{Hematin content } (\mu g/g) \times AW/MW$$

where AW was the atomic weight of iron, and MW was the molecular weight of hemin chloride.

Iron in total heme fraction

A modification of the procedures of Lewis (1954) and Hornsey (1956) was used to determine the total heme content in the meat samples. Meat slurry (≈10g) was acidified to pH <3 with addition of 2.5 mL of 1N HCl before homogenization with 37.5 mL acetone for 2 min. Homogenates were centrifuged at 3000 × *g* for 20 min in covered centrifuge tubes. The acetone extract was then filtered through No. 3 Whatman filter paper, and the absorbance was read spectrophotometrically at 540 nm (Model U-2000 UV/Vis Spectrophotometer). Total heme concentration in samples was calculated from the standard curve of hemin chloride (Sigma). Iron content in the total heme fraction was calculated for hematin iron.

Iron in ferritin fraction

Ferritin in meat slurries was separated from other water-soluble proteins by Ultrogel AcA 34 column (Spectrum, Los Angeles, CA) (Hazell, 1982). Supernatant (10 mL aliquot) was loaded on the column (100 cm and 2.5 cm diam) and diluted with 0.2M potassium orthophosphate buffer (pH 6.8) at 0.42 mL/min (Hazell, 1982). Twelve hr after loading the sample, fractions were collected at 10 min intervals. Ferritin from horse spleen (Sigma) was used as the standard to identify the ferritin peak, since ferritin from beef and chicken was not available commercially. Fractions were pooled and wet-ashed with the mixture of nitric acid and perchloric acid. Iron content in the ferritin fractions was then determined by AAS.

Statistical analysis

The experimental design was a randomized block design (RBD) with replication as block and temperature as treatment for analyses of variance (ANOVA; SAS Institute, Inc., 1985). Least-squares means (LSM) were compared using t-tests when analyses of variance indicated temperature effects at $p \leq 0.05$.

RESULTS & DISCUSSION

THE COMPOSITION of ground beef samples was determined to be 71.23 ± 0.15% moisture and 4.05 ± 0.08% crude fat. The moisture and crude fat contents of ground chicken samples were 75.15 ± 0.23% and 3.26 ± 0.09%. Total iron content and the iron content in dialyzable, hematin, total heme, and ferritin iron fractions at different temperatures were compared in beef longissimus (Table 1) and chicken thigh muscles (Table 2). Total iron in beef longissimus and chicken thigh muscles did not change ($p<0.05$) with increased temperature. Total iron content in beef longissimus muscle was about 10 μg/g greater than in chicken thigh. The concentrations of total iron were higher for beef longissimus and chicken thigh than reported previously. Range of total iron in beef longissimus was reported as 17.5–24.5 μg/g (Schricker et al., 1982; Hazell, 1982; Love, 1988), while that reported in chicken thigh was 8.8–13.2 μg/g (Hazell, 1982; Love, 1988). Incomplete bleeding during slaughtering, lower fat content, different breed types, nutritional status, animal maturity, and sex might contribute to different iron content of the muscle sample.

Water soluble iron in raw meat sample includes diffusate iron, heme iron in hemoproteins and ferritin iron (Torrance et al., 1968; Hazell, 1982). In cooked meat samples, water soluble iron only would include diffusate iron, heme iron in undenatured hemoproteins and undenatured ferritin iron, since denatured hemoproteins and ferritin are not water soluble. All iron which cannot be extracted by water would be considered water insoluble iron. Raw beef contained much higher soluble iron (68.9% total iron) and lower insoluble iron (33% total iron) than did raw chicken, which contained 37.9% soluble iron and 63.4% insoluble iron. The main iron-containing proteins in the water extracts from meat are the hemoproteins, Mb and Hb (Hunt and Hedrick, 1977; Hazell, 1982; Love, 1988). Beef has a higher pigment content than chicken (Fleming et al., 1960; Saffle, 1973) resulting in the higher water-soluble iron in raw beef longissimus muscle we found. The contents of water-soluble iron and insoluble iron of beef and chicken muscle followed similar patterns of change with temperature (Fig. 1). Iron content in the water-soluble fraction decreased, while that of the insoluble fraction increased in beef

Table 2—Iron content[a] in various fractions of heated chicken thigh muscles

Iron type	Internal temperature					
	Control[b]	55°C	70°C	85°C	100°C	S.E.M.[c]
Total Fe	14.54	14.49	14.54	14.61	14.93	0.69
Water soluble Fe	5.51[e]	4.87[e]	2.93[f]	2.83[f]	2.82[f]	0.23
Water insoluble Fe	9.22	10.12	11.40	12.07	12.67	0.98
Diffusate Fe	1.01[e]	1.05[e]	1.22[ef]	1.46[f]	1.46[f]	0.09
Fe in hematin	0.15[e]	0.16[e]	0.18[e]	0.20[e]	0.31[f]	0.02
Fe in total heme	2.44[e]	2.40[e]	2.19[g]	1.98[h]	1.87[h]	0.05
Fe in ferritin	0.50[e]	0.40[f]	0.23[g]	ND[d]	ND[d]	0.02

[a] (μg Fe/g sample) least-squares means of four measurements.
[b] Controls maintained at 27°C.
[c] S.E.M. = standard error of least-squares means.
[d] ND = not detectable.
[e][f][g][h] Means in same row bearing common superscript are not different ($p<0.05$).

muscle with increased temperature, presumably due to denaturation of heme proteins. Compared with the iron in raw beef, that in water-soluble fractions decreased 66.6% and in insoluble fractions increased 126% at 100°C. The largest rate change in iron content in the water-soluble and insoluble fractions of beef and chicken muscles during heating occurred from 55 to 70°C which suggested that most hemoproteins must denature in that range.

The iron content in water-soluble and insoluble fractions of chicken muscle changed less than similar fractions in beef muscle with increased temperature. These results may be because chicken initially had higher insoluble iron than soluble iron, while beef had higher soluble iron than insoluble iron (Tables 1 and 2). Heating chicken muscle did not increase insoluble iron content ($p<0.05$). The precise nature of insoluble iron in muscle is unknown. Some iron may be hemosiderin, an iron storage compound detected in muscle which occurs in large concentrations in the liver (Martinez-Torres et al., 1976; Torrance et al., 1968). Insoluble iron in cooked meats also included the iron in denatured heme proteins.

The amount of diffusate iron for both beef and chicken increased with increased temperature of heating (Tables 1 and 2). The concentration of diffusate iron increased ($p<0.05$) more as temperature increased from 70 to 85°C than from 55 to 70°C. There was no further increase in diffusate iron as temperature further increased to 100°C. Such increased diffusate iron could come from the breakdown of the heme ring and/or release from ferritin.

Heme compounds in meat include hematin, Hb, Mb, cytochrome c, and several heme-containing enzymes such as catalase and peroxidase (Apte and Morrissey, 1987; Stryer, 1988). Hematin is a free heme group with two coordinate water molecules or hydroxyl groups (Ladikos and Wedzicha, 1988) and is insoluble in water but soluble in acetone. Hb and Mb are water-soluble proteins complexed with the heme moiety (Dickerson and Geis, 1983). The heme moiety in hemoproteins is not a fixed prosthetic group, and reversible dissociation into heme and apoproteins may occur (Ladikos and Wedzicha, 1988; Rossi-Fanelli et al., 1958). However, in both Hb and Mb, the affinity of heme for the protein at neutral pH is very high (Fronticelli and Bucci, 1963).

Dissociation of heme increases considerably at acidic pH (Lewis, 1954). If the pH of a hemoprotein solution is <3, the characteristic linkage of the prosthetic group with the protein is ruptured, and the protein is denatured (Lewis, 1954; Ladikos and Wedzicha, 1988). Cleaved heme dissolves in acetone and has a well defined spectrum band at 540 nm, while free protein precipitates (Lewis, 1954). These properties of heme were applied to determine the hematin and total heme content in muscle. Although hemoproteins in meat denatured and became insoluble in water at high temperature, most of the heme moiety remained even at 100°C (Tables 1 and 2). Changes in total heme in beef and chicken were similar. Concentrations of total heme iron at 100°C in beef decreased 18.8% and in chicken

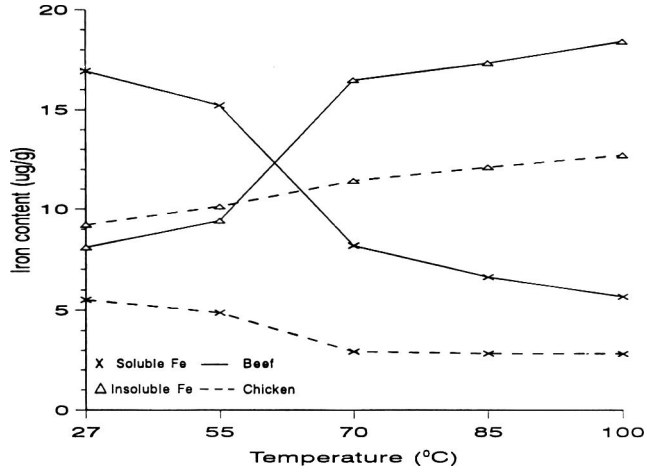

Fig. 1—Effect of internal temperature on iron content in water-soluble and insoluble fractions of beef longissimus and chicken thigh muscle.

23.4%, compared with corresponding total heme iron contents in raw samples. Most of the decrease occurred at 55–85°C. The greater reduction of heme iron at these temperatures was consistent with results of Chen et al. (1984), who showed that the optimal temperature for release of iron from heme was between 63 and 70°C.

Schricker et al. (1982) indicated that the decrease of heme iron in meats by heat was due to oxidative cleavage of the porphyrin ring of heme. In our study, oxidative cleavage of the heme porphyrin ring due to heat should result in an increase in diffusate iron. However, the amount of increased diffusate iron was smaller than the decrease in heme iron at each temperature level in both beef and chicken samples (Tables 1 and 2). Heating might also cause changes in heme other than, or in addition to, the oxidative cleavage of the porphyrin ring to decrease heme iron content. Also, iron released from heme during cooking might be bound ionically to coagulated proteins which would lead to a lesser increase in diffusate iron.

When the temperature reached 85°C, hematin content in beef samples began to show a slight ($p<0.05$) increase compared with controls. Hematin content increased to a greater ($p<0.05$) extent in both beef and chicken samples when the temperature reached 100°C. The initial increase in hematin in beef and chicken with increased temperatures suggested hemoproteins did not readily release their heme moiety during denaturation with most of the heme still associated with globin in cooked meat. However, as temperature increased from 85°C, more bonds between heme and globin could have broken which could explain the greater increase in hematin in beef and chicken heated to 100°C

Decker and Welch (1990) reported that temperature had a

marked effect on the amount of iron released from ferritin by the reducing agents, ascorbate and cysteine. Ascorbate-released iron increased 3.3-fold and cysteine-released iron increased fivefold as temperature increased from 2 to 37°C. In our study, extractable ferritin iron decreased more in the meat samples heated from room temperature to 70°C than in those heated from room temperature to 55°C. Heating to 70°C might partially denature ferritin (Frenkel et al., 1983) in the meat samples which would result in a decrease of extractable ferritin iron. Heating might also stimulate the release of iron bound to ferritin by weakening the bond between them, which would cause a decrease in amount of ferritin iron. Iron in ferritin fractions in samples heated above 85°C could not be determined by the chromatographic technique due to denaturation of ferritin which resulted in insolubility. The denaturation product of ferritin was probably hemosiderin (Linder, 1985). Nonheme iron has been reported to be a major catalyst of lipid oxidation in cooked meat (Sato and Hegarty, 1971; Love and Pearson, 1974; Igene and Pearson, 1979).

CONCLUSIONS

HEAT affected the distribution of iron in different fractions in both beef and chicken muscles. Iron content in water-soluble fractions decreased, while that in water-insoluble fractions increased as temperature increased to 100°C with greatest rate change from 55 to 70°C. Heat decreased the content of heme iron and extractable ferritin iron and increased the amount of diffusate iron. Increased diffusate iron by heating might be partially responsible for the rapid development of oxidative rancidity in cooked meat.

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Dietary Vitamin E Enhances Color and Display Life of Frozen Beef from Holstein Steers

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ABSTRACT

We studied effects of freeze-thaw treatments, storage time, light and film permeability on color of frozen longissimus lumborum (LL) muscle from control and vitamin E supplemented (2100 IU/head/day) Holstein steers. Color changes with time were modeled using an exponential decay equation. Dietary vitamin E supplementation increased color stability of frozen LL samples kept at −20°C. After 3 mo of dark storage, saturation index and a* for control LL wrapped in polyethylene were 11.4 and 8.8; for supplemented meat, same conditions, they were 19.0 and 17.0, respectively. Illumination and vacuum packaging increased color changes. Predisplay dark storage of 30 days reduced discoloration of supplemented LL. Repeated freeze-thaw cycles caused fading of semimembranosus muscle (SM) and the effect was more pronounced in meat from control animals.

Key Words: vitamin E, beef, color, storage stability

INTRODUCTION

THE APPEARANCE of beef, in particular its color, is important for acceptability to consumers. Beef discoloration is mainly due to the oxidation of purple deoxymyoglobin and bright red oxymyoglobin to form brown metmyoglobin. Several researchers reported that lipid and pigment oxidation in fresh beef were interrelated (Greene, 1971; Kanner et al., 1987; Andersen et al., 1990; Andersen and Skibsted, 1991); thus, retarding the breakdown of lipids should result in a similar delay in metmyoglobin accumulation. Attempts have been made to reduce pigment and lipid oxidation in meat by dietary vitamin E supplementation of poultry (Bartov et al., 1983) and pigs (Buckley and Connolly, 1980). Reported studies (Faustman et al., 1989a; b; Mitsumoto et al., 1991; Arnold et al., 1992) concluded that dietary supplementation of Holstein steers with vitamin E produced considerable improvement in lipid and color stability of fresh retail cuts.

Marketing fresh meat in the frozen state has great advantages of cost, convenience and long term stability, but can sometimes result in deterioration in appearance of meat (Renerre, 1990). Freezing and thawing had adverse effects on surface color of fish (Chow et al., 1988), lamb (Moore and Young, 1991) and beef (Lanari et al., 1989). Light is an important factor in frozen meat discoloration during retail display. Lentz (1971) and MacDougall (1982) reported that color of frozen beef remained attractive for 3 mo when stored in the dark but only 3 days under illumination. The rate of fading was affected by illumination level, storage temperature, packaging methods and muscle type (Kropf, 1982; MacDougall, 1982; Bertelsen and Skibsted, 1987; Andersen et al., 1989; Lanari et al., 1989; Lanari and Zaritzky, 1991).

Arnold et al. (1993) reported that discoloration in steaks which had been bloomed for 48 hr prior to freezing was less when they had been derived from vitamin E-supplemented as compared with control steers. Our general objective was to evaluate the validity of the earlier report. Specific goals were to determine the efficacy of vitamin E dietary supplementation in enhancing color stability of frozen beef after several freeze-thaw cycles and during simulated commercial dark storage and illuminated display.

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MATERIALS & METHODS

Samples

Meat samples were obtained from the longissimus lumborum (LL) and semimembranosus (SM) muscles of 6 Holstein steers fed 90% high-moisture corn-plus-supplement 10% corn silage diet formulated to contain 0.1 ppm selenium. Three animals per treatment received 0 (control) or 2100 (supplemented) IU of α-tocopheryl acetate (vitamin E, Hoffmann-La Roche, Inc., Nutley, NJ) per head daily for 126 days prior to slaughter. The right strip loin and top round from each carcass were removed at 24 hr postmortem, vacuum packaged and aged an additional 9 days at 4°C. Samples 5 mm thick and 50 mm diameter were cut, wrapped with fresh meat PVC film (permeability = 15,500–16,200 cm^3/m^2/day at 23°C, Filmco Ind. Inc., Aurora, OH) and exposed to air during 48 hr at 4°C to allow blooming. During this period, samples were illuminated continuously with cool white fluorescent lights (1614 lux).

Packaging

After blooming, samples were skin packaged in polyethylene (thickness = 0.098 mm, permeability = 2271 cm^3/m^2/day at 23°C) or wrapped under vacuum with a film constructed of Nylon/Saran/curpolymer-polyethylene (Trade name: Curlon 863, thickness = 0.082 mm, Oxygen permeability < 15.5 cm^3/m^2/day at 25°C and relative humidity = 0%, Curwood Inc., New London, WI, USA); Packaging was done with a SuperVac Smith machine (Smith Equipment Co., Clifton, NJ, USA) with single chamber and thermal sealing.

Freezing and thawing

Samples wrapped in polyethylene or under vacuum were frozen to −20°C in a freezer at −70°C. They were thawed in still air at 22°C for 30 min. Thermal histories were monitored with thermocouples inserted on the periphery and the center of the samples and connected to a thermometer Bat-4 (Bailey Inst. Inc., Saddle Brook, NJ, USA). Freezing and thawing rates were expressed by local characteristic freezing (t_{7f}) and thawing (t_{7t}) times. t_{7f} was defined as the period in which the temperature at the surface changed from −1°C to −7°C; t_{7t} was interpreted likewise but inverting initial and final temperature levels. In these experiments freezing and thawing rates were t_{7f} = 21 min and t_{7t} = 1 min.

Effect of repeated freezing and thawing on surface color

Samples of SM randomly chosen from superficial and deep locations were wrapped in polyethylene, frozen to −20°C with t_{7f} = 21 min and thawed 30 min at 22°C (t_{7t} = 1 min) 5 times. After each frozen/thawed cycle, surface color was measured.

Storage

Samples of LL packaged in polyethylene or under vacuum were either stored in the dark or displayed at −20°C for 126 days. During display, samples were illuminated continuously with cool white fluorescent lights (1614 lux) in an area enclosed with white cardboard.

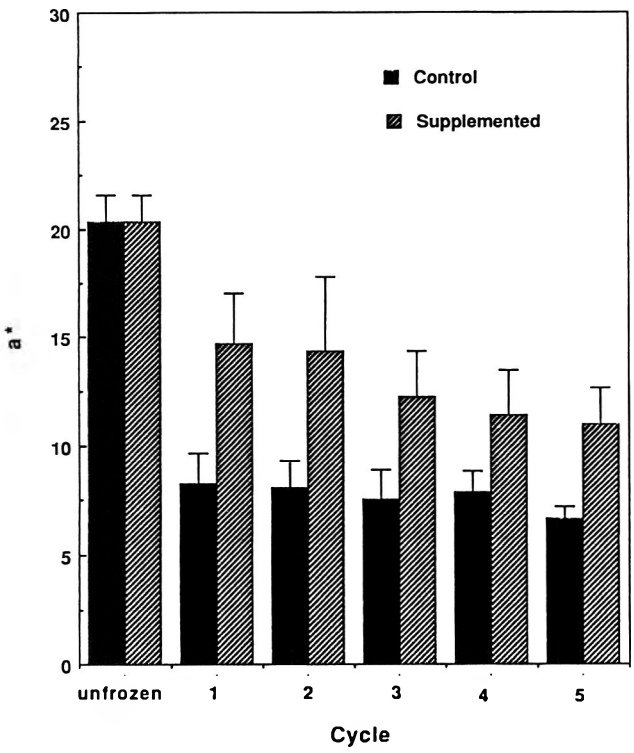

Fig. 1—a levels for control and supplemented SM unfrozen and after each freeze-thaw cycle.*

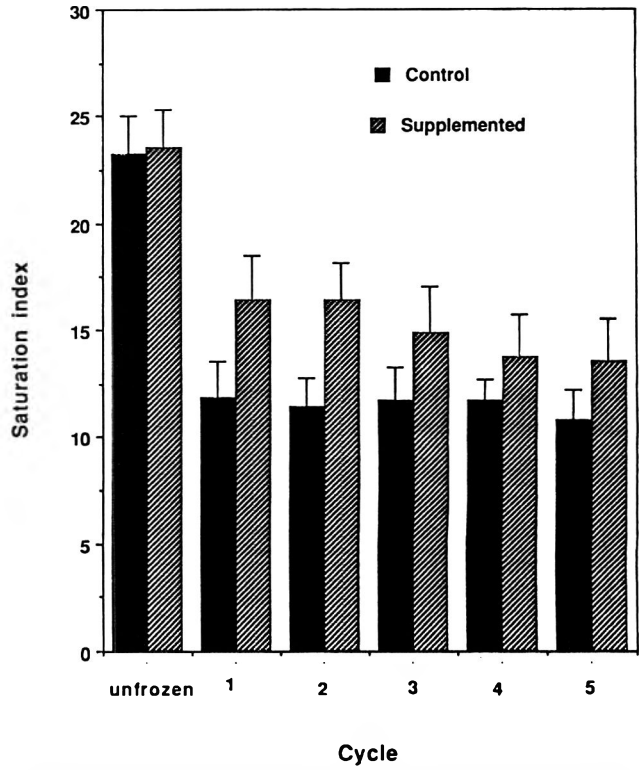

Fig. 2—Saturation index levels for control and supplemented SM unfrozen and after each freeze-thaw cycle.

Meat color was measured at 0, 3, 7, 14, 27, 31, 34, 41, 55, 69, 84 and 126 days after freezing.

To analyze effects of previous dark storage on display life of polyethylene or vacuum-packaged LL, samples were stored in the dark 30 days at −20°C and then displayed 96 days in the illuminated conditions described. Color was measured at days 31, 34, 41, 55, 69, 84 and 126 after freezing.

Analytical techniques

Analysis for vitamin E content in meat was performed in duplicate by the methods of Cort et al. (1983) and Burton et al. (1985) with modification introduced by Arnold et al. (1993). Color measurements were in duplicate samples before and after thawing with a Minolta Chroma Meter CR-200. Meat color was expressed by the L*a*b* system (L* represents luminosity, a* redness and b* yellowness). Saturation Index ((SI), a measure of color intensity or "colorfulness," was computed as (MacDougall, 1982):

$$SI = (a^{*2} + b^{*2})^{0.5}$$

Statistical analysis

Data were analyzed by the General Linear Models (GLM) procedure of SAS Institute, Inc. (1985) as a split-plot design. For comparisons pertaining to measurements over time, E supplementation was considered the whole plot. Treatment combinations of light, time and frozen state were analyzed in the subplot. For the freeze/thaw experiment, the vitamin E supplementation effect was determined in the whole plot and the influence of freeze/thaw cycles in the subplot. Variations of a* and saturation index with time were fitted by a regression model. The parameters were estimated by nonlinear regression analysis using Marquardt and DUD minimization methods (SAS Institute, Inc., 1985).

RESULTS & DISCUSSION

Vitamin E content

α-tocopherol concentrations in μg/g of meat for control and supplemented LL were 0.48 (± 0.19) and 5.25 (± 0.27) μg/g of meat, respectively. In SM, these values were 0.54 (± 0.08) μg/g of meat for control and 6.49 (± 1.00) μg/g of meat in supplemented muscle. Thus, tissue α-tocopherol levels of supplemented animals were more than 10 times greater than control steers.

Effect of repeated freezing and thawing on surface color

For control and supplemented SM, freezing and thawing produced a considerable decrease ($P<0.0001$) in both a* and saturation index levels (Fig. 1,2). Fennema (1973) reported that during thawing, the temperature of meat rose rapidly to near the melting point and remained there throughout the course of thawing, thus providing opportunity for chemical reactions including myoglobin autoxidation (Ledward and Macfarlane, 1971; Zachariah and Satterlee, 1973; Lanari et al., 1989). Supplementation with vitamin E highly influenced ($P<0.0001$) both a* and saturation index. For control SM, these parameters reached their lowest value after being frozen-thawed once (a* = 7.84, saturation index = 11.70). Further freeze/thaw cycles did not affect ($P>0.20$) surface color. For supplemented SM, a* and saturation index remained constant ($P>0.20$) after two freeze-thaw cycles (a* = 14.51, saturation index = 16.41); however, additional freezing and thawing caused discoloration ($P<0.05$). MacDougall et al. (1986) reported that saturation index = 16 could be considered as the limit of acceptability. Thus the color of supplemented samples remained acceptable after two freeze-thaw cycles.

Color variations during storage or display

Vitamin E effects were highly significant. The a* and saturation index levels for supplemented LL samples wrapped in polyethylene were considerably greater ($P<0.01$) than for control samples (Fig. 3,4). Exposure to light and thawing increased the rate of color change ($P<0.01$) in both control and supplemented LL. A predisplay dark storage period of 30 days led to higher values ($P<0.01$) of a* and saturation index during subsequent illuminated display (data not shown). Raw data for

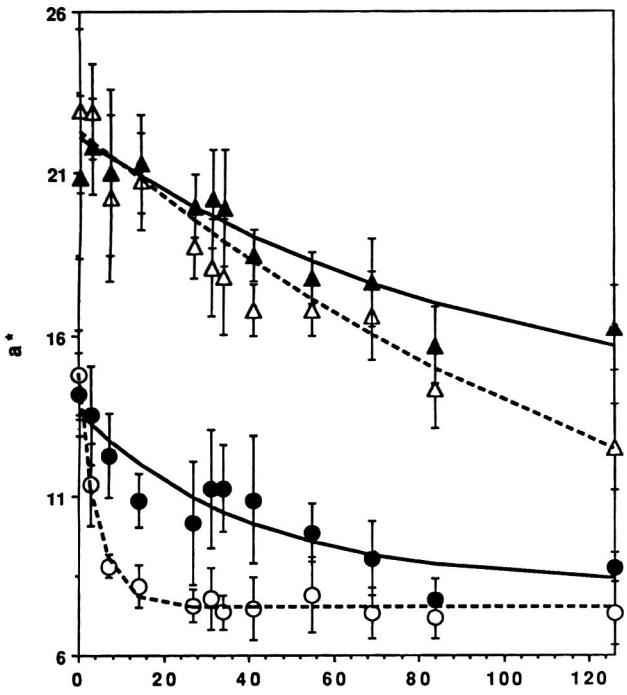

Fig. 3—a values for control (○, ●) and vitamin E-supplemented LL (△, ▲) during frozen dark storage (●, ▲) and illuminated display (○, △). Solid and dashed lines represent values predicted by the kinetic model for the respective treatments.*

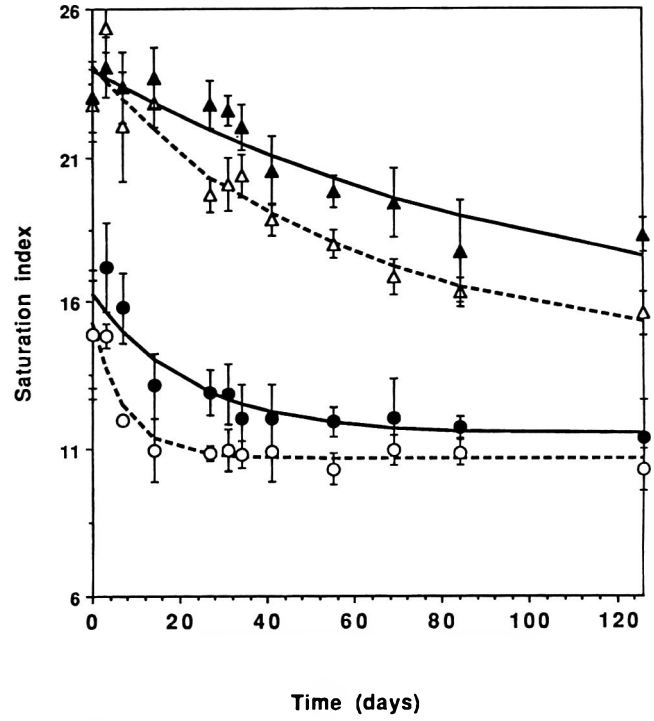

Fig. 4—Saturation index values for control (○, ●) and vitamin E-supplemented LL (△, ▲) during frozen dark storage (●, ▲) and illuminated display (○, △). Solid and dashed lines represent saturation index values predicted by the kinetic model for the respective treatments.

the rest of the experimental conditions analyzed in this study are not shown; however, the same trends were observed.

Improvement of beef color with dietary vitamin E was less noticeable in vacuum packaged LL (data not shown). Differences in saturation index between control and supplemented LL were significant ($P<0.05$) but no effect ($P>0.05$) was observed in a*. Effects of illumination and of a predisplay dark storage period were significant ($P<0.05$) but less than for samples wrapped in polyethylene. No differences were observed between frozen and thawed samples ($P>0.30$) therefore these data were pooled. Supplementation with vitamin E did not modify b* ($P>0.63$); however, illumination, time and thawing produced a decrease ($P<0.05$) in yellowness. L* showed no changes due to supplementation ($P>0.90$), illumination ($P>0.19$), thawing ($P>0.53$) or storage time ($P>0.53$).

Table 1—Rate constant (day^{-1}) and equilibrium values of a and saturation index (SI) for LL stored in the dark or displayed*

		Control				Supplemented			
	pdt[a]	Dark storage		Displayed		Dark storage		Displayed	
	(days)	Y_{eq}[b]	k[c]	Y_{eq}	k	Y_{eq}	k	Y_{eq}	k
a*[d]	0	8.16 (0.89)[e]	0.03 (0.08*10^{-1})	7.49 (0.09*10^{-1})	0.22 (0.02)	13.17 (2.15)	0.01 (0.03*10^{-1})	10.13 (2.01)	0.01 (0.03*10^{-1})
	30	–	–	6.35 (0.23)	0.07 (0.06*10^{-1})	–	–	11.45 (1.38)	0.04*10^{-1} (0.03*10^{-2})
a*[f]	0	8.61 (0.10)	0.14 (0.01)	6.09 (0.31)	0.07 (0.01*10^{-1})	12.53 (1.38)	0.013 (0.04*10^{-1})	7.14 (1.45)	0.01 (0.03*10^{-1})
	30	–	–	7.37 (0.27)	0.11 (0.02)	–	–	7.51 (0.75)	0.01 (0.02*10^{-1})
a*[g]	0	6.59 (0.32)	0.025 (0.06*10^{-1})	6.27 (0.34)	0.03 (0.06*10^{-1})	7.01 (1.18)	0.01 (0.06*10^{-1})	7.47 (0.59)	0.03 (0.06*10^{-1})
SI[d]	0	11.49 (0.56)	0.05 (0.01)	10.67 (0.19)	0.14 (0.03)	14.77 (2.95)	0.09*10^{-1} (0.03*10^{-1})	14.21 (1.74)	0.02 (0.04*10^{-1})
	30	–	–	11.77 (0.17)	0.17 (0.04)	–	–	14.81 (1.91)	0.02 (0.05*10^{-1})
SI[f]	0	11.30 (0.19)	0.18 (0.04)	9.67 (0.25)	0.19 (0.07)	15.41 (1.04)	0.02 (0.06*10^{-1})	12.66 (1.42)	0.03 (0.05*10^{-1})
	30	–	–	9.10 (0.68)	0.19 (0.05)	–	–	12.81 (1.31)	0.02 (0.04*10^{-1})
SI[g]	0	9.41 (0.21)	0.02 (0.05*10^{-1})	9.19 (0.25)	0.03 (0.06*10^{-1})	10.99 (0.58)	0.03 (0.06*10^{-1})	9.97 (0.47)	0.04 (0.06*10^{-1})
	30	–	–	8.83 (0.24)	0.03 (0.06*10^{-1})	–	–	10.95 (0.38)	0.04 (0.05*10^{-1})

[a] pdt = predisplay storage time.
[b] Y_{eq} = equilibrium values of a* and saturation index (SI).
[c] k = rate constant.
[d] Frozen LL packaged in polyethylene.
[e] Standard deviations are given in parenthesis.
[f] Thawed LL packaged in polyethylene.
[g] Vacuum packaged LL, frozen and thawed data were pooled.

Table 2—Shelf-life (days) of frozen LL before and after thawing

		Control		Supplemented	
	pdt[a] (day)	Dark storage	Displayed	Dark storage	Displayed
LL Frozen[b]	0	1	1	214	101
	30	-	1	---	103
LL Thawed[b]	0	1	1	111	38
	30	-	1	---	57
LL Vacuum[c]	0	9	0	16	12
	30	-	0	---	14

[a] Predisplay storage time.
[b] Frozen and thawed LL packaged in polyethylene.
[c] Frozen and thawed data were pooled.

Assuming that the decay of a* or saturation index with time followed first order kinetics, the following expression was derived:

$$dY/dt = -k^*Y \text{ with } Y = Y_{eq} \text{ at } t = t_{eq} \quad (1)$$

Y represents a* or saturation index at time t, Y_{eq} the equilibrium value of Y and k the rate constant (days $^{-1}$). Integration of the differential equation led to:

$$Y = Y_1{}^*e^{(-k^*t)} + Y_{eq} \quad (2)$$

Saturation index and a* data were satisfactorily fitted by equation (2). Rate constants and equilibrium values for both parameters are given (Table 1).

For stored and displayed LL wrapped in polyethylene, supplementation with vitamin E raised Y_{eq} and reduced k. The effect on these parameters in vacuum packaged samples was smaller. In both control and supplemented samples, illumination had a darkening effect on meat color, represented by a decrease in Y_{eq} and an increase in k in displayed LL compared to LL kept in the dark. These results confirmed previous reports (Lentz, 1971, 1979; Kropf, 1982; Andersen et al., 1990). A predisplay time of 30 days produced an increase in color stability.

Considering a saturation index of 16 as the limit of acceptability for frozen beef (MacDougall et al., 1986), we calculated the shelf-life for each experimental condition (Table 2). Meat color of control animals was unacceptable after 1 day of dark storage or illuminated display. However, shelf lives of frozen supplemented LL wrapped in polyethylene and stored in the dark or continuously displayed under illumination were 214 and 101 days, respectively. When saturation index was determined on thawed samples, shelf-life decreased to 111 days for stored samples and 38 days for those kept under illuminated display. Shelf-life of vacuum packaged supplemented LL was considerably shorter. Kropf (1982) and Moore (1990) working with beef and lamb, respectively, reported similar conclusions. MacDougall et al. (1986) exposed LL 24 hr to air and then vacuum-packaged and froze the meat in a moderately high permeability film (Surlyn). They reported shelf-life values of 90 days for dark storage conditions but only 21 days when meat was displayed.

A predisplay storage period of 30 days increased the shelf-life of LL samples compared to those that had been displayed from day 0. This effect was more noticeable (Table 2) when saturation index was determined in polyethylene wrapped samples previously thawed. In that case, shelf-life increased 50%, from 38 to 57 days, whereas for frozen samples packaged in polyethylene or under vacuum, the increment was 2 days. Moore (1990) reported that shelf-life of frozen lamb ribs at −20°C was 51 days and 61 days when meat was previously stored in the dark for 35 days.

Dietary supplementation of Holstein steers with vitamin E delayed surface discoloration after repeated freeze-thaw cycles and during dark storage or illuminated display. This technique when coupled with an effective blooming period, may provide a way of marketing frozen meat in a low-cost packaging film with bright red color.

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Substituting Olive Oil for Pork Backfat Affects Quality of Low-Fat Frankfurters

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ABSTRACT

Low-fat frankfurters (10% fat), formulated for 10%, 12% and 14% protein, were made with olive oil. Compared to control (27.6% all animal fat, 10.9% protein) they had similar flavor, lower ($P<0.05$) TBA values and reduced (44.7–47.6%) caloric content, but had lower ($P<0.05$) processing yield (5.5–6.5%) and overall palatability. Among low-fat treatments, samples with 12% protein had better quality characteristics. The 12% protein frankfurters compared to the control (except for palatability), had similar ($P>0.05$) sensory attributes and higher ($P<0.05$) skin strength and improved texture. The treatment with 10% protein had undesirable color and was very soft. That with 14% protein had the same ($P>0.05$) red color as the control but higher ($P<0.05$) firmness, skin strength and textural traits and lower ($P<0.05$) juiciness.

Key Words: olive oil, frankfurters, fat substitution, low fat, meat products

INTRODUCTION

IN MOST industrialized societies consumers are recommended to reduce energy intake and to reduce fat intake to 30% or less of total caloric intake (AHA, 1986). Manufacturing calorie-reduced foods, which include low-fat meat products, is of both economic and health interest (Wirth, 1988). Frankfurter type sausages produced with pork fat have up to 30% fat. Pork fat has about 40% saturated fatty acids (Briggs and Schweigert, 1990) while cholesterol is the most important sterol present.

Saturated fat is considered a primary cause of hypercholesterolemia (Mattson and Grundy, 1985) and oxidation products of cholesterol also have adverse human health effects (Pearson et al., 1983; Addis, 1986; Maerker, 1987). Although polyunsaturated fatty acids decrease plasma LDL-cholesterol (Mattson and Grundy, 1985), they promote carcinogenesis in experimental animals (Clinton et al., 1984). In contrast to saturated and polyunsaturated fats, diets high in monounsaturated fat have been associated with decreases in coronary heart disease. Prevalence of heart disease was relatively low in areas of the Mediterranean region in which diets high in monounsaturated fat are typically consumed (Keys, 1970; Keys et al., 1986; Aravanis and Dontas, 1978). Thus incorporation of monounsaturated fats in meat products may have a positive effect on consumer health.

St. John et al. (1986) increased the monounsaturated/saturated fatty acid ratio in low-fat frankfurters using the lean and fat from pigs fed elevated levels of canola oil which contains 64% oleic acid. Shackelford et al. (1991) studied the acceptability of low-fat frankfurters as influenced by feeding of elevated levels of monounsaturated fats to growing-finishing swine. They reported that the high-oleate treatments were comparable to the control in all sensory characteristics. Riendeau (1990) incorporated canola oil into smoked sausages and found that fat and calorie-reduced products were acceptable in quality. Park et al. (1989, 1990) studied the properties of low-fat frankfurters manufactured by direct incorporation of high-oleic sunflower oil (HOSO) as a source of monounsaturated fat. They reported that low-fat frankfurters with maximum allowable added water and HOSO could be manufactured without adverse effects on processing yield, texture or sensory properties.

Virgin olive oil is the most monounsaturated vegetable oil. It contains 56.3–86.5% monounsaturated fatty acids, 8–25% saturated and 3.6–21.5% polyunsaturated fatty acids (IOOC, 1984). It also has tocopherols and phenolic substances which act as antioxidants. Olive oil has a high biological value attributed to its high ratio of vitamin E to polyunsaturated fatty acids (Viola, 1970). It also has a lower ratio of saturated to monounsaturated fatty acids and the presence of antioxidant substances at an optimum concentration (Christakis et al., 1980).

Our objectives were to evaluate quality of low-fat frankfurters (<10% fat) produced by direct incorporation of virgin olive oil as a sole source of monounsaturated fat, and to study effects of protein level in the finished product on quality characteristics.

MATERIALS & METHODS

Ingredients and formulation

Commercial frozen beef meat, fresh pork meat and pork backfat were obtained from the local meat market. Partially thawed beef and the fresh pork were trimmed of separable fat to provide extra lean meats. The lean meat and the pork backfat were separately ground through a 12 mm plate and then through a 3 mm plate. The ground meats and pork backfat were vacuum packaged and frozen at −20°C for 1–2 wk until product formulation. Representative samples were analyzed for moisture, fat and protein (AOAC, 1984) prior to freezing. All raw materials were tempered at 0°C for 24 h prior to use.

Virgin commercial olive oil containing 0.71% free fatty acids (as oleic) was pre-emulsified the day of use. Eight parts of hot water were mixed for 2 min with one part sodium caseinate. The mixture was emulsified with 10 parts oil for 3 min (Hoogenkamp, 1989a, b).

Four treatments were prepared (Table 1). The control was produced using only pork back fat formulated to 28% fat and 11% protein. These values represent about the mean fat and protein content of commercial frankfurters in Greece (Bloukas and Paneras, 1986). The

Table 1—Formulation ingredients

Ingredients (g)	Control[a]	Low-fat treatments[b]		
	A	B	C	D
Protein (%)	11	10	12	14
Beef lean (1.32% fat)	700	830	1020	1200
Pork lean (3.87% fat)	1000	1170	1430	1700
Pork backfat (75.84% fat)	1700	–	–	–
Olive oil[c]	–	415	405	395
Ice / water[d]	1630	2615	2175	1735
Sodium chloride	95	87	87	87
Sodium nitrite	1	1.2	1.2	1.2
Sodium ascorbate	3	4	4	4
Phosphates	12	12	12	12
Sodium caseinate	50	50	50	50
Starch	200	200	200	200
Seasoning	24	32	32	32

[a] Prepared with pork backfat and formulated for 28% fat and 11% protein.
[b] Prepared with virgin olive oil and formulated for <10% fat and 10%, 12% and 14% protein.
[c] Percent in batter composition: 7.6%, 7.4% and 7.2%, respectively.
[d] Percent in batter composition: 30.9%, 48.2%, 40.1% and 32.0%, respectively.

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other three treatments were produced with olive oil formulated to give a final product with less than 10% fat and 10%, 12% and 14% protein, respectively. In low-fat treatments the added salt was reduced while the amount of seasonings was increased as suggested by Wirth (1988, 1991) and Hoogenkamp (1989b). All treatments were replicated three times from separate meat and fat sources at three different time periods.

Frankfurter manufacture

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

Immediately after chopping the batter of each treatment was vacuum stuffed into 24 mm diameter Nojax cellulose casings. Each treatment was handlinked at 15 cm intervals and the frankfurters were heat processed and smoked in a smokehouse to internal temperature 72°C (Hoogenkamp 1989a and b, Wirth 1988, 1991). The frankfurters were showered for 15 min and chilled at +2°C for 24 hr. After chilling the frankfurters were peeled, vacuum packaged (vacuum level 650 mmHg) in film pouches with a reported oxygen permeability rate of $\approx 116 cm^3/m^2/24$ hr/1 atm (23°C, 0% RH) and stored in the dark in a cooler at +4°C until subsequent analysis.

Batter properties

Immediately after processing the following parameters of batters were determined: **pH** was determined with a WTW digital pH meter with corrections for temperature differences. **Viscocity** was measured immediately after batter preparation with a Brookfield digital viscometer, model DV-II, set at 2.5 rpm and equipped with a spindle No 5. Frankfurters were weighed before heat processing and smoking and after chilling at +2°C for 24 hr. The processing yield (%) was determined from the weights.

Chemical analysis

Representative samples from each treatment were homogenized and analyzed, prior to vacuum packaging (0 week), for percentage moisture, fat (ether—extractable), protein, ash, starch and sodium chloride according to standard AOAC (1984) procedures. Percent added water was also calculated according to AOAC (1984) formula. Sodium nitrite was determined by the ISO (1975) method. All analyses were performed in duplicate.

Purge loss

Two vacuum packages (≈ 250–300g each) per treatment were used to determine purge loss of frankfurters the 1st, 3rd and 5th week of storage in the dark at 4°C. Before packaging each link of frankfurters was dried with paper tissue and all links per package were weighed. After removing sausages from the package each link was again dried with paper tissue and all links per package were reweighed. Purge loss was determined from the difference in weights between the two measurements expressed as percentage of initial weight.

Color measurements

Color measurements were performed the 0 and 5th week of storage. A True—Color Neotec colorimeter was used to evaluate L, a and b (Hunter color system). The instrument was standardized using a white ceramic tile calibrated to tristimulus values of L = + 96.0, a = −1.03, and b = + 2.4. Two frankfurters per treatment were used. The surface of the glass tray was completely covered with sections of the frankfurters and four measurements were taken per link by rotating the glass tray one-quarter after each measurement. Data are means of eight measurements.

Rancidity determination

The 2-Thiobarbituric acid (TBA) test according to Tarladgis et al. (1960) was used to determine extent of oxidative rancidity after the 0, 1st, 3rd and 5th week. Two frankfurters were randomly sampled from each treatment. The frankfurters were ground in a chopper for 1 min and two 10-g portions were removed for TBA analysis. Duplicate determinations were conducted on each treatment. The amount of residual nitrite in each sample was taken into account and the amounts of sulfanilamide were added in the samples for TBA analysis according to the modifications of Shahidi et al. (1985). Readings were made on a LKB Ultrospec II spectrophotometer at 538 nm. The conversion factor 7.8 was used in calculation of TBA numbers.

Sensory evaluation

Sensory evaluation was conducted the 1st and 5th week of storage by a five-member trained panel. The panelists were chosen on the basis of previous experience in evaluating frankfurters. The following attributes were evaluated on a 5-point or 8-point scale: color (5 = very intensive, 1 = very poor), springiness (5 = extremely springy, 1 = not springy), firmness (8 = extremely firm, 1 = extremely soft), juiciness (8 = extremely juicy, 1 = extremely dry), flavor intensity (8 = extremely strong, 1 = extremely weak to unpleasant), overall palatability (8 = palatable, 1 = unpalatable). Each attribute was discussed and tests were initiated after panelists were familiarized with scales. Samples were prepared by steeping frankfurters in boiling water in individual pans 2 min. Warm, 2.5 cm long pieces from each treatment were randomly distributed for evaluation. Tap water was provided between samples to cleanse the palate.

Texture profile analysis

An Instron Universal Testing Machine, model 1140, was used to conduct texture profile analysis, as described by Bourne (1978), after 1 wk storage. Samples were prepared by steeping frankfurters in boiling water for 2 min and cooling to ambient temperature. Four 20 mm long sections per treatment were axially compressed by a two cycle compression test to 75% of original height. Force-time deformation curves were recorded at a crosshead speed 5 cm/min, chart speed 5 cm/min and full scale 50 kg. Texture variables of force and area measurements were: FF = force to fracture; F1 = maximum force for first compression; A1 = total energy for first compression; F2 = maximum force for second compression; A2 = total energy for second compression; springiness (S) = height sample recovered between end of first compression and start of second; gumminess = F1 × A2/A1; chewiness = F1 × A2/A1 × S; and cohesiveness = A2/A1. Peak areas were determined by using the Ladd Graphic Data Analyzing System.

Skin strength

Skin strength of frankfurters was measured with a penetrometer Sur-Berlin, model PNR 6, equipped with a half-scale aluminum cone of 45 g and 20 g load weight. Samples were prepared by steeping frankfurters in boiling water for 2 min and cooling to ambient. The pointed part of the cone was placed at the surface of the frankfurters and the instrument was turned on for 10 sec to produce a puncture. The depth of puncture was measured in mm and higher depth means less skin strength. The same procedure was applied to five surface areas of each of two links of frankfurters per treatment. Data reported are means of ten measurements.

Statistical analysis

Data collected for batter characteristics, processing yield, chemical composition, sensory and instrumental texture profile values were analyzed by one-way analysis of variance. Data collected for purge losses, pH, TBA values and instrumental color were analyzed by a two factor factorial arrangement in a completely randomized design. The factors were: treatments (A,B,C,D) and storage time. Means were compared by using the $LSD_{0.05}$ test. Data analyses were performed using the MSTAT program.

RESULTS & DISCUSSION

MEAN pH and viscosity for uncooked batter of control and low-fat frankfurters containing olive oil were compared (Table 2). No differences ($P > 0.05$) were found between pH of control and low-fat batters. The Brookfield viscosity of uncooked batter in low-fat frankfurters was higher ($P < 0.05$) in treatments

Table 2—pH and viscocity for uncooked batter of control and low-fat frankfurters containing olive oil[f]

Parameters	Control[a] 11%	Low-fat treatments[b] 10%	12%	14%
pH	6.60 (0.25)[c]	6.51 (0.23)[c]	6.41 (0.12)[c]	6.33 (0.11)[c]
Brookfield viscocity (cp X 10^3)	414 (17.21)[c]	251 (14.93)[e]	339 (59.65)[d]	456 (38.16)[c]

[a] Prepared with pork backfat and formulated for 28% fat and 11% protein.
[b] Prepared with virgin olive oil and formulated for < 10% fat and 10%, 12% and 14% protein.
[c-e] Means within the same row with different superscript letters are different ($P < 0.05$).
[f] Means (standard deviation).

Table 3—Processing yield and proximate composition of control and low-fat frankfurters containing olive oil[g]

Parameters	Control[a] 11%	Low-fat treatments[b] 10%	12%	14%
Processing yield (%)	86.6 (3.8)[d]	80.2 (7.2)[e]	80.5 (5.9)[e]	80.5 (4.7)[e]
Moisture (%)	55.0 (0.8)[d]	70.6 (0.4)[e]	69.7 (0.5)[e]	68.0 (0.6)[f]
Protein (%)	10.9 (0.4)[d]	10.7 (0.1)[d]	12.4 (0.2)[e]	14.3 (0.2)[f]
Fat (%)	27.6 (0.7)[d]	11.6 (0.1)[e]	10.8 (0.4)[e]	10.6 (0.7)[e]
Ash (%)	2.5 (0.1)[d]	2.6 (0.1)[d]	2.7 (0.1)[de]	2.8 (0.1)[e]
Starch (%)	3.8 (0.4)[d]	4.3 (0.6)[d]	4.1 (0.8)[d]	4.1 (0.7)[d]
Sodium chloride (%)	1.8 (0.1)[d]	1.8 (0.1)[d]	1.8 (0.1)[d]	1.8 (0.1)[d]
Sodium nitrite (ppm)	112 (6.8)[d]	117 (7.5)[d]	125 (23.0)[d]	110 (13.0)[d]
Added water (%)[h]	12.6 (2.6)[d]	38.6 (0.5)[e]	24.9 (1.4)[f]	11.8 (0.8)[d]
Caloric content (Kcal/100g)[c]	312	163	168	172
Caloric content reduction (%)		47.6	46.1	44.7

[a] Prepared with pork backfat and formulated for 28% fat and 11% protein.
[b] Prepared with virgin olive oil and formulated for < 10% fat and 10%, 12% and 14% protein.
[c] Calculations based on 9.1 Kcal/g for fat and 4.1 Kcal/g for protein and carbohydrates (Wirth, 1988).
[d-f] Means within same row with different superscript letters are different ($P < 0.05$).
[g] Means (standard deviation).
[h] Percent added water = $(W - 4P)/(1 - 0.01W + 0.04P)$, where W = moisture %, P = protein % (AOAC, 1984).

with higher protein. No differences were found in viscosity between controls and low-fat treatments with 14% protein. The added water in both treatments was similar, 12.6% and 11.8% respectively (Table 3). These results agreed with Claus et al. (1989) who found that added water had greater effect than fat or protein on Brookfield viscosity.

Processing yields (Table 3) for control (86.6%) were 5.5–6.5% higher ($P < 0.05$) than for low-fat treatments (80.2–80.5%). These results were in accordance with Townsend et al. (1971) who found that frankfurters with vegetable oil had lower processing yield than those prepared with animal fat. Preliminary experiments have shown that the small reduction of added salt in low-fat treatments, (16.1g/kg of batter instead of 17.5 g/kg in the control) had no effect on processing yield. Park et al. (1989) also reported that control frankfurters with 30% animal fat had 5–6% higher yield than low-fat treatments with ≈17% oil and the same added salt.

The proximate composition of control frankfurters was very near the targeted values. Total fat and protein concentrations of low-fat frankfurters were higher than targeted values, due to higher moisture loss during processing. For purposes of discussion, references to protein concentrations will be made according to formulated levels. The higher the protein content the lower the moisture content of the low-fat frankfurters except for the frankfurters with 10% and 12% protein where there was no difference ($P > 0.05$). No differences ($P > 0.05$) were found in sodium chloride and sodium nitrite content although added quantities in low-fat treatments were slightly different.

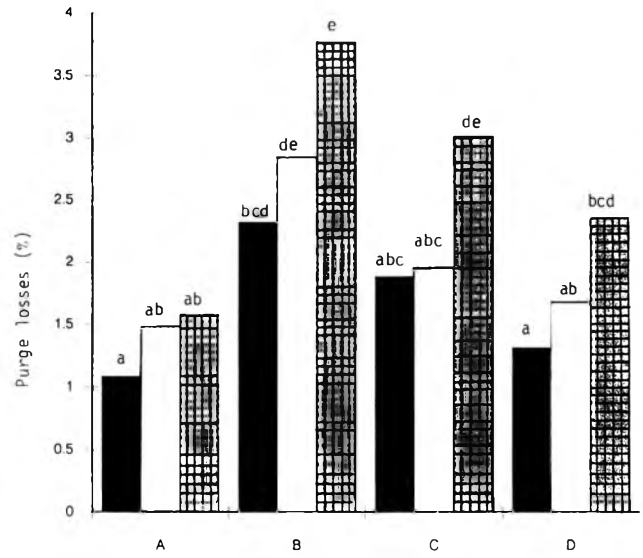

Fig. 1—Effect of storage time on purge losses of control (A) and low-fat frankfurters (B,C,D) containing olive oil. (A) Prepared with pork backfat and formulated for 28% fat and 11% protein. (B,C,D) Prepared with virgin olive oil and formulated for <10% fat and 10%, 12% and 14% protein, respectively. [a-e] Bars with different superscript letters are different ($P < 0.05$). ■ 1st wk, □ 3rd wk, □ 5th wk.

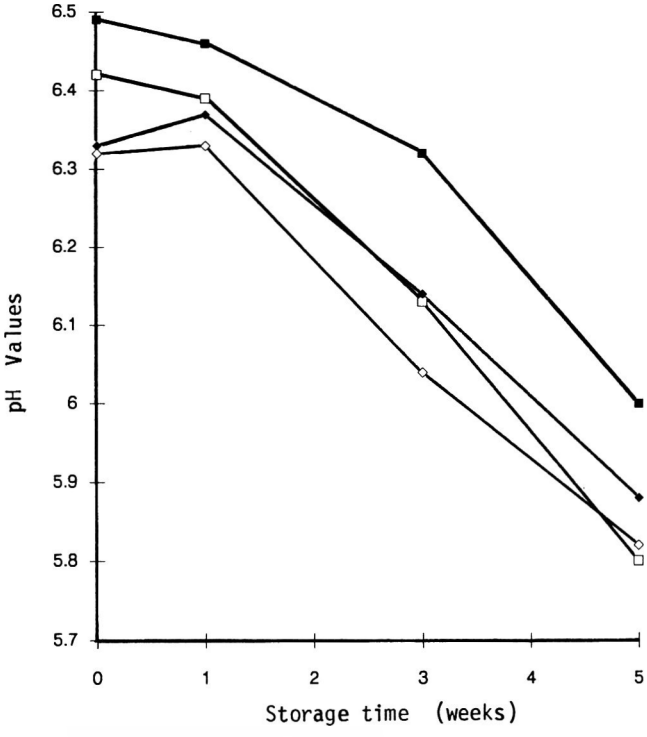

Fig. 2—pH values of control (A) and low fat frankfurters (B,C,D) containing olive oil. (A) ■———■ Prepared with pork backfat and formulated for 28% fat and 11% protein. (B) □———□, (C) ♦———♦, (D) ◇———◇ prepared with only virgin olive oil and formulated for <10% fat and 10%, 12% and 14% protein, respectively.

The total reduction in caloric content of low-fat frankfurters ranged from 44.7% to 47.6% compared to controls.

The low-fat treatment with 10% protein had higher ($P < 0.05$) purge loss than all other treatments. Storage time had a significant effect on purge losses, especially in low-fat treatments (Fig. 1). The lower the protein level the higher the purge losses. The low-fat treatment with 14% protein was not different

Table 4—Effect of storage time on TBA values (mg malonaldehyde/kg) of control and low-fat frankfurters containing olive oil

Storage time at 4°C	Control[a] 11%	Low-fat treatments[b] 10%	12%	14%
0 week	0.81[d]	0.62[c]	0.59[c]	0.45[c]
1st week	0.94[d]	0.48[c]	0.55[c]	0.35[c]
3rd week	0.97[d]	0.97[c]	0.66[c]	0.62[c]
5th week	0.85[d]	0.63[c]	0.53[c]	0.42[c]

[a] Prepared with pork backfat and formulated for 28% fat and 11% protein.
[b] Prepared with virgin olive oil and formulated for <10% fat and 10%, 12% and 14% protein, respectively.
[c-d] Means within same row with different superscript letters are different ($P < 0.05$).

($P > 0.05$) in purge loss from the control during the storage period of 5 wk. Claus et al. (1990) found that the low-fat frankfurters had higher consumer shrink and purge losses. Higher purge losses of low-fat frankfurters were due to lower ionic strength. In our experiment the added salt in low-fat treatments was purposely reduced slightly. This probably contributed to further decrease of ionic strength in low-fat treatments. The increase in purge losses during storage was due to the decrease in pH. The correlation coefficient between purge losses and pH after the 1st week of storage was $r = -0.644$ ($P < 0.05$). The pH of control was reduced from 6.5 to 6.0 and that of low-fat treatments from 6.4 to 5.8 during the 5 wk storage of vacuum-packed frankfurters at 4°C (Fig. 2). Paneras and Bloukas (1988) reported a decrease in pH from 6.3 to < 5.8 during the 9 wk storage of vacuum packed frankfurters at 3°C. Kempton and Bobier (1970) also found a decrease in pH from 6.3 to 5.4 during storage of frankfurters under vacuum at 5°C for 28 days. Simard et al. (1983) reported a decrease in pH from 6.18 to 5.42 during 7 wk storage of frankfurters under vacuum at 7°C. The pH decrease was attributed to activity of lactobacilli, and/or dissolution of CO_2 into meat tissue.

TBA values of refrigerated vacuum-packaged frankfurters over 5 wk were compared (Table 4). All low-fat treatments containing olive oil had lower ($P < 0.05$) TBA values than control, initially and during 5 wk storage. The lower TBA values observed in olive oil containing frankfurters was attributed to tocopherols and phenolic substances with antioxidant activity in addition to nitrite. The TBA values of control treatment although higher than low-fat treatments were lower than acceptable range (<1.0) for oxidative rancidity (Ockerman, 1976). Storage time did not affect TBA values, probably due to the presence of curing ingredients, such as nitrite, phosphate and ascorbate, which also act as antioxidants.

Means for color measurements (Table 5) showed no difference ($P > 0.05$) in Hunter L and b values between treatments and storage time. These results were in agreement with Ahmed et al. (1990) who found that decreasing fat content in fresh pork sausages with simultaneous increase in added water, did not affect Hunter L values. The lower the protein level of low-fat frankfurters the lower ($P < 0.05$) the redness. The low-fat treatment with 14% protein level had the same ($P < 0.05$) Hunter a value as the control. Differences in redness between low-fat treatments were due to different added water and protein levels. In low-fat treatments, added water increased from 12.4% to 39.2% while protein content was inversely reduced from 14.3% to 10.7% (Table 3). Reduced protein content resulted in dilution of myoglobin and consequently less red color. During the 5 wk refrigerated storage under vacuum no decreases in redness were observed.

Data on sensory scores and instrumental texture profiles of control and low-fat frankfurters containing olive oil were compared (Table 6). The low-fat treatment with 10% protein had lower ($P < 0.05$) color, firmness and overall palatability scores. The treatment with 12% protein had similar ($P > 0.05$) sensory attributes except palatability. The higher the protein content

Table 5—Hunter color values of control and low-fat frankfurters containing olive oil

Hunter color numbers	Storage time (wk)	Control[a] 11%	Low-fat treatments[b] 10%	12%	14%
L (lightness)	0	55.0[c]	55.7[c]	54.4[c]	54.2[c]
	5	54.8[c]	55.7[c]	54.2[c]	53.8[c]
a (redness)	0	14.4[e]	11.1[c]	12.4[d]	14.7[e]
	5	13.6[e]	10.6[c]	11.8[d]	14.0[e]
b (yellowness)	0	12.9[c]	13.6[c]	13.2[c]	13.1[c]
	5	13.2[c]	13.9[c]	13.5[c]	13.1[c]

[a] Prepared with pork backfat and formulated for 28% fat and 11% protein.
[b] Prepared with virgin olive oil and formulated for <10% fat and 10%, 12% and 14% protein.
[c-e] Means within rows of same numbers with different superscript letters are different ($P < 0.05$).

Table 6—Sensory scores and instrumental texture profile of control and low-fat frankfurters containing olive oil[c]

Parameters	Control[a] 11%	Low-fat treatments[b] 10%	12%	14%
Sensory attribute:				
Color[g]	4.0[d]	3.0[e]	4.0[d]	4.5[d]
Springiness[h]	4.2[d]	4.1[d]	4.2[d]	4.3[d]
Firmness[i]	4.5[d]	2.7[e]	4.2[d]	6.5[f]
Juiciness[j]	7.2[d]	6.8[de]	6.4[de]	5.9[e]
Flavor intensity[k]	5.7[d]	5.6[d]	5.8[d]	5.8[d]
Overall palatability[l]	7.3[d]	5.7[e]	6.5[f]	6.4[f]
Skin strength (mm)	155.6[d]	168.0[d]	120.3[e]	77.0[f]
Texture profile:				
Fracturability (FF)[m]	34.0[d]	46.7[d]	61.1[e]	68.0[e]
1st bite hardness (F1)[m]	47.4[d]	43.8[d]	80.7[e]	109.2[f]
2nd bite hardness (F2)[m]	32.6[d]	24.8[d]	56.5[e]	87.6[f]
Springiness (S)[m]	15.1[d]	12.7[f]	15.4[de]	17.0[e]
Cohesiveness (A2/A1)	0.2[d]	0.1[d]	0.2[d]	0.2[d]
Gumminess (F1XA2/A1)	9.2[d]	6.7[d]	16.4[e]	23.7[f]
Chewiness (F1XA2/A1XS)	140.2[d]	87.6[d]	254.0[e]	403.6[f]

[a] Prepared with pork backfat and formulated for 28% fat and 11% protein.
[b] Prepared with virgin olive oil and formulated for 10% fat and 10%, 12% and 14% protein.
[c] Data presented are means
[d-f] Means within row with different superscripts are different ($P < 0.05$).
[g] 5 = very intensive, 1 = very poor
[h] 5 = extremely springy, 1 = not springy
[i] 8 = extremely firm, 1 = extremely soft
[j] 8 = extremely juicy, 1 = extremely dry
[k] 8 = extremely strong, 1 = extremely weak to unpleasant
[l] 8 = palatable, 1 = unpalatable
[m] Expressed in Newtons

the higher ($P < 0.05$) the firmness in low-fat frankfurters. Simon et al. (1965) and Claus et al. (1989) reported the same effects. Differences in flavor intensity between the control and low-fat treatments were not significant.

The 1st week of storage the control treatment had higher ($P < 0.05$) overall palatability scores while differences between low-fat frankfurters with 12% and 14% protein were not significant. The frankfurters with 10% protein were very soft while those with 14% protein were harder and less juicy than the control. During the 5 wk cold storage a ($P < 0.05$) reduction in overall palatability was found in all treatments (Fig. 3). The control treatment had higher ($P < 0.05$) overall palatability while in low-fat treatments containing olive oil the higher the protein level the higher the overall palatability. The observed decrease in palatability during storage was probably due to microbial activity of lactic acid bacteria, which is in agreement with pH reduction (Fig. 2).

The control treatment had higher skin strength and fracturability and not significant changes in bite hardness, gumminess and chewiness with 10% protein low-fat frankfurters. This was probably due to the similar protein level of the 2 treatments

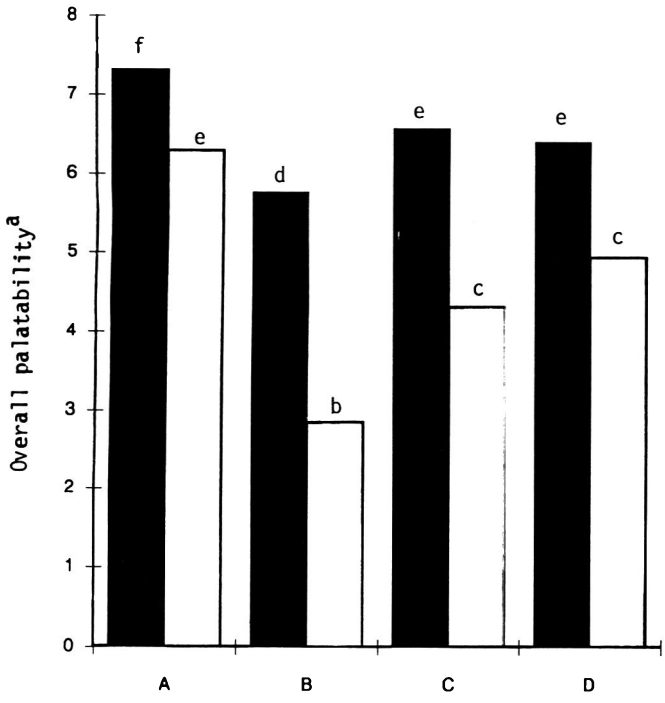

Fig. 3—Overall palatability scores the 1st and 5th week of storage of control (A) and low-fat frankfurters (B,C,D) containing olive oil. (A) Prepared with pork backfat and formulated for 28% fat and 11% protein (B,C,D) Prepared with virgin olive oil and formulated for < 10% fat and 10%, 12% and 14% protein, respectively. ■ 1st wk, □ 5th wk. [a]8 = palatable, 1 = unpalatable; [b-e] Bars with different superscript letters are different (P<0.05).

(Table 3). According to Saffle et al. (1964) the skin strength is developed by the migration of protein to the surface of frankfurters and subsequent denaturation during smoking. Differences between the control and low-fat treatments with 12% and 14% protein for skin strength, fracturability, 1st and 2nd bite hardness, springiness, gumminess and chewiness were significant. The higher the protein in low-fat treatments the higher ($P<0.05$) was the skin strength, the 1st and 2nd bite hardness, gumminess and chewiness. Low-fat treatments with 12% and 14% protein had no significant differences for fracturability and springiness while all treatments had the same ($P<0.05$) cohesiveness.

CONCLUSIONS

LOW-FAT FRANKFURTERS (10% fat) could be manufactured with olive oil and without added animal fat. The low-fat frankfurters would be highly desirable from a diet/health standpoint as they contain monounsaturated vegetable oil, have lower caloric value, reduced cholesterol and a higher protein content. Among low-fat treatments with olive oil, that with ≈ 12% protein had quality characteristics most comparable to the control.

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End-point Temperature (EPT) Affects N-Acetyl-β-D-Glucosaminidase Activity in Beef, Pork and Turkey

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ABSTRACT

End-point temperature (EPT) affected N-acetyl-β-D-glucosaminidase (NAGase) activity in nonfrozen and previously frozen cores and ground samples of beef, pork, turkey breast and ground turkey leg muscle tissue. There was little loss of NAGase activity when heating the products to 40°C; however, as EPT increased from 40–70°C, there was ($P<0.05$) loss of activity. At 70°C, 90% of activity was lost in beef, 98% in pork, and 93 to 98% in turkey. Inactivation temperature values, IT_{50}, (50% inactivation) were: beef, 59.8°C; pork, 53.4°C; turkey breast, 55.6°C; and turkey leg, 56.6°C.

Key Words: N-acetyl-β-D-glucosaminidase, thermal-processing, beef, pork, turkey.

INTRODUCTION

THE CODE of Federal Regulations, Title 9, Chapter III, Meat Inspection Regulations, establishes prescribed thermal treatment for meat and poultry products (USDA-FSIS, 1985a, 1985b). Examples are: imported canned hams, picnics and luncheon meat (69°C); rare roast beef (63°C); uncured poultry products (71.1°C) and cured/smoked poultry products (68.3°C). The USDA has regulatory authority of a public health nature and the Food Safety and Inspection Service (FSIS) is especially concerned with *Escherichia coli* 0157:H7, *Salmonella* and *Listeria monocytogenes* in uncured meat and poultry products (USDA-FSIS, 1990). The need for an analytical laboratory method to determine adequacy of thermal treatment of meat and poultry products has been expressed in personal communications from FSIS.

The enzyme, N-acetyl-β-D-glucosaminidase (NAGase, 3.2.1.30) is found in mammalian muscle tissue and egg products. Henderson and Robinson (1969) and Jackle et al. (1989) used a NAGase assay procedure for determining adequacy of heat pasteurization of egg whites. Spanier et al. (1990) investigated the effect of end-point cooking temperature on NAGase activity and soluble protein in beef and its relationship to development of meat flavor. However, the effect of thermal treatment on NAGase activity in pork and turkey has not been reported. Rawn (1988) reported that the reduction in enzyme activity was due to the heating of protein which increased the vibrational and rotational energy of the protein molecules. It also weakens the interactions that stabilize the folded conformation. Thus the loss of the native structure causes the protein to uncoil and lose biological activity.

The objective of our study was to evaluate the Boehringer Mannheim clinical test kit (normally used for determining NAGase activity in urine) for determining the influence of thermal processing on NAGase activity in nonfrozen (NF) and previously frozen (PF) beef, pork, turkey breast and ground turkey leg, and to determine if this enzyme assay procedure could be used as an end-point indicator.

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MATERIALS & METHODS

Preparation of filtrate

Whole top beef rounds (semimembranosus/adductor) containing less than 10% fat; pork loin (longissimus muscle); whole turkey breast (pectoralis major and minor) and whole turkey legs were purchased from a local supermarket. Fatty tissue was removed from the beef and pork samples and the skin and fatty tissue from the turkey samples. About 18 to 20, 2.5 cm diameter by 7.5 to 9.0-cm long core samples (25–27g) were removed from each whole muscle piece and the remaining muscle tissue was finely chopped for 45 sec in a Model 702-3 Hamilton Beach food processor (Hamilton Beach Food Processor, Division of Scovill, Inc., Miami, FL).

Half the core and ground samples were studied as nonfrozen (NF) samples and the remaining half were frozen at −20°C until ready to use (usually within 1 mo). The frozen samples were labeled as "previously frozen" (PF) samples. NF and PF core or ground samples (25–27g) were placed in 25 mm × 200 mm glass tubes, the tubes placed in ice to equilibrate to 0.6°C to achieve uniform starting temperature, and then thermally processed in a water bath at 1°C above the target end-point temperatures (EPTs) which were 23, 30, 40, 50, 60, 62.8, 66.7, 67.8, 68.8, 70, and 80°C. Temperature was monitored using a Bailey Model BAT-12 digital thermometer (Bailey Instruments, Saddle Brook, NJ) equipped with a five-probe copper constantan thermocouple switch box. Thermocouple probes were positioned in the geometric center of each sample. After the desired EPT was reached, samples were immediately chilled in ice and held overnight at 3°C. Calculation of the percent loss of NAGase activity was based upon 2 determinations from each of 2 lots of product.

Enzyme analysis

Preliminary results, as well as those reported by Spanier et al. (1990), showed little change in NAGase activity at < 30°C. Therefore, the NAGase enzyme was extracted from 23°C (raw) muscle tissue and used as the initial NAGase activity value for conversion to percent loss of NAGase activity. The enzyme was extracted from the raw and heated treated muscle tissue by adding to 20 mL of 20 mM, pH 7.2 HEPES buffer, 5g of finely chopped tissue and homogenized with an Omni Homogenizer (Omni International Waterbury, Ct). The slurry was centrifuged at 12,000 × *g* for 10 min at 5°C in a refrigerated Sorvall RC-5B centrifuge (DuPont Company, Newtown, CT). The supernatant fluid was then filtered through a #588 Schleicher and Schuell pre-pleated filter paper (Schleicher and Schuell, Inc., Keene, NH).

A modification of the Boehringer Mannheim assay (Boehringer Mannheim Biochemicals, Indianapolis, IN) procedure for determination of NAGase activity in urine was used, but 0.05 mL of the filtrate from extracts of the muscle was used instead of urine. Incubation of filtrate samples was conducted at 37°C. The procedure is a colorimetric assay based on the principle that 3-cresolsulfonphthalcinyl-N-acetyl-β-D-glucosaminide (sodium salt) is hydrolyzed by N-acetyl-β-D-glucosaminidase (NAGase) with the release of 3-cresosulfonphthalein (3-cresol purple) which is measured spectrophotometrically at 580 nm. Results are expressed as percent loss of activity based on initial NAGase value [U/L] of a 23°C (raw) muscle sample.

Statistical analysis

The NAGase activity values (U/L) were converted to percent loss from the initial (U/L) value of the 23°C sample. Selecting the linear proportion of the response curve for each product (beef, pork, turkey breast and leg) - method (core, ground) - condition (NF, PF), slopes and estimated 50 percent loss (IT_{50}) values were analyzed using The General Linear Model procedure of the SAS Institute Inc. (1987).

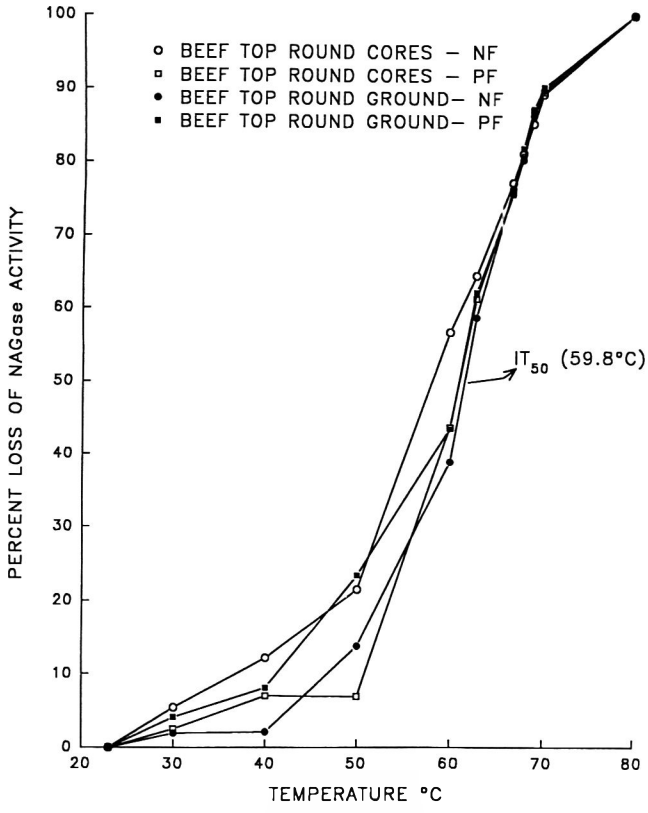

Fig. 1—Influence of end-point temperature (EPT) on percent loss of N-Acetyl-β-D-glucosaminidase (NAGase) activity in non-frozen (NF) and previously frozen (PF) cores and ground samples of beef. IT_{50} (59.8°C) represents temperature for 50% inactivation.

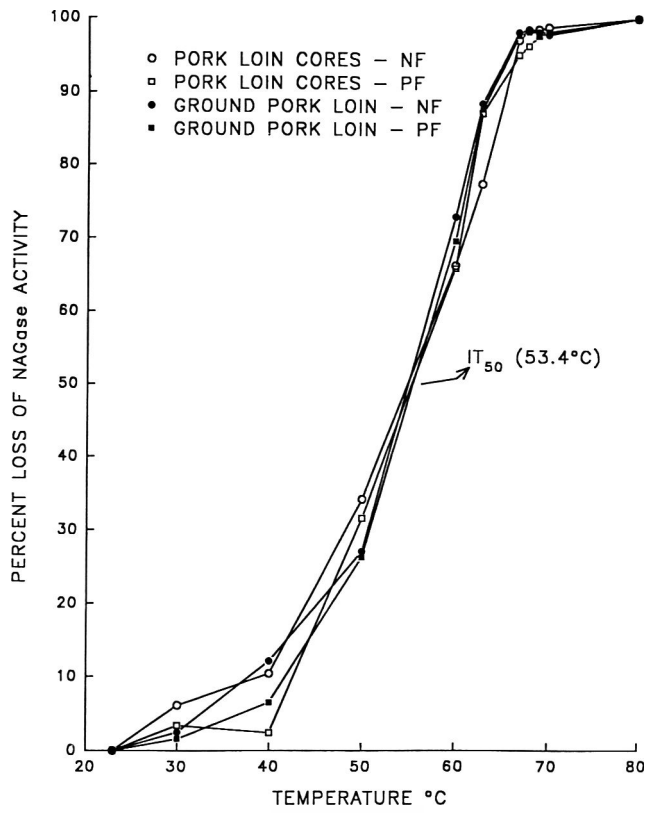

Fig. 2—Influence of end-point temperature (EPT) on percent loss of N-Acetyl-β-D-glucosaminidase (NAGase) activity in non-frozen (NF) and previously frozen (PF) cores and ground samples of pork. IT_{50} (53.4°C) represents temperature for 50% inactivation.

RESULTS & DISCUSSION

Laboratory samples

The effects of end-point temperatures (EPT) on N-acetyl-β-D-glucosaminidase (NAGase) activity in nonfrozen and previously frozen cores and ground beef top round muscle tissue samples were compared (Fig. 1). Assays from product temperatures of 23–50°C showed considerable variation in percent loss of enzyme activity. The causes for such variation are not clear. Donovan and Hansen (1971) reported low reproducibility and precision in determining NAGase activity in egg white and whole egg. They also reported that the variation in enzyme activity remaining at different temperatures resulted from inequalities in energies for inactivation of the enzyme. Spanier et al. (1990) also observed variations in enzyme activity at < 50°C. From 50–60°C, there was less variation in percent loss of activity; and, from 40 to 70°C, there was significant ($P < 0.05$) loss of NAGase activity. At 60°C, ≈ 40% of enzyme activity was lost, and at 70°C nearly 90% was lost. This was slightly less than the 92.6% loss reported by Spanier et al. (1990). That study reported IT_{50} (temperature for 50% inactivation/denaturation) of 62°C for beef. Using that system of data analysis, our results (Fig. 1) showed an IT_{50} of 59.8°C in general agreement.

The effects of EPT on loss of NAGase activity in nonfrozen and previously frozen core and ground pork loin muscle tissue samples were compared (Fig. 2). In contrast to beef (Fig. 1), there was little variation in percent loss of activity among samples at EPT < 40°C, and less at higher EPTs. Pork samples heated to 50°C lost 23–33% of activity, while beef heated to 50°C lost 6–23%. This difference between pork and beef samples was also evident at 60°C: 68–74% for pork and 38–56% for beef. At 66.7°C more than 95% of activity was lost in pork and at 70°C more than 98% of the activity was lost. Using Spanier's IT_{50} system, the IT_{50} for pork would be 53.4°C compared to 59.8°C for beef. This indicates that the NAGase in pork is less stable to heat than that in beef. No NAGase activity was detected in beef or pork samples heated to 80°C. Townsend and Davis (1991) reported differences in lactate dehydrogenase (LDH) activity between light and dark chicken muscle tissue. Dark meat appeared to be more stable to heat than light meat. Therefore, the NAGase in muscle tissue from beef and pork may have different stabilities to heat.

The effects of EPT on loss of NAGase activity in nonfrozen and previously frozen cores and ground samples of turkey breast muscle tissue were also compared (Fig. 3). There was less variation in activity among turkey breast samples heated to 70°C than for beef and pork. The percent loss of enzyme activity in the turkey breast samples heated to 70°C was greater ($P < .05$) than in those heated to 40°C. However, there were some differences between core and ground turkey breast samples. At 40°C, only 0.5 to 6% of NAGase activity was lost in turkey breast core samples while 8–9% was lost in the ground turkey breast samples. At 50°C, the NAGase activity loss for turkey breast meat (27–33%) was similar to that for pork (23–33%. At 60°C, about 65% of the enzyme activity was lost and at 70°C about 93 to 96% of activity was lost. The IT_{50} for turkey breast was 55.6°C, similar to pork, (53.4°C) which indicated that the NAGase in pork and turkey are both less stable to heat than the NAGase in beef.

The effects of EPT on loss of NAGase activity in nonfrozen and previously frozen ground turkey leg muscle tissue were compared (Fig. 4). As observed with turkey breast, there was little variation in loss of NAGase activity among samples heated from 23 to 70°C. However, there was loss ($P < 0.05$) of activity as turkey leg samples were heated from 40 to 70°C. At 40°C, 6–8% of the activity was lost, slightly higher than the 0.5 to 6% for turkey breast. At 50°C, 28 to 31% of activity was lost, very similar to the activity lost for turkey breast (22–

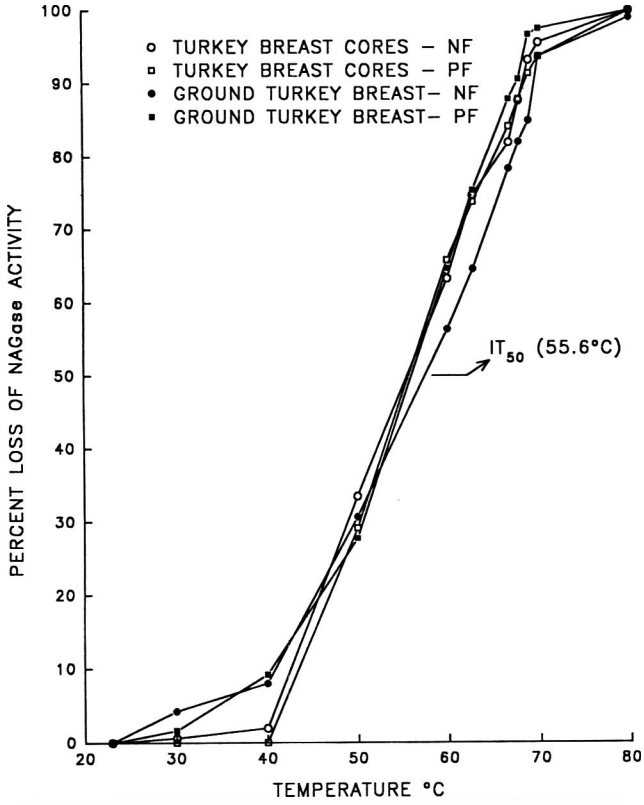

Fig. 3—Influence of end-point temperature (EPT) on percent loss of N-Acetyl-β-D-glucosaminidase (NAGase) activity in non-frozen (NF) and previously frozen (PF) cores and ground samples of turkey breast. IT_{50} (55.6°C) represents temperature for 50% inactivation.

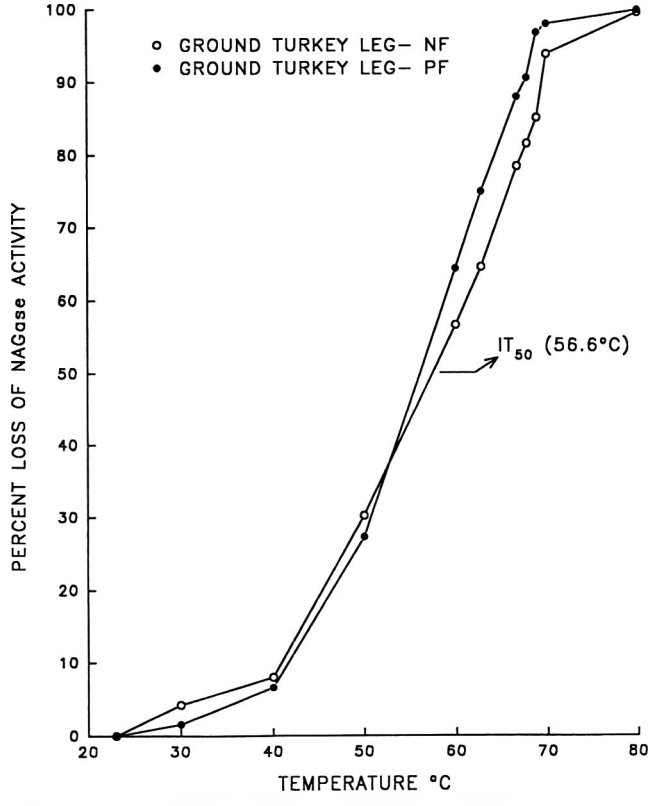

Fig. 4—Influence of end-point temperature (EPT) on percent loss of N-Acetyl-β-D-glucosaminidase (NAGase) activity in non-frozen (NF) and previously frozen (PF) ground samples of turkey leg muscle. IT_{50} (56.6°C) represents temperature for 50% inactivation.

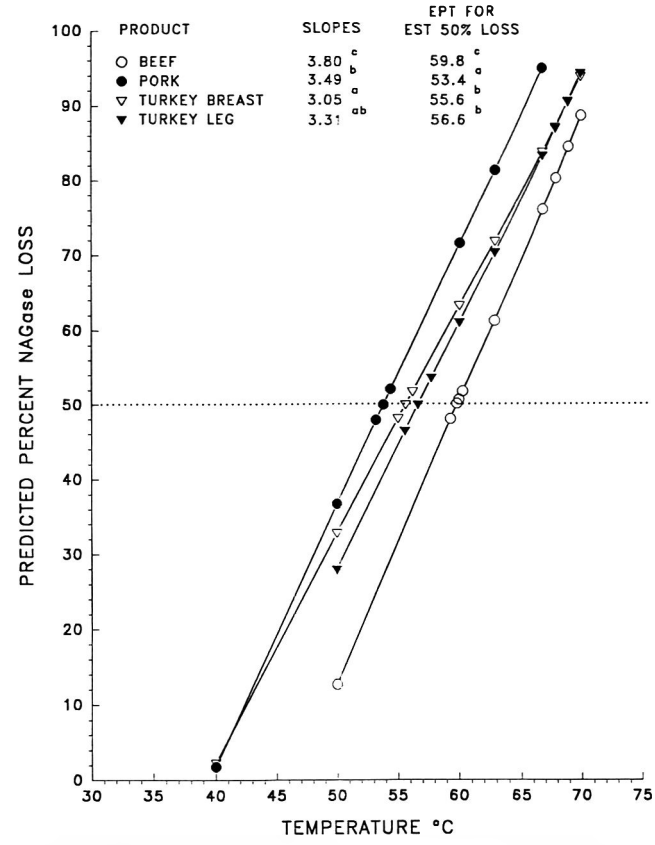

Fig. 5—Slope values and EPT values for estimated 50% activity loss for the linear portion of the response curve (40–70°C) for beef, pork, turkey breast and turkey leg muscle tissue. Product slope and predicted IT_{50} values with the same superscript are not significantly different at the 0.05 level.

34%). At 60°C, ≈ 56–64% of NAGase activity had been lost and at 70°C about 94–98% was lost. The IT_{50} value for ground turkey leg muscle tissue was 56.6°C similar to turkey breast (55.6°C), but slightly higher than pork muscle.

The data (Fig. 1, 2, 3 and 4) showed little loss of NAGase activity (< 12%) when beef, pork and turkey were heated up to 40°C. Slopes and estimated 50% loss values were analyzed for the linear portion of the response curve (40–70°C) for each product (beef, pork, turkey) - method (core, ground) - condition (PF, NF) (Fig. 5). All main effects for method and condition were nonsignificant. Slope calculations were 3.80, 3.49, 3.31 and 3.05 for beef, pork, turkey leg and turkey breast, respectively, with significant ($P<0.05$) differences between pork loin and turkey breast and beef. Turkey leg did not differ from turkey breast or pork loin.

Estimated EPTs for 50% loss of NAGase activity (IT_{50}) were predicted to be 53.4°C for pork; 55.6°C for turkey breast; 56.6°C for turkey leg; and 59.8°C for beef. The estimated EPT difference between pork and beef was significant ($P<.05$). The two turkey products were significantly ($P<0.05$) different from pork and beef products, but not different from each other for estimated EPT for IT_{50}.

CONCLUSIONS

THOUGH there was a decrease in NAGase activity as EPT increased, the use of the NAGase enzyme assay procedure may have limitations for determining the EPT of domestic meat and poultry products (patties, nuggets, etc.) due to residual enzyme activity in samples heat processed at <71.1°C. However, the NAGase assay could be used as an end-point temperature indicator providing EPT criteria could be established to indicate the percent loss of activity at any given increment of EPT.

—Continued on page 716

Bovine longissimus muscle tenderness as affected by postmortem aging time, animal age and sex

E.J. HUFF and F.C. PARRISH, JR.

ABSTRACT

Strip loins were removed from 10 bulls and 10 steers ≈14 mo old and from 10 cows ≥ 44 mo. Samples were removed at 3, 7, 14, and 28 days postmortem. Shear-force values were measured. Sensory evaluation was done by a 15-member trained panel. Animal age and postmortem aging time had more influence on tenderness attributes than did sex of the animal. Increased postmortem aging time improved tenderness attributes regardless of sex or age.

Key Words: beef, aging, tenderness, shear force

INTRODUCTION

SEVERAL FACTORS have been identified as general predictors of whether beef will be acceptably tender. Most notable among these factors are age and sex of the animal. Beef from more mature animals repeatedly has been found less tender than beef from younger animals (Tuma et al., 1963; Dikeman and Tuma, 1971; Smith et al., 1982). Sex of the animal (castrated vs noncastrated males), however, has somewhat less definitive effects upon tenderness. Albaugh et al. (1975) showed that beef from bulls was less tender than that from steers, whereas Hedrick et al. (1969) reported no difference in tenderness of beef from bulls and steers < 16 mo of age. Postmortem aging is widely known to improve tenderness of beef. Field et al. (1971) reported shear-force values declined from 2 to 21 days of postmortem storage. Jennings et al. (1978) also showed a decline in shear-force values during postmortem storage up to 20 days.

A potential measure of tenderness that has been examined is the myofibril fragmentation index (MFI). This is a measure of the amount of fragmentation undergone by myofibrils during postmortem storage (Culler et al., 1978). MFI is positively correlated with sensory and Warner-Bratzler measures of tenderness. MFI increases with longer postmortem aging times (Olson et al., 1976) and is higher for A maturity animals than for older maturities (B and older) (Parrish et al., 1987). Degree of marbling (Traces and higher) does not significantly affect MFI (Parrish et al., 1979).

Quantifying differences in palatability attributes by using sensory and mechanical methods has required much time and effort. Because meat is a multiple component system, any method to quantify tenderness and other palatability attributes should be differentiated into several components. Cover et al. (1962) proposed that tenderness descriptors used in sensory panel studies should be partitioned into several categories, namely, softness to tongue and cheek, softness to tooth pressure, ease of fragmentation, mealiness, adhesion, and tenderness of connective tissue. Likewise, it has been suggested that traditional mechanical methods to assess tenderness, such as Warner-Bratzler shear device, should be re-evaluated. Bouton and Harris (1972) compared several instrumental methods of measuring meat tenderness with measures that directly determine specific texture characteristics of meat. They suggested that compression as measured by the Instron and adhesion measurements made on muscle samples were highly related, as were the peak (maximum) force measurements made with the Warner-Bratzler shear device and fiber tensile measurements. Other researchers have used instrumental methods (Brady and Penfield, 1981; Brady and Hunecke, 1985; Zayas and Naewbanij, 1986) and evaluated such parameters as shear firmness and shear cohesiveness. Shear firmness is defined as the slope of a line from the origin of the curve to its peak, expressed in kg/min. Shear cohesiveness is measured as the peak force on the shear deformation curve. Møller (1981) proposed another way to analyze deformation curves using the Warner-Bratzler attachment on the Instron by dividing it into multiple components. He proposed that the initial yield on the curve represented the myofibrillar component, whereas the final yield point on the curve represented the connective tissue component, of tenderness.

Our objective was to use multiple sensory descriptors and multiple Warner-Bratzler shear-force measurement components to determine effects of postmortem aging, animal age, and sex (castrated vs noncastrated males) on the tenderness attributes of beef. This was done to provide detailed sensory and mechanical data to separate some differences in subcellular aspects of muscle that may affect tenderness of beef.

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MATERIALS & METHODS

Sources and storage of muscle

Strip loins were obtained from carcasses of 10 bulls and 10 steers from a single management group. The bulls and steers in this study were all ≈14 mo old (A maturity). Strip loins also were obtained from carcasses of 10 cows 44 mo old to 108 mo old (C^{50} to E maturity). Marbling scores for carcasses in all groups ranged from $Slight^{60}$ to $Small^{70}$. None of the carcasses was electrically stimulated. Strip loins were removed from carcasses at ≈24 hr postmortem. Each of the 30 strip loins were individually vacuum packaged and stored at 2°C. Two 2.86 cm steaks and two 1.27 cm steaks were removed from each strip loin at 3, 7, 14 and 28 days postmortem. Each sex and age/aging period was replicated 10 times. All steaks were vacuum packaged, frozen and stored at −20°C until analysis. Steaks were held frozen to facilitate serving the 120 total samples to the sensory panel in a manner that would allow meaningful assessment.

Sample preparation

Steaks were removed from the freezer and thawed at 2°C for 36 hr. After thawing, steaks were removed from vacuum bags and trimmed to ≤ 0.51 cm external fat. Steaks were cooked in an electric (General Electric CB60, Chicago Heights, IL) broiler oven previously preheated a minimum of 30 min to 204°C. Samples were placed on aluminum broiler pans with the surface of the meat between 12.7 cm and 11.0 cm away from the heat source. During cooking, each steak had a copper constantan thermocouple in the geometrical center. Temperatures were monitored with an Omega Digital Trendicator (Omega Engineering, Inc. Stamford, CN). Steaks were turned at half doneness (32.5°C) and cooked to final endpoint of 65°C. Steaks were wrapped in aluminum foil and placed in a preheated oven (65°C) until served to panelists. Samples were held for ≤ 20 min before serving. Immediately prior to being served, steaks were removed from the oven and cut into 1.27 × 1.27 × 2.86 cm cubes by using an 11.4 × 11.4 cm Plexiglass mold. Two cubes/steak/panelist were removed at random, placed into preheated 6.0-cm diameter aluminum pans and served to a 15 member trained sensory panel.

Sensory analysis

Sensory panelists were selected from the staff and students of the Animal Science Department. Panelists were trained in a series of eight training sessions, each ≈45 min long. Training sessions were held over the period of 1 wk. The first five training sessions were used to familiarize panelists with characteristics being evaluated and the scale used. During this period, samples representing the range of characteristics were used so panelists would be familiar with types of samples they could encounter. The final three sessions were used to determine homogeneity of panelists' response. A total of 15 panelists were selected. Panelists were seated in individual booths during evaluation. Red fluorescent lights were used to eliminate biases due to color. Panelists used a 15-cm line scale (anchored at 1 cm from each end) to evaluate samples by placing a vertical mark on the horizontal line to indicate their score for each of the following attributes: softness to tooth pressure, fiber fragmentation, mealiness, residue, and juiciness. Softness to tooth pressure was a measure of how easily the sample compressed between the molars on the first one or two chews. Fiber fragmentation was defined as how easily the sample broke down into smaller pieces upon chewing. Mealiness was described as the breakdown of the sample while chewing into small, hard and dry particles that clung to the cheek and gums. Residue was an estimation of the amount of insoluble connective tissue and/or other insoluble material that remained in the mouth after the sample was thoroughly chewed. Juiciness was an estimation of the amount of free fluids released while chewing. Scores for softness to tooth pressure, fiber fragmentation, mealiness, residue, and juiciness were recorded in mm and had a possible range from 0 (very hard, very difficult, very mealy, large amount, or very dry, respectively) to 150 (very soft, very easy, none, none, and very juicy, respectively). Panelists were asked to cleanse the palate between samples by eating an apple slice or unsalted soda cracker and drinking room-temperature (≈23°C) water. Order of presentation of samples during each session was randomized, with a total of six steaks (representing one cow, one bull and one steer, each at two aging periods) being served. The two aging periods and the three individual animals were selected at random for each panel session. Two panel sessions were held each day, one at 10:00 am and the other at 2:00 pm. A total of 20 panel sessions were held over 4 wk to accommodate the 120 samples evaluated.

Warner-Bratzler shear-force determinations

Cores were taken from each of three sections (central, medial and lateral locations) of the cooked steaks after they had been allowed to equilibrate to room temperature (25°C). The three 1.27-cm cores were removed parallel with the axis of the muscle fiber and were used for Warner-Bratzler shear-force determinations of tenderness. Measurements were made using the shear-force attachment of the Instron Universal Testing Machine (Instron Corporation, Canton, MA). A 50-kg load cell and a cross-head speed of 200 mm/min were used. Shear-force deformation curves were recorded at a chart speed of 200 mm/min. Each core was sheared twice along the long axis, and values were averaged. The Warner-Bratzler curves for each sample were divided into two parts. The first yield point of the curve was identified as the compression force and corresponded to the myofibrillar component of tenderness. The second yield point of the curve was identified as the residual force and corresponded to the connective tissue component of tenderness (Møller 1981). These values are reported as kg force/cm^2.

Statistical analysis

Data were analyzed in a split-plot design Cochran and Cox (1968). The Statistical Analysis System (SAS, Institute Inc., 1985) was used to determine means, standard errors and analysis of variance. Least Significant Differences (LSD) were calculated and used to separate means. An alpha level $P \leq 0.05$ was used to determine significance.

RESULTS & DISCUSSION

Sensory-panel studies

Results of the sensory-panel study showed that steaks from carcasses of young steers were scored as softer ($P < 0.05$) than steaks from older animals (cows) (Table 1). Samples from bull and steer carcasses were not different ($P > 0.05$) for the attribute of softness to tooth pressure. A similar trend was noted for fiber fragmentation. Steaks from young steers were scored as easier ($P < 0.05$) to fragment across the fiber than those from older animals (cows) (Table 1). Sensory panelists found no differences ($P > 0.05$) in samples from bull and steer carcasses for fiber fragmentation. These results indicate that, for attributes that reflect some component of muscle tenderness (softness to tooth pressure and fiber fragmentation), those steaks from more mature animals tended toward lower scores than did those samples from A-maturity animals. This indicates maturity is important in determining tenderness. Smith et al. (1982) also reported large differences in sensory-panel tenderness ratings for more mature beef (C maturity) compared with young beef (A and B maturity). Similarly, Dikeman and Tuma (1971) reported a significant decrease in tenderness scores between samples from A and C maturity animals, but little difference between samples from C and E maturity animals.

Table 1—Means of sensory scores for loin steaks from different sex and age categories over four postmortem aging times

Classification	Softness to tooth pressure[a]	Fiber fragmentation[b]	Mealiness[c]	Residue[d]	Juiciness[e]
Cow	102.7[f]	98.4[f]	124.0[f]	108.1[f]	107.4[f]
Bull	107.8[fg]	107.9[fg]	119.8[f]	120.7[g]	98.4[g]
Steer	118.3[f]	119.7[g]	112.7[g]	128.4[g]	95.9[g]
SEM[h]	0.79	0.86	0.73	0.81	0.81

[a] Possible range of scores: 0 = very hard to 150 = very soft.
[b] Possible range of scores: 0 = very difficult to 150 = very easy.
[c] Possible range of scores: 0 = very mealy to 150 = none.
[d] Possible range of scores: 0 = large amount to 150 = none.
[e] Possible range of scores: 0 = very dry to 150 = very juicy.
[f-g] Means with different superscripts within the same column are significantly different ($P < 0.05$).
[h] SEM is the standard error of the mean.

Steaks from young steers were more mealy ($P < 0.05$) than those from old animals (cows) or from bulls (Table 1). Since mealiness indicates degree to which meat is broken into small pieces, these higher scores, associated with easier to fragment, softer samples from steers, may reflect more myofibrillar fragmentation.

There was no difference ($P > 0.05$) between steaks from bulls and steers for connective tissue residue (Table 1). Hunsley et al. (1971) also reported that sex (bulls vs steers) did not significantly affect collagen amounts in steaks as determined by hydroxyproline analysis. The sensory panel scored steaks from older animals as having more detectable connective tissue residue ($P < 0.05$) than steaks from either young bulls or steers. Cross et al. (1973) also reported that as animal age increased, so did sensory-panel scores for amount of connective tissue after chewing. Detectable connective tissue was defined as the amount of insoluble connective tissue that remained after thorough chewing. Thus, this parameter measured the amount of relatively indestructible muscle components.

The samples from carcasses of older animals were more juicy ($P < 0.05$) than samples from carcasses of young bulls and steers, but there was no significant difference ($P > 0.05$) between steaks from bulls and steers (Table 1). Reagan et al. (1976) reported that percentage moisture did not correlate with sensory panel evaluations of juiciness, but collagen content of samples was related to panel scores of juiciness. When the collagen content rose, so did panel juiciness scores. Likewise, in our study, steaks from the most mature animals (cows) had more detectable connective tissue and also were most juicy. This may be explained by the fact that those samples containing the most detectable connective tissue would require more mastication and thus more salivation, thereby increasing perceived juiciness.

For all ages and sexes of cattle, steaks aged 3 days postmortem had the least ($P < 0.05$) softness to tooth pressure and the most ($P < 0.05$) resistance to fiber fragmentation (Table 2). Conversely, steaks aged 28 days were softer ($P < 0.05$) and easier to fragment than steaks from the other three aging

Table 2—Means of sensory scores for loin steaks for four postmortem aging periods over all sexes and ages

Days postmortem	Softness to tooth pressure[a]	Fiber fragmentation[b]	Mealiness[c]	Residue[d]	Juiciness[e]
3	99.2[f]	97.7[f]	120.9[f]	110 5[f]	99.2[f]
7	109.7[g]	107.9[g]	123.5[f]	120.3[g]	150.0[g]
14	110.1[g]	109.7[g]	114.1[g]	119.5[g]	94.4[h]
28	121.1[h]	121.7[h]	115.9[g]	127.9[h]	103.1[g]
SEM[i]	0.79	0.86	0.73	0.81	0.81

[a] Possible range of scores: 0 = very hard to 150 = very soft.
[b] Possible range of scores: 0 = very difficult to 150 = very easy.
[c] Possible range of scores: 0 = very mealy to 150 = none.
[d] Possible range of scores: 0 = large amount to 150 = none.
[e] Possible range of scores: 0 = very dry to 150 = very juicy.
[f-h] Means with different superscripts within the same column are significantly different ($P < 0.05$).
[i] SEM is the standard error of the mean.

Table 3—Means for Warner-Bratzler shear-force values for loin steaks from different sex and age classifications over all postmortem aging times

Classification	Compression (kg/cm²)	Residual (kg/cm²)
Cow	3.3[a]	3.2[a]
Bull	2.7[b]	2.6[b]
Steer	2.5[b]	2.3[b]
SEM[c]	0.03	0.04

[a-b] Means with different superscripts within the same column are significantly different ($P < 0.05$).
[c] SEM is the standard error of the mean.

periods. Panelists reported no differences ($P > 0.05$) between steaks aged 7 and 14 days postmortem (over all ages and sexes) for softness to tooth pressure and fiber fragmentation. These results further support the concept of myofibrillar fragmentation tenderness (MacBride and Parrish, 1977). As postmortem aging time increased, myofibrils became fragmented as the Z-disks became more degraded (Parrish et al., 1973; Olson et al., 1976). Parrish et al. (1973) suggested that the reduced size of myofibrils brought about by fragmentation caused enough reduction in force to cut through the sample that the force reduction could be detected by panelists.

The steaks aged 3 and 7 days were less mealy ($P < 0.05$) than those aged 14 and 28 days over all sexes and ages. Again, as this parameter describes the amount of very small pieces of meat detected, it may be an indication of breakdown of myofibrils into smaller pieces due to increased fragmentation over longer postmortem aging.

Steaks aged 3 days postmortem had most ($P < 0.05$) detectable connective tissue residue, and those aged 28 days postmortem had the least ($P < 0.05$). Stanton and Light (1987) showed they could extract greater amounts of collagen or its fragments from muscles aged 14 days than from unaged muscles, suggesting that limited proteolysis of muscle collagen may occur upon postmortem aging. Whether our panelists detected a decrease in tensile strength of collagen or were responding to a general increase in tenderness over time could not be determined. No consistent trend for juiciness was observed. Juiciness was scored highest ($P < 0.05$) for steaks aged 7 and 28 days postmortem, whereas steaks aged 14 days postmortem were scored lowest ($P < 0.05$) for this attribute.

Shear-force results

The Warner-Bratzler shear-force results revealed that samples from carcasses of more mature animals (cows) had greater ($P < 0.05$) compression (initial yield) and residual shear (final yield) force values than did samples from young bull and steer carcasses (Table 3). However, the compression and residual shear-force values from steaks from young bull and steer carcasses were not different ($P > 0.05$), indicating essentially no differences in tenderness between the two sexes. Effects of maturity seemed more important than differences caused by sex condition, in agreement with results reported by Prost et al. (1975). They found that sex of the animal (bulls vs. heifers and steers) did not affect tenderness from sensory panel or shear-force tests. Age of animal had an effect, however, with steaks from older animals being less tender than those from younger animals.

Table 4—Means for Warner-Bratzler shear-force values for loin steaks from four postmortem aging periods over all sexes and ages

Days Postmortem	Compression (kg/cm²)	Residual (kg/cm²)
3	3.1[a]	2.8
7	2.9[b]	2.7
14	2.8[c]	2.8
28	2.3[d]	2.2
SEM[e]	0.03	0.04

[a-d] Means with different superscripts within the same column are significantly different ($P < 0.05$).
[e] SEM is the standard error of the mean.

Analysis of shear-force values for postmortem aging (Table 4) showed days of postmortem aging (over all ages and sexes) was significant ($P < 0.05$) in the model for the compression (initial yield). However, days of postmortem aging (over all ages and sexes) was not significant ($P > 0.05$) in the model for residual (final yield) values. A comparison of means for compression values indicated all four postmortem aging periods were ($P < 0.05$) different from each other, with 3-day postmortem aging requiring the greatest amount of force. Values for the other 3 periods (7, 14, and 28 days postmortem) showed a downward decline ($P < 0.05$) through time in the amount of force required to produce the initial yield value. This was consistent with results of Jennings et al. (1978) who showed that tenderness of beef samples continued to increase through time.

The compression (initial yield) values decreased steadily as time postmortem increased, a response that could be expected if the myofibrillar component underwent structural disruption with increasing time as several researchers have reported (Schmidt and Parrish, 1971; Davey and Gilbert, 1967; Olson et al. 1977). In addition, days of postmortem aging were not significant with respect to residual shear-force values. This may support the hypothesis that the residual shear-force value relate to the connective tissue component of tenderness suggested by Møller (1981). This suggests that division of the deformation curve in the manner described could aid in prediction of whether myofibrillar components or connective tissue components are more important for determining tenderness. The response of connective tissue to aging is generally theorized to be less than that of the myofibrillar component (Bailey and Light, 1989). The decreased response in that parameter seen in our study may be reflected in the lack of difference in residual values of samples between the four postmortem aging times. These results further indicate that connective tissue had less to do with postmortem tenderization than did myofibrillar tenderization that occurred as myofibrils became more fragmented with increasing time postmortem (Olson et al., 1976; Olson and Parrish, 1977). No significant interactions were observed between animal age and postmortem aging time. There were no significant interactions found between sex (intact males vs castrates) and postmortem aging time for either sensory-panel results or shear-force results.

Separating trained sensory panel descriptors into several categories and more selectively analyzing curves from shear-force measures may help determine whether differences in tenderness between types of samples (very old animals vs young animals, or aged vs unaged) are due to muscle fiber characteristics or connective tissue characteristics. Animal age and postmortem aging time had more influence on tenderness attributes than did sex of the animal. More importantly, increased postmortem aging time improved myofibrillar tenderness attributes regardless of sex or age of animal. This improved

tenderness as postmortem aging time increased supports the concept of myofibrillar fragmentation tenderness of beef steaks.

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NAGase ACTIVITY IN BEEF, PORK AND TURKEY MUSCLE. . .From page 712

Since no NAGase activity was present at about 79–80°C, it could possibly be useful for determining adequacy of heat treatment of imported cooked beef (which must be heat processed to 79.4°C).

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Low-dose Gamma Irradiation and Refrigerated Storage *in vacuo* Affect Microbial Flora of Fresh Pork

D. W. THAYER, G. BOYD, and R. K. JENKINS

ABSTRACT

Vacuum-packaged ground fresh pork samples absorbed gamma radiation doses of 0, 0.57, 1.91, 3.76, 5.52, or 7.25 kGy at 2°C. Samples were analyzed after 1, 7, 14, 21, 28, or 35 days storage at 2°C for presence and number of aerobic and anaerobic mesophiles and endospore formers, and aerobic psychrotrophs. Conventional plate counts did not detect surviving microflora in any sample that received an absorbed dose of 1.91 kGy or higher, even after refrigerated storage for up to 35 days. The microflora in the control were predominantly Gram-positive for the first 21 days; however, *Serratia* predominated at 28 and 35 days. *Staphylococcus, Micrococcus,* and yeast species predominated in samples that received 0.57 kGy.

Key Words: pork, vacuum storage, low dose gamma irradiation

INTRODUCTION

THE U.S. Food & Drug Administration (FDA) approved the treatment of pork meat and products with a minimum dose of 0.3 kGy and a maximum of 1.0 kGy of ionizing radiation to control *Trichinella spiralis* (Anon., 1985). The USDA Food Safety and Inspection Service likewise approved the processing regulation (Anon., 1986). Although doses of ionizing radiation below 1 kGy are adequate to control helminths, they are not generally considered adequate to control many food-borne pathogens or to provide notable extension of shelf life. Government and industry have indicated interest in ionizing radiation doses in excess of 1.0 kGy but less than 10 kGy to increase shelf life and improve safety of products for consumers. Two important concerns remained about the effects of a 1 to 10 kGy treatment of meats: the effect on nutrients and the impact of such processing on microflora especially when the product was vacuum packaged.

Mattison et al. (1986) studied the effects of a 1 kGy gamma radiation dose at ≈ 0°C on microflora, sensory characteristics, and fat stability of vacuum-packaged pork loin. Microbial evaluations were performed at 2, 7, 14, and 21 days on samples stored at 4°C. Radiation treatment reduced the numbers of mesophiles, psychrotrophs, anaerobic bacteria, and staphylococci. Differences between irradiated and nonirradiated populations tended to increase during refrigerated storage. They reported clostridial counts were significantly lower for irradiated pork than for nonirradiated pork.

Ehioba et al., (1987, 1988) characterized bacterial cultures from vacuum-packaged irradiated and nonirradiated ground pork stored up to 12 days at 5°C. Treating the meat with radiation decreased the numbers of mesophiles, psychrotrophs, and anaerobes and extended the shelf life by 2–3 days (from 8 to 11.5 days). Initial microflora gradually shifted from Gram-negative to Gram-positive in nonirradiated samples. In contrast, the majority of microflora in irradiated (1 kGy) samples were Gram-positive shortly after irradiation and increased to 97% after 9 days at 5°C. On the day of preparation *Pseudomonas* and *Enterobacter* species predominated in nonirradiated meat. After 12 days refrigerated storage *Lactobacillus* and coryneform bacteria predominated in the irradiated meat.

Lebepe et al., (1990) studied the effects of a 3 kGy radiation dose on microflora and other characteristics of fresh, vacuum-packaged pork loins stored at 2°C up to 98 days. Treating fresh pork with 3 kGy reduced populations of aerobic, psychrotrophic, and mesophilic bacteria. The anaerobic and facultative anaerobic mesophiles were below detection level. Surviving populations increased during the next 40 days of refrigerated storage at 2–4°C. Gram-positive isolates predominated in the irradiated samples. *Hafnia* and *Serratia* were the most prevalent genera among the *Enterobacteriaceae* in nonirradiated samples. *Staphylococcus aureus* was detected consistently in nonirradiated samples only. The microbial shelf life of vacuum-packaged pork was extended from 41 days to 90 days by a 3 kGy radiation dose.

Jenkins et al. (1989) investigated the effects of radiation dose, cooking, and post-irradiation storage on thiamin content of ground pork loin and microbial flora of raw meat. Results of treatments on thiamin content were reported (Jenkins et al., 1989). The results of microbiological analyses of the irradiated samples are presented here. The objectives of these studies were to determine the effects of gamma radiation dose (0, 0.57, 1.91, 3.76, 5.52, and 7.25 kGy at 2 ± 2°C) and storage time of treated samples *in vacuo* at 2°C on the microbial populations and to determine if the treatments caused shifts in composition of those populations. The 0.57 kGy dose was selected to represent pork irradiated to control *Trichinella spiralis* since the minimum dose would be 0.30 kGy and the maximum dose 1.0 kGy. The higher doses would be more appropriate to control food-borne pathogens and to extend shelf life of irradiated pork.

MATERIALS AND METHODS

Experimental design

The experimental design included 6 gamma radiation doses and 6 storage periods of vacuum-packaged ground fresh pork for a total of 36 treatments. Each treatment was performed in triplicate for a total of 108 independent samples.

Sample preparation and packaging

Samples were prepared and packaged as described previously (Jenkins et al., 1989). Briefly, samples (15 g each) of lean ground pork from two matched pairs of pork loins, obtained 2 days post-mortem, were placed in 13 cm × 18 cm plastic barrier pouches (All-Vak #13; International Kenfield Dist. Co., Rosemont, IL). The pouches had a food contact layer of polyethylene and an oxygen permeability of 15.5 $cm^3/m^2/24$ hr at 25°C. The pouches were vacuum sealed to a dial reading of −690 mm Hg. All samples were refrigerated at 2 ± 2°C.

Irradiation and storage

Samples were irradiated at 0.129 kGy/min using a self-contained cesium-137 gamma source (Shieh et al., 1985). The temperature was maintained at 2 ± 2°C during irradiation. Absorbed dose measurements were made using ferrous-cupric sulfate dosimetry (Jarrett and Halliday, 1979). Variation between maximum and minimum calculated doses was ≈ 4.6% The measured treatment doses were 0, 0.57,

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Table 1—Storage at 2°C and/or gamma radiation effects on the microbial flora of ground pork

Colony type	Dose kGy	Log number of colony forming units ± SD[a]					
		0	7	14	21	28	35 days
Aerobic	0	2.76 ± 0.06	2.35 ± 0.08	2.81 ± 0.83	2.30 ± 0.44	4.11 ± 0.04	6.50 ± 0.35
Mesophile	0.57	2.50 ± 0.15	2.30 ± 0.19	1.95 ± 0.62	2.97 ± 0.80	5.15 ± 0.41	3.88 ± 0.50[d]
Anaerobic	0	0.91 ± 0.55	2.09 ± 0.15	2.79 ± 0.83	2.85 ± 0.75	4.07 ± 0.28	5.38 ± 1.12
Mesophile[b]	0.57	2.22 ± 0[d]	1.76 ± 0.49	1.73 ± 0.77	3.36 ± 0.18	5.00 ± 0.28	2.61 ± 2.24
Growth at	0	2.05 ± 0.24	2.27 ± 0.05	3.04 ± 0.52	3.56 ± 0.38	3.84 ± 0.07	6.79 ± 0.10
10°C	0.57	ND[c]	ND[c]	3.26 ± 0.08	4.28 ± 0.84	5.32 ± 0.35[d]	7.44 ± 0.10[d]

[a] SD represents standard deviation of means.
[b] Anaerobic mesophile include facultative organisms.
[c] ND represents not detected (< 10 CFU/g).
[d] Indicates that the value for the irradiated sample differs significantly ($P < 0.05$) from that for the nonirradiated sample in the same data set.

Table 2—Distribution of microbial types on nonirradiated and irradiated (0.57 kGy) vacuum-packaged pork stored at 2°C for 1 to 35 days

Storage (Days)	kGy	Percentage distribution of microflora[a]								
		Bacillus	Entero-bactor	Micrococcus	Pseudo-monas	Serratia	Staphylo-coccus	Strepto-coccus	Yeast	Unknown
1	0	8					64	14		14
1	0.57			18			82			
7	0			7			69		17	7
7	0.57			14			78		4	4
14	0			15			59		11	15
14	0.57			4			79		17	
21	0		11			29	57			4
21	0.57			9			30		61	
28	0					27	10		30	33
28	0.57			3			7		86	3
35	0				8	83				8
35	0.57						94			6

[a] Pooled analysis of isolates from three replicate samples per treatment.

1.91, 3.76, 5.52, and 7.25 kGy. The samples were stored in the dark at 2 ± 2°C for the required storage periods of 1 day, 1, 2, 3, 4, and 5 wk post irradiation.

Microbiological analysis

At time of withdrawal from refrigerated storage, sub-samples of 5.0 g of the ground pork were removed aseptically from each pouch for microbiological analysis. The remaining portion was retained for vitamin and sensory analyses, as reported (Jenkins et al., 1989). Each sample was vacuum sealed within an All-Vac #13 barrier pouch, frozen rapidly at −50°C and stored frozen until analysis. Preliminary experiments with commercial ground pork revealed less than one log reduction in microbial population of small amounts of meat frozen rapidly in this manner. (The means of five determinations of logarithms of the number of colony forming units (cfu) of aerobic mesophilic bacteria of a commercial ground pork loin before and after freezing were 5.91 ± 0.06 and 5.93 ± 0.02, respectively.) Each sample pouch was opened aseptically and the sample macerated for 90 sec with a Stomacher 400 (Tekmar Co., Cincinnati, OH) in enough 0.1% peptone water (Difco) for a dilution of 1/10. Appropriate serial dilutions were prepared in 0.1% peptone water. Aerobic mesophilic cfu (35°C/96 hr), anaerobic (or facultative) mesophilic CFU (35°C/96 hr) and aerobic microorganisms capable of growth at 10°C (120 hr) were counted after growth in Tryptic soy agar (TSA, Difco, Detroit, MI) pour plates. Aerobic or anaerobic (or facultative) endospore forming cfu were enumerated using TSA spread plates prepared with heat-shocked 1 to 10 dilutions of stomachates (97°C/15 sec); these were incubated for 96 hr at 35°C. Anaerobic conditions were established where necessary with a BBL Microbiology Systems Gas Pack Plus (Cockeysville, MD). The CFU on three petri plates giving 30 to 300 colonies were counted using a New Brunswick Scientific Biotran II automated colony counter (New Brunswick Scientific Co., Inc., Edison, NJ 08818) and averaged.

Aerobic mesophilic colonies (≈35) were selected from the same areas on replicate TSA plates from each treatment. All were recloned on TSA at 35°C and stored on TSA slants at 5°C. A gram stain was made when the slant was streaked, and results were used in selection of appropriate identification methods. About 200 isolates were analyzed taxonomically using the Vitek AMS Automicrobic System (bioMéricux Vitek, Inc., USA, Hazelwood, MO), (Knight et al., 1990); API 20E System (API Analtab Products, Plainview, NY), (Lennette et al., 1985); API Staph Trac System (API Analtab Products, Plainview, NY), (Gemmell and Dawson, 1982); and API Rapid Strep System (API Analtab Products, Plainview, NY), (Tillotson, 1982). Yeasts were identified from morphology. No attempt was made to identify yeast to genus. Many colonies were pink. Plate-count data were transformed into logarithms, and all data were analyzed by using the Statistical Analysis System (SAS Institute, Inc., 1985).

RESULTS

No CFU were observed in plate counts of any sample that received a radiation dose of ≥ 1.91 kGy. Populations of aerobic mesophilic bacteria in control nonirradiated samples were not significantly ($p > 0.05$) different from those in samples that received a radiation dose of 0.57 kGy, and population increases were not apparent during refrigerated storage until after 21 days (Table 1). Populations of anaerobic of facultative mesophilic bacteria in control samples did not differ ($p > 0.05$) from those in samples that received a radiation dose of 0.57 kGy (Table 1). Populations of the anaerobic or facultative mesophilic bacteria of the samples increased during the first 28 days refrigerated storage (Table 1). Aerobic microorganisms capable of growth at 10°C were not detected in irradiated samples at 1 and 7 days refrigerated storage (Table 1). Populations of aerobic microorganisms that multiplied at 10°C increased in both irradiated and nonirradiated samples from 14 days through 35 days refrigerated storage. The population of the microorganisms that multiplied at 10°C was higher ($p < 0.05$) at 28 days and 35 days of refrigerated storage in irradiated than in nonirradiated samples. In nonirradiated samples, 25 aerobic and 10 anaerobic or facultative cfu of endospore former gram were detected after 1 day refrigerated storage. None was detected in samples stored 7, 14, 21, and 28 days. After 35 days refrigerated storage, aerobic and anaerobic or facultative endospore forming bacterial counts of nonirradiated meat samples were 1 CFU/g each. In the irradiated (0.57 kGy) meat samples aerobic endospore formers (3/g) were detected only in samples stored 7 days.

Bacillus cereus, B. coagulans, and *B. macerans* were identified from colonies picked from the nonirradiated samples at 1 day (Table 2). No *Bacillus* isolates were found in any other sample. *Enterobacter agglomerans* was identified among selected isolates only in the nonirradiated samples after storage 21 days (Table 2). All eleven isolates had identical taxonomic

characteristics. *Micrococcus* isolates from several samples were identified to the genus level (Table 2). No clear pattern to the isolations was identified. *M. roseus* and *M. varians* were identified. The remainder of the isolates were not identified to species. *Pseudomonas fluorescens* and *P. stutzeri* were identified among the isolates (Table 2) from the nonirradiated samples that had been stored for 35 days. *Serratia liquefaciens* became an important component of the total population in nonirradiated samples stored 21 and 28 days and the predominant isolate from samples stored 35 days (Table 2). *Serratia* was not identified among the isolates from irradiated samples.

Staphylococcus species were frequently identified among the isolates from both irradiated and nonirradiated pork meat during the first 21 days of refrigerated storage (Table 2). Twelve *Staphylococcus* species were identified. The ratios of *S. aureus* to total *Staphylococcus* isolates from nonirradiated refrigerated samples were as follows: 0/23, 4/20, 1/16, 0/28, 0/3, and 0/0 after 1, 7, 14, 21, 28, and 35 days refrigerated storage, respectively. The ratios of *S. aureus* to total *Staphylococcus* isolates from the irradiated refrigerated samples were as follows: 3/12, 1/21, 1/13, 0/7, 0/2, and 0/17 after 1, 7, 14, 21, 28, and 35 days refrigerated storage, respectively. The following species were identified: *S. aureus, S. auricularis, S. epidermidis, S. capitis, S. haemolyticus, S. hominis, S. warneri, S. sciuri, S. lentus, S. saprophyticus,* and *S. xylosus.* Several isolates were not identified to species. Four *Streptococcus agalactiae* isolates and one *Enterococcus faecalis* isolate were obtained from the nonirradiated pork at 1 day (Table 2).

DISCUSSION

OUR RESULTS were unexpected in that no surviving microflora were detected in samples exposed to gamma radiation doses >0.57 kGy even after storage at 2°C up to 35 days. Perhaps by chance, the composition and size of the population of microflora of these pork samples were such that none could survive a dose of 1.91 kGy or higher. The initial contamination level of this meat was very low ($<10^3$/g) compared with a commercial ground pork loin. The number of viable vegetative cells would be substantially reduced by a dose of 1.91 kGy. The very small number of bacterial endospores detected in the nonirradiated samples, though substantially more resistant than vegetative cells (D-value for *Clostridium botulinum* in meat at 25°C is ≈ 3.6 kGy), would also be reduced in number. Thayer et al., (1992) found no difference in the response of *Salmonella typhimurium* to gamma radiation when present at 10 or 100 CFU/chicken wing and only small differences when present at 1,000 or 10,000 cfu/wing. Such results might be expected for meat obtained 2 days postmortem from a local abattoir and then carefully prepared in a research facility with due precautions to prevent contamination. Two factors nevertheless make this study relevant when evaluating effects of gamma radiation treatments for the control of trichina. First, the applied radiation dose of 0.57 kGy more closely approximated that which would be used by industry than would studies with an average dose of 1.0 kGy. Second, both the irradiation temperature and absorbed dose were controlled within very narrow limits in our study. Regulations state that maximum dose must not exceed 1.0 kGy, and minimum dose must exceed 0.3 kGy. Since the processor would be working with commercial-sized samples, some parts of the product would approach 1.0 kGy; and some may receive only slightly more than 0.3 kGy. The variation between the minimum and maximum calculated doses in our study was 4.6%. Rather small variations in irradiation temperature (i.e., 10°C) may have significant effects on survival of some food-borne pathogens, such as salmonellae (Thayer and Boyd, 1991).

The predominant genera of the microflora in both the nonirradiated and irradiated (0.57 kGy) meat were Gram-positive at the beginning of the study, as were samples analyzed at 7 and 14 days. The nonirradiated samples opened on the 21st day of refrigerated storage had significant populations of *Serratia,* as did those opened on the 28th day. Gram-positive genera, however, still predominated. *Serratia* represented 83% of all aerobic mesophilic isolates from nonirradiated samples stored *in vacuo* at 2°C for 35 days. Ehioba et al. (1988) reported mainly Gram-positive organisms in irradiated vacuum-packaged ground pork immediately following a dose of 1 kGy and during 12 days of refrigerated storage, as did we. However, they did not store samples beyond 14 days. Our results differed from those of Ehioba et al. (1988) in that *Staphylococcus* represented a major portion of the microflora in the irradiated (0.57 kGy) samples at each sampling period, whereas they found a predominance of *Lactobacillus* spp. and coryneform bacteria. These results could be simply the results of chance. However, Thayer and Boyd (1992) reported that 90% of *S. aureus* CFU in mechanically deboned chicken meat were killed by a dose of 0.36 kGy. Since the total population of aerobic mesophiles did not exceed 10^3 CFU/g at the beginning of our study, possibly the small population of *Staphylococcus* could have been eliminated by a radiation dose of 1.9 kGy and not by 0.57 kGy. The absence of competitive species may have allowed its growth. *S. aureus* was isolated only during the first 2 wk refrigerated storage of the pork. Its presence could be a food safety concern. However, neither *S. aureus* nor any other microorganism was found in the pork that had received a gamma radiation dose of 1.91 kGy or higher.

We did not observe a marked reduction in total population of aerobic mesophiles with a dose of 0.57 kGy, but did in the populations of anaerobic or facultative mesophiles and bacteria capable of growth at 10°C. With 10^7 CFU/g of aerobic mesophiles as an indicator of bacterial spoilage, then none of the samples including the nonirradiated control spoiled during the 35 days storage at 2°C. Conventional plate counts did not detect surviving cfu in the ground pork following treatments of 1.91 kGy and higher. Consequently, those samples would be expected to have greater shelf life. Because neither enrichment procedures nor abuse tests were performed, we cannot state that there were no microbial survivors at doses of 1.91 kGy or higher. It is also possible that addition of pyruvate or other resuscitative agents to the assay media might have altered results. However, preliminary experiments did not indicate any advantage from addition of such agents. Niemand et al. (1981) reported that a dose of 2 kGy almost doubled the shelf life of fresh meat. Our results do not contradict their conclusion but afford no clear support since the nonirradiated control did not reach a level (10^7 cfu/g) of aerobic mesophilic microflora indicative of spoilage within the 35 day period.

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Functional Properties of Myofibrillar Proteins from Cold-Shortened and Thaw-Rigor Bovine Muscles

YOULING L. XIONG and SUZANNE P. BLANCHARD

ABSTRACT

Prerigor bovine sternomandibularis muscles were stored at 15, 0 and -29°C to examine cold-shortening and thaw-rigor effects on myofibrillar protein extractability and gelation properties of myofibrils and salt-soluble protein (SSP). Frozen muscle that underwent severe contraction at thawing showed greater protein extractability (35%) than muscles stored at 0 and 15°C (27% extractability). Of the three tempered muscles, thaw-rigor muscle produced the strongest myofibril gel and cold-shortened muscle formed the most elastic SSP gel as determined by dynamic shear and penetration measurements. However, thermally induced changes in gel viscoelastic moduli for all protein samples followed similar patterns. Results indicated that physicochemical changes accompanying muscle contraction affected protein network formation during gelation.

Key Words: bovine, myofibrils, cold-shortened, thaw-rigor, protein

INTRODUCTION

MUSCLE cold-shortening and thaw-rigor are associated with low-temperature postmortem storage of prerigor meat. Cold-shortening occurs when muscle is rapidly chilled immediately after slaughter (Locker and Hagyard, 1963), while thaw-rigor results from thawing muscle that was frozen prerigor (Marsh and Thompson, 1958). The term "thaw-shortening" is also used to describe thaw-rigor muscle when muscle fiber shortens substantially during thawing or rigor development (Davey and Gilbert, 1973). However, shortening can be largely eliminated if the prerigor muscle is stored at ≈ 15°C (Locker and Hagyard, 1963; Honikel et al., 1983). It has been suggested that at low temperatures, or during thawing, muscle undergoes severe contraction resulting from the inability of the sarcotubular and mitochondrial systems to restore the released calcium ions (Bendall, 1960; Pearson et al., 1973; Buege and Marsh, 1975). Such muscle contraction is most severe for red meat (e.g., beef, lamb and chicken thigh) that contains a high percentage of red fibers (Buege and Marsh, 1975; Newbold, 1979; Cornforth et al., 1980).

Meat subjected to cold-shortening and thaw-rigor showed increased toughness and dripping loss (Davey and Gilbert, 1973; Smulders et al., 1990). For muscle that is unrestrained (e.g., hot-boned meat), prerigor chilling presents a particularly adverse effect. However, for microbiological and safety reasons (which have received much attention), it is necessary to rapidly chill meat to refrigerator temperatures after slaughter. Thus, techniques such as electrical stimulation (Carse, 1973), mechanical tenderization and pressurization (Macfarlane, 1973, 1976), carcass posture and delayed freezing (Davey and Gilbert, 1973), have been suggested to minimize cold-shortening or thaw-rigor. All such techniques have been partially effective, but none has enabled complete elimination of the shortening problem.

In spite of much research on fresh meat toughness in relation to cold-temperature storage and freeze-thaw, little is known about the shortening effect on textural attributes and protein functionality of further processed meat products. For example, the extractability of myofibrillar protein and the ability of the protein to form gels during cooking affect meat binding and the texture of processed meat products (Acton et al., 1983). Information on the physical and biochemical changes in cold-shortened and thaw-rigor muscle as related to protein functional characteristics is limited. A few reports exist on the quality of comminuted and processed products made from cold-shortened and thaw-rigor meat (Honikel and Fischer, 1980; Honikel et al., 1980). These showed that sausages prepared from cold- or thaw-contracted muscle tended to have softer texture and less consistency. However, softening mechanisms were not determined. The objectives of our study were to assess the impact of rapid chilling and freeze-thaw of prerigor muscle on the extractability and gelation properties of myofibrillar proteins. We also examined the relationships between gel strength and certain physical/biochemical changes occuring during postmortem storage.

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MATERIALS & METHODS

Muscle sample preparation

Seven Brangus steers (223 ± 21 kg carcass weight) were slaughtered at the University of Kentucky Meat Laboratory. Sternomandibularis muscles from the left and right sides of the neck were excised 30–40 min post-mortem, trimmed of excessive external fat, and each cut along the fibers into three strips of equal size. After the length was measured, muscle strips were wrapped loosely in plastic film. Two strips from each animal were placed separately in plastic pouches and incubated at 0 (crushed ice) and 15°C for 22 hr. The third strip of muscle sample was blast-frozen at -29°C and stored in a freezer (-29°C) up to 30 days before use.

Thaw and muscle shortening measurement

Frozen muscle samples were placed in sealed plastic bags and thawed by submersing in a 20°C waterbath 5 hr, followed by storage 2°C for 17 hr. The length of each sample (muscle strip) was remeasured after incubation, and compared to the original length to determine the degree of shortening. Muscle sarcomere lengths were not determined. Percent shortening was defined as the net reduction in muscle length (cm) after storage divided by the original muscle length (cm) × 100.

pH measurement

Muscle pH was determined in triplicate within each replicated experiment. Finely minced muscle sample (3g) was mixed with 30 mL of 0.1M NaCl solution. The slurries were homogenized for 15 sec using a PT 10/30 Polytron homogenizer equipped with a low-foam PTA 20TS generator (Brinkmann Instruments Inc., Westbury, NY) at speed set 6. The pH of the homogenates was determined immediately thereafter at 23°C using a combination probe connected to a Model 110 Ohaus pH meter (Ohaus Corp., Florham Park, NJ).

Preparation of myofibrils and salt-soluble protein (SSP)

Myofibrils and SSP were prepared from pooled sternomandibularis muscles of both sides of the carcass as described previously (Xiong, 1992). Each preparation was performed using muscles excised from

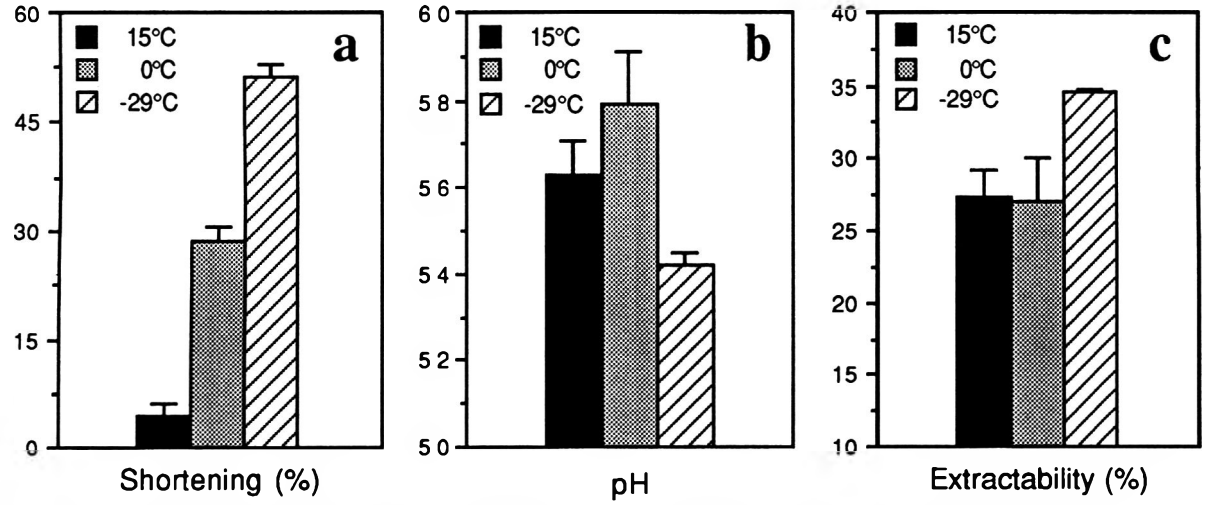

Fig 1—Degree of contraction (a), final pH (b) and myofibrillar protein extractability (c) of cold-shortened (0°C), thaw-rigor (-29°C), and control (15°C) bovine sternomandibularis muscles. Bars represent means of seven replicates with standard errors.

a single animal, and all steps were at 2–4°C. SSP was extracted from purified myofibrils (10 mg/mL, suspended in 0.6M NaCl and 50 mM sodium phosphate at pH 6.0) by centrifuging at 15,000 X *g* for 15 min, and recovered by diluting the supernatants to an ionic strength of 0.1 and subsequent centifugation at 10,000 X *g* for 15 min. Protein concentration was determined by the biuret method (Gornall et al., 1949) using bovine serum albumin (Sigma Chemical Co., St. Louis, MO) as standard.

Protein extractability

The method outlined by Xiong and Brekke (1989) was followed to determine protein extractability from the isolated myofibrils.

SDS-polyacrylamide gel electrophoresis (SDS-PAGE)

SDS-PAGE was carried out as described by Laemmli (1970) using an SE 250 Mighty Small II slab gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, CA). The stacking gel and the resolving gel were 3% and 10% acrylamide, respectively.

Dynamic viscoelastic measurements

Myofibrils and SSP were suspended to 2% (w/v) protein concentration in 50mM sodium phosphate buffer containing 0.6M NaCl (final) at pH 6.0. Dynamic oscillatory measurements of protein suspensions during gelation were performed using a Bohlin VOR rheometer (Bohlin Reologi, Inc., Cranbury, NJ). Samples were loaded in the 1-mm gap between two parallel plates (upper plate diameter 3.0 cm). The exposed sample perimeter was covered with a thin layer of silicon oil to prevent dehydration. The gelling unit (both plates and protein sample) was enclosed in two temperature-insulated shells to minimize heat loss. After equilibration at the initial temperature of 20°C for 5 min, the sample was heated at 1°C/min to a final temperature 75°C, during which the sample was sheared at a fixed frequency of 0.1 Hz with a maximum strain of 0.02. The sample temperature was verified using a thermocouple connected to the surface of the lower plate.

Rheological data were collected after each 30 sec during the course of gel formation (sol → gel). The complex modulus G*, calculated by the supplied software from the measured storage modulus G' (an elastic element which was a measure of the energy stored and recovered) and loss modulus G" (a viscous element which was a measure of the heat dissipated), was recorded. G* was calculated using the equation: $G^* = (G'^2 + G''^2)^{0.5}$.

Gel penetration test

The procedures described previously (Xiong, 1992) were followed to prepare myofibril and SSP gels (2% protein in 0.6M NaCl, 50mM sodium phosphate, pH 6.0), and an empirical penetration test was used to determine the ultimate strength of the thermoset gels. The force required to disrupt the gel (force at the first peak) by a probe attached to the Instron universal testing instrument (Model 1122, Instron Corp., Cantor, MA) was used to describe gel strength.

Statistical analysis

Measurements of muscle shortening, pH, and protein extractability were replicated 7 times, and gelation and gel property measurements were replicated three times. A replication was defined as a complete set of experiments using muscle samples from a single animal on a different day. All measurements were performed at least in duplicate. Analysis of variance was performed on all data using General Linear Models procedures of the Statistix 3.5 program (Analytical Software Inc., St. Paul, MN). Least significance difference test (LSD) was performed to determine significant differences between means (Snedecor and Cochran, 1989).

RESULTS & DISCUSSION

THE DEGREE of muscle shortening at varying temperatures (Fig 1a) showed muscle that was frozen prerigor and then thawed was stiff (rigor) and had a drastic reduction in length (51%), indicating severe fiber contraction during thawing. At 0°C, relatively mild shortening (29%) was induced, and at the "control" temperature of 15°C very little shortening was observed (5%). These results confirmed previous findings on beef muscle (Locker & Hagyard, 1963; Macfarlane et al., 1976) and verified that muscle temperature treatments were properly performed to represent "cold-shortening" and "thaw-rigor" for subsequent protein preparations. Although muscle stored at 0°C shortened much more than that at control temperature, the ultimate pH of both muscle samples was similar ($P > 0.05$)(Fig 1b). However, thaw-rigor muscle had a lower pH ($P < 0.05$) than cold-shortened and control muscles. This supported the findings of Macfarlane (1973) and suggested that the glycolytic events and, possibly, the breakdown of some energetic nucleotides during the thaw-induced muscle supercontraction differed from those at 0 or 15°C.

The influence of prerigor storage temperature on protein extractability (Fig 1c) showed myofibrillar protein in thaw-shortened muscle was more ($P < 0.05$) extractable (35% extractability) than cold-shortened or control samples (both 27% extractabilty). This seemed to contradict the hypothesis that protein extractability is high for more relaxed muscle and low for contracted muscle in which myosin and actin could be highly crosslinked. However, based on evidence that thaw-shortening could severely damage the structure of muscle fibers (Okubanjo et al., 1975), we postulated that the improved protein extraction in thaw-shortened myofibrils resulted from

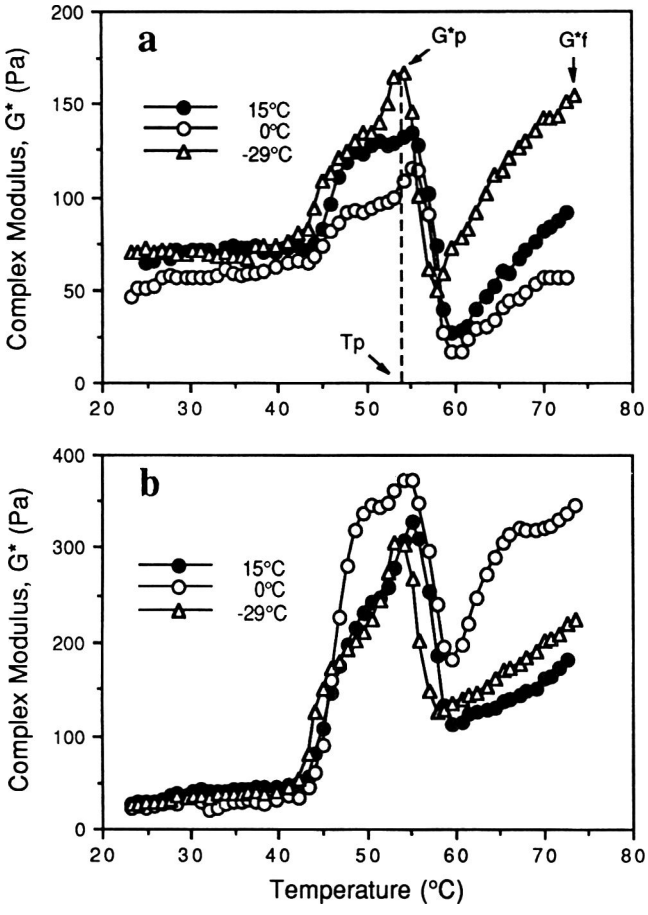

*Fig 2—Typical viscoelastic curves showing dynamic changes in the complex moduli of myofibril (a) and salt-soluble protein (b) suspensions of cold-shortened (0°C), thaw-rigor (-29°C), and control (15°C) bovine sternomandibularis muscles during thermal gelation. G*p is the complex modulus at transition peak at temperature Tp; G*f the complex modulus at the end of heating. Gelling solutions contained 2% (w/v) protein, 0.6M NaCl, 50 mM sodium phosphate, pH 6.0.*

Table 1—Dynamic rheological parameters of 2% gels made from myofibrils and salt-soluble proteins of contracted muscles[a]

	Myofibrils			Salt-soluble proteins		
Parameters[b]	15°C	0°C	−29°C	15°C	0°C	−29°C
G*p	145.3[ef]	125.7[f]	162.3[e]	358.8[cd]	381.3[c]	319.5[d]
G*f	111.7[ef]	86.7[f]	124.8[e]	223.8[d]	310.5[c]	235.3[d]
Tp	54.9[c]	55.2[c]	53.3[d]	55.3[c]	54.7[c]	53.0[d]

[a] Gels were formed in 0.6M NaCl, 50 mM sodium phosphate (pH 6.0) by heating from 20 to 75°C at 1°C/min; the three temperatures represent control (15°C), cold-shortening (0°C) and thaw-rigor (−29°C).
[b] G*p and G*f are defined in Fig. 2 and represent the complex moduli at the peak and at the end of the heating, respectively. Tp is temperature at peak.
[c-f] Means within the same row bearing no common superscript are significantly different at $P < 0.05$.

physical disruption and loosening of muscle fibers due to supercontraction.

Dynamic rheological measurements revealed considerable differences in viscoelastic properties of myofibrils and SSP of the three tempered muscle samples. The complex modulus G* (a measure of the total force or energy required to deform a sample) increased rapidly between 40 and 55°C for myofibrils of all three muscles (Fig 2a), suggesting the formation of a viscoelastic gel matrix. Above 55°C, G* declined drastically to a minimum at about 60°C, and then recovered at higher temperatures. Similar viscoelastic curves were reported by Egelandsdal et al. (1986) for myosin gels. The marked decrease in G* above 55°C was probably a result of protein

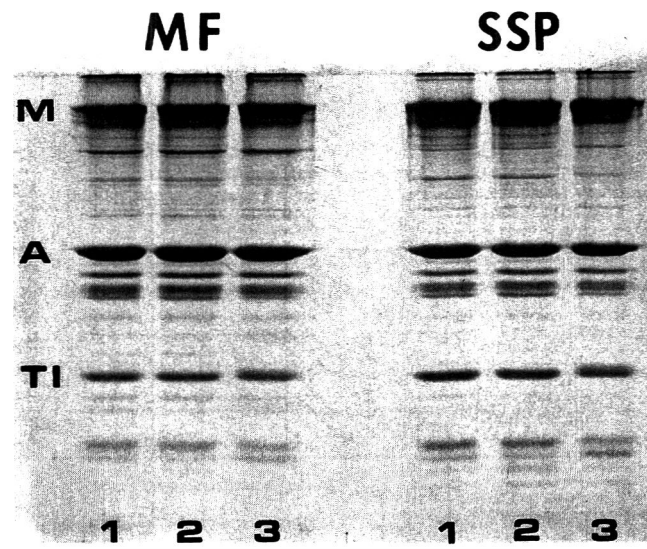

Fig 3—SDS-Polyacrylamide gel electrophoretic patterns of myofibrils (MF) and salt-soluble proteins (SSP). Lanes 1 = control, 2 = cold-shortened and 3 = thaw-rigor samples. M = myosin heavy chain; A = actin; TI = troponin-I. All electrophoretic samples contained 10% 2-mercaptoethanol, and 20 μg of protein was loaded in each lane.

conformational changes and dissociation of protein complexes that led to a rearrangement of intermolecular linkages. The peak temperature (Tp) of the thaw-rigor myofibril sample was less ($P < 0.05$) than that of the cold-shortened and control samples (Table 1). However, the G* values of the peak (G*p) and at the end of heating (G*f) were greater for the thaw-rigor samples. These results suggested that myofibrils from thaw-contracted muscle could form more rigid gels.

Electrophoretic analysis showed no appreciable differences between thaw-rigor and cold-shortened or control myofibril samples (Fig 3). Hence, the free-thaw process did not facilitate proteolytic degradation within the 22-hr storage. Furthermore, the electrophoretic patterns of all three myofibril samples were similar to those of myofibrils isolated from at-death muscle (not shown). Possibly the release of calcium due to thawing or rapid chilling did not differentiate the activation of calpain (a calcium-dependent muscle endogenous protease) on the basis of production of new polypeptides from myofibrillar components (Koohmaraie et al., 1988). Factors other than protein composition including protein extractability and possible protein conformational changes due to freeze-thaw damage, may have made major contributions to different viscoelastic behaviors between thaw-rigor myofibrils and cold-shortened or control samples. Hamm (1982) reported that sarcomeres of bovine sternomandibularis muscle shortened to about 1.0 and 0.7 μm for prerigor chilled (0°C) and thaw-rigor samples, respectively, from the original length of approximately 1.9 μm. The exact influence of sarcomere length on myofibril gelation, however, was not clear.

Changes in G* of SSP samples during dynamic aggregation and gelation were quite similar to those of the myofibril samples, although G* of SSP was about twice that of myofibrils (Fig 2b). Note that the pre-gelling protein solutions of all 3 muscles had the same "apparent viscosity" (calculated from G′ and G″, results not shown), which partially supported early findings by Chaudhry et al. (1969). They reported no difference in viscosity of myofibrillar extracts between cold-shortened (0°C) and control (16°C) muscles. But, upon heating up to 46°C, SSP of cold-shortened muscle exhibited a greater apparent viscosity. Furthermore, cold-shortened muscle produced stronger dynamic SSP gel networks than muscle stored at -29 or 15°C. This differed from myofibril gelation in which the thaw-rigor samples had the largest G* value (Fig 2a). The

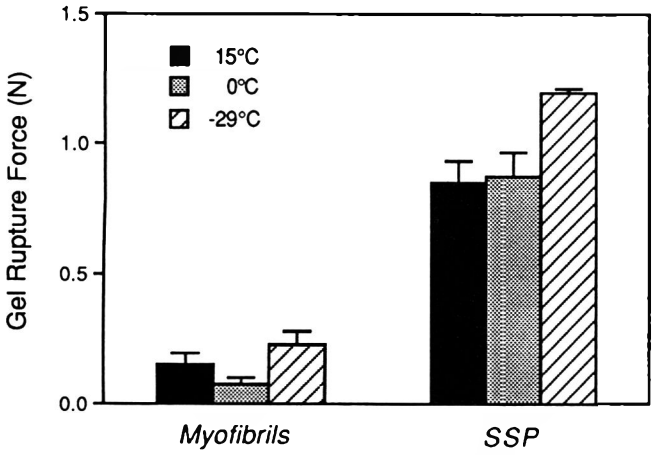

Fig 4—Instron penetration test for gels made from 2% (w/v) myofibrils and salt-soluble proteins (SSP) isolated from cold-shortened (0°C), thaw-rigor (-29°C), and control (15°C) bovine sternomandibularis muscles. Gels were pre-formed in 0.6M NaCl and 50 mM sodium phosphate at pH 6.0. Bars represent means of three replicates with standard errors.

large G* in thaw-rigor myofibrils could be partially attributed to the great extractability of SSP for the thawed muscle. However, it is not clear whether the functionality of extracted SSP was also affected by the muscle pH prior to protein extraction. That SSP formed stronger gel networks than myofibrils was expected because: (1) SSP consisted of all solubilized protein molecules so that the number of basic crosslinking units was larger in SSP gel matrices, and (2) the effective volume of the gelling components (protein) would be larger for SSP than for myofibrils. The myosin and troponin-I bands appeared intense but the actin band appeared light for SSP samples, in comparison with those for myofibrils (Fig 3). Such compositional variations could also contribute to different SSP and myofibril gel rigidities.

Protein gels cooled to 23°C were penetrated to assess the structural properties. Both myofibril and SSP gels of thaw-rigor muscle were more resistant to structural failure than the respective gels of cold-shortened or control muscle samples (Fig 4). This suggested that gels of thaw-rigor samples contained stronger protein matrices. No significant difference in gel rupture force was found between muscles stored at 0 and 15°C. Therefore, the physical and chemical changes in muscle and protein constituents resulting from freeze-thaw treatment were important for developing and maintaining a rigid gel structure. The gelling properties of myofibrillar proteins are critical to meat-and water-binding and emulsion-stabilizing in comminuted and restructured products (Acton et al., 1983). Thus, the fact that neither cold-shortening nor thaw-rigor impaired the protein gel properties would be important to consider during meat processing and product formulation.

CONCLUSIONS

Although generally accepted that prerigor meat (hot-boned) has more desirable functional properties than postrigor meat (cold-boned), it is difficult to maintain the prerigor state during storage and use of the product. Thus, much hot-boned meat is susceptible to cold-shortening if not used immediately, or produces thaw-rigor if frozen. For such contracted meat, questions have arisen as to whether the meat or protein has functional characteristics comparable to cold-boned meat (less shortening). Prerigor chilling or freezing was not detrimental to protein functionally (solubility, gelation) under the conditions used (pH 6.0, 0.6M NaCl). It therefore seems feasible to manufacture good-quality communited products from freshly deboned chilled or frozen muscle.

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Porcine Aminopeptidase Activity as Affected by Curing Agents

FIDEL TOLDRÁ, M-CARMEN CERVERÓ, and CAROLINA PART

ABSTRACT

The effect of common curing agents (salt, nitrate, nitrite, ascorbic acid and glucose) on porcine muscle leucyl, arginyl, alanyl, pyroglutamyl and tyrosyl hydrolyzing activities was studied. Salt, ascorbate and nitrite had inhibitory effects on most enzyme activities. All activities decreased as salt increased (to 1.25 M) and/or water activity decreased (from 1.00 to 0.81). Nitrite inhibited only alanyl, pyroglutamyl and tyrosyl hydrolyzing activities. An in-vitro study using model conditions of three stages of dry-curing of ham, revealed that leucyl hydrolyzing activity may be important through the complete process. Other enzymes may also be involved in later stages of curing but are less important.

Key Words: aminopeptidases, inhibition, curing, curing agents, dry-cured ham

INTRODUCTION

DRY-CURED HAMS develop a characteristic texture and flavor as a result of biochemical changes (Bellatti et al., 1983; Toldrá et al., 1992a). Most of them are due to the action of muscle enzymes which are active throughout the process (Toldrá and Etherington, 1988; Toldrá et al., 1991; Toldrá, 1992). Dry-cured hams also have an important increase in free amino acid concentration (Bellatti et al., 1983; Aristoy and Toldrá, 1991). Although their origin has not been thoroughly investigated, it is attributed to muscle aminopeptidases (Nishimura et al., 1988).

Aminopeptidases (1-aminoacylpeptide hydrolases) are present in skeletal muscle (McDonald and Barrett, 1986). Leucyl and alanyl aminopeptidase activities have been determined in human muscle (Lauffart and Mantle, 1988) and liver (Ledeme et al., 1983), bovine lens (Taylor et al., 1984) and hog kidney (Shen and Melius, 1977; Taylor et al., 1984). Tyrosyl aminopeptidase has been also found in human skeletal (Lauffart and Mantle, 1988) and human serum (Lalu et al., 1986). Arginyl aminopeptidase has been determined in rat (Kirschke et al., 1983) and human skeletal muscles (Mantle et al., 1985; Lauffart and Mantle, 1988), while pyroglutamyl aminopeptidase has also been reported in human skeletal muscle (Lauffart and Mantle, 1988). All these aminopeptidase activities have been reported in muscle and adipose tissue from both raw and dry-cured ham and had good stability even after 8 mo of curing (Toldrá et al, 1992b). Optimal pH (neutral) and temperature (around 37 °C) for maximal activity was also determined (Toldrá et al., 1992b). However, there is no available information about how curing agents might affect enzyme activity during processing.

Our objective was to determine the effects of curing agents (salt, nitrate, nitrite, ascorbic acid and glucose) and water activity, in the ranges usually found during dry-curing, on porcine muscle aminopeptidase activities.

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MATERIALS & METHODS

Muscle extract

Muscle extracts were prepared according to the method described by Lauffart and Mantle (1988) with slight modifications. Five grams of Biceps femoris was removed 8 to 10 hr postmortem from six raw hams obtained from six pigs (6-mo-old), free of visible fat and connective tissue, and the muscle was immediately homogenized in 50 mL of 50 mM phosphate buffer containing 5 mM EGTA, pH 7.5, using a Polytron homogenizer (3 × 10 sec strokes at 27000 rpm with ice cooling). The homogenate was centrifuged at 10000 × *g* for 20 min at 4 °C. The resulting supernatant was filtered through glass wool and collected as enzyme extract for further activity assays.

Aminopeptidase activities

The reaction mixture (3.2 mL) for the assay of leucyl hydrolyzing activity contained 1.5 mL of 0.1% polyoxyethylenlauryl ether (Brij 35), 0.8 mL 50 mM Tris-acetate buffer, pH 6.5, 0.8 mL 10 μM L-leucyl 7-amino-4-methyl coumarin (Sigma, St Louis, MO) and 100 μL enzyme extract. For testing tyrosyl hydrolyzing activity, the reaction mixture was identical except the addition of 1 mM of $CaCl_2$, the previous dilution 1:10 of the enzyme extract with the assay buffer and the use of 10 μM L-tyrosyl 7-amino-4-methyl coumarin (Sigma, St Louis, MO) as substrate. Phosphate buffer (50 mM) instead of tris-acetate, with no added $CaCl_2$, and 10 μM L-arginyl 7-amino-4-methyl coumarin (Sigma, St. Louis, MO) as substrate were used for testing arginyl hydrolyzing activity. Cathepsin H did not interfere in the assay (Rico et al., 1991a). The reaction mixture (3.2 mL) for testing alanyl hydrolyzing activity contained 1.2 mL of 100 mM phosphate buffer, pH 6.8, containing 4 mM dithiothreitol, 1.1 mL of 0.1% Brij 35, 0.8 mL of 10 μM L-alanyl 7-amino-4-methyl coumarin (Sigma, St. Louis, MO) and 100 μL of enzyme extract. The pyroglutamyl hydrolyzing activity was assayed in a reaction mixture containing 1.2 mL of 100 mM phosphate buffer, pH 7.4, 4 mM EDTA, 1.1 mL 0.1% Brij 35, 0.8 mL 10 μM L-pyroglutamic acid 7-amino-4-methyl coumarin (Sigma, St. Louis, MO) and 200 μL of enzyme extract.

All enzymes were incubated at 37 °C for 30 min except in tests for leucyl and pyroglutamyl hydrolyzing activities which were incubated for 15 and 60 min, respectively. The reaction was stopped by addition of 3.2 mL of 100 mM sodium acetate, 100 mM chloroacetate buffer, pH 4.3 (Barrett, 1980) and fluorescence was measured at excitation and emission wavelengths of 360 and 440 nm, respectively. One unit (U) of enzyme activity was defined as the release of 1 μMol of 7-amino-4-methyl coumarin/hr at 37 °C. Activities were expressed as a percentage of control (U/g raw muscle): 1.32 ± 0.07 for leucyl, 0.97 ± 0.06 for alanyl, 3.12 ± 0.08 for arginyl, 2.46 ± 0.09 for tyrosyl and 0.06 ± 0.01 for pyroglutamyl hydrolyzing activities. In all cases, four repeated measures were made on the same muscle for each experimental point.

Curing agents

Assay buffer (2.3 mL) was prepared containing individual curing agents and appropriate enzyme reactants at the following final concentrations in the reaction mixture: sodium chloride (0, 0.10, 0.25, 0.50, 0.75, 1.00, and 1.25M) sodium nitrate (0, 200, 400, 600, and 800 mg NO_3^-/L), potassium nitrite (0, 50, 100, and 200 mg NO_2^-/L), glucose (0, 0.25, 0.50, 1.00, and 2.00 g/L) and ascorbic acid (0, 200, 400, and 800 mg/L).

Water activity

Seven different water activity (a_w) levels in the range 0.81–1.00 were assayed for each enzyme. Each level of a_w was fixed by addition

Table 1—Effect of combined curing agents as a simulation of three stages in the processing of dry-cured ham on the amino acids hydrolyzing activities

Fixed parameters							Enzyme activities[a]				
Stages[m]	pH	a_w (%)	KNO_3 (mg/L)	NaCl (%)	Ascorb (mg/L)	Glucose (g/L)	Leucyl	Alanyl	Arginyl	Tyrosyl	Pyroglutamyl
1	5.6	0.98	400	8.0	500	1.0	38.6 ± 3.1[c]	11.2 ± 0.9[d]	3.4 ± 0.4[e]	0.0[g]	0.0[i]
2	6.0	0.95	250	6.0	300	0.5	39.9 ± 2.9[c]	12.3 ± 0.8[d]	7.5 ± 0.8[f]	6.7 ± 1.1[h]	8.3 ± 1.5[k]
3	6.2	0.90	100	6.0	100	0.2	40.0 ± 3.0[c]	12.8 ± 0.7[d]	8.0 ± 0.9[f]	21.7 ± 3.2[i]	13.1 ± 2.1[l]

[a-l] Amino acid hydrolyzing activities (means of four samples ± s.e.m.) measured at 37 °C and expressed as a percentage of control (optimal conditions and absence of curing agents). Any two means in the same column having the same letters are not significantly different at $P<0.05$.

[m] Stage 1 would represent the external muscle Semimembranosus after the salting step. Stage 2 and 3 would represent the muscle Biceps femoris in the middle and the end, respectively, of the dry-curing period.

Table 2—Inhibitory effect of salt on aminopeptidase activities

NaCl (M)	Amino acid hydrolyzing activities				
	Leucyl	Alanyl	Arginyl	Tyrosyl	Pyroglutamyl
0.00	100.0 ± 5.3[a]	100.0 ± 6.2[a]	100.0 ± 2.6[a]	100.0 ± 3.6[a]	100.0 ± 6.5[a]
0.10	81.1 ± 3.1[b]	98.3 ± 3.3[a]	83.1 ± 3.2[b]	55.5 ± 5.1[b]	100.0 ± 5.3[a]
0.25	64.5 ± 3.5[c]	85.4 ± 2.2[b]	54.2 ± 2.5[c]	28.7 ± 4.6[c]	89.9 ± 5.5[a]
0.50	32.3 ± 2.8[d]	42.3 ± 1.0[c]	28.7 ± 4.3[d]	10.5 ± 3.9[d]	52.5 ± 4.3[b]
0.75	20.5 ± 3.0[e]	28.8 ± 0.9[d]	16.6 ± 3.6[e]	7.3 ± 2.1[d]	37.8 ± 4.2[c]
1.00	17.7 ± 2.9[e]	17.7 ± 1.1[e]	12.4 ± 2.1[e]	5.8 ± 1.6[d]	22.2 ± 3.2[d]
1.25	9.6 ± 2.1[f]	9.2 ± 0.9[f]	11.1 ± 2.0[e]	0.5 ± 0.5[e]	14.4 ± 3.5[e]

[a-f] Amino acid hydrolyzing activities (means of four samples ± s.e.m.) measured at 37 °C and expressed as a percentage of control. Any two means in the same column having the same letters are not significantly different at $P<0.05$.

Table 3—Effect of potassium nitrite on aminopeptidase activities

Nitrite (mg/L)	Amino acid hydrolyzing activities				
	Leucyl	Alanyl	Arginyl	Tyrosyl	Pyroglutamyl
0	100.0 ± 5.3[a,b]	100.0 ± 6.2[a]	100.0 ± 2.6[a]	100.0 ± 3.6[a]	100.0 ± 6.5[a]
50	111.3 ± 4.9[a]	101.1 ± 2.3[a]	106.5 ± 3.2[a]	86.1 ± 4.2[b]	94.2 ± 2.5[a]
100	96.2 ± 3.2[b]	88.8 ± 2.1[b]	104.3 ± 2.9[a]	85.2 ± 3.9[b]	93.1 ± 2.7[a]
200	94.8 ± 5.1[b]	72.5 ± 1.9[c]	102.1 ± 4.4[a]	85.4 ± 4.0[b]	86.6 ± 2.1[b]

[a-c] Amino acid hydrolyzing activities (means of four samples ± s.e.m.) measured at 37 °C and expressed as a percentage of control. Any two means in the same column having the same letters are not significantly different at $P<0.05$.

Table 4—Effect of glucose on aminopeptidase activities

Glucose (mg/L)	Amino acid hydrolyzing activities				
	Leucyl	Alanyl	Arginyl	Tyrosyl	Pyroglutamyl
0	100.0 ± 5.3[a]	100.0 ± 6.2[a]	100.0 ± 2.6[a]	100.0 ± 3.6[a]	100.0 ± 6.5[a]
0.25	115.5 ± 4.5[b]	96.5 ± 3.1[a]	107.4 ± 4.3[a,b]	102.1 ± 2.1[a]	98.7 ± 3.3[a]
0.50	132.2 ± 7.6[c]	95.6 ± 3.3[a]	112.1 ± 3.9[b]	108.8 ± 1.5[b]	100.5 ± 3.1[a]
1.00	161.3 ± 2.3[d]	91.3 ± 3.7[a]	121.2 ± 12.2[b]	103.2 ± 4.6[a,b]	96.4 ± 3.4[a]
2.00	175.6 ± 2.6[e]	94.6 ± 5.6[a]	124.5 ± 10.1[b]	99.9 ± 8.8[a,b]	95.5 ± 3.3[a]

[a-d] Amino acid hydrolyzing activities (means of four samples ± s.e.m.) measured at 37 °C and expressed as a percentage of control. Any two means in the same column having the same letters are not significantly different at $P<0.05$.

Table 5—Effect of ascorbic acid on aminopeptidase activities

Ascorbic acid (mg/L)	Amino acid hydrolyzing activities				
	Leucyl	Alanyl	Arginyl	Tyrosyl	Pyroglutamyl
0	100.0 ± 5.3[a]	100.0 ± 6.2[a]	100.0 ± 2.6[a]	100.0 ± 3.6[a]	100.0 ± 6.5[a]
200	103.3 ± 3.1[a]	98.8 ± 3.1[a]	87.1 ± 3.5[b]	103.2 ± 2.9[a]	77.5 ± 3.1[b]
400	66.5 ± 4.5[b]	99.2 ± 2.7[a]	85.2 ± 4.5[b]	112.1 ± 4.9[b]	73.4 ± 2.9[b]
800	47.8 ± 4.3[c]	100.3 ± 3.0[a]	82.1 ± 4.1[b]	111.5 ± 2.3[b]	66.4 ± 3.2[c]

[a-c] Amino acid hydrolyzing activities (means of four samples ± s.e.m.) measured at 37 °C and expressed as a percentage of control. Any two means in the same column having the same letters are not significantly different at $P<0.05$.

of different amounts of glycerol to the final reaction mixture containing the muscle extract. Reaction mixtures for a_w determination were identical to those used for enzyme assays. Water activity was determined at 37 °C by using a Humidat-RC (Novasina, Zürich, Switzerland).

Combined curing agents and processing conditions

Three mixtures of curing agents, pH and water activity were prepared as a simulation *in vitro* of three typical stages in processing of dry-cured ham. The combination are reported in the first part of Table 1. Stage 1 would correspond to the external Semimembranosus of the ham, where curing agents had initiated penetration, after the salting step. Aw was high (0.98) at this stage because drying had not been initiated yet and the hams still retained most of the moisture. Stages 2 and 3 represent the Biceps femoris in the middle and end, respectively, of the dry-curing period. As drying progressed, the a_w decreased.

Statistics

Three replicates were tested for each parameter. Statistical analysis (t-test) was applied on the data for each enzyme for significant differences ($P<0.05$) among concentration levels.

RESULTS & DISCUSSION

SODIUM CHLORIDE, sodium nitrite, potassium nitrate, glucose and ascorbic acid are often used in curing of hams and sausages. The effect of these agents on leucyl, arginyl, alanyl, tyrosyl and pyroglutamyl aminopeptidase activities was studied in concentration ranges usually found in the dry-curing process

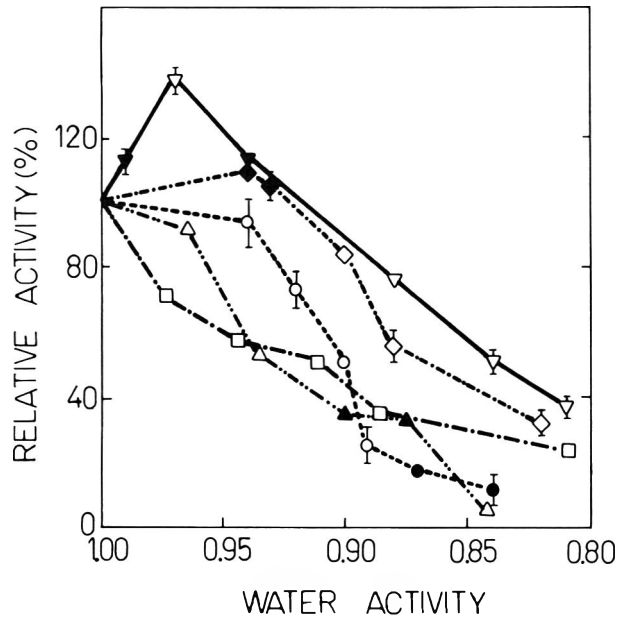

Fig. 1—Effect of water activity on aminopeptidase activities. Means of four samples ± s.e.m. (represented as vertical bars. No bars shown when interval was smaller than symbol). Filled symbols for each enzyme are not significantly different at P<0.05; Leucyl (◇), alanyl (△), arginyl (▽), pyroglutamyl (□), and tyrosyl (○) hydrolyzing activities.

(Flores and Toldrá, 1992). Salt is a main substance in the dry-curing of meat products. As previously shown with cathepsins B, D, H and L (Rico et al., 1990, 1991b), salt was also a powerful inhibitor of muscle aminopeptidases (Table 2). Thus, at salt concentrations between 0.75 and 1M, typical of Spanish dry-cured hams, only 5 to 40% of initial activity was recovered. However, alanyl and pyroglutamyl hydrolyzing activities still showed high levels of activity below 0.25M of salt.

Nitrate and nitrite are common in curing mixtures because they act as preservatives as well as contribute to color and flavor. Nitrate, in the range 0 to 200 mg/L did not seem to affect activities of the enzymes. Arginyl hydrolyzing activity was not ($P < 0.05$) affected by nitrite but the other enzymes were slightly, ($P < 0.05$), inhibited (Table 3).

Glucose is also a curing agent because it contributes to a more desirable color and flavor. Leucyl and, to a less extent, arginyl hydrolyzing activities were ($P < 0.05$) increased while tyrosyl hydrolyzing activity was different at an intermediate level and the other enzymes remained unaffected (Table 4).

Ascorbic acid mainly contributes to color development. Arginyl, pyroglutamyl and, specially, leucyl hydrolyzing activities (Table 5) were ($P < 0.05$) inhibited by ascorbic acid while alanyl remained unaffected and tyrosyl hydrolyzing activity was increased.

Decrease in water activity (a_w) below 0.95 reduced ($p < 0.05$) the activities of the enzymes (Fig. 1) as reported for other muscle enzymes such as cathepsins B, D, H, and L (Rico et al., 1990, 1991b). Usual a_w values in dry-cured hams at the end of the process were between 0.85 and 0.90 where all enzymes are still active: 10–30% for alanyl, pyroglutamyl and tyrosyl and 40–80% for leucyl and arginyl hydrolyzing activities. The arginyl enzyme showed increased activity ($P<0.05$) at a_w between 0.95 and 1.00.

In previous work (Toldrá et al., 1992b), optimal pH was 6.5 for leucyl, arginyl and tyrosyl, 7.0 for alanyl and 7.5 for pyroglutamyl hydrolyzing activities. The enzymes were found still quite active around pH 6.0, a usual pH in the middle of the dry-curing process. Regarding temperature, optimal activity was ≈ 35 °C except the leucyl hydrolyzing activity which was greatest at 45 °C. All enzymes were quite active in the range 15–30 °C, quite usual during the dry-curing process (Toldrá et al., 1992b).

A model system combining all curing ingredients, pH and a_w was prepared as a simulation of different stages in the processing of dry-cured hams. Stage 1 (Table 1) represents the external part of the ham, muscle Semimembranosus, just after the salting step. In that stage, there was complete inhibition of tyrosyl and pyroglutamyl, almost complete inhibiton of arginyl (3.4%) enzymes and less inhibition of leucyl and alanyl hydrolyzing activities (38.6 and 11.2%, respectively). These low activities were probably due to the high content of salt and low pH value, a very important factor (Toldrá et al., 1992b). The activation of leucyl and arginyl enzymes by glucose seemed to be counteracted by inhibition to ascorbic acid, salt and low pH. Conditions 2 and 3 (Table 1) would represent the Biceps femoris, which is the inner part of the ham, at the middle and end of the process. Arginyl and alanyl hydrolyzing activities showed similar activities (7.5–8.0% and 12.3–12.8%, respectively) as dry-curing progressed. This was probably due to the increase in pH and the slight decrease in salt content. However, leucyl hydrolyzing activity maintained a steady activity, around 39% of the control, at all three conditions. In general, the important inhibition by sodium chloride seemed to be counterbalanced by the other curing agents, specially in the case of the leucyl hydrolyzing activity activated by nitrite and glucose. In view of these simulations, leucyl hydrolyzing activity might be important throughout processing of dry-cured ham while the other enzymes may be also important, but mainly at the middle/end stages.

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—Continued on page 747

Gelation of Turkey Breast and Thigh Myofibrils: Effects of pH, Salt and Temperature

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ABSTRACT

Salt concentration >0.1M and pH were important to the development of gel fracture properties. However, meat type and salt concentration (0.5–1.0M) did not influence gel shear stress or shear strain. Isothermal heating temperature (55°C or 70°C) affected only gel shear strain. Rheological properties at fracture and nonfracture did not respond alike to changes in gelation conditions. General similarities between breast and thigh myofibril gels implied that protein isoforms were not the main factor influencing gel structure formation.

Key Words: turkey, breast, myofibrils, gelation, salt concentration

INTRODUCTION

THE TEXTURE of comminuted muscle foods primarily results from the three-dimensional gel network that forms as proteins unfold and aggregate during heating. Elucidation of the mechanisms associated with gelation could help in understanding how to control the texture of muscle foods. Texture of muscle food gels can be measured using fundamental or empirical rheological tests. Fundamental tests define properties in physical units and are preferred over empirical tests since it is not clear what aspects empirical tests measure. Three types of fundamental fracture tests may be used to deform gel samples: compression, tension and torsion. Of these, the twisting deformation of torsion is preferred since it does not change sample shape or volume (Hamann, 1991). Fracture parameters in such testing are shear stress (gel strength) and shear strain (gel deformability). These correlated with sensory notes of hardness and cohesiveness (Montejano et al., 1985).

Amato et al. (1989) used torsion testing to evaluate fracture properties of comminuted turkey breast and thigh gels formed at natural pH (i.e., pH 6.0 and 6.4, respectively). They reported that thigh gels had higher shear stress and shear strain values than breast gels. However, they did not determine whether the variance in pH was responsible for observed fracture trends. To determine the effect of pH, Daum-Thunberg et al. (1992) used torsion testing to evaluate fracture properties of comminuted turkey breast and thigh gels adjusted to pH 6.0 and 6.4. Their results showed that at pH 6.0 thigh gels had higher shear stress values and shear strain values than breast gels. At pH 6.4, shear stress of the two types of gels were equivalent, but breast gels had higher shear strain values than thigh gels. Comminuted breast and thigh gels also have different levels of lipids and connective tissue which could affect gel fracture properties. To determine functionality of myofibrillar proteins without these components, Northcutt et al. (1993) investigated fracture properties of turkey breast and thigh myofibril gels, using torsion testing, at pH 6.4 and 7.0. They reported that shear stress and strain values of breast and thigh myofibril gels were equivalent at each of the pH values.

Studies that have investigated gelation of myofibrillar proteins using rheological tests other than torsion have reported opposing trends as to whether breast or thigh gels had higher rheological values. Morita et al. (1987) and Asghar et al. (1984) found gels from chicken breast myosin had higher rigidity, determined by a small strain nonfracture rheological test, than leg myosin gels when at pH 5.5 to 6.0. They reported no isoform-associated differences in rigidity of gels at pH 6.0 to 7.0. Using compression testing, Dudziak et al. (1988) and Foegeding (1987) found gels from myosin/actomyosin (pH 7.0) or salt-soluble proteins (pH 6.0) from turkey breast had higher shear stress and strain values than those of similar proteins from turkey thigh. Xiong and Brekke (1990, 1991) reported the same trend when they used back-extrusion (empirical test) to measure properties of chicken salt-soluble protein gels. The contradictory trends between investigations that used torsion testing (Amato et al., 1989; Daum-Thunberg et al., 1992; Northcutt et al., 1993) and other rheological methods (Morita et al., 1987; Asghar et al., 1984; Dudziak et al., 1988; Foegeding, 1987) may indicate rheological tests measured different physical properties, differences existed in solution/heating conditions or in amounts and types of proteins.

Similarities Northcutt et al. (1993) observed between breast and thigh myofibril gels suggested that turkey myofibrils had protein isoform-independent gelation properties. However, the gels in that study were formed under limited solution conditions. Salt concentration, pH (Asghar et al., 1984) and isothermal heating temperature (Wu et al., 1991) affect gelation. The objective of our study was to determine whether turkey breast and thigh myofibril gels had the same fracture properties when salt concentration, pH and isothermal heating temperature were varied.

MATERIALS & METHODS

Meat

Boxes (40 kg) of fresh whole turkey breasts and thighs (deboned and skinned) were obtained from a local processor on the day of processing. The meat was from tom turkeys that had an average live weight of 12.4 ± 0.5 kg. Treating breast and thigh separately, excess fat and epimysial tissue were trimmed, and whole meat pieces were cut into smaller chunks before being mixed and ground through a series of plates with orifice diameters of 9.0 mm and 4.5 mm. The ground meat was divided into 1 kg portions, double-wrapped in freezer paper (Poly-Wrap, Plastic Coated Paper, Inc., Pensacola, FL), frozen and held at −20°C until needed. Overnight tempering at 4°C partially thawed the meat before use.

Myofibril isolation

Myofibrils were isolated from ground turkey meat according to the method of Northcutt et al. (1993). Ground turkey meat was chopped with a pH 7.0, 20 mM EDTA buffer (1:2 w/v) for 6 min at 2500 rpm in a Stephan Chopper (Model 2889, Germany). The resulting slurry was centrifuged at 6000 × *g* for 20 min and the supernatant discarded. The pellet, with added EDTA buffer, was chopped again and the slurry centrifuged. The pellet was resuspended (1:2 w/v) in a pH 7.0 rigor buffer (0.1M NaCl, 0.2 mM $MgCl_2$, 5 mM EDTA, 5 mM DTT, 10 mM sodium phosphate) by stirring for 5 min. The slurry was centrifuged at 6000 × *g* for 5 min and the supernatant discarded. The rigor buffer "rinse" was repeated three additional times. The pellet was suspended a fifth time in rigor buffer, and the mixture was filtered through cheese cloth. The isolated myofibrils were recovered by centrifuging at 6000 × *g* for 5 min and stored (covered) overnight at

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4°C. Integrity of myofibrils was verified by light microscopy (Olympus BH-2, Tokyo, Japan).

Adjustment of pH and salt concentration

Dialysis was used to adjust pH of myofibril suspensions from about 7.0 to 6.0 or 6.4 and a salt concentration of 0.1, 0.5 or 1.0M NaCl producing six combinations. The myofibril pellet was loaded into dialysis tubing (Spectrapor, MW cutoff: 12,000–14,000, dry cylinder diameter 1.6 cm) using a modified 60 mL plastic syringe and dialyzed (1:10 w/v) overnight at 4°C in an appropriate pH, 50 mM sodium phosphate buffer that contained the necessary molar concentration of sodium chloride and 0.02% sodium azide. A pH meter was used to determine the pH of a 10% (w/v) slurry of dialyzed myofibrils in water.

Gel formation

The protein concentration of the myofibrils after dialysis was determined using the macro-Kjeldahl method (AOAC, 1984), and the myofibrils were diluted to 8% (w/v) protein with a 50 mM sodium phosphate buffer of appropriate pH and salt concentration. The myofibril solutions were loaded into 12.0 mm i.d. glass tubes lubricated with SigmaCote (Sigma, Inc., St. Louis, MO). A rubber stopper was placed in one end of the glass tube and the other end was covered with aluminum foil. Gentle tapping of the stoppered end of the glass tube removed entrapped air bubbles. Gels were formed by heating isothermally in a 55°C or 70°C water bath for 45 min. After heating, the tubes were cooled under running tap water. The gels were removed from the glass tubes and stored covered overnight at 4°C.

Torsion

Samples were allowed to warm at room temperature for 1 hr before being cut to a length of 28.7 mm (about 3.3 mL gel or 0.3g myofibrils). Plastic disks were attached with a cryanoacrylate glue to each end of the samples. The samples were ground with a bench grinder (Gel Consultants, Inc., Raleigh, NC) to capstan shapes with minimum diameters 5.25 mm and twisted to fracture at 2.5 rpm using a Torsion Gelometer (Gel Consultants, Inc., Raleigh, NC). At least three samples from each treatment were tested. Shear stress and shear strain at fracture were calculated using equations of Diehl et al. (1979). Nonfracture moduli were calculated at a shear strain of 0.8.

Statistical analysis

Breast and thigh meat were obtained from three separate processing days, i.e., three lots of meat. The data were analyzed as a split-split plot. The whole plots were the three lots of meat. The whole plot treatment was meat type. The sub-plot treatments were both pH and salt concentration since these variables were adjusted in the same step. The sub-sub plot treatment was temperature. The error term for lot and meat was lot × meat. The error term for the sub-plot was rep × meat × pH × salt. The sub-sub plot was tested against the residual. Analysis of variance was determined using the General Linear Model procedure (SAS Institute, Inc., 1982). Treatment means were compared using Waller-Duncan k-ratio t-tests. Significance was established at $P \leq 0.05$.

RESULTS & DISCUSSION

THE FRACTURE parameters in torsion testing are shear stress (which indicates gel strength) and shear strain (deformability). Torsion testing has several advantages. Sample shape and volume do not change during testing. The gel can fracture in compression, tension, shear or a combination of these modes. The mode of fracture (tension or shear) can be determined (Hamann, 1991). However, two limitations associated with torsion were encountered. First, the 0.1M NaCl gels could not be tested using torsion since they did not withstand grinding to the capstan shape. Those gels had a coarse, particulate appearance and crumbled when handled. The frailty of the 0.1M NaCl gels may have been due to incomplete extraction of proteins, which are essential to formation of gel strength and deformability (Foegeding, 1990). It could also be related to limited swelling of myofibrils at such low salt concentrations (Offer and Trinick, 1983). Since 0.1M NaCl gels could not be tested, results were not reported. The second limitation with torsion testing was that the pH 6.4, 55°C gels did not fracture. Those gels were so deformable that as they were twisted, the middle of the capstan knotted or twisted upon itself resulting in the sample slipping from the torsion device without fracturing. However, the shear stress and shear strain values of the gels were estimated at maximum deformation achieved prior to changes in the capstan shape. The shear strain for each gel sample was recorded as >3.0, an approximate maximum fracture value reported in previous torsion studies (Hamann and Lanier, 1987). The shear stress was determined from force/deformation curves at the point where the middle of the capstan shape began to visually crease and change shape.

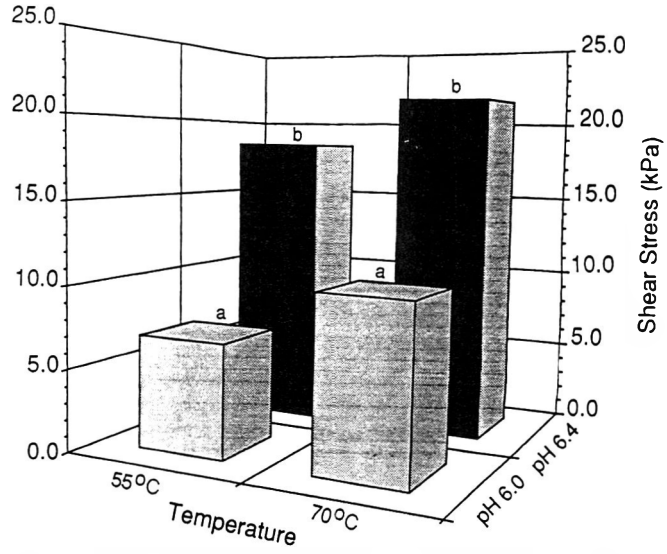

Fig. 1—Effect of temperature and pH on shear stress of gels prepared at 0.5M NaCl. Mean values pooled for meat type and replication. Bars with different letters are different (P<0.05).

Fracture properties

Shear strain differed ($P<0.05$) between lots of meat. However, Waller-Duncan K-ratio t-tests indicated that within each lot of meat, for a particular treatment, fracture values were independent of meat source used (i.e., breast or thigh). Therefore, breast and thigh fracture data from each lot were combined by treatment for statistical analysis. Results indicated that shear stress and shear strain were not significantly affected by salt concentration (i.e., 0.5M and 1.0M), however, these values were influenced ($P<0.05$) by pH and isothermal heating temperature. The discussions that follow are based on statistical results from combined data for meat source. The data in Fig. 1 to 4 were also combined for meat source in addition to replication.

A minimum NaCl concentration between 0.1 and 0.5M was required to form machinable gels. The weakest gel tested by torsion had a shear stress of 3.0 kPa and a shear strain of 0.55. However, salt did not continue to influence fracture parameters as molar concentration increased from 0.5 to 1.0. The function of NaCl in processed meats is to aid swelling of myofibrils (Hamm, 1960; Ranken, 1976; Lawrie, 1979) and to extract myofibrillar protein (Acton, 1972; Froning and Sackett, 1985). The lack of difference between fracture values of gels containing 0.5 or 1.0M NaCl gels could have resulted from optimal swelling ability of the myofibrils and/or extractability of proteins for gelation being reached prior to (or at) 0.5M. An increase in gel rheological properties at low salt concentrations followed by a plateau at higher concentrations was also reported for gels from myosin (Ishioroshi et al., 1979) and comminuted meat (Amato et al., 1989; Trout and Schmidt, 1986).

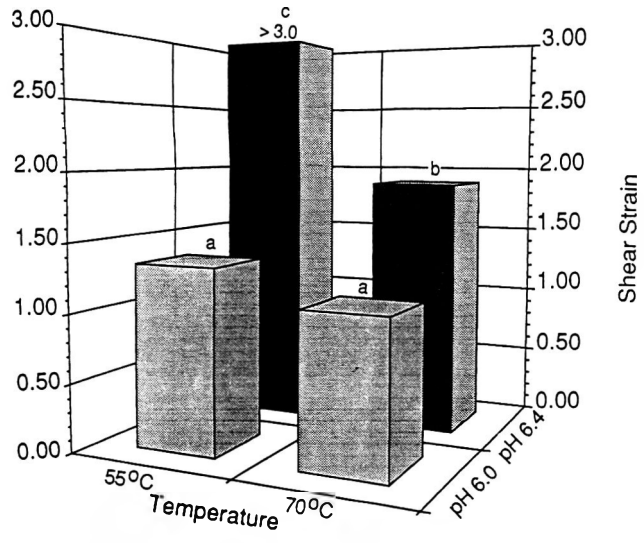

Fig. 2—Effect of temperature and pH on shear strain of gels prepared at 0.5M NaCl. Mean values pooled for meat type and replication. Bars with different letters are different (P<0.05).

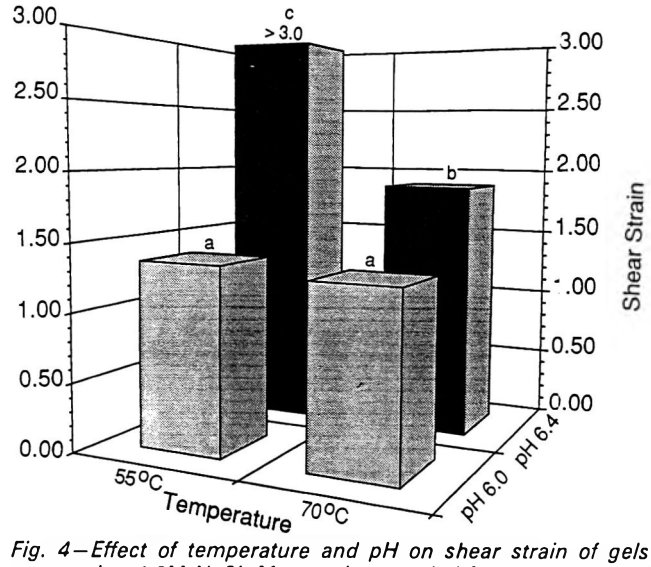

Fig. 4—Effect of temperature and pH on shear strain of gels prepared at 1.0M NaCl. Mean values pooled for meat type and replication. Bars with different letters are different (P<0.05).

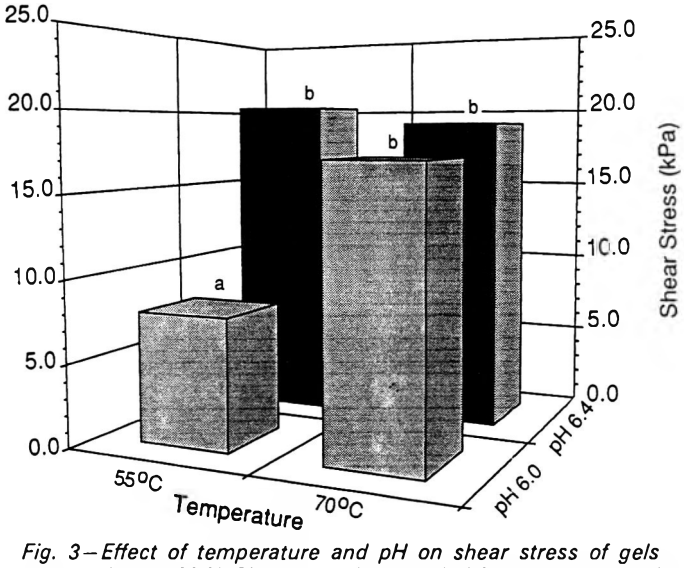

Fig. 3—Effect of temperature and pH on shear stress of gels prepared at 1.0M NaCl. Mean values pooled for meat type and replication. Bars with different letters are different (P<0.05).

The shear stress and shear strain of the gels increased between pH 6.0 and 6.4 (Fig. 1 to 4). This confirmed pH effects observed by Daum-Thunberg et al. (1992). However, note that researchers using rheological methods other than torsion fracture have reported that pH 6.0 gels were stronger and more deformable compared to gels made at higher pH values (Foegeding, 1987; Morita et al., 1987; Xiong and Brekke, 1990, 1991). Contradictions concerning the pH at which optimum gel functionality occurs could be due to different gel properties measured by various rheological methods or to differences in types of proteins and concentrations used to form gels.

The lower functionality of myofibrils at pH 6.0, could be related to water-holding (or myofibril swelling). Daum-Thunberg et al. (1992) reported a positive relationship between held-water, shear stress and shear strain between pH 6.0 and 6.4. Offer and Trinick (1983) also reported more swelling of the myofibril structure when pH increased.

Isothermal heating temperature primarily affected shear strain of the pH 6.4 gels, with less deformable gels resulting at the higher isothermal heating temperature (Fig. 2 and 4). The pH 6.4 gels cooked at 55°C visually had no fluid loss and had to be forced from the glass tubes. In contrast, the pH 6.4 gels cooked at 70°C had fluid loss and gel shrinkage. The decrease in deformability of pH 6.4, 70°C gels could be associated with tightening or shrinking of the gel network or an increase in protein concentration, as water was lost during heating.

Experiments indicated that the high deformability of pH 6.4, 55°C gels was not an artifact due to extent of heating since those gels retained high shear strain values after isothermal heating at 55°C for 40 hr (results not shown). Properties of the gels formed by heating at 55°C and 70°C corresponded to those characteristics that Wu et al. (1991) reported for myosin gels from chicken breast meat. They described gels formed below 58°C as elastic and those formed above 58°C as rigid, with a high degree of viscosity (energy loss). The pH 6.0, 1.0M, 70°C gels had high shear stress values relative to gels of other pH 6.0 treatments (Fig. 1 and 3). We did not know why those gels had such high shear stress values, but they appeared to be the result of combined high salt (1.0M) and high temperature (70°C) conditions.

Similarities in fracture values of breast and thigh myofibril gels implied that mechanisms by which the two types of gels were formed were similar. This indicated that gelation of turkey breast and thigh myofibril suspensions were isoform-independent. Moreover, fracture properties of breast and thigh myofibril gels responded similarly to changes in salt concentration, pH and isothermal heating temperature.

Northcutt et al. (1993) also reported similarities in fracture values of turkey breast and thigh myofibril gels at pH 6.4 and 7.0. The fracture trends for breast and thigh myofibril gels differed from those reported by Daum-Thunberg et al. (1992) for comminuted turkey breast and thigh gels. They found that comminuted thigh gels had higher fracture values than breast gels at pH 6.0. However, at pH 6.4, the shear stress values of the two types of gels were equivalent, but breast gels had higher shear strain values than thigh gels. To determine if variances in experimental conditions were responsible for different fracture trends for comminuted meat gels and myofibril gels, conditions that differed between studies were examined. Those conditions included: the diameter of the gel, the type of heating, protein concentration of gels and whether fresh or frozen meat was used. For the myofibril gels, the diameter of the tube and type of heating used to form gels (i.e., isothermal vs incremental) affected the magnitude of shear stress and shear strain values (results not shown). Gels formed by heating from 25°C to 70°C at 0.5°C/min had higher values for shear stress and shear strain than those formed by heating for 45 min at

Table 1 – Shear moduli values and corresponding standard deviations[s]

	Shear moduli (kPa)			
	55°C		70°C	
pH	0.5M NaCl	1.0M NaCl	0.5M NaCl	1.0M NaCl
6.0	4.7[a]	4.7[a]	8.6[b,f]	11.4[c]
6.4	7.5[d]	7.4[d]	9.7[e,f]	8.6[d,e]

[a-f] Means in same row or column with different superscripts are significantly different ($P < 0.05$).

[s] Averages of 3 replications combined for meat source.

70°C. However, there were no rheological differences between breast and thigh myofibril gels for either type heating. We could not determine what effect protein concentration had on gelation since the maximum consistent concentration for the myofibrils was 8% (w/v). The comminuted meat gels of Daum-Thunberg et al. (1992) were formulated to 13% (w/v) protein. Protein concentration has been shown to affect gel shear stress (Hamann, 1987; Daum-Thunberg et al., 1992; Foegeding, 1990). In dilute protein solutions (5 mg/mL–40 mg/mL), breast and thigh gels responded differently to changes in protein concentration (Asghar et al., 1984; Foegeding, 1987; Dudziak et al., 1988). A preliminary study with myofibril gels from fresh, rather than frozen, turkey breast and thigh meat indicated that freezing affected rheological properties of the gels (results not shown). Freezing may be detrimental to breast gels but beneficial, especially to shear strain, of thigh gels.

Nonfracture properties

Studies that have used nonfracture rheological techniques have differed in findings on rheological properties of myosin gels from dark or white meat. Asghar et al. (1984) reported that chicken white meat myosin gels (0.6M KCl) had higher rigidity than dark meat myosin gels between pH 6.0 and 6.5, while between pH 6.8 and 7.0 the two types had similar rigidity. Morita et al. (1987) found myosin gels (0.6M KCl) prepared from dark and white chicken meat had the same rigidity values between pH 6.0 and 6.5. For bovine myosin, Fretheim et al. (1986) found myosin gels from fast twitch muscles (0.6M NaCl) had greater moduli values than those from slow twitch muscles. To determine if turkey breast and thigh myofibril gels prepared in our study had the same rheological properties at nonfracture strains as at fracture strains, shear moduli values (i.e., stress/strain ratios) were determined from torsion force-deformation curves.

Shear moduli were different ($P < 0.05$) among lots of meat, but shear moduli corresponding to a particular treatment within a lot were not influenced by source of meat (i.e., breast or thigh). These results were in agreement with those of Morita et al. (1987). Since meat source did not affect ($P < 0.05$) shear moduli, the data were combined for meat source and lot (Table 1). Paired comparisons of treatment means indicated salt concentration (0.5 and 1.0M) did not influence ($P < 0.05$) shear moduli values, with exception of the pH 6.0, 70°C gels. Under those conditions, the 1.0M gels had higher ($P < 0.05$) moduli than 0.5 M gels. Increasing the pH of gels cooked at 55°C (both 0.5 and 1.0M NaCl gels) from 6.0 to 6.4 resulted in higher shear moduli. For gels cooked at 70°C, effects of pH were mixed. Shear moduli of the 0.5M, 70°C gels were not affected by pH, while 1.0M, 70°C gels had higher shear moduli values at pH 6.0 (Table 1). Nonfracture and fracture data showed the same response to changes in pH at 55°C, but not at 70°C. Temperature affected ($P < 0.05$) shear moduli of the gels, with the exception of the pH 6.4, 1.0M NaCl gels where temperatures did not influence shear moduli (Table 1). These trends were not the same as those at fracture. Comparison of results could possibly be limited by overestimation of shear stress values for gels that did not fracture (i.e., pH 6.4, 55°C gels). In general, the shear stress of protein gels would be expected to increase and shear strain decrease as final heating temperature increased (Foegeding and Ramsey, 1987).

Rheological trends for large and small strain testing differed depending on conditions. However, in all cases, rheological values of the breast and thigh myofibril gels, for any specific treatment, were not different ($P < 0.05$). These results indicate that direct comparison of shear stress and shear strain to shear moduli (i.e., stress/strain ratio) would not always yield similar trends.

CONCLUSION

Breast and thigh myofibril gels had equivalent rheological properties at fracture and nonfracture strains and responded similarly to alterations in conditions used to form the gels. Increasing pH from 6.0 to 6.4 resulted in higher shear stress and shear strain values and isothermal cooking temperature affected shear strain at fracture of the pH 6.4 gels. The mechanisms by which the two types of myofibrils formed gels appeared to be similar.

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–Continued on page 760

Selenium Content of Bison, Elk and Mule Deer

L.C. MEDEIROS, R.P. BELDEN, and E.S. WILLIAMS

ABSTRACT

Selenium (Se) content of elk, mule deer, bison and beef skeletal muscle was measured and compared. Selenium content was determined by graphite furnace after wet nitric acid digestion. Game samples did not differ in Se content but contained more Se than beef (wet weight) ($P<0.05$). On dry weight basis, deer contained more Se than elk, bison or beef ($P<0.05$). Within samples from male elk and deer, and elk and bison of both genders, there were interactions between species and muscles ($P<0.05$). Type of muscle and gender did not influence Se content ($P<0.05$). Selenium toxicity from consuming game that foraged in high selenium environments is remote.

Key Words: selenium, game meat, wild game, beef, minerals, elk, mule deer, bison

INTRODUCTION

SELENIUM (Se) toxicity in humans is rare (Levander, 1988). Ingestion of foods produced in regions with high Se containing soils increased clinical indicators of Se in humans, but apparent toxicity was not reported (Longnecker et al, 1991). A limit of 500 μg per day has been suggested as the maximum acceptable intake (Levander, 1988). The current Recommended Daily Allowance (RDA) for Se is 70 μg/day for adult males and 55 μg/day for females (25–50 yr) (National Research Council, 1989).

Average selenium intake nearly parallels the Se content of foods produced and distributed intra-regionally, such as meat and dairy products (Snook et al., 1987). The likelihood of over-ingestion of Se is greater when soil Se is high and foods of local origin are frequently consumed. Se content of fruits, vegetables, cereals and other foods in wide food distribution systems are less related to average Se intake.

Selenium content of a variety of foods has been reported from several studies (Morris and Levander, 1970; Olson and Palmer, 1984; Snook et al., 1987). Meat and seafood samples from the Washington D.C. area contained between 0.1-0.36 μg/g Se, wet weight (Morris and Levander, 1970). Organ meats, such as kidney and liver, contained considerably more Se than muscle. Snook et al. (1987) reported Se content of foods purchased or produced in Ohio. Selenium content of various forms of meat, fish and poultry ranged between 0.05 and 0.18 μg/g with most cuts containing 0.06 to 0.14 μg/g (all values, wet weight). Snook et al. (1987) indicated that high soil iron content and acidity were responsible for decreasing the bioavailability of soil Se in Ohio (Snook et al., 1987). Selenium deficiency among Ohio livestock has been reported (Mahan and Moxon, 1979). Meat samples from South Dakota, including antelope, bear, deer, buffalo and pheasant (exact species unknown), contained 0.2-1.1 μg/g Se (Olson and Palmer, 1984). Areas of South Dakota have high Se soil content where Se toxicity has been reported in some livestock, (Moxon, 1937). Except data for pheasant, Olson and Palmer (1984) had few samples for analysis. Few data were reported for different muscles and genders.

Soils in Rocky Mountain areas have high Se, especially in some localized areas where levels may be up to 100 ppm water soluble Se (Case and Cannia, 1988). The recommended critical limit in soil to limit feed toxicity is 0.1 ppm ABDTPA (Ammonium bicarbonate-DTPA) extractable Se (Soltanpour and Workman, 1980). Residents may be dependent on food produced from these areas. Feeding regimens of domesticated animals can be controlled more closely than can the diet of free-roaming game species. The possibility of isolated cases of selenium toxicity in humans exists, although it is remote and no history of toxicity in the region has been documented. Our objectives were to determine the Se content of muscles of game animals and beef produced in the same general geographic regions to assess whether over-ingestion of Se might be possible, and to provide information regarding Se content of game species.

MATERIALS & METHODS

MUSCLE samples were obtained from animals with access to forage grown on soils with medium (0.6–5.0 ppm water soluble) to high (10 ppm and up) Se. Some animals were supplemented with hay grown in medium-selenium areas (Se content unknown). Beef was taken from steers from the University of Wyoming herd. Longissimus and semitendinosus muscle samples came from steers grazed on improved pasture land between Laramie, Wy and the Snowy Mountain Range. Cattle finished on grain (unknown Se) were selected so that Se content of the meat would more nearly represent retail market beef. Muscle samples were longissimus, semimembranosus, shoulder (omotransversarius/brachiocephalicus or supraspinatus) and abdomen (external abdominus) from 9-mo-old elk (*Cervus elaphus nelsoni*, four males and nine females) and 9-mo-old mule deer (*Odocoileus nemionus*, four males). The elk and deer were held in isolation pens in the Wyoming Game and Fish Department Sybille Wildlife Research and Conservation Education Unit. Animals were part of an unrelated research study and were fed alfalfa hay grown in the same general geographic region as the Sybille Unit (hay grown in Wheatland, WY) and supplemented with commercial grains (origin unknown). Deer had access to a small pasture seeded in grass and elk were in dirt paddocks with no access to pasture. As fawns and calves, the animals had access to mineral salt blocks and fawns had access to calf manna (Manna Pro, Los Angeles, CA) when they were young.

Longissimus, semimembranosus, shoulder (omotransversarius/brachiocephalicus or supraspinatus) and abdomen (external abdominus) muscles from eight male and nine female bison (*Bison bison*) were obtained from animals culled on the National Elk Refuge, Jackson, WY. Animals were free-roaming within the park but were fed on the National Elk Refuge during winter. Bison consumed native vegetation during all seasons but were fed pelleted alfalfa during winter. Hay for pellets was grown in Idaho. Bison ranged in age from 1 to 10 yr.

Table 1—Overall least square means for moisture content (%) and selenium content of game meat and beef (μg/g)

Species	Moisture	Selenium	
		Wet wt	Dry wt
Elk	73.7[a]	0.40[b]	1.6[b]
Mule deer	73.1[a]	0.64[c]	2.5[c]
Bison	73.3[a]	0.49[bc]	1.7[b]
Beef longissimus	70.8[b]	0.10[a]	0.4[a]
semitendinosus		0.10[a]	0.5[a]

[abcd] Within each column, different means indicated by different superscript ($P<0.05$).

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Table 2—Selenium content of elk, mule deer, and bison (μg/g)

Muscle/ Gender	Elk					Mule deer					Bison				
	Wet wt			Dry wt		Wet wt			Dry wt		Wet wt			Dry wt	
	N[a]	Mean	SEM[b]	Mean	SEM	N	Mean	SEM	Mean	SEM	N	Mean	SEM	Mean	SEM
Abdomen															
Male	3	0.4	0.24	1.6	0.92	4	0.7	0.06	2.6	0.21	8	0.6	0.08	2.1	0.30
Female	7	0.7	0.04	2.6	0.15	–[c]	–	–	–	–	8	0.7	0.09	2.2	0.30
Shoulder															
Male	4	0.7	0.17	2.4	0.59	4	0.4	0.05	1.4	0.26	8	0.4	0.03	1.4	0.15
Female	7	0.7	0.05	2.7	0.20	–[c]	–	–	–	–	9	0.4	0.07	1.5	0.26
Longissimus															
Male	4	0.1	0.07	0.5	0.17	4	0.9	0.03	2.9	0.09	–[c]	–	–	–	–
Female	7	0.1	0.02	0.4	0.07	–[c]	–	–	–	–	1	0.3	–	1.0	–
Semimembranosus															
Male	3	0.1	0.00	0.4	0.10	4	0.7	0.06	2.4	0.33	1	0.3	–	1.2	–
Female	9	0.1	0.02	0.6	0.10	–[c]	–	–	–	–	1	0.6	–	2.0	–

[a] Number of samples used for analysis.
[b] Standard error of mean.
[c] no data available.

Moisture content of samples were determined in duplicate, using the standard AOAC method (AOAC, 1984). Data for Se content were expressed on both a wet and dry weight basis. Sample preparation and wet ashing were conducted by a modification of a published method (Schelkoph and Milne, 1988). Intact muscles were minced with a meat grinder to achieve a uniform sample. About 1.5 to 2.0g wet sample (≈0.5g dry matter) were dried in a 120 mL nitric acid rinsed Teflon PFA vessel (CEM Corp, Indian Trail, NC) for 24 hr or to constant weight. Wet ashing of the tissue was in 10 mL of 70% nitric acid inside the pressurized digestion vessel. Digestion took place in a microwave digestion system (CEM Corp, Indian Trail, NC) with a 600 W magnetron. The microwave system was programmed to process the sample 4 min at 65% power and then 15 min at 40% power. This program ensured digestion of all organic material. A bovine liver sample from the National Bureau of Standards (Reference No. 1577a) was used as external standard. Liver was processed in a manner identical to the muscle samples.

Selenium was analyzed by graphite furnace (Perkin-Elmer model 5000 AA Spectrophotometer with a Model HGA, Perkin-Elmer Corporation, Norwalk, CT). A sample (40 μL) of digest plus 10 μL of 2000 ppm palladium, added as a matrix modifier, was analyzed. The 204 nm line was used to limit Fe interference with background correction.

Data were analyzed by analysis of variance (ANOVA). One-way ANOVA was used to determine differences among species. Least square means were calculated. Species and muscle differences were determined by two-way ANOVA using species (male elk and deer) and muscle (longissimus, semimembranosus, shoulder and abdomen) as factors. ANOVA was also used to determine differences among gender (male and female), species (elk and bison), and muscle (shoulder and abdomen muscles only). A separate ANOVA was calculated to detect gender (males and females) and muscle (longissimus, semimembranosus, shoulder and abdomen) differences for elk data. The Statistical Analysis Software (SAS) system was used for data calculations (SAS Institute, Inc., 1985). Significant means were identified using the least significant difference test.

RESULTS & DISCUSSION

PUBLISHED data on the Se content of game is either lacking or very limited. Various studies contain other information on nutrient composition of wild game. Miller et al. (1986) determined moisture and various lipid components of antelope (*Antilocapra americana*), mule deer, and elk. Marchello et al. (1989) reported the proximate composition and mineral content of raw and cooked bison, but Se was not included in the study. Olson and Palmer (1984) included game in their report on Se content of foods, but only single observations of several species were reported. Because only one other study has reported Se content of game (Olson and Palmer, 1984), Se content of beef was included in our study to enable comparison with others.

Deer, bison and elk contained more moisture and Se (wet weight) than did beef ($P<0.05$) (Table 1). Mule deer also contained more Se (wet weight) than elk ($P<0.05$) and bison, though there was no difference between bison and deer ($P>0.05$). On a dry weight basis, deer contained more Se than the other species ($P<0.05$), and elk and bison were different from beef ($P<0.05$). Se content of elk, deer, and bison by species, muscle and gender is shown in Table 2.

Muscle and species differences were determined using data for longissimus, semimembranosus, shoulder and abdomen muscles and male elk and deer. Se content differed between species ($P<0.05$) with deer samples containing more Se than elk. These animals were reared in the same research facility and were fed a similar diet, except deer had access to pasture grass and elk were housed in dirt paddocks. This indicated either a species difference or the grass consumed by the deer contained enough Se to cause the change in Se content of the muscle. Selenium content in various muscles did not differ ($P>0.05$) but there was a species by muscle interaction ($P<0.05$).

Species, muscle and gender differences were statistically analyzed for male and female elk and bison for shoulder and abdomen muscles. There was a significant species main effect ($P<0.05$) and species by muscle interaction ($P<0.05$). Elk contained more Se than bison ($P<0.05$). No other significant relationships were detected.

We studied any influence of gender on muscle Se content with the elk data. Gender differences were not detected ($P>0.05$) but muscle type was a significant main effect ($P<0.05$). Se content of abdomen and shoulder were higher than both longissimus and semimembranosus muscles. Abdomen and shoulder Se did not differ nor did longissimus and semimembranosus. The best comparison between these data and published values was for deer muscle, although the comparison is inexact because species source of previously published data are unknown. Olson and Palmer (1984) reported an average Se content of 0.49 μg/g (wet weight) for two deer samples. Muscle type was not stated. With exception of shoulder muscle, this value was lower than the Se values for deer we found (Table 2). In contrast, Se content of beef muscle (specific muscle unknown) in the South Dakota study (Olson and Palmer, 1984) was higher (0.27 to 0.46 μg/g, wet weight) than the beef Se content we found (Table 1). High Se content in beef (0.36 μg/g) was also reported by Morris and Levander (1970) but exact origin of samples was not known. Snook et al. (1987) reported 0.14 μg/g (wet weight) of Se in miscellaneous cuts of cooked beef. Values for uncooked samples were not reported. Because of a possible concentration effect from cooking, we could assume Se content of raw muscle would have been lower. Se content of the beef we studied (Table 1) compared most closely to values reported by Snook et al. (1987) which was unexpected

since Ohio is a low-selenium area. However, the exact muscle types analyzed by Snook et al. (1987) are unknown. Another unknown and uncontrolled factor possibly influencing Se content of beef would be Se content of the diet of the animal.

Toxicity from Se in individuals who consume game meat from high selenium soil areas is not likely. The game animals used in this study consumed hay or forage from areas with soil selenium exceeding the critical limit for toxicity in animal feeds by 10 to 100 fold (Soltanpour and Workman, 1980). Humans living in high seleniferous areas may be at risk for Se toxicity if foods originating from the same area were frequently consumed. Longnecker et al. (1991) studied the Se intake and clinical health of individuals living in an area known for high Se soil content. Average daily Se intake was ≈239 μg/d, much higher than the reported average which ranges from 20-90 μg/d (Pennington et al., 1989). Although Longnecker et al (1991) reported individual Se intake as high as 724 μg/day, no compelling evidence of Se toxicity was detected. Yang et al. (1989) reported clinical signs of toxicity in a Chinese population when intake was 850 μg/day.

The game we studied contained mildly elevated Se compared to beef and game from another region (Olsen and Palmer, 1984). Se intake from consumption of similar game would not likely result in toxicity. However, two of the species in our study were captive and may not be similar to free-roaming game. Also our sample numbers were relatively low, so generalizations to all game cannot be made. Our data adds to the limited and incomplete information regarding Se content of game. A clinical study similar to that of Longnecker et al. (1991) on hunters and others using single sources of meat in the diet would be needed to determine unequivocal risk of potential cases of Se toxicity from consuming wild game.

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Edible Meat Casing from Reconstruction of Collagen-Elastin Matrix

KOJI TAKAHASHI and MAKOTO HATTORI

ABSTRACT

Pepsin-solubilized elastin (PSE) was prepared from insoluble elastin (IE) preparation by digesting it with pepsin [IE:pepsin, 100:1(w/w)] at 25°C for 5–30 hr prior to dialyzing against water and lyophilizing. PSE showed amino acid compositions similar to that of IE and relative molecular weight (M_r) of 6-640 × 10^3. PSE accelerated the matrix reconstruction of pepsin-solubilized collagen (PSC) and reduced the redissolution of the PSC matrix under several solvent conditions. PSE elevated the matrix thermal denaturation temperature from about 50°C to 55°C, leading to decreased breaking strength and reduced elongation of the PSC film, as compared with the control.

Key Words: meat, edible casing, collagen, elastin, enzymes

INTRODUCTION

FOR STUFFING SAUSAGE, edible collagen casings are usually used in place of natural casings because of uniformity in size and physical characteristics. However, they have some disadvantages. These include greater strength than natural casings, resulting in undesirable chewing properties. They do not always have a proper denaturation temperature for the drying of wet casings from a collagen solution or dough under a hot air stream.

Since elasticity affects breaking properties, the first defect, breaking strength too great could be averted by forming casings with a proper elasticity in reconstructed collagen fibrils. The second defect, low denaturation temperature, could be overcome by increasing intermolecular adhesion of the fibrils. Thus, the addition of an elastic substance interacting with collagen in the collagen solution or dough may be effective for reconstructing a collagen matrix with desired elasticity and denaturation temperature. This could lead to improved chewing properties for edible collagen casings. Since elastin is an edible protein resource with good elasticity, we investigated its use as a textural modifier for collagen casings.

For this purpose, it was necessary to obtain soluble elastin peptide chains because elastin is highly insoluble. Several solubilizations have been studied. Hot oxalic acid (Partridge et al., 1955) and alcoholic alkali (Moschetto et al., 1974) are ordinarily used. However, in those methods, it is difficult to regulate cleavage of the peptide chain, resulting in the production of a wide distribution of peptide fragments, as indicated by amino acid sequence and molecular weight. On the other hand, an enzyme treatment could result in specific amino acid sequences, depending on substrate specificity. Our objective was to solubilize insoluble elastin (IE) with pepsin, and apply the pepsin-solubilized elastin (PSE) to regulate matrix reconstruction from soluble collagen and thus improve the denaturation behavior and breaking properties of the collagen film.

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MATERIALS & METHODS

Preparation of pepsin-solubilized collagen (PSC)

Insoluble pigskin collagen cubes prepared according to Takahashi et al. (1987) were pulverized with a POLYTRON homogenizer (Kinematica, Switzerland) and solubilized by pepsin (Sigma, 3200 U/mg) digestion. Pepsin (10 mg) was added to 200 mL 0.5M acetic acid containing 1g of pulverized insoluble collagen and the reaction mixture was incubated at 25°C for 24 hr with gentle stirring according to Takahashi et al. (1989). From the centrifuged supernatant, PSC was obtained by salting out and redissolving in 0.05 M acetic acid three times, dialyzing against water and lyophilizing. PSC was kept at −20°C until used for collagen matrix reconstruction.

Preparation of PSE

IE was prepared from minced bovine *Ligamentum nuchae* by extracting it with 10% sodium chloride, acetone and ether, followed by autoclaving at 2 kg/cm^2 for 1 hr. This step was repeated seven times according to the method of Partridge et al. (1955) and IE was recovered by lyophilization. IE, pulverized with dry ice-acetone, was solubilized by digesting it with pepsin as described above except a 5–30 hr digestion time was adopted. The enzymic reaction was stopped by adjusting pH to 12 with 30% sodium hydroxide following centrifugation. The supernatant was dialyzed against water and lyophilized to recover PSE.

Collagen matrix reconstruction with PSE

Reconstruction of the collagen matrix (collagen gel with three-dimensional network structure) with PSE was performed according to the procedure of Nomura et al. (1989). PSC was dissolved in 0.5M acetic acid at 4°C to give a concentration of about 1 mg/mL and the mixture was dialyzed against 0.067M phosphate buffer at pH 8.0 before centrifuging at 18,000 rpm for 30 min. After degassing, PSE, dissolved in the same phosphate buffer, was combined with the PSC solution to give the following concentrations: 0.67 mg/mL PSC and 0.076 mg/mL PSE. This mixed solution was incubated at 37°C to reconstruct the PSC matrix and the reaction was monitored by absorbance at 310 nm.

Preparation of collagen film containing PSE (PSC-PSE film)

The PSC matrix, reconstructed with PSE (PSC-PSE matrix) on an acrylic resin tray (10 × 10 × 0.5 cm^3) at 33°C for 18 hr [PSC conc, 0.3%; PSE:PSC, 1:9 or 1:2(w/w)], was dehydrated and desalted in ethanol with stepwise increases in concentration of 40, 70 and 99.5%. PSC-PSE film was obtained by drying it under air stream at 33°C for 24 hr.

Solubility of PSC-PSE matrix

The PSC-PSE matrix was dialyzed against water at 4°C for 24 hr, heated in water at 45, 48 or 50°C for 20 min, dissolved in 0.25M acetic acid at 4°C for 1 hr or dissolved in 0.5% sodium dodecyl sulfate (SDS)/25 mM acetate buffer at pH 7.0 at 25°C for 48 hr. In each solubilization, 20 times the amount of solvent based on the matrix weight was used. After centrifugation at 18,000 rpm (35,500 × g) for 30 min, the residual matrix was dried at 110°C to constant weight. Solubility was evaluated from the dry weight.

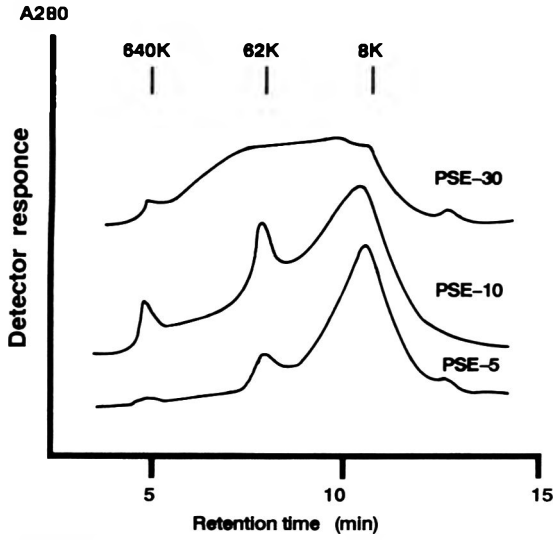

Fig. 1—HPLC patterns of pepsin-solubilized elastins (PSE). PSE-5, PSE-10 and PSE-30, elastins solubilized with pepsin-digestion at 25°C for 5, 10 and 30 hr, respectively. Absorbance was monitored at 280 nm by a Tosoh UV-8000 detector connected with a Shimadzu C-R6A integrator.

Table 1—Amino acid composition and saccharides contents of pepsin-solubilized elastins[a]

Amino acid[b]	IE	PSE-5	PSE-10	PSE-30
Hydroxyproline	10	11	11	11
Aspartic acid	6	19	12	9
Threonine	9	18	14	9
Serine	9	19	14	10
Glutamic acid	15	23	19	18
Proline	115	131	138	131
Glycine	328	304	315	300
Alanine	221	166	177	224
Cystine	0	2	2	<1
Valine	128	133	136	134
Methionine	1	2	1	1
Isoleucine	25	28	27	24
Leucine	63	78	71	57
Tyrosine	10	13	8	11
Phenylalanine[c]	48	39	45	53
Unknown	3	2	2	3
Hydroxylysine	0	0	0	0
Lysine	3	4	4	4
Histidine	1	1	<1	<1
Arginine	6	7	7	4
Desmosine[d]	2.6	2.0	1.7	3.1
Isodesmosine[d]	1.8	1.4	1.2	2.3
Total saccharide[e]	0.3	0.8	0.6	0.5
Uronic acid[f]	–	0.6	0.3	–

[a] IE, insoluble elastin; PSE-5, PSE-10 and PSE-30, elastins solubilized with pepsin-digestion at 25 for 5, 10 and 30 hr, respectively.
[b] Residues per 1,000 amino acid residues.
[c] Containing presumably desmosine and isodesmosine.
[d] Mol/10^5 g.
[e] % as glucose.
[f] % as glucuronic acid.

Size exclusion chromatography (SEC)

SEC of PSE was carried out using a HPLC pump (Tosoh CCPM, Japan) and a TSK gel G3000SW column (7.8 × 300 mm) (Tosoh, Japan) equilibrated with 0.067M phosphate buffer at pH 7.0 containing 0.3M sodium chloride. The PSE sample (100 μg/100; μL) was applied to the column and eluted at 1.0 mL/min. Absorbance was monitored at 280 nm.

Electron microscopic examination

PSC-PSE (1:2) matrix in 0.05 M acetate buffer, pH 7.0, was washed to remove the free PSC and PSE with fresh buffer solution by centrifuging it 5 times at 3,000 rpm for 20 min. The supernatant was removed after every wash. The washed matrix was fixed with 2% glutaraldehyde/0.05 M acetate buffer at pH 7.0 at 4°C for 15 hr, washed thoroughly with water and dehydrated in ethanol with stepwise concentrations of 50, 70, 80, 90, 95, 99, and 99.5%. After critical point drying with carbon dioxide and Pt coating with an ion sputter, samples were examined by a Hitachi S-800 scanning electron microscope at an accelerating voltage of 5 kV. For transmission electron microscopy (TEM), the washed sample was dispersed with a glass homogenizer, allowed to settle on the microgrids coated with carbon and positively stained with 1% uranyl acetate and 1% phosphotungstic acid. After washing, TEM was done using a JEOL JEM-100C microscope at direct magnification of 33,000.

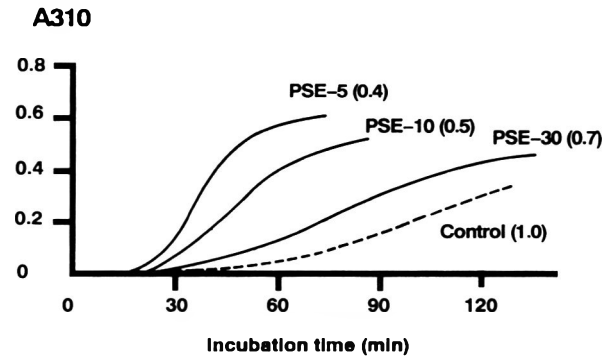

Fig. 2—Changes in collagen matrix reconstruction as affected by pepsin-solubilized elastins (PSE) and incubation time. PSE-5, PSE-10 and PSE-30, elastins solubilized with pepsin-digestion at 25°C for 5, 10 and 30 hr, respectively. ( ): ration of Tmax (the time at maximum velocity) to that of the control.

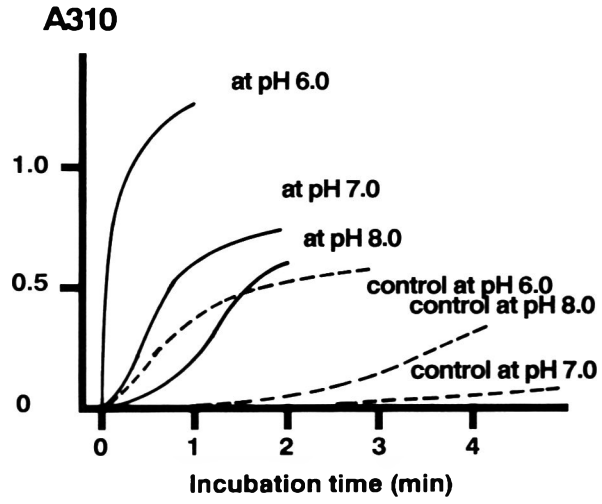

Fig. 3—Effect of pH on the collagen matrix reconstruction with pepsin-solubilized elastin (PSE). PSE-5 (pepsin-digestion time, 5 hr) was used.

Table 2—Solubility of collagen matrix with pepsin-solubilized elastin (PSE) under different solvent conditions (%)

Matrix (PSE:PSC[a])	Dialysis at 4°C[b]	Water at 45°C[c]	Water at 48°C[c]	Water at 50°C[c]	0.25 M acetic acid[d]	0.5% SDS /25mM acetate buffer[e]
Control	39	14	80	92	83	84
1 : 9	19	11	57	81	87	45
1 : 2	12	4	31	75	36	36

[a] PSC, pepsin-solubilized collagen.
[b] Dialysis against water for 24 hr.
[c] Dissolution for 20 min.
[d] Dissolution at 4°C for 1 hr.
[e] Dissolution at pH 7.0 at 25°C for 48 hr.

Differential scanning calorimetry (DSC)

DSC of the PSC-PSE matrix was performed using a DSC apparatus (Seiko SSC-5020 DSC 100, Japan) as described by Takahashi et al.

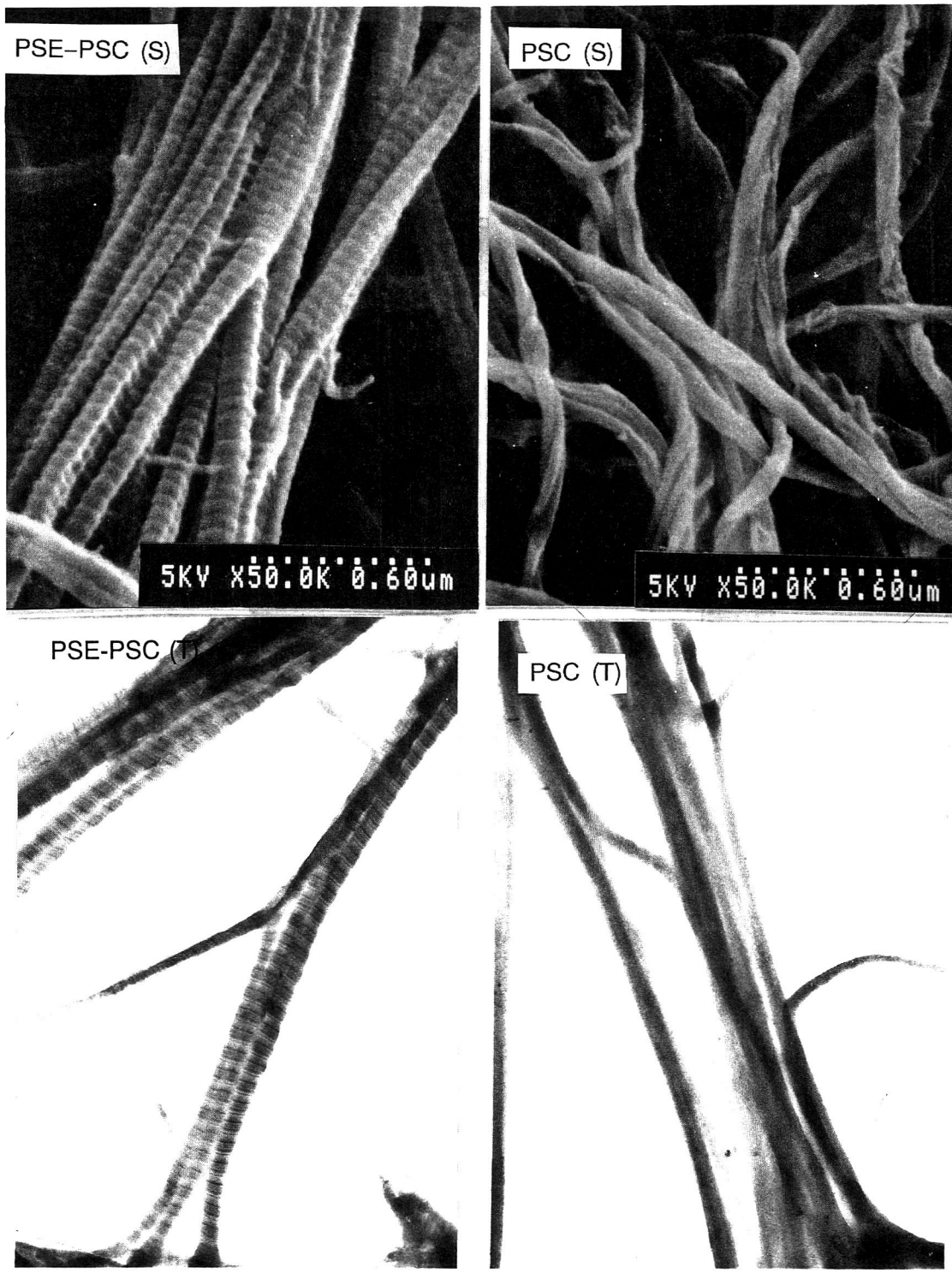

Fig. 4—Electron micrographs of collagen matrix with pepsin-solubilized elastin (PSE). PSE-10 (pepsin-digestion time, 10 hr) and pepsin-solubilized collagen (PSC) was used. PSE-PSC, matrix with PSE; PSC, control matrix; (S), SEM observation; (T), TEM observation (direct magnification, 33000).

(1988). The sample, scaled in a cell, was dissolved in 0.05M acetate buffer at pH 7.0 at 100°C for 20 min after DSC and the protein concentration was measured by the microbiuret method. From DSC curves, denaturation temperatures [onset temp (T_o), peak temp (T_p) and final temp (T_f)] and denaturation enthalpy were estimated as characteristics of the thermal denaturation process.

Measurement of tensile strength

Tensile strength of PSC-PSE film was measured in air or water using a thermal mechanometer (Seiko SSC-5020 TMA/SS 100, Japan). Sample (2.0 × 5.0 × 0.013 mm^3) was chucked on the probe and extended at a loading rate of 40 g/min in air or 2 g/min in water at 25°C in order to obtain the stress-strain curve.

Analytical methods

Amino acid composition of PSE was determined as described previously (Takahashi et al., 1987). Desmosine and isodesmosine were quantified by ion-pair chromatography. After hydrolyzing in 6N HCl at 110°C for 48 hr in vacuo, hydrolyzate was analyzed on a column of TSK gel ODS-80™ (4.6 × 150 mm) (Tosoh, Japan) equilibrated

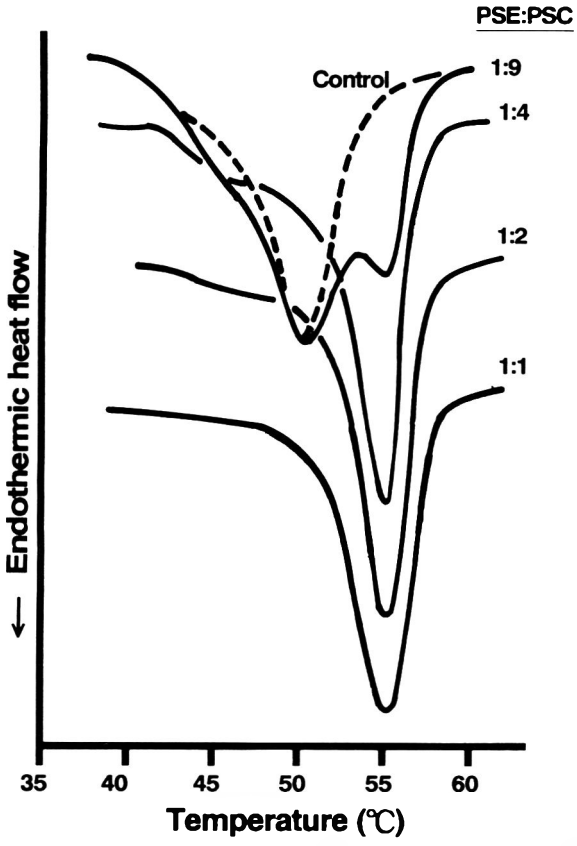

Fig. 5—Thermal denaturation of collagen matrices with pepsin-solubilized elastin (PSE) determined by DSC.

with 0.1M methanesulfonic acid (pH 2.0) containing 6 mM sodium 1-heptanesulfonate and 10% (v/v) acetonitrile at 1.0 mL/min at 25°C. Absorbance was monitored at 275 nm.

Total saccharides, uronic acid and protein of PSE were determined by the phenol-sulfuric acid method (Dubois et al., 1956), the sulfuric acid-carbazole method (Bitter and Muir, 1962) and the microbiuret method (Itzhaki and Gill, 1964).

RESULTS & DISCUSSION

Chemical composition of PSE

Yields of PSE after 5, 10 and 30 hr digestion (PSE-5, PSE-10 and PSE-30) were 11, 23 and 73%, respectively. Amino acid compositions of these PSEs demonstrated the absence of hydroxylysine (Table 1), indicating complete removal of collagen from the elastic tissue. All PSEs showed high contents of nonpolar amino acids (750–790 residues) similar to that of IE, but PSE had slightly higher proline content than that of IE. This suggested that the PSE preparations had the amino acid sequence specific to IE. However, PSEs, in particular, PSE-5, had somewhat higher content (42 residues) of acidic amino acids (aspartic acid, glutamic acid) than IE, although little difference in cross-linked amino acids was observed. A small amount of total saccharides and uronic acids was found, suggesting the presence of hexuronate-containing components such as glycosaminoglycan or proteoglycan in ease PSE.

PSE-5 and PSE-10 were composed of three peak components with *Mr* 10×10^3, 62×10^3 and 640×10^3 (as globular protein) without low molecular components, while PSE-30 indicated distributed molecular weight of *Mr* $8 \times 10^3 - 640 \times 10^3$ (Fig. 1).

Reconstruction of PSC-PSE matrix

Changes in absorbance of a 1:9, PSE:PSE solution were measured to investigate the effect of PSE on the reconstruction of PSC matrix, because the progressive aggregation of PSC increased absorbance of the reaction mixture. As shown (Fig. 2), the absorbance/incubation time curves, indicative of collagen matrix reconstruction, ordinarily have three or four steps corresponding to the lag phase, the growth phase and the stationary phase of molecular rearrangement. The time required for maximum velocity of the matrix reconstruction (*T*max) corresponds to the second step reaction. Then, the values of *T*max were recorded. *T*max of the control was 105 min. However, in the presence of PSE, *T*max was reduced in all cases compared with control. In particular, with PSE-5 and PSE-10, *T*max were reduced to about 2/5 and 1/2 of the control. It appeared that PSE accelerated the molecular rearrangement of PSC.

Using PSE-5, further experiments were performed to investigate the effect of pH on the reconstruction of PSC-PSE matrix. At any pH, the matrix was rapidly reconstructed with PSE as compared with the control, and with decreasing pH, reconstruction was accelerated (Fig. 3). Especially at pH 6.0, no lag phase was observed on the matrix reconstruction curve. Since the isoelectric points of PSC and PSE were about 9 and about 4, based on precipitation behavior, the decrease in pH from 8 to 6 resulted in increase in the positive charge of PSC. Therefore, the primary driving force for the acceleration of molecular rearrangement probably was the electrostatic interaction.

Amounts of reconstructed matrix and incorporated PSE were estimated by determination of PSC and PSE concentrations in the centrifugal supernatants after matrix reconstruction. PSE concentration was determined from the desmosine content. The collagen matrices reconstructed without PSE were about 47, 51 and 62% at pH 6, 7 and 8, respectively, while with PSE, about 52, 58 and 64%. This indicated that, with PSE, collagen matrices could be reconstructed over a wide pH range as compared with the control. PSE incorporated per PSC matrix was low (0.01–0.02%), suggesting that the interactive site of PSE was quite limited. For further experiments, we used PSE-10 because the yield of PSE-5 was relatively low (11%).

Solubility of PSC-PSE matrix

The stability of PSC-PSE matrix to redissolution was examined under a wide range of conditions. Solubility of the control matrix in water increased as temperature increased (Table 2). However, the presence of PSE resulted in a marked decrease in solubility. For example, solubility of PSE-PSC (1:9) and PSE-PSC (1:2) matrix at 48°C was about 70% or 40% of the control. Solubility in acetic acid and SDS solutions also showed similar results. Therefore, it appears that PSE could improve the stability of a PSC matrix as indicated by reduced solubility.

Electron microscopic observation of PSC-PSE matrix

Electron micrographs of the PSC-PSE matrix (Fig. 4) show the control matrix by SEM had slightly twisted fibrils (diameter, about 60-100 nm) with the coarse surface forming a well-developed network structure. TEM of the control matrix showed a no band pattern on the fibrils. This suggested that random molecular rearrangement of PSC occurred. In the case of added PSE (PSE:PSC, 1:2), the predominant thick fibrils (diameter, 80–140 nm) with a constriction at regular intervals (about 50 nm) and orderly fibril aggregates (fibers) were clearly observed on SEM. TEM demonstrated a periodic cross-striated structure and subbands of nine lines in one period on the fibrils, indicative of ordered molecular rearrangement like the native type. This response suggested that PSC matrix with PSE produced high intermolecular cohesion compared with the control. Therefore, PSE is important for the ordered molecular rearrangement of PSC and the orderly assembly of microfibrils and fibrils.

Table 3—Thermal denaturation characteristics[a] of collagen matrix with PSE evaluated by DSC

Matrix (PSE:PSC)	Denaturation temperature[b] (°C)				Denaturation enthalpy (mJ/mg)
	T_o	T_{p_1}	T_{p_2}	T_f	
Control	46.8 ± 0.4	50.1 ± 0.1		53.4 ± 0.4	68.0 ± 1.6
1 : 9	45.0 ± 0.9	50.6 ± 0.1	55.2 ± 0.1	57.3 ± 0.1	67.7 ± 0.5
1 : 4	51.8 ± 0.3		55.1 ± 0.1	56.9 ± 0.2	56.6 ± 0.8
1 : 2	52.1 ± 0.3		55.3 ± 0.1	57.3 ± 0.2	69.9 ± 2.2
1 : 1	51.5 ± 0.1		55.3 ± 0.1	58.2 ± 0.1	70.5 ± 3.4

[a] Mean of three determinations with standard deviation.
[b] T_o, onset temperature; T_{p_1}, peak temperature of low temperature peak; T_{p_2}, peak temperature of high temperature peak; T_f, final temperature.

Table 4—Breaking properties[a] of PSC-PSE films evaluated by thermal mechanometry

Breaking property	Medium	PSC-PSE film		
		Control	PSC:PSE = 1:9	PSC:PSE = 1:2
Breaking strength (Pa)	Air	(8.6 ± 0.3) × 10^7	(8.0 ± 1.4) × 10^7	(6.2 ± 0.9) × 10^7
	Water	(9.3 ± 0.8) × 10^6	(6.9 ± 0.4) × 10^6	(2.7 ± 0.3) × 10^7
Elongation at break (%)	Air	14 ± 1.1	19 ± 2.3	20 ± 0.8
	Water	33 ± 2.5	25 ± 2.9	25 ± 2.2
Young's modulus at break (Pa)	Air	(6.1 ± 0.7) × 10^8	(4.3 ± 1.3) × 10^8	(3.1 ± 0.6) × 10^8
	Water	(2.9 ± 0.5) × 10^7	(2.7 ± 0.5) × 10^7	(1.1 ± 0.2) × 10^7

[a] Mean of four determinations with standard deviation.

Thermal denaturation of PSC-PSE matrix

Thermal denaturation of the control matrix occurred in a region of about 47–53°C and with a relatively sharp single peak about 10°C higher than that of collagen molecules in solution (Fig. 5 and Table 3). PSC-PSE matrix at the ratio of 1:9 indicated two endothermic peaks around 50°C and 55°C, unlike the control. An increase in PSE resulted in disappearance of the low temperature peak and expansion of the high temperature peak. Thus, a matrix with a stable macrostructure could be reconstructed with PSE compared to the control. This was compatible with results of the reduced solubility and reconstruction of the ordered structure. Proteoglycan such as proteodermatan sulfate accelerates the reconstruction of collagen matrix with a less stable macrostructure (Nomura et al., 1989). Thus, the increase in thermal stability observed was considered to be caused not by the hexuronate-containing components in PSE, but by PSE itself. On the other hand, it seemed that there was little difference in the denaturation enthalpy among different matrices.

Breaking properties of PSC-PSE film

PSC film without PSE indicated a relatively high breaking strength, while PSC-PSE film had a very low breaking strength (Table 4). The decrease in breaking strength was more notable in water than in air because the fibrils in film can slip more easily in water. The breaking strength of PSC-PSE film at the 1:2 ration was only 30% that of the control. The elongation at break of PSC-PSE films in water also decreased noticeably compared to the control, leading to a decrease in Young's modulus at break. Because the collagen fibrils with random molecular orientation in the control film could be straightened or aligned by increased tension (Myers et al., 1988), the control film showed a relatively large elongation and a high breaking strength. PSC-PSE film had limited elongation depending on its ordered molecular rearrangement, and thus it was easy to break. These results suggest that PSE could be applied to improve the breaking properties and the thermal denaturation behavior of edible collagen casings.

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IgG Antibody from Hen Egg Yolks: Purification by Ethanol Fractionation

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ABSTRACT

A procedure was developed for large-scale preparation of IgG antibodies from egg yolks. The supernatant from egg yolks was obtained after an initial 9-fold dilution with water. The lipids in the supernatant were then almost completely eliminated from the water-soluble protein fraction containing the antibody, by precipitation with 60% ethanol and filtration. Yolk antibody was purified from the lipid-free water-soluble protein fraction by ethanol fractionation at final concentration 30% (pH unadjusted), and again at 25% (pH 7.4). The purified fraction was composed of >99% pure IgG. Recovery of antibody was calculated as 40%.

Key Words: IgG, hen-egg-yolk, ethanol precipitation, *Streptococcus mutans*

INTRODUCTION

ORAL PASSIVE IMMUNIZATION using specific IgG antibody produced in other animal species has been attempted to prevent oral or gastrointestinal infections induced by certain bacteria and viruses in animals or humans (Lehner et al., 1978, 1985; Ebina et al., 1985; Ma et al., 1987, 1990; Michalek et al., 1987; Fayer et al., 1990; Filler et al., 1991). Polyclonal antibodies were isolated from sera, milk or colostrum of hyperimmunized animals such as rabbits, cows, and goats. In one study, mouse monoclonal antibodies were used for passive immunization (Lehner et al., 1985). Egg yolk has been suggested as a good source of IgG antibody for oral passive immunization since it is cheap and convenient to handle. Egg yolk IgG (yIgG) has been successfully used for protection of experimental animals against infectious diseases and toxemias (Bartz et al., 1980; Yolken et al., 1988; Ebina et al., 1990; Thalley and Carroll, 1990; Hamada et al., 1991).

For application of passive immunization in humans, a large amount of IgG would be required. Large-scale preparation of IgG antibody from human plasma for clinical use stems from classical studies on separating plasma proteins by Cohn et al. (1946). Although several methods have been developed to purify yIgG from egg yolk (Polson et al., 1980, 1985; Jensenius et al., 1981; Bade and Stegemann, 1984; Hassl and Aspöck, 1988; Hatta et al., 1990; Polson, 1990; Wallmann et al., 1990; Akita and Nakai, 1992), they were too complicated for preparation of yIgG on a large scale. Ethanol is an effective protein precipitant with favorable chemical properties such as low toxicity, high volatility, chemical inertness, low cost and ready availability (Kistler and Friedli, 1980). It is the most widely used precipitating agent for large-scale production of major plasma proteins, i.e., albumin and immunoglobulins.

Since egg yolk is composed of phospolipid-protein complex and water-soluble proteins, the major problem is to separate lipoproteins from egg yolk prior to purification of yIgG from water-soluble protein. Our objective was to develop a procedure for removing yolk lipoproteins from eggs and purification of yIgG from the separated water-soluble protein by ethanol fractionation.

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MATERIALS & METHODS

Immunization

White leghorn hens (24 wk old) were immunized intramuscularly with 1 mg of cell-associated glucosyltransferase (CA-GTase) of *Streptococcus mutans* MT8148 (Hamada et al., 1989) emulsified in 1 mL of Freund's complete adjuvant (Difco Laboratories, Detroit, MI). Booster immunization was done 5 wk after primary immunization. Eggs from the hyperimmunized hens were stored at 4°C until processed.

Removal of lipoproteins from egg yolk

The scheme of the method for purification of yIgG from egg yolk is shown in Fig. 1. Pooled egg yolks (100 mL) after separation from the albumen was diluted with 800 mL distilled water (10°C, pH 6.6), mixed and incubated 10 min at 10°C. The supernatant (Sup I) containing yIgG was separated from the precipitate by centrifugation at 1,600 × *g* for 10 min at 10°C. Precooled 95% ethanol (−20°C) was added to Sup I (763 mL) to a final ethanol concentration of 60% (v/v) and mixed by stirring for 30 min at 4°C. The resultant precipitate (Ppt II) was collected by centrifugation at 22,000 × *g* for 20 min at 4°C, dissolved in 763 mL of 30 mM NaCl solution (4°C, pH 6.6).

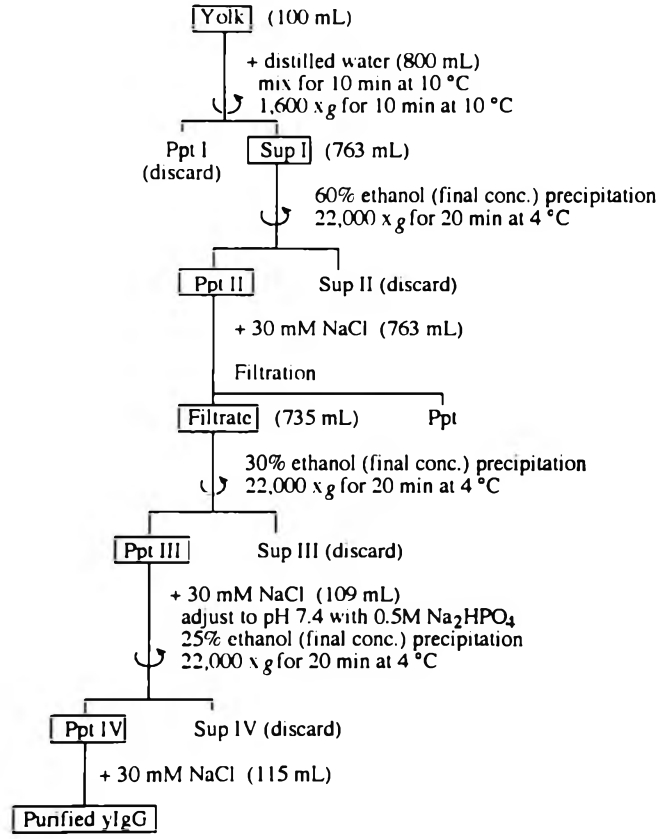

Fig. 1—Scheme for purification of yIgG from egg yolk.

The homogeneous suspension was passed through filter paper (No. 2; Advantec Toyo, Tokyo) at 4°C to remove precipitates (lipoproteins), and water-soluble protein fraction containing yIgG (Filtrate) was obtained. Delipidated egg yolk proteins were also prepared by use of chloroform according to the method of Aulisio and Shelokov (1967). This preparation was used for comparison with the other preparations obtained by ethanol precipitation.

Purification of yIgG from Filtrate

Precooled 50% ethanol (−20°C) was added to the Filtrate (735 mL) to a final ethanol concentration of 30% (v/v) and stirred for 30 min at 4°C. The precipitate (Ppt III) was collected by centrifugation at 22,000 × *g* for 20 min at 4°C, and dissolved in 30 mM NaCl solution (109 mL) at a concentration of 3 mg protein/mL (Ppt III). To purify the antibody further, the above solution was adjusted to pH 7.4 by adding 0.5M Na_2HPO_4 solution, and then by adding precooled 50% ethanol (−20°C) to a final ethanol concentration of 25% (v/v). The mixture was stirred for 30 min at 4°C and centrifuged at 22,000 × *g* for 20 min at 4°C. The precipitate (Ppt IV) was suspended uniformly in 30 mM NaCl solution (115 mL), and tested by immunodiffusion assay using rabbit anti-chicken IgG (Cappel, Cochranville, PA).

Lipid and protein determination

Lipids were extracted from yolk fractions by chloroform-methanol (2:1). After solvent was evaporated from the extract, lipids were determined by weighing. The protein concentration was determined by the method of Bradford (1976).

ELISA

Individual wells of microtiter plates (Immulon 2; Dynatech Laboratories, Chantilly, VA) were coated with 100 μL antigen solution (pH 9.6) containing 0.125 μg purified CA-GTase of *S. mutans* MT8148 (Hamada et al., 1989) per well and the plates were incubated overnight at 4°C. Unbound antigen was removed by washing the wells 6 times with saline containing 0.02% Tween 20. To prevent nonimmunological reactions in the microtiter wells, 150 μL blocking solution containing 3% bovine serum albumin (fraction V; Miles Inc., Kankakee, IL) in 10 mM phosphate-buffered saline (pH 7.4, PBS) was added to each well, and the plates were then incubated for 1 hr at 37°C. Then the wells were washed again, and 100 μL of serial twofold dilutions of samples in PBS containing 0.05% Tween 20 were added to the wells, and the plates were incubated for 1 hr at 37°C and then washed 6 times. Horseradish peroxidase-labeled rabbit anti-chicken IgG (heavy and light chain specific) antibody (Cappel, Cochranville, PA) was added to each well and the plates were incubated for 30 min at 25°C. After the wells were washed 6 times, freshly prepared substrate solution of 0.02% H_2O_2 and 0.04% *o*-phenylenediamine in citrate-phosphate buffer (pH 4.8; 100 μL/well) was added to each well, and incubated for 20 min. The reaction was then stopped with 3N H_2SO_4 (100 μL/well), and the intensity of color was measured at 492 nm using a microplate reader (MTP-32; Corona Electric Co., Katsuta, Japan). The ELISA titer was defined as the maximum dilution giving an A_{492} of ≥ 0.2.

SDS-PAGE

SDS-PAGE was carried out as described by Laemmli (1970). Samples (0.75 to 6 μg protein) were treated for 5 min at 100°C in 50 mM Tris-HCl buffer (pH 6.8) containing 2% (w/v) SDS, 1% 2-mercaptoethanol (ME), 20% (v/v) glycerol and 0.01% bromophenol blue. 2-ME was omitted when samples were prepared for nonreducing gels. Reduced protein samples were analyzed by electrophoresis in a 12.5% (w/v) acrylamide gel and the nonreduced samples in a 7.5% (w/v) acrylamide gel. Proteins were stained with Coomassie brilliant blue R-250 (Sigma, St. Louis, MO).

Gel permeation chromatography

Purity of yIgG was determined by high-performance liquid chromatography using TSKgel 3000SW_{XL} column (7.5 mm × 30 cm; TOSOH, Tokyo). The solvent was a 10 mM phosphate-buffered saline (pH 6.8), at 1.0 mL/min. The elution profile was monitored at 280 nm. Bovine thyroglobulin (669 kDa), apoferritin (443 kDa), β-amylase (200 kDa), alcohol dehydrogenase (150 kDa), bovine serum albumin (66 kDa), and carbonic anhydrogenase (29 kDa) (Sigma) were used as marker proteins for estimation of molecular weight.

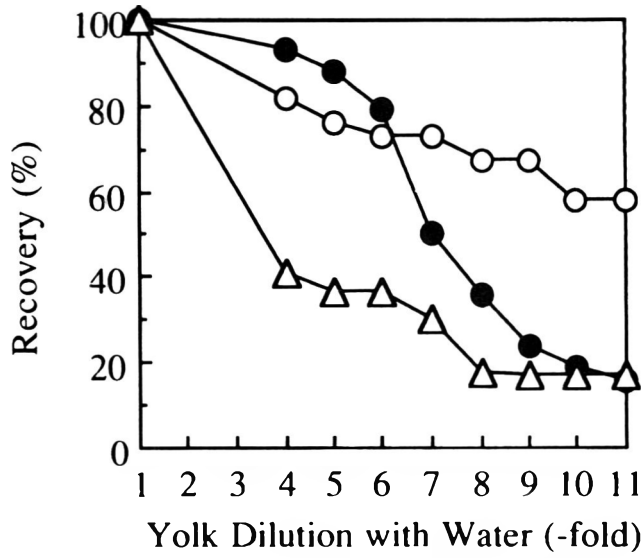

Fig. 2—Effects of diluting egg yolk on selective separation of antibodies and other components from hyperimmunized hens with CA-GTase of S. mutans. ○—○, % antibody activity, ●—●, lipophilic component, △—△, *proteinaceous substances. Antibody titer was measured by ELISA.*

RESULTS & DISCUSSION

Partial removal of lipoproteins from egg yolk

Since egg yolk is composed of phospolipid-protein complex and water-soluble proteins, it was necessary to remove lipoproteins from egg yolk prior to purification of yIgG from water-soluble protein. We first tried dilution of yolk with water. Egg yolks were suspended with increasing quantities of water to extract the antibody fraction containing less lipophilic components in the yolks. Lipid, protein and antibody titer in the Sup I were measured after dilution of yolk with increasing volumes of water (Fig. 2). The lipid and protein contents in Sup I decreased up to 9-fold dilution of yolk with water, while at 9-fold dilution, the antibody titer in Sup I remained ca. 70% of the undiluted yolk. The excellent recovery of antibodies with the lowest lipid content was obtained when egg yolks were diluted 1 : 8 with water. The supernatant thus obtained (Sup I) was used as starting material for the next step.

Complete removal of lipoproteins from Sup I

Akita and Nakai (1992) also used simple dilution of egg yolk with water to separate plasma proteins from granular proteins. However, they concluded that direct ethanol precipitation of yIgG in the supernatant was not efficient to isolate yIgG with high purity. We assumed that the purification of yIgG was disturbed by lipoproteins in the supernatant, and then investigated complete removal of lipoproteins from Sup I using ethanol. Sup I was precipitated with increasing volume of ethanol, and the precipitate (Ppt II) was dissolved in 30 mM NaCl to the same volume of Sup I. The suspensions retained antibody activities when >40% (final concentration) ethanol was added to Sup I, while the lipid content gradually decreased when higher concentrations (>50%) of ethanol were added to Sup I (Fig. 3-A). However, when the NaCl suspension was passed through filter paper, the filtered liquid (Filtrate) retained ca. 85% antibodies present in Sup I, and was almost completely free from lipid when Sup I had been treated with >60% ethanol (Fig. 3-B).

Although the complete removal of lipoprotein from yolk was

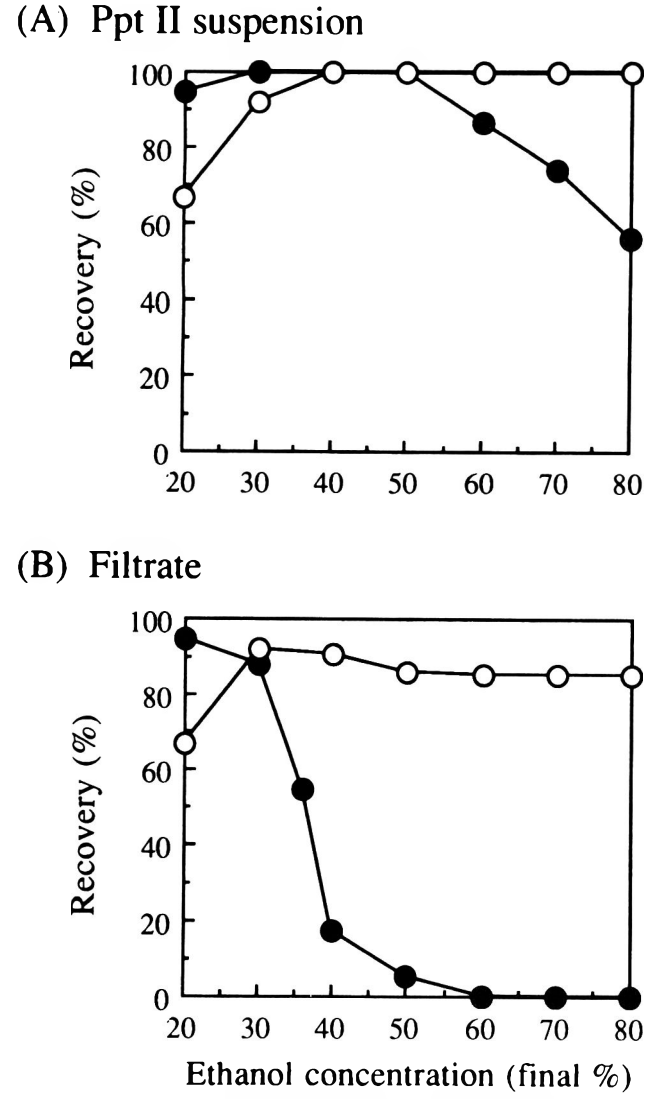

Fig. 3—Recoveries of antibody activity (○) and lipophilic substances (●) in Ppt II suspension and Filtrate (cf. Fig. 1) obtained by ethanol precipitation.

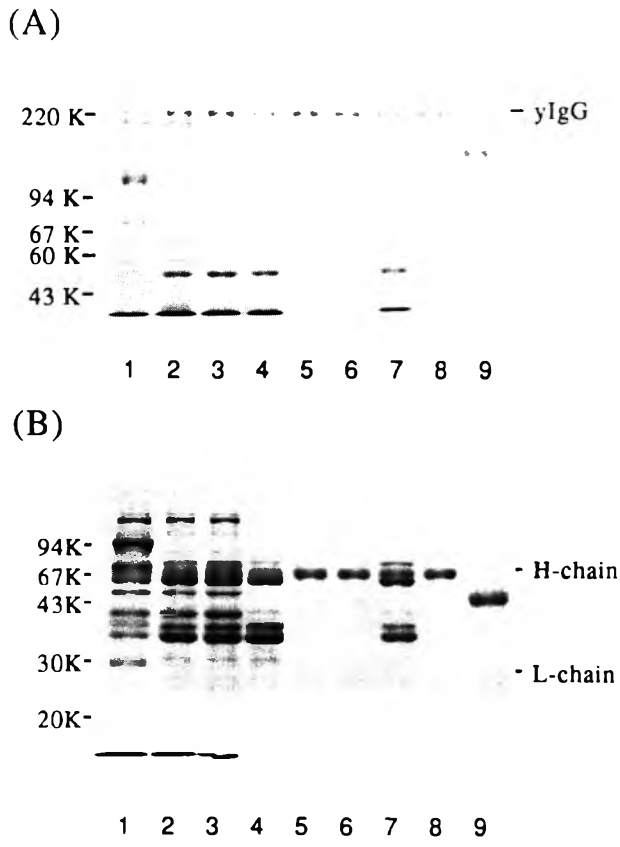

Fig. 4—SDS-PAGE of yIgG. (A) Nonreducing gel. Protein samples were prepared in the absence of 2-mercaptoethanol. Lane 1, egg yolk (4.5 μg of protein); lane 2, Sup I (3 μg); lane 3, Ppt III (3 μg); lane 4, Filtrate (3 μg); lane 5, Ppt III (0.75 μg); lane 6, purified yIgG (0.75 μg); lane 7, chloroform-delipidated water-soluble protein fraction (3 μg); lane 8, purified chicken serum IgG (0.75 μg); lane 9, purified rabbit serum IgG (0.75 μg). (B) Reducing gel. Protein samples were prepared in the presence of 2-mercaptoethanol. Lane 1, egg yolk (6 μg); lane 2, Sup I (4 μg); lane 3, Ppt II (4 μg); lane 4, Filtrate (4 μg); lane 5, Ppt III (1 μg); lane 6, purified yIgG (1 μg); lane 7, chloroform-delipidated water-soluble protein fraction (4 μg); lane 8, purified chicken serum IgG (1 μg); lane 9, purified rabbit serum IgG (1 μg). Proteins were stained with Coomassie brilliant blue R-250.

carried out by precipitation with 60% ethanol and filtration we investigated whether the first dilution of yolk with water was necessary. The supernatant from 2-, 4- or 9-fold diluted yolk was used as starting material in this experiment and then 60% ethanol precipitation followed by filtration of the suspension was carried out to obtain respective filtrates. The recovery of antibody was high (57% of yolk) in the filtrate when the supernatant from 9-fold diluted yolk was used as starting material, while it was very low (15 or 17%, respectively) when the supernatant from 2- or 4-fold diluted yolk was used. No lipoprotein was contained in any filtrate. Thus, we found that the first dilution step with water (9-fold dilution) was necessary for yielding a high recovery of antibody in the filtrate. SDS-PAGE analysis revealed that the filtrate showed very similar protein bands to those of chloroform-delipidated water-soluble protein fraction of egg yolk (Fig. 4). This was the first demonstration of the removal of lipids with high recovery of antibody by using ethanol. Consequently, a lipid-free, water-soluble fraction, i.e., filtrate (Fig. 1), was obtained from Sup I after 9-fold dilution of yolk, and it was used for further purification of yIgG.

Purification of yIgG from the filtrate

To obtain the yIgG fraction, the Filtrate was further purified by precipitation with ethanol at a final concentration of 30%. The sediment (Ppt III) was dissolved in 30 mM NaCl and reprecipitated with 25% (final concentration) cold ethanol at pH 7.4 (Ppt IV). Ppt IV was dissolved again in 30 mM NaCl, and the purity of yIgG was determined by gel permeation chromatography.

The chromatographic profile revealed that Ppt IV gave a single, symmetric peak, indicating that the purity was >99% (Fig. 5). Furthermore, SDS-PAGE analysis gave a single band (Fig. 4-A) and when the sample was reduced with 2-ME, SDS-PAGE yielded two bands (Fig. 4-B) as previously described (Hamada et al., 1991). Ppt IV was reactive with rabbit anti-chicken IgG, forming a single precipitin band in agar immunodiffusion. These results showed that Ppt IV was purified IgG. Purification and recovery of yIgG were summarized (Table 1). The final recovery was 40% of total antibody activity of the starting yolk, somewhat lower than that obtained by pilot-scale production (Jensenius et al., 1981; Polson et al., 1985; Hatta et al., 1990). The immunological activity of purified yIgG was stable in a lyophilized form at 4°C, 20°C or 40°C for at least 6 mo (data not shown). We could obtain highly purified yIgG fraction by ethanol precipitation from the lipid-free filtrate, indicating that complete removal of lipoproteins from yolk was necessary for isolating yIgG with high purity by ethanol fractionation.

With this study we established a method for purification of

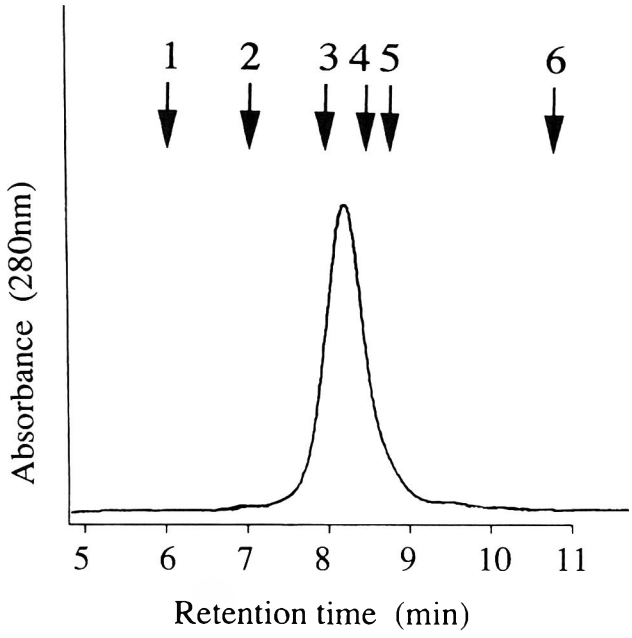

Fig. 5—Profile of high-performance gel permeation chromatography of yIgG. Markers: (1) bovine thyroglobulin (669 kDa), (2) apoferritin (443 kDa), (3) β-amylase (200 kDa), (4) alcohol dehydrogenase (150 kDa), (5) bovine serum albumin (66 kDa), (6) carbonic anhydrogenase (29 kDa).

Table 1—Changes in activity and purity of yIgG during purification

Purification step	Volume (mL)	Total lipid (g)	Total protein (g)	Total activity (Unit)[a]	Recovery of activity (%)	Purity of yIgG (%)
Yolk	100	44	20	171×10^4	100	—[b]
9-fold dilution	763	8	2.8	122×10^4	71	—[b]
60% ethanol ppt	763	7	2.3	122×10^4	71	—[b]
Filtration	735	0	2.0	93×10^4	54	26
30% ethanol ppt	109	0	0.3	80×10^4	47	85
pH 7.4, 25% ethanol ppt	115	0	0.2	68×10^4	40	>99

[a] Total antibody activity (Unit) was defined as ELISA titer × volume.
[b] Not tested.

yIgG by cold ethanol fractionation on a large scale. For separation of yIgG from egg yolks, polyethylene glycol (Polson et al., 1980, 1985; Polson, 1990), dextran sulfate (Jensenius et al., 1981) or carrageenan (Hatta et al., 1990) has been used. However, use of ethanol for purification of yIgG resulted in no residual and/or contaminating chemical reagents, which may provide advantages for manufacturing yIgG preparations as food, pharmaceutical or diagnostic products in terms of safety and purity. Various organic solvents and chemicals have been used to separate antibody proteins from lipophilic substances in egg yolks. For example, Aulisio and Shelokov (1967) isolated water-soluble proteins after removing lipoproteins from egg yolk using chloroform. Bade and Stegemann (1984) isolated water-soluble protein fraction containing yIgG from egg yolk after complete removal of lipoproteins with organic solvents, propane-2-ol and acetone. However, such solvents as those may not be practical for mass preparation of yIgG because of toxicity to the environment as well as humans.

CA-GTase of *S. mutans* synthesizing water-insoluble glucan from sucrose, a virulent factor in dental caries development, was used as antigen in our study. We prepared 700 g of purified yIgG from 20,000 eggs from 1,000 hens immunized with CA-GTase. With it the development of experimental dental caries in rats was significantly inhibited when it was applied orally with a caries-inducing diet (Hamada et al., 1991).

Thalley and Carroll (1990) reported that antivenom yIgG purified from hen eggs immunized with rattlesnake and scorpion venoms neutralized the toxicity of the venoms *in vivo* in mice. However, they chose polyethylene glycol fractionation and affinity chromatography to remove nonimmunoglobulin proteins, which are higher cost and not suitable for large-scale production. Our method could be applied to large-scale production of yIgG, and would be useful for oral passive immunization and intravenous administration to prevent some infectious diseases and toxemias.

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Sialyloligosaccharides of Delipidated Egg Yolk Fraction

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ABSTRACT

Delipidated egg yolk (DEY) was homogenized and centrifuged. The supernatant was dialyzed by ultrafiltration using a molecular weight cut-off of 1,000. The asparagine-linked oligosaccharides in the concentrate were liberated from protein by hydrazinolysis and labeled with UV-absorbing *p*-amionbenzoic ethyl ester (ABEE). The ABEE-derivatized oligosaccharides were fractionated by anion exchange and reverse-phase HPLC. The total sialyloligosaccharides from the water-soluble DEY fraction, were about 47.7% monosialyl and 50.6% disialyloligosaccharides. The structures of 3 predominant sialyloligosaccharides were determined by NMR. They were of the biantennary complex-type, accounting for about 33.6% of the total sialyloligosaccharides of the DEY water-soluble fraction.

Key Words: egg, yolks, sialyloligosaccharides, sialic acid

INTRODUCTION

The nonreducing termini of carbohydrate chains are generally occupied by sialic acid and are binding sites for enzymes, hormones, toxins, lectins, bacteria and viruses. Sialic acid is mainly responsible for the charge, including that of an organism's cell surface. Sialic acid also is important in the transport of ions, amino acids and viruses through membranes (Schauer, 1982). Sialic acid has been reported to have bioactivities, such as serving as influenza virus receptor (Paulson et al., 1984) and binding with pathogenic *Escherichia coli* (Morschhäuser et al., 1990). The terminal glycosylation sequences, in particular sialylated oligosaccharides, which are cell type specific and developmentally regulated, have been implicated in a variety of complex biological events (Feizi, 1985; Rademacher et al., 1988; Paulson, 1989). The bioactivity of erythropoietin, a glycoprotein drug, has been reported to be dependent on glycoside regions (Tsuda et al., 1988).

The role of various sialyloligosaccharides, e.g., sialyl-Le^x and sialyl-Tn, is being explored to create novel carbohydrate-based drugs (Phillips et al., 1990; Lowe et al., 1990). In addition, food companies formulate functional foods by addition of sialyloligosaccharides. Sialyl-Le^x is a tetrasaccharide often found at the terminus of cell surface oligosaccharides of neutrophil and tumor cells (Ichikawa et al., 1992) and has been identified as a ligand of endothelial leukocyte adhesion molecule (ELAM-1). It is involved in inflammatory response. Investigation of the biological function of cell surface oligosaccharides requires efficient methods for preparation of a variety of oligosaccharides, especially sialyloligosaccharides. Chemical methods as well as enzymatic methods are being explored. The synthesis of sialyloligosaccharides is necessary in order to clarify their vital functions (Vliegenthart and Kamerling 1982), but methods are cumbersome and laborious.

Egg yolk is not only nutritionally balanced but a chemical storehouse. Egg yolk has been reported to contain very-low-density lipoprotein (Kocal et al., 1980a, 1980b), phospholipids and proteins such as phosvitin, livetin (Kwan et al., 1991). We reported on the large-scale preparation of N-acetylneuraminic acid from chalaza and egg yolk membrane (Juneja et al., 1991) and delipidated hen egg yolk (DEY) (Koketsu et al., 1992). Our current objective was to isolate and characterize the major sialyloligosaccharide moieties of the water-soluble fraction of DEY as a step toward elucidation of their biological and physiological functions.

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MATERIALS AND METHODS

Materials

The DEY, as reported previously (Koketsu et al., 1992), was used in this study. ABEE (*p*-aminobenzoic ethyl ester) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Sodium cyanoborohydride and *Arthrobacter ureafaciens* neuraminidase were purchased from Nacalai Tesque Inc. (Kyoto, Japan). Bio-gel P-4 (200-400 mesh) was purchased from Bio-Rad (Richmond, CA).

Methods

Water-soluble fraction of delipidated egg yolk (DEY). Egg yolk powder was homogenized with three volumes of ethanol. The suspension was filtered. The DEY was obtained as residue (Koketsu et al., 1992). DEY (50g) homogenized with 150 mL water and then centrifuged. The water-soluble DEY of the supernatant (1.4g) was dialyzed by ultrafiltration (nominal molecular weight cut-off of 1,000).

Ultrafiltration. The water-soluble fraction of DEY was dialyzed with a Filtron OMEGA 1K ultrafiltration unit (Fuji Filter Co., Ltd., Tokyo), which had a membrane of nominal molecular weight cut-off of 1,000, using Master Flex pump PA-25B (Cole-Parmer Co., Chicago, IL).

Liberation of oligosaccharides. Oligosaccharides were liberated from water-soluble DEY by hydrazinolysis and N-acetylation (Takasaki et al., 1982; Bendiak and Cumming, 1985). Water-soluble DEY was suspended in 2 mL freshly distilled anhydrous hydrazine and heated in a sealed tube at 100°C for 10 hr. The reaction mixture was evaporated to dryness under reduced pressure over conc. H_2SO_4 at ambient temperature (≈23°C). The residue was dissolved in saturated $NaHCO_3$ solution, then acetylated by adding acetic anhydride. The reaction mixture was applied to a column of Dowex HCR-W2 (H^+ form, 200-400 mesh).

Preparation of ABEE derivatives. The labeling of oligosaccharides with ABEE was previously reported (Wang et al., 1984; Matsuura and Imaoka, 1988; Ohta et al., 1990). The oligosaccharide fraction was dissolved in 50 μL water. To this solution, 200 μL of a reagent mixture freshly made by mixing 35 mg ABEE, 3.5 mg $NaBH_3CN$, 41 μL acetic acid and 350 μL methanol was added and the mixture was heated at 80°C for 30 min. The reaction mixture was suspended in 1 mL water and extracted 5 times with 1 mL portions of diethyl ether. The ABEE-derivatized oligosaccharides in the aqueous layer were separated into acidic and neutral oligosaccharides by a Presep C18 cartridge (Tessek, Mountain View, CA) chromatography as described by Matsuura and Imaoka, (1988).

Bio-Gel P-4 chromatography. The ABEE-derivatized oligosaccharides were separated by Bio-gel P-4 (1.5 cm × 50 cm, 200-400 mesh), using 0.1 M pyridinium-acetate buffer (pH 4.0). Each fraction was measured at 304 nm, and sugar contents of each fraction was analyzed with phenol-H_2SO_4 reagent. All fractions showing typical UV spectra for ABEE-oligosaccharides were pooled and lyophilized.

HPLC method. HPLC analysis was performed with a HLC-803D (Tosoh Co., Ltd., Tokyo) attached to a Hitachi D-2500 integrator operated at 304 nm. Anion exchange HPLC was performed on a TSKgel DEAE-5PW column (0.75 cm × 7.5 cm, Tosoh Co., Ltd.,

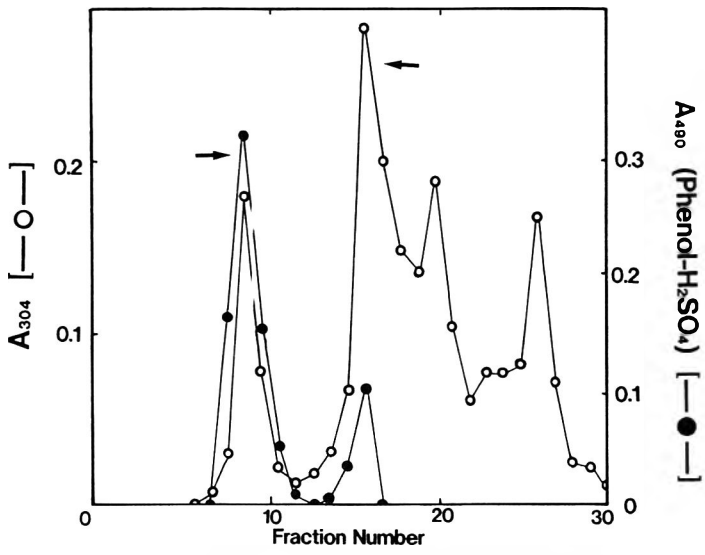


Fig. 1—Elution profile of ABEE-derivatives from Bio-gel P-4 (1.5 × 50 cm, 200–400 mesh, using 0.1 M pyridinium acetate buffer, pH 4.0). Each fraction was measured at 304 nm (- ○ -), and sugar of each fraction was analyzed with phenol-H_2SO_4 reagent (- ● -).

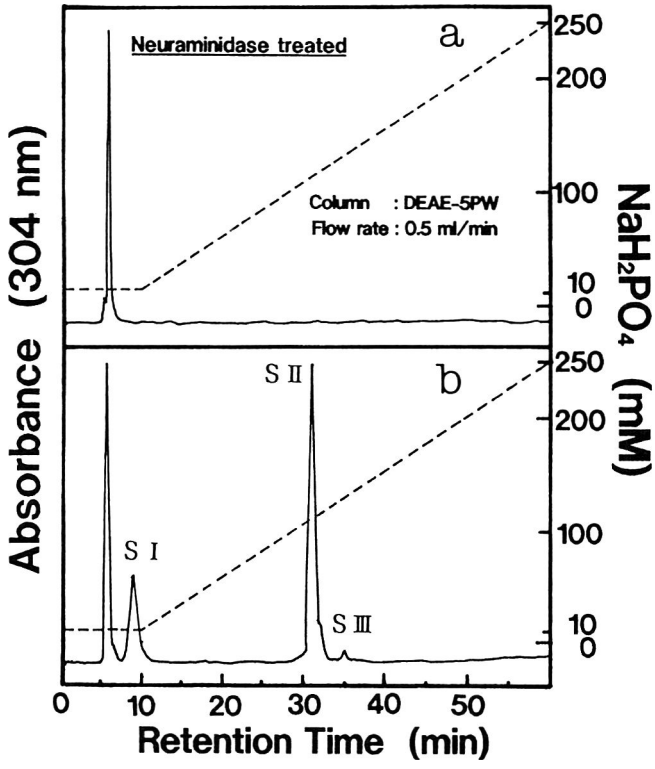


Fig. 2—Separation of ABEE-sialyloligosaccharides from the delipidated egg yolk fraction on a DEAE-5PW anion exchange column (0.75 cm × 7.5 cm) with a linear gradient from 10–250 mM NaH_2PO_4. Flow rate, 0.5 mL/min.; detection, 304 nm. (a), The elution profile of a sialyloligosaccharide fraction obtained by neuraminidase digestion of (b); (b), the elution profile of acidic oligosaccharide fraction. (SI, SII and SIII indicate the elution position of standard mono-, di- and trisialyloligosaccharides).

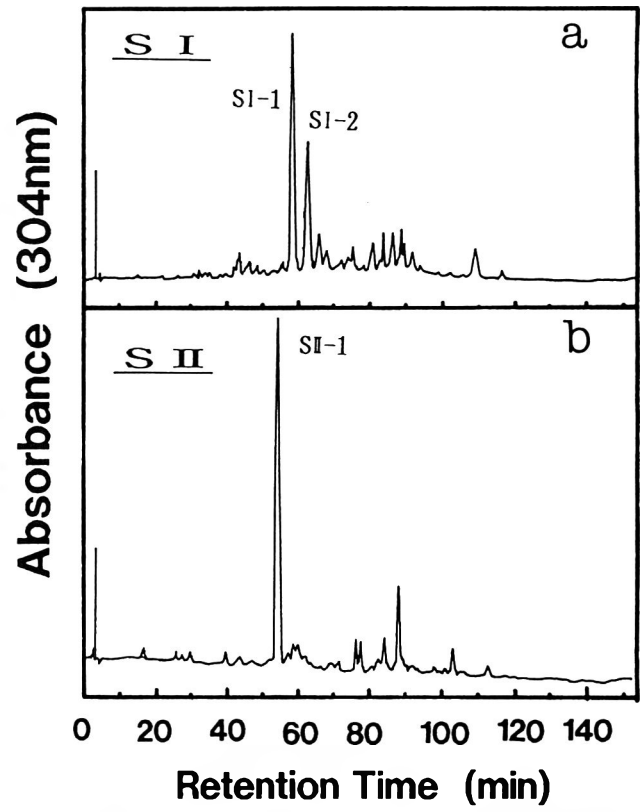


Fig. 3—Separation of SI (Mono-) and SII (Disialyl-oligosaccharides) from the delipidated egg yolk fraction on a Wakosil 5C18–200 reverse-phase column (0.4 cm × 25 cm). Eluant, NaH_2PO_4-acetonitrile (92:8), flow rate, 0.5 mL/min.; detection, 304 nm. (a) The elution profile of SI; (b), the elution profile of SII.

Tokyo). The column was pre-equilibrated with 10 mM NaH_2PO_4 and elution was performed isocratically for 10 min with 10 mM NaH_2PO_4 followed by a linear gradient to the final concentration of 250 mM NaH_2PO_4 over 60 min, flow rate 0.5 mL/min at ambient temperature (≈23°C). The ABEE-derivatized acidic oligosaccharides were fractionated on a Wakosil 5C18-200 column (0.4 cm × 25 cm, Wako Pure Chemical Industries, Osaka, Japan). The column was eluted isocratically with 50 mM NaH_2PO_4-acetonitrile (92:8, v/v) at 0.8 mL/min at 35°C. Elution was monitored at 304 nm. ABEE neutral oligosaccharides were analyzed by two-dimensional mapping techniques as reported (Matsuura et al., 1992).

NMR spectrometry. ABEE-oligosaccharides were repeatedly treated with 99.96% deuterated D_2O (Aldrich, Milwaukee, WI) through intermediate lyophilization. The 400 MHz ^{1}H-NMR spectra were recorded on a JEOL-GSX-400 spectrometer (JEOL Co., Ltd., Tokyo), operating in the pulsed Fourier transform mode. The chemical shifts (δ) were expressed in ppm downfield from external sodium-2,2-dimethyl-2-silapentane-5-sulfonate (DSS), but were actually measured by reference to internal acetone (δ = 2.225 ppm).

Sugar analysis. Hexoses were determined with phenol-H_2SO_4 reagent (Hodge and Hofreiter, 1962) prepared by adding 100 μL of 50% phenol solution to 100 μL of sample and mixing. Then 500 μL of conc. H_2SO_4 was immediately added, mixing vigorously using a vortex mixer until a clear solution was obtained and cooled to ambient temperature (≈23°C). The reaction mixture was measured at 490 nm using an MPS-2000 spectrophotometer (Shimadzu Co., Kyoto, Japan). Sialic acid was quantified by the periodate-resorcinol method (Jourdian et al., 1971).

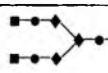
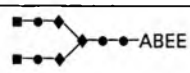
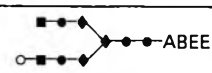
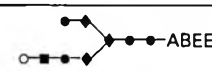
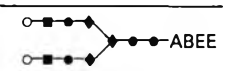
Neuraminidase digestion. The ABEE-derivatized acidic oligosaccharides (3 nmol) were digested with 50 mU of neuraminidase in 0.1 M sodium acetate buffer at pH 4.5 and 37°C for 24 hr under a toluene atmosphere.

RESULTS & DISCUSSION

Preparation of oligosaccharides

An undetectable amount of sialic acid (molecular weight of NeuAc 309) was in the fraction obtained from the UF cut-off with molecular weight < 1,000; however, the fraction having molecular weight > 1,000 contained sialic acid. The latter fraction was subjected to hydrazinolysis/N-acetylation to re-

Table 1—1H chemical shifts of structural-reporter group of monosaccharides for ABEE-derivatives of major sialyloligosaccharides[a] from the delipidated egg yolk fraction

Structure reporter group		 Asialo-GlcNAc-ol	 Asialo-ABEE	 SI-1		 SI-2	 SII-1	
		Reported value[c] (ppm)	Reported value[d] (ppm)	Actual value (ppm)	Reported value[c] (ppm)	Actual value (ppm)	Actual value (ppm)	Reported value[c] (ppm)
H-1	GlcNAc-2	4.637	4.625	4.629	4.642	4.624	4.625	4.643
	Man-3	4.773	4.762	ND[e]	4.78	ND[e]	ND[e]	4.780
	Man-4	5.120	5.117	5.132	5.138	5.133	5.132	5.136
	Man-4′	4.925	4.925	4.926	4.930	4.916	4.946	4.950
	GlcNAc-5	4.580	4.576	4.602	4.608	4.604	4.60	4.606
	GlcNAc-5′	4.580	4.576	4.581	4.583	4.547	4.60	4.606
	Gal-6	4.466	4.467	4.445	4.446	4.444	4.442	4.445
	Gal-6′	4.471	4.467	4.466	4.473	–	4.442	4.445
H-2	Man-3	4.248	4.230	4.237	4.257	4.235	4.240	4.258
	Man-4	4.190	4.185	4.192	4.194	4.188	4.193	4.199
	Man-4′	4.110	4.108	4.108	4.11	4.099	4.115	4.121
H-3a	NeuAc	–	–	1.723	1.718	1.720	1.718	1.716
	NauAc′	–	–	–	–	–	1.718	1.716
H-3e	NeuAc	–	–	2.667	2.673	2.668	2.669	2.669
	NeuAc′	–	–	–	–	–	2.669	2.674
NAc	GlcNAc-1	2.056	1.907	1.902	2.057	1.903	1.903	2.057
	GlcNAc-2	2.080	2.068	2.071	2.081	2.067	2.071	2.083
	GlcNAc-5	2.053	2.052	2.068	2.071	2.065	2.071	2.070
	GlcNAc-5′	2.046	2.043	2.044	2.048	2.048	2.062	2.065
	NeuAc	–	–	2.028	2.031	2.028	2.028	2.030
	NeuAc′	–	–	–	–	–	2.028	2.030

[a] See structure SII-1, Fig. 5.
[b] Structures are represented as follows: NeuAc (○), Gal (■), GlcNAc (●) and Man (◆).
[c] E.D. Green et al., 1988. J. Biol. Chem. 263: 18253–18268.
[d] M. Ohta et al., 1991. Glycoconjugate J. 8: 400–413.
[e] Value could not be determined merely by inspection of the spectrum.

lease oligosaccharides and then the oligosaccharides were labeled with ABEE.

Fractionation of sialyloligosaccharides

The ABEE-derivatized oligosaccharides were purified by Pre-Sep C18 cartridge followed by Bio-Gel P-4 chromatography (Fig. 1). The first fraction contained sialic acid as well as hexose, indicating the presence of sialyloligosaccharides. The second fraction contained hexose but not sialic acid, indicating the presence of neutral oligosaccharides. The other peaks, which absorbed at 304 nm, could be amino acids, peptides, or proteins. The fraction containing acidic oligosaccharides was further separated by anion exchange column HPLC into neutral (N) and two acidic oligosaccharide fractions (SI and SII). The eluant positions of SI and SII corresponded to those of mono- and disialylated ABEE-oligosaccharides (Ohta et al., 1990), respectively. The molar ratios of SI and SII in total sialyloligosaccharides were calculated as 47.7% and 50.6%, respectively, on the basis of UV absorbance at 304 nm (Fig. 2b). Both SI and SII were converted to neutral oligosaccharides by neuraminidase treatment (Fig. 2a). Therefore, their negative charge was attributed to the carboxylic group of neuraminic acid residues. This result suggested there were no sulfate and/or other acidic carbohydrate residues besides sialic acid in this acidic oligosaccharide fraction.

Fractionation and characterization of SI and SII

The fractionated SI and SII were further fractionated by HPLC using an ODS column (Fig. 3). Three subfractions SI-1, SI-2 and SII-1 were isolated and subjected to structural characterization. When these fractions were digested with neuraminidase, SI-1 and SII-1 gave an ABEE-oligosaccharide which eluted at the position of Galβ 1-4GlcNAcβ1-2Manα 1-3 (Galβ 1-4GlcNAcβ 1-2Manα1-6) Man β1-4GlcNAcβ 1-4GlcNAc-ABEE on a two-dimensional map and SI-2 Galβ1-4GlcNAcβ 1-2Manα1-3 (GlcNAcβ 1-2Manα1-6) Man β1-4GlcNAcβ 1-4GlcNAc-ABEE.

In order to characterize the complete structures, ABEE-oligosaccharides SI-1, SI-2 and SII-1 were subjected to analysis by 400 MHz NMR. Chemical shifts of structural reporter group protons of the samples and reference compounds were summarized (Table 1) and the NMR spectrum of SI-1 is shown in Fig. 4.

Four signals from GlcNAc CH_3 group and the chemical shifts of H-1 and H-2 for Man-3, -4 and -4′ indicated all saccharides were biantennary complex-type oligosaccharides (Vliegenthart et al., 1983; Green et al., 1988; Ohta et al., 1991). In the spectrum of SII-1, shift decrements for H-1 of Gal-6 and -6′ and shift increments for H-1 of GlcNAc-5, GlcNAc-5′, man-4 and Man-4′ indicated the introduction of sialic acid residues to Gal-6 and -6′, as compared to asialobiantennary oligosaccharides (Vliegenthart et al., 1983, Table 1). The set of chemical shifts for H-3a and H-3e of NeuAc residues indicated both NeuAc residues were α2,6-linked to the Gal residues. Therefore, the structures of SII-1 was proposed as in Fig. 5.

In the spectrum of monosialyloligosaccharide SI-1, the chemical shift values for H-1 of Man-4 ($\delta = 5.132$ ppm) and Man-4′ ($\delta = 4.926$ ppm) and for H-3a and H-3e of NeuAc ($\delta = 1.723$ and 2.667 ppm, respectively) indicated that a NeuAc residue was linked to Gal-6 residue via α2,6 linkage (Vliegenthart et al., 1983). From these results, the structure of SI-1 was proposed (Fig. 5).

In the spectrum of monosialyloligosaccharide SI-2, chemical shifts for H-3a and H-3e of a NeuAc residue and H-1 of Man-4 were similar to those in SI-1. These results demonstrated that the NeuAc residue was α2,6-linked to Gal-6. The intensity of the signal at $\delta = 4.444$ ppm demonstrated the presence of a single galactose residue in SI-2. Signals for the H-1 of Man-4′ and GlcNAc-5′ were shifted downfield as compared with those in SI-1. We, therefore, concluded that SI-2 did not possess the Gal residue linked to GlcNAc-5′ (Fig. 5).

Ratio of three major sialyloligosaccharides

The UV intensity of ABEE derivatives is dependent upon the concentration of its compounds and can be calculated (Mat-

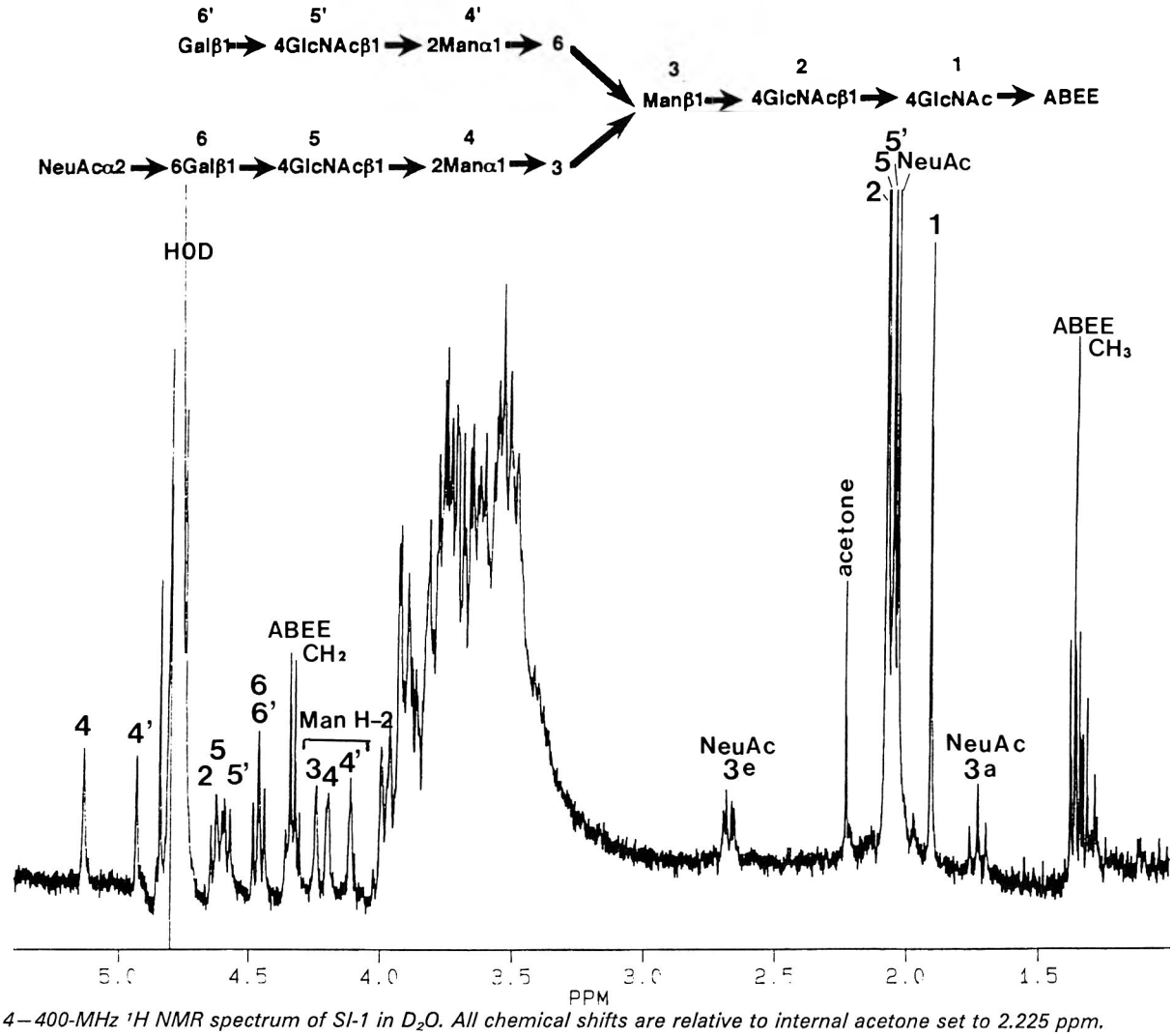


Fig. 4—400-MHz ^1H NMR spectrum of SI-1 in D_2O. All chemical shifts are relative to internal acetone set to 2.225 ppm.

SI-1

Galβ1→4GlcNAcβ1→2Manα1→6
NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3
Manβ1→4GlcNAcβ1→4GlcNAc→ABEE

SI-2

GlcNAcβ1→2Manα1→6
NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3
Manβ1→4GlcNAcβ1→4GlcNAc→ABEE

SII-1

NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→6
NeuAcα2→6Galβ1→4GlcNAcβ1→2Manα1→3
Manβ1→4GlcNAcβ1→4GlcNAc→ABEE

Fig. 5—The structure of major sialyloligosaccharides from the delipidated egg yolk fraction.

suura and Imaoka, 1988). The concentrations of SI-1, SI-2 and SII-1 were calculated as 9.6, 6.5 and 17.5%, respectively, on the basis of UV absorbance at 304 nm.

CONCLUSIONS

THE MAJOR sialyloligosaccharides of the water-soluble fraction of delipidated egg yolk are of the biantennary complex-type, which account for about 33.6% of the total sialyloligosaccharides of the water-soluble fraction. The structure of sialyloligosaccharides of egg yolk has not been reported. Such knowledge could help in clarifying their functions. These data may lead to industrial applications, e.g., formulation of new functional foods.

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*EFFECT OF CURING AGENTS ON AMINOPEPTIDASES. . .*From page 726

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Anhydrous Milk Fat Fractionation with Continuous Countercurrent Supercritical Carbon Dioxide

A.R. BHASKAR, S.S.H. RIZVI, and J.W. SHERBON

ABSTRACT

A continuous pilot-scale supercritical carbon dioxide system was designed and built for separation and fractionation up to 400 g/hr of anhydrous milk fat. This fat was separated into five fractions (S1–S5) in the pressure range 24.1–3.4 MPa at 40–75°C. The solvent/feed ratio was 62 with extraction yield 78 wt%. The short-chain (C4-C8) and medium-chain (C10-C12) fatty acids increased from S1–S5. The long-chain and unsaturated fatty acids (C14–C18) decreased gradually from S1–S5. The triglycerides followed the same trend as fatty acids. Cholestrol content decreased by 51% and β-carotene concentration increased by 145% in the raffinate (S1).

Key words: milk-fat, fractionation, supercritical-carbon dioxide

INTRODUCTION

A STEADY decline in milk fat consumption throughout much of the Western world has posed a considerable problem to the dairy industry. By the year 2000, the milk fat surplus in the U.S. may be up to 500,000 M.T. per year (Williams and Lyones, 1989). The reasons for such decreasing consumption of milk fat include both its limited dietary value and functional properties in the native form. Milk fat is a complex mixture of triglycerides composed of at least 60 fatty acids of a distinctive range of molecular weights and degree of unsaturation. The majority of fatty acids of milk fat range in carbon number (C) from 4–18 and are both saturated and unsaturated moieties (Rizvi et al., 1989). About 66 wt% of the fatty acids in milk fat are saturated (USDA, 1976), and milk fat contains 0.25–0.4% cholesterol (Rizvi, 1991).

An increase in utilization of milk fat might result if its properties could be tailored to specific applications. Some common methods of fractionation of milk fat involve crystallization at different temperatures, with or without use of solvents (deMan, 1968; Fjaervoll, 1970; Norris et al., 1971; Larsen and Samuelsson, 1979). However, separation of crystalline from uncrystallized fat is more difficult when solvents are not used. The shift, for example, in major fatty acid composition remains within the range of natural variations of milk fat (Timmen et al., 1984). The use of solvents or surfactants produces better separation of triglycerides but solvent removal becomes a problem. Modification of milk fat by chemical methods such as interesterification (deMan, 1961; Richardson, 1968) and hydrogenation (Yoncoskie et al., 1969) causes losses of many desirable characteristics and destroys natural flavors (Norris et al., 1971). Milk fat fractions obtained by these methods show substantial differences in physical and functional properties but relatively small variations in chemical composition.

Such restrictions are not found with supercritical fluid extraction (SFE). Applications of SFE for fractionation and cholesterol reduction of milk fat offer advantages (Shishikura et la., 1986; Arul et al., 1987; Rizvi et al., 1989). The preferred solvent in the food industry is carbon dioxide because it is nontoxic, non-corrosive, low cost, non-flammable, readily available and has low critical temperature and pressure. Milk fat extraction and fractionation can be done in conjunction with cholesterol reduction. Shishikura et al. (1986) used a single-pass unit with operating pressure between 12.8–24.8 MPa at two different temperatures (40 and 60°C). They reduced cholesterol by 95%, but also removed 75% of the triglycerides from butter oil. They also found that cholesterol removal could be increased by passing the solute-rich solvent through a silica gel column which preferentially adsorbed cholesterol. Arul et al. (1987) carried out experiments to fractionate milk fat with SC-CO_2 at 10–35 MPa and 50–70°C. They produced eight different fractions and found that cholesterol tended to concentrate in the low- and intermediate-melting fractions. Bradley (1989) used an ascending-pressure profile. He removed 90% of the cholesterol, but the process yield was low, 47%. Recently Lim et al. (1991) achieved an overall cholesterol reduction of 92.6% with a process yield of 88.5%. They used magnesium silicate as an adsorbent. The extraction was done at 40°C and 24.1–27.5 MPa.

Research on milk fat fractionation with supercritical carbon dioxide (SC-CO_2) has been applied mostly on batch systems. With fluids, such as milk fat, which can be pumped at high pressures, the processing time can probably be minimized and the economics more favorable with continuous processing (Rizvi, 1991). Specific objectives of our research were to examine the effects of operating conditions on distribution of fatty acids, triglycerides and cholesterol in anhydrous milk fat (AMF) fractions and to determine the physicochemical properties of AMF and its fractions. The performance of the packed column for AMF extraction was previously reported (Bhaskar et al., 1993).

MATERIALS & METHODS

FIGURE 1 shows a schematic of the continuous/batch, pilot-scale supercritical fluid extraction system used. This unit could be adapted for liquid-liquid as well as solid-liquid extraction.

The system consisted of a packed column (6) and four separation vessels (8,9,10,11). The column was packed with SS 304 Goodloe knitted mesh packing and was 1.8 m long and 4.9 cm in internal diameter with six inlet/outlet ports. The continuous system could be operated in either cocurrent or countercurrent mode. A Milton Roy Type B reciprocating pump (2) (max flow rate: 8.0 L/hr) was used for feeding AMF. The feed could be introduced from the top or from the center of the column. The packed column and the separators were equipped with sampling valves and heating jackets. A positive displacement, reciprocating pump (3) (max flow rate: 113 L/hr) was used to compress the gas to the desired operating pressure. System pressure was maintained by back pressure regulators. The AMF and CO_2 flow rates were monitored by rotameters (1,12).

Commercial grade butter was converted into AMF by melting at 60 °C, decanting the top layer and filtering through Whatman No. 1 filter paper. The extraction was studied over a pressure range of 24.1–3.4 MPa and temperatures of 40–75 °C. In a typical run, the SC-CO_2 with the dissolved fat passed into the first separation vessel, S2, through a pressure reduction valve. Those triglycerides becoming insoluble at the reduced pressure precipitated in the separation vessel. The mother liquor was passed to the next vessel, S3, and the pressure reduced again. The process continued until the remaining triglycerides were precipitated in S5. The volumetric flow rate of CO_2 was measured by a dry test meter and the gas was then vented to the atmosphere. The raffinate was removed at S1 and represented those triglycerides insoluble in SC-CO_2 at the pressure, temperature, and solvent-to-feed ratio in the extraction column.

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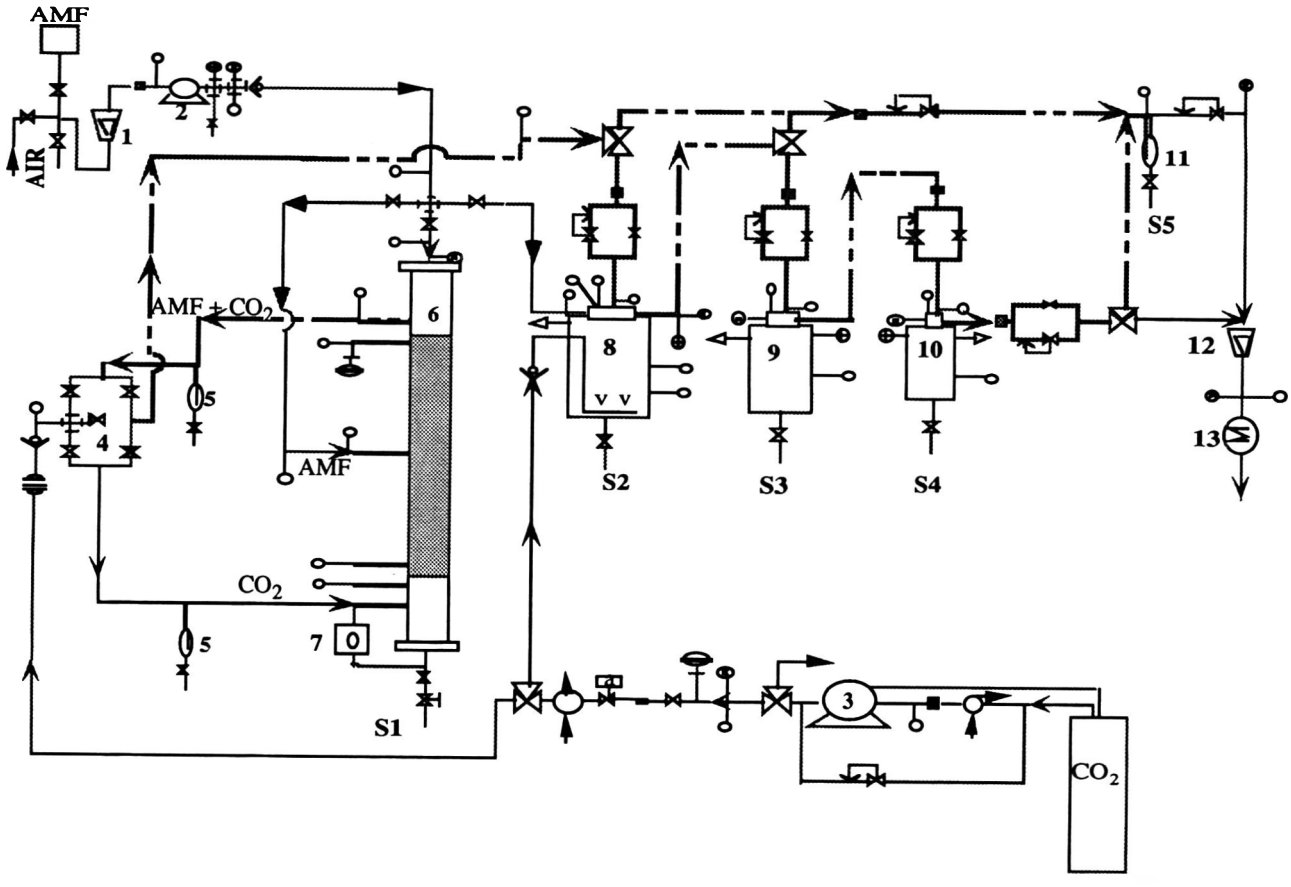

Fig. 1.—Schematic diagram of pilot-scale continuous SFE system. 1—AMF flow meter; 2—AMF pump; 3—CO_2 pump; 4—CO_2 flow loop; 5—entrainment vessels; 6—packed column; 7—view cell; 8—separator 1; 9—separator 2; 10—separator 3; 11—separator 4; 12—CO_2 flow meter; 13—dry test meter; ■ filter; pressure gauge; thermocouple; safety valve; check valve; back pressure regulator; heat exchanger; CO_2 flow; AMF flow; Extract flow; rupture disc; 3-way valve.

Data were collected periodically for feed and solvent flow rates and amount of fractions from each separator and raffinate. A material balance check was determined at the end of each run. When the material balance accountability ≥ 90%, the samples were analyzed. The packed column was considered to be in steady state when the amount of fractions and flow rates (solvent and feed) were constant for 3 successive time intervals. A typical run required 1 hr to reach steady state.

Analysis

Fatty acids, triglycerides and cholesterol. The AMF and its fractions were analyzed for fatty acids, triglycerides and cholesterol on a gas chromatograph fitted with a flame ionization detector (HP 5890, Hewlett Packard Co., Avondale, PA). The carrier gas was helium, 1.5 mL/min.

The fatty acids were first converted to methyl esters (AOCS, 1989) and analyzed using a capillary glass column, 30 m × 0.25 mm, Durabond-225 (J & W Scientific Co., Folsom, CA). The oven temperature was held at 60 °C for 2 min, then increased at 4 °C/min to 220 °C and held for 10 min. The injector was 200 °C and detector 260 °C.

Triglycerides were analyzed by a modified method of Amer et al. (1985). The triglycerides in AMF and its fractions were directly analyzed using a capillary glass column 30 m × 0.25 mm, Durabond-5 (J & W Scientific Co., Folsom, CA). The oven temperature was programmed in three stages: from 50 to 240 °C at 25 °C/min; from 240 to 345 °C at 3 °C/min, then held 25 min; finally, from 345 to 350 °C at 0.1 °C/min. Injector was at 330 °C and detector 345 °C.

Cholesterol was determined by a modified method of Lynch and Barbano (1988). Sterols were derivatized to form trimethylsilyl (TMS) esters and then chromatographed using a capillary column coated with SE-30 (Chrompack Co., Middelburg, The Netherlands). Temperatures were: oven 250 °C, injector 260 °C and detector 270 °C.

Physicochemical properties. Iodine number (IN) and saponification number (SN) were determined by AOCS (1989) methods Cd 1-25 and Cd 3-25, respectively. Melting and crystallization curves were determined using a differential scanning calorimeter (DSC) (DSC-1, Perkin Elmer, Norwalk, CT) as outlined by Norris et al. (1971). The variation in solid fat content with temperature was calculated from DSC melting thermograms (Norris et al., 1971). The AMF and raffinate were also analyzed for flash point determined by AOCS (1989) method Cc 9a-48 and carotenoids (determined as β-carotene) determined by AOAC (1990) method 938.04. Based on duplicate analysis the standard error of the results was < 5%.

RESULTS AND DISCUSSION

INITIAL experiments were done to determine the best conditions which yielded fractions showing marked differences in composition. Pressure and temperature in the first separator affected the composition of fractions in subsequent separators. As the mass precipitating in the first separator (S2) was reduced by increasing pressure at constant temperature or decreasing temperature at constant pressure, the fatty acid composition of the collected fraction became more like the raffinate, and the fractions in the subsequent separators (S3, S4, S5) more like the original AMF. Thus changes in pressures and temperatures yielded fractions marked by different compositions. These changes with conditions used in this research are recorded in Table 1. The economics of operating and fractionating AMF continuously has been studied by Raj and Rizvi (1993) and shown to be commercially viable.

The fatty acid composition of AMF and its fractions (Table 2 and Fig. 2) indicated short-chain (C4–C8) and medium-chain (C10–C12) fatty acids increased from S1–S5. The long-chain (C14–C18) and unsaturated fatty acids decreased gradually from

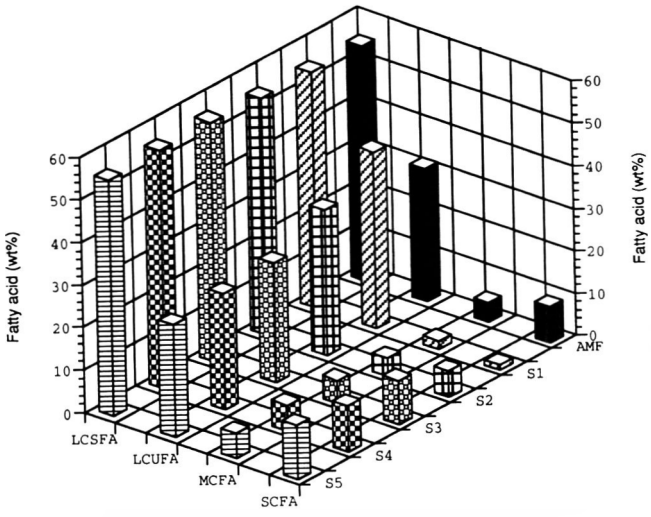

Fig. 2—Distribution of fatty acids in AMF and its fractions (SCFA:short-chain fatty acids; MCFA:medium-chain fatty acids; LCUFA:long-chain unsaturated fatty acids; LCSFA:long-chain saturated fatty acids).

Table 1—Fatty acid, triglyceride and cholesterol composition of AMF and its fractions with SC-CO_2

Solvent/feed (g/g): 62
Extract loading (wt%): 1.27
Fat recovery (wt%): 89

	Feed	Raffinate	Fractions			
	AMF	S1	S2	S3	S4	S5
Temp (°C)	40	40	60	75	60	60
Pressure (MPa)	24.1	24.1	24.1	17.2	6.9	3.5
Fat yield (wt%)	100	21.0	15.0	48.0	4.0	11.0
Fatty acids						
C 4:0 – C 8:0	8.55	1.22	5.97	10.14	10.67	12.42
C10:0 – C12:0	4.60	1.95	4.17	5.34	5.91	5.88
C14:0 – C18:3	86.85	96.83	89.86	84.52	83.42	81.69
Unsaturated	31.28	41.57	34.37	28.19	27.23	26.32
Saturated	55.01	55.26	55.49	56.33	56.19	55.59
Unsat/sat ratio	0.57	0.75	0.62	0.50	0.48	0.47
Triglycerides						
C24 – C34	16.72	Traces	10.18	18.82	24.30	26.39
C36 – C40	50.85	17.07	49.94	56.19	53.62	54.22
C42 – C54	32.93	82.93	39.88	24.99	22.08	19.39
Cholesterol (mg/100g)	240.6	117.6	234.6	251.8	363.6	353.7
Cholesterol[a] change (%)	–	−51.1	−2.5	+4.7	+50.7	+47.0
Carotenoid[b] (IU/100g)	314	768	N/A[c]	N/A	N/A	N/A

[a] Compared to original AMF
[b] Determined as β-carotene
[c] not analyzed

S1–S5. The unsaturated to saturated fatty acid ratio decreased from S1–S5, with a range of 0.75–0.47 as compared to 0.57 for AMF. However, the concentration of long-chain saturated fatty acids remained constant in AMF and its fractions. Although the ratio of unsaturated to saturated fatty acids changed, the long-chain saturated fatty acids remained constant irrespective of operating conditions, indicating that at least one of the long-chain saturated fatty acids was part of each triglyceride.

The triglyceride composition of AMF and its fractions (Table 1 and Fig. 3) showed triglycerides followed the same trend as fatty acids. The low-melting (LMT) and medium-melting (MMT) triglyceride concentrations increased from S1–S5, while the high-melting (HMT) triglycerides decreased from S1–S5. Almost all LMT were extracted from AMF and their concentration increased from S1–S5 with a 145% increase in S5. The fractions S4 and S5 showed very little differences in compositions. The trend of data for fatty acid and triglyceride compositions of the fractions confirmed other reported studies (Shishikura et al., 1986; Arul et al., 1988).

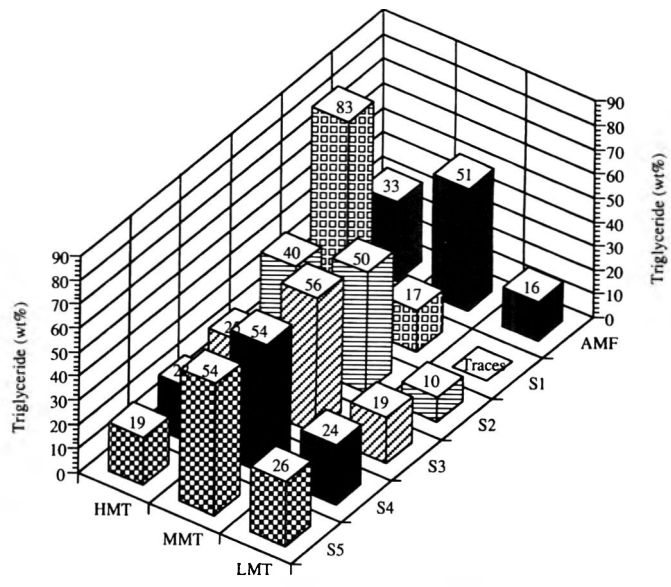

Fig. 3.—Distribution of triglycerides in AMF and its fractions (LMT:low-melting triglycerides; MMT:medium-melting triglycerides; HMT:high-melting triglycerides).

Table 2—Melting range and % area from DSC melting thermograms for AMF and its fractions

	Low-melting triglycerides		Medium-melting triglycerides		High-melting triglycerides	
Sample	Temp range, K	Area %	Temp range, K	Area %	Temp range, K	Area %
AMF	250–283	31	283–295	35	295–302	34
S1	263–287	14	287–290	3	290–318	83
S2	264–282	14	282–293	36	293–312	50
S3	255–288	61	288–293	20	293–305	19
S4	242–288	63	288–292	20	292–304	17
S5	260–287	61	287–293	23	293–303	16

Cholesterol is a minor constituent of milk fat, and was expected to partition into liquid, intermediate and solid fractions according to its affinity towards their triglycerides (Arul et al., 1988). The cholesterol content of AMF and its fractions (Table 1) tended to concentrate in the more soluble fractions (S4 and S5). The cholesterol content decreased in the raffinate (S1) by 51% but increased in fractions S3, S4, S5. In parallel, there was a gradual increase in LMT and MMT as well as short- and medium-chain fatty acids from S1-S5. Likewise, there was a gradual decrease in HMT and long-chain fatty acids. This suggested that cholesterol had a higher affinity for LMT and MMT and to a lesser extent for HMT. Arul et al. (1988) also measured the activity coefficient (γ_1) of cholesterol in triglycerides. In all cases, $\gamma_1 > 1$ and it was closer to 1 in short-chain simple triglycerides and in HMT with an increasing number of double bonds. Thus cholesterol would be miscible in the LMT and MMT to a large extent and to some degree in the HMT. Our qualitative data agreed with reports of Arul et al. (1988) and Kaufmann et al. (1982) that the liquid (soluble) fractions were enriched in total cholesterol and its concentration decreased with increasing melting point of the fractions. Cholesterol has low selectivity in SC-CO_2 and could be reduced in the fractions only by a secondary treatment such as in-line adsorption (Rizvi et al., 1989). The use of these fractions in various dairy products is currently being investigated in our laboratory.

The β-carotene concentration increased by 145% in the raffinate (Table 1) relative to AMF. Since β-carotene ($C_{40}H_{56}$) is predominantly hydrocarbon in nature it is fat-soluble. Favati et al. (1988) studied SC-CO_2 extraction of carotene from leaf protein concentrates (LPC). Higher extraction pressures (> 30

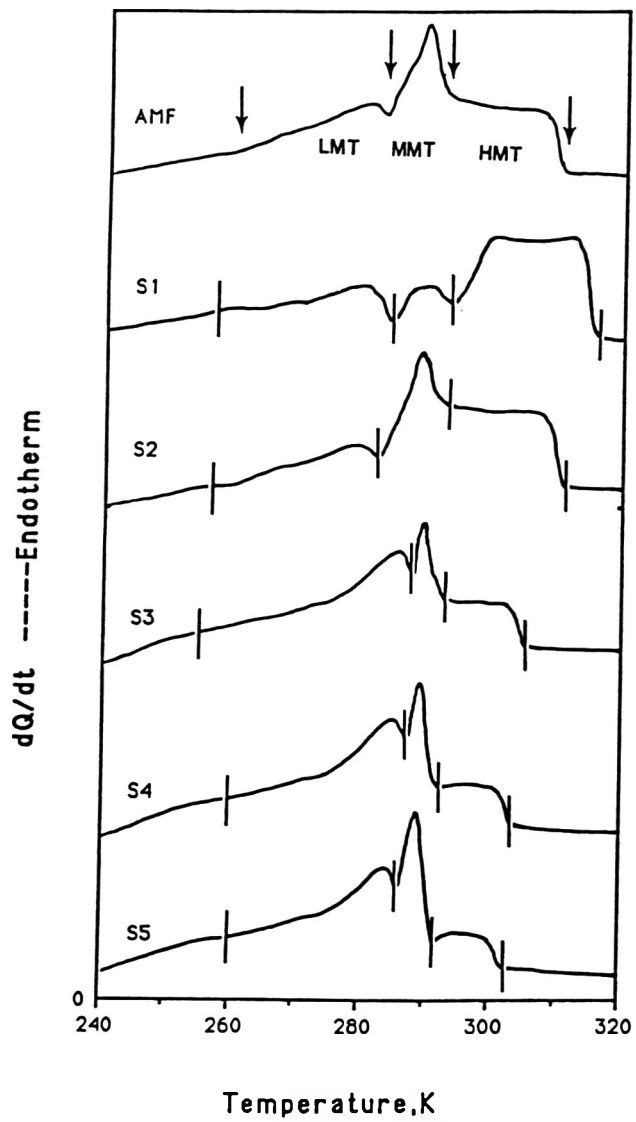

Fig. 4.—Melting thermograms of AMF and its fractions by DSC (The areas defining LMT, MMT, and HMT are represented between consecutive arrows).

MPa) at 40 °C were required to recover > 90% of the carotene from LPC. This suggested that β-carotene may be less soluble in SC-CO_2 at the extraction conditions we used. Lorenzo et al. (1991) studied supercritical fluid extraction of carotenoids from *Dunaliella* algae. They found that the contact time was a limiting factor in reaching equilibrium for the measurements of pure β-carotene solubility. In our experiments, we used a high solvent-to-feed ratio, thus the contact time was less and this may be another reason for low solubility of β-carotene in SC-CO_2.

These results indicate a definite improvement in nutritional properties of the raffinate, which is rich in unsaturated fatty acids and β-carotene and low in cholesterol. The raffinate could be incorporated into skim milk to make nutritionally improved low-fat or whole milk and also used as an ingredient for ice cream and cheese. The other fractions became more saturated but had more short- and medium-chain fatty acids making them potentially useful for specific dietary formulations. The short-chain fatty acids are used by the body for energy. MMT are used in special medical formulations for people who cannot absorb long-chain fatty acids but need ready energy from fat. MMT's are also important in infant formulas to duplicate MMT found in human milk.

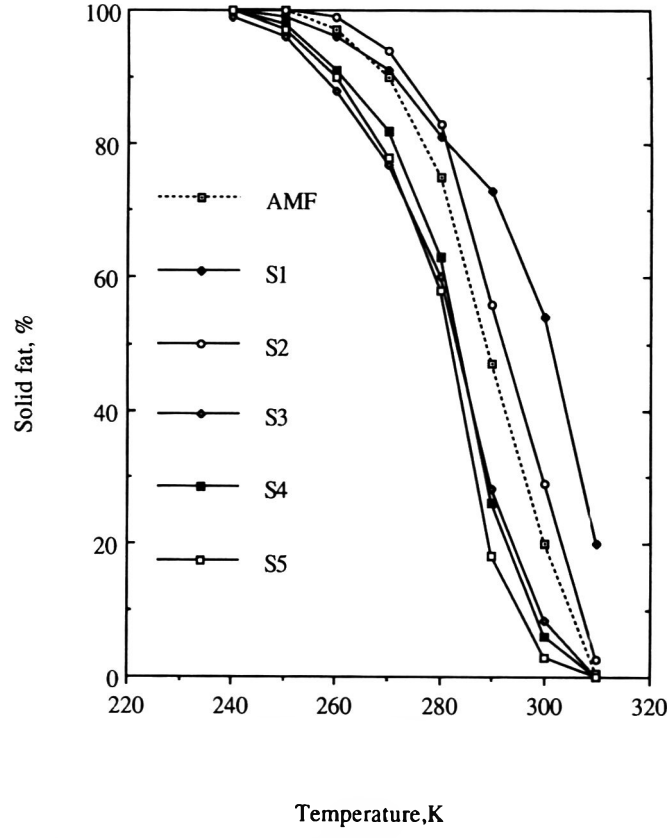

Fig. 5.—The variation in solid fat content with temperature of AMF and its fractions.

Physical properties for AMF and its fractions

Selected physiochemical properties of AMF and its fractions obtained with SC-CO_2 were measured and compared to illustrate the effectiveness of fractionation. The melting thermograms of AMF and its fractions (Fig. 4) showed sharp upper temperature limits but lower temperature limits were not well defined. The fractions showed three major melting zones [low- (LMT); medium- (MMT); and high-melting (HMT)] and the percentages of triglycerides in each zone were different. The low- and medium-melting fractions (S2,S3,S4,S5) showed two partially resolved peaks with a shoulder plateau. The plateau decreased from S2 – S5. The high-temperature melting plateau was more pronounced for the raffinate. The percentage area and melting range for AMF and its fractions (Table 2) indicate AMF contained 66% as LMT and MMT. The amount of LMT increased from S2 – S5 (14 –63%) and this was mainly due to a shift from the MMT. This may be the reason for the narrow melting range of MMT and lower melting limits of HMT for these fractions. The raffinate had significantly higher levels of HMT (83%) and lower levels of MMT (3%) resulting in a narrow melting range (287–290K). On the other hand, a significantly wider range was observed for HMT (290–318K). The major shift was from MMT to LMT for S3 – S5 and to HMT for S1 and S2. But in S1 and S2 there were also lowered levels of LMT.

The solid fat content is an indication of product hardness. It is an important parameter for determining consistency of plastic fat. The percentage of solid and liquid fat at different temperatures were calculated from thermograms of AMF and its fractions (Fig. 5). The solid fat content increased in the order of S5–S4–S3–AMF–S2–S1 fractions. This was consistent with the proportion of calculated triglycerides (Table 2). For example, at 300K the solid fat content in the fractions S5 – S1 ranged from 5 – 65% compared to 20% for AMF. Curves

Table 3—Selected physicochemical properties of AMF and its fractions.

Sample	Melting point[a] (°C)	Iodine number	Saponification number
AMF	40	35.20	222
S1	45	42.90	212
S2	38	39.68	217
S3	32	32.05	234
S4	31	28.72	236
S5	30	28.36	241

[a] Melting temperature from DSC melting thermograms at which all the fat is in liquid state.

like Fig. 5 may generally indicate the suitability of a fat for particular purposes.

Physiochemical properties

Table 3 compares the iodine number (IN) and saponification number (SN) for AMF and its fractions with SC–CO_2. The IN increased from S5 – S1 with a range of 28.36 – 42.90 compared to 35.20 for AMF. As expected from the chromatograms, the saponification number increased from S1 – S5. The upper melting limit from the DSC thermograms ranged from 30 – 45°C for S1 – S5, compared to 40°C for AMF (Table 3). Fjaervoll (1970) fractionated milk fat into two fractions (low- and high-melting) by a continuous process. The fractions showed a difference of 16°C in melting point between the low- and high-melting fractions. The melting properties of fat may be affected by 3 factors: the component acids, distribution of acids in the glycerides, and the polymorphic forms of the fat crystals (Jenness and Patton, 1959). Any decrease in melting temperature of the raffinate due to more unsaturated fatty acids was largely compensated by increased proportions of long-chain saturated fatty acids. The flash point of a fatty material is a measure of its thermal stability when heated in contact with air. It is the temperature at which volatile products evolved can be ignited but would not support combustion (Mattil et al., 1979). The flash point temperature of the raffinate increased to 329°C compared to 313°C for AMF. This may be advantageous for cooking or deep frying.

CONCLUSIONS

ANHYDROUS MILK fat was separated into five fractions with SC-CO_2. The concentration of short- and medium-chain fatty acids increased from S1 – S5, while the concentration of long-chain and unsaturated fatty acides decreased from S1 – S5. The triglycerides followed the same trend as fatty acids. Cholesterol content decreased by 51% in the raffinate.

SFE affords opportunities for obtaining milk fat fractions with distinctive differences in chemical and physical properties which may satisfy requirements of many food applications.

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Milk Fat Globule Membrane Substances Inhibit Mouse Intestinal β-Glucuronidase

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ABSTRACT

The β-glucuronidase inhibitory action of milk fat globule membrane (MFGM) was studied. By increasing the MFGM concentration to 0.2%, nearly 90% of the β-glucuronidase activity was inhibited. The trypsin-derived digests of MFGM inhibited β-glucuronidase activity by ≈ 42%. The inhibitory effect of MFGM on intestinal β-glucuronidase of mouse was studied. We administered MFGM (groups I: 5%; group II: 10%; group III: 20%) to mice and investigated the time course of changes in fecal β-glucuronidase activity. In group I and II, enzyme activity had decreased 15–20% on the 5th day of treatment, but in group III, a decrease in enzyme activity was seen with an inhibition rate of 50%, showing a marked intracolonic efficacy of MFGM. Thus MFGM may have potential in explaining, treating or preventing intestinal cancers.

Key Words: βeta-glucuronidase, mouse-intestine, milk fat, globule membrane

INTRODUCTION

THE HUMAN intestinal flora consist of about 100 species of bacteria that inhabit the gastrointestinal tract (Reddy, 1981). Among these enterobacteria are species that are beneficial to the host, and others that have harmful effects. These enterobacteria produce a variety of enzymes, and some, such as nitrate reductase, β-glucuronidase and steroid 7-α-hydroxylase, are especially important because they transform chemical carcinogen precursors into carcinogens within the colon (Simon and Gorbach, 1984). Inclusion in our daily food of a compound that inhibits the action of such enzymes would lead to a decrease in the level of these carcinogenic substances in the colon. β-Glucuronidase is inhibited by sialoglycoproteins from the sublingual and the submandibular salivary glands (Sakamoto et al., 1974). Sialoglycoproteins are widely distributed in nature, and those found within the human body have a wide range of physiological activities (Schultze et al., 1962; Donaldson and Evans, 1963; Heimburger and Haupt, 1965; Barret and Starkey, 1973; Rosenberg, 1975). Cow milk contains sialoglycoproteins, namely, κ-casein and milk fat globule membrane (MFGM) substances. MFGM contains many glycoproteins (Shimizu et al., 1976) that could be expected to demonstrate diverse physiological activities. Our objective was to determine the effect of MFGM on β-glucuronidase, after we found that it has potent enzyme-inhibitory action. The question of whether this could be repeated under *in vivo* conditions prompted us to administer MFGM in food to mice and observe the time course of changes in fecal β-glucuronidase activity as a marker of intracolonic β-glucuronidase inhibition.

MATERIALS & METHODS

Materials

The β-glucuronidase from *Escherichia coli* and p-phenolphthalein-β-D-glucuronide were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin was purchased from Difco.

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Preparation of MFGM

To prepare the MFGM substances, 50% raw cream was used. The cream was washed three times with distilled water, triple volume each time. The yield was kept at 4°C overnight for aging, then put into a small diamond-shaped stainless steel churn. The resultant buttermilk was collected, to which was added enough solid ammonium sulfate to make the resultant mixture half saturated. The mixture, after being salted out, was left overnight and centrifuged at 3000 rpm for 30 min. The condensate in the upper layer was collected, dialyzed at 4°C against distilled water until the dialysate gave a negative reaction to $BaCl_2$, and freeze-dried for storage. The recovery of MFGM substance was about 1.2% of the milk fat.

Selective extraction of glycoprotein from MFGM was performed by the method of Tomita et al. (1978). The MFGM preparation was suspended in 0.3M lithium diiodosalicylate and 0.05M tris-HCl buffer, pH 7.5, at 25 mg MFGM protein/mL and stirred at room temperature (≈23°C) for 15 min. Two volumes of distilled water were then added, and the turbid suspension was stirred an additional 10 min at 4°C. After centrifugation at 45,000 × *g* for 90 min at 4°C, the supernatant was decanted and dialyzed against distilled water for 3 days. The precipitate produced during dialysis was removed by centrifugation at 10,000 × *g* for 30 min. The supernatant was collected and freeze-dried for storage. The protein (Kjeldahl method), lipid (Folch et al., 1957), and carbohydrate (Dreywood, 1949) contents of MFGM were determined to be 48.8% (protein), 49.0% (lipid) and 1.05% (carbohydrate).

κ-casin and protein

Crude κ-casein was prepared by the method of Zittle and Custer (1963). It was then purified by a method of Takeuchi et al. (1985). Protein was determined by the method of Lowry et al. (1951) using crystalline bovine serum albumin as a standard.

Assay of enzyme activity

β-Glucuronidase activity was measured according to a modified method (Sigma method) of Fishman (1974), utilizing hydrolysis of p-phenolphthalein β-D-glucuronide. The reaction mixture contained 0.5 mL of 75 mM potassium phosphate buffer (pH 6.8), 0.25 mL of 3 mM p-phenolphthalein β-D-glucuronide, 0.1 mL β-glucuronidase solution (500 Sigma units/mL) and 0.65 mL distilled water. It was immediately mixed and incubated at 37°C. After exactly 30 min, 5.0 mL of 200 mM glycine buffer, pH 10.4, was added to stop the reaction. The liberated phenolphthalein was spectrophotometrically measured at 540 nm. One unit of enzyme activity was defined as that amount of 1μM phenolphthalein liberated in 1 hr from p-phenolphthalein β-D-glucuronide. To determine β-glucuronidase activity in feces, freeze-dried feces (200 mg) was dissolved in 2 mL of 75 mM potassium phosphate buffer (pH 6.8) and centrifuged at 15,000 rpm for 30 min. β-Glucuronidase activity in the supernatant was measured according to the method described.

HiLoad column chromatography

The MFGM solution (200 mg/4 mL) was applied to a HiLoad column (1.6 × 60 cm, Pharmacia) equilibrated with 0.05M phosphate buffer, pH 7.2, containing 0.1M sodium chloride. Elution also was with the same buffer, and 2 mL fractions were collected. Individual fractions were monitored by absorbance at 280 nm and assayed for β-glucuronidase activity.

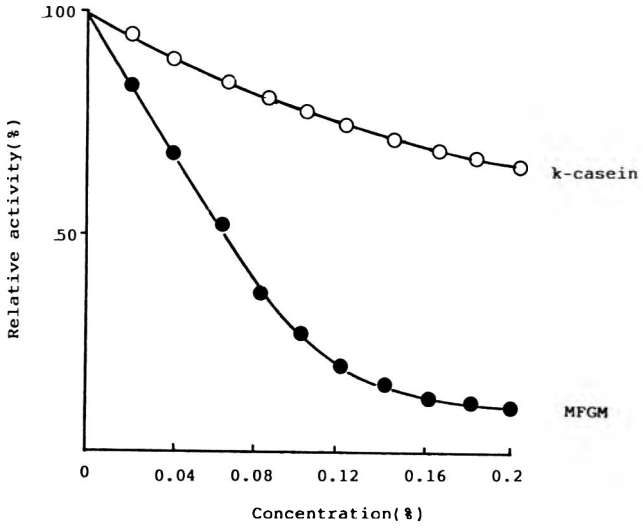

Fig. 1—Effect of MFGM or κ-casein concentration on β-glucuronidase activity. Measurement of the activity was carried out in the presence of various MFGM or κ-casein concentrations (0–0.2%) and β-glucuronidase in the reaction mixture. Conditions were described in the estimation of β-glucuronidase activity.

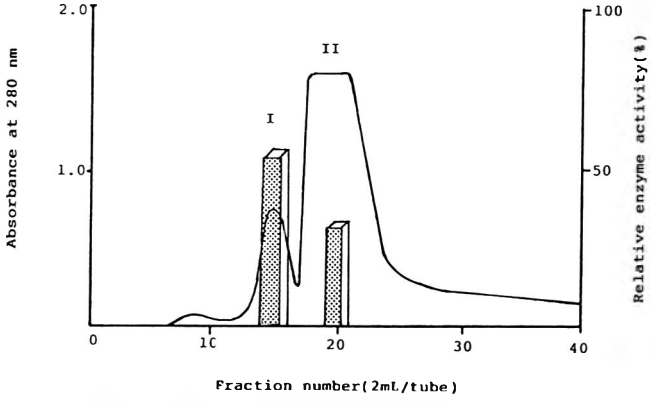

Fig. 2—Elution pattern of MFGM on HiLoad column chromatography. Column: HiLoad 16/60 Superdex 200 prep grade; Sample: 200mg MFGM; Buffer: 0.05M sodium phosphate containing 0.1M sodium chloride; pH 7.2; Flow rate: 9 mL/hr; ▨ : β-Glucuronidase inhibition of fraction I and II.

Table 1—Inhibition of β-glucuronidase activity of trypsin digests of MFGM.

Fraction	Conc (mg)	Enzyme activity Unit	Relative activity (%)
None	0	204.0	100
MFGM digests	1.5	117.4	57.5

Trypsin treatment of MFGM

The MFGM material was treated with trypsin as described by Kornfeld and Kornfeld (1970). One volume of MFGM solution (20 mg/mL) was added to 1 volume of 0.9% NaCl-0.05M phosphate buffer, pH 7.5, containing 0.25 mg/mL of trypsin 1/250 (Difco). The MFGM suspension was incubated with shaking at 37°C for 1 hr. The precipitate was removed by centrifugation at 40,000 × *g* for 90 min and to the chilled supernatant fluid 1/8 volume of cold 50% trichloracetic acid was added. The resulting precipitate was removed by centrifugation at 110,000 × *g* for 30 min and the supernatant fluid, containing all the trypsin-released glycopeptides, was neutralized with NaOH, dialyzed overnight at 4°C, and then freezed-dried.

Animals

Experimental animals consisted of 3-wk-old male mice. They had been fed on standard diets (Saitama Experimental Animal) for 1 wk, and those that showed normal growth were selected, separated into groups consisting of 10 mice each, housed in individual cages. The standard diet plus MFGM (I:5%; II:10%; III:20%) was given to the test group for 5 days. The feces of each day were collected and freeze-dried for storage. The time course of changes in fecal β-glucuronidase activity was monitored.

RESULTS

Effect of κ-casein and MFGM on β-glucuronidase activity

κ-casein or MFGM with a protein content of 0–0.2% were added to a β-glucuronidase solution in order to investigate effects on enzyme activity (Fig. 1). Relative to β-glucuronidase activity (100%) without κ-casein or MFGM, its activity in the solution with 0.1% MFGM concentration was reduced to 26.1% and that in the solution with κ-casein was reduced to 34.2%. β-Glucuronidase activity with MFGM concentration of 0.2% was inhibited by about 90%. Thus both substances inhibited β-glucuronidase activity, and particularly potent inhibitory action was exhibited by MFGM.

HiLoad column chromatography

We fractionated a 4-mL aliquot of MFGM solution using a HiLoad column (Fig. 2). The first eluted fraction, (absorption peak 280 nm) was termed I, and the second peak, II. These fractions, (I and II) were collected, freeze-dried, and 1.5 mg of each added to a β-glucuronidase solution. Both fractions I and II led to a substantial decrease in β-glucuronidase activity (Table 1). Specifically, fraction I inhibited β-glucuronidase activity by 44.83%, and fraction II by 71.59%. Inhibition of β-glucuronidase activity by trypsin hydrolysate of MFGM.

The effect of MFGM's inhibitory action was also followed after dissolving 1.5 mg of trypsin-treated MFGM in 0.65 mL distilled water, and adding it to a β-glucuronidase solution (Table 1). The results show that the trypsin digests inhibited β-glucuronidase activity by ≈ 42%.

Effect of MFGM on intestinal β-glucuronidase in mouse

For 5 days, male mice were fed a diet mixed with 5% (group I), 10% (group II), and/or 20% (group III) MFGM, and we investigated the time course of changes in fecal β-glucuronidase activity over the treatment period (Fig. 3). In groups I and II, enzyme activity had decreased 15–20% on the 5th day of treatment, but in group III, (MFGM at higher dose 20%), a decrease in enzyme activity was seen with an inhibition rate of 50%.

DISCUSSION

THE STUDY to determine effects that cow milk sialoglycoproteins, MFGM and κ-casein had on β-glucuronidase (Fig. 1) showed the addition of 0.2% MFGM or κ-casein led to inhibition of β-glucuronidase by 90.0% and 65.8%, respectively. Since MFGM exhibited a particularly high inhibition, we conducted enzyme inhibition tests using MFGM in another study consisting of several proteins that exhibit only slight solubility, similar to biomembranes. If used in this state, most of the structural components would remain insoluble, thereby preventing full expression of potential activity. Consequently, we partially modified MFGM by the method of Tomita et al. (1978) to solubilize the structural components, such as glycoproteins. As a supplemental experiment, we confirmed the heat stability (80°C, 10 min) of MFGM. Results showed MFGM was heat-stable. It has been reported that solubilized MFGM can be separated by SDS-polyacrylamide gel electrophoresis into 16 polypeptides and 9 glycopeptides (Shimizu et al., 1976). The MFGM component with inhibitory activity is likely to be one of those nine glycopeptides. We used gel filtration by

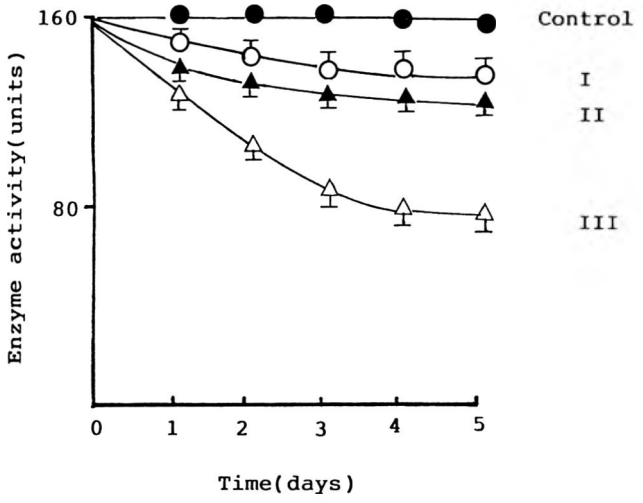

Fig. 3—Time course of changes of β-glucuronidase activity in mouse feces. The standard diet containing MFGM (I:5%; II:10%; III:20%) was given to the test group for 5 days. The time course of changes in fecal β-glucuronidase activity was measured. Results were analyzed by using the Mann-Whitney u-test and expressed as mean values and SD of 10 mice. Significant difference: $p<0.02$; Initial mean body weight: 33-35g; Control group (Standard diets): I group (Control + MFGM 5%); II group (Control + MFGM 10%); III group (Control + MFGM 20%).

HiLoad column chromatography to separate MFGM into two fractions. Since the glycoprotein components of MFGM have molecular weights of 10,000–88,000, we used a HiLoad Superdex prep grade (Pharmacia) column, capable of fractionating molecular weights in the range of 10,000–600,000. This HiLoad column chromatography is recommended as a high-performance carrier with a good separation rate. MFGM was separated by this column into two peaks (I, II). Further, the β-glucuronidase inhibitory action of each of these fractions (I, II) was investigated, (Fig. 2). Both fractions I and II inhibited β-glucuronidase activity, with an inhibition rate of ≈ 45% shown by I and ≈ 72% by II, (i.e., both fractions had a notable effect on enzyme action). This clearly associated the inhibition of enzyme action with the glycoprotein components of MFGM. In particular, the finding of a higher inhibition rate by fraction II pointed to potent enzyme inhibitory action of a glycoprotein with a relatively low molecular weight. Although β-glucuronidase is widely distributed throughout the organs of the body, it has been proposed that the enzyme, implicated in colon cancer, is produced by microorganisms of the colon flora. An inhibitor of this enzyme administered orally, would be influenced by diverse factors before it reaches the colon. To ensure such inhibitor would act effectively in the colon, its essential inhibitory function must remain stable regardless of such diverse effects. The first effect encountered by a protein through the gastrointestinal tract is the dissociating action of protease trypsin. When trypsin was allowed to act on MFGM, it converted the substance into digests of lower molecular weight by action on the amino acid residues. Investigation of the magnitude of inhibition by these trypsin-derived digests on β-glucuronidase activity under *in vivo* conditions would be needed to fully clarify the mechanism of enzyme inhibition by MFGM. We purified the trypsin digests and examined their action on β-glucuronidase. The digests inhibited β-glucuronidase activity (Table 1) by ≈ 42%, demonstrating retention of enzyme inhibitory activity despite trypsin treatment. Following trypsin dissociation in the gastrointestinal tract, proteins are further subjected to peptidase enzymes, which cleave the digests to smaller fragments, which are finally dissociated into amino acids. After digestion by trypsin, MFMG is acted on by peptidases. However, MFGM is a sialoglycoprotein which, unlike other proteins, is probably less affected by action of peptidase. We have confirmed this in another experiment. In fact, by adding the MFGM (0.1% conc) in the reaction mixture with aminopeptidease, nearly half the aminopeptidase activity was inhibited, thus indicating a marked effect. Similar results have been reported on cow milk sialoglycoprotein, namely, κ-casein (Ito et al., 1991). Many reports on *in vivo* inhibition of enzymes by glycoproteins have been published (Schultze et al., 1962; Donaldson and Evans, 1963; Heimburger and Haupt, 1965; Barret and Starkey, 1973; Rosenberg, 1975). Based on this reasoning, we propose that several enzyme-inhibiting peptide components of MFGM are not dissociated into amino acids by peptidases, but reach the colon where they inhibit β-glucuronidase. That MFGM inhibits the action of β-glucuronidase within the gastrointestinal tract is of considerable importance. This raises the question of whether MFGM would inhibit β-glucuronidase activity within the colon with the same degree of efficacy as observed *in vitro*. We considered that a practical method to investigate the effectiveness of MFGM *in vivo* might be to administer it to test animals in food, then measure β-glucuronidase activity in the feces with the passage of time. In theory, this method would allow the efficacy of MFGM *in vivo* to be determined with relative ease. For instance, observation of a remarkable decrease in fecal β-glucuronidase activity would indicate inhibition of β-glucuronidase by MFGM within the colon. To test this hypothesis, we administered MFGM (I: 5%; II: 10%; III: 20%) to mice by mixing it with the feed supplied to the animals each day for 5 days, and followed the changes in fecal β-glucuronidase activity over the treatment period (Fig. 3). The results showed a decrease in β-glucuronidase activity in all groups over the treatment period. In groups I and II, enzyme activity had decreased 15–20% on the 5th day of treatment, but in group III, (given MFGM at the higher 20%), a remarkable decrease in enzyme activity was seen with inhibition rate of 50%. This showed the intracolonic efficacy of MFGM in inhibiting β-glucuronidase. The mean daily food consumption/mouse during treatment was 4.8g for group I, 5.2g for group II, and 4.4g for group III, which was equivalent to MFGM doses of 0.24g, 0.52g, and 0.88g, respectively. These results showed that an MFGM dose of ≈0.88 g/day was needed to exhibit a strong inhibitory effect (group III). Identification of the active component using affinity chromatography method is under investigation.

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Soluble and Lipid-Bound Calcium and Zinc During Processing of Infant Milk Formulas

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ABSTRACT

Samples were withdrawn during the manufacture of infant formulas to determine steps which may affect soluble and lipid-bound Ca and Zn, which were estimated after centrifugal separation of *in vitro* digested samples. Pasteurization did not significantly reduce solubility of Ca and Zn. Blends forewarmed at low temperature contained a smaller proportion of soluble Ca than skim milk, but Zn was not affected. Forewarming at high temperature reduced solubility of Ca. The solubility of Ca and Zn was lower in sterilized and spray-dried formulas than in blends forewarmed at low temperature. Though initial Ca and Zn levels were greater in infant formulas than in human milk, percentages of soluble Ca and Zn were not higher.

Key Words: infant formula, calcium, zinc, lipids.

INTRODUCTION

FULL and complete breast-feeding is accepted as best for the human infant (Sai, 1991). Surveys from 1984 to 1989 in the United States have shown declines in breast-feeding, up to six months of age (Ryan et al., 1991). There are circumstances in which breast-feeding cannot be realized: premature and milk-sensitive infants, inborn errors of metabolism (Gurr, 1981; Coveney, 1985), insufficient milk syndrome and breast-feeding failure (Miller and Chopra, 1984), and there are mothers who do not choose to breast-feed. The aim of manufacturers is to produce milk-based formulas which closely resemble human milk, and to make sure adequate utilizable nutrients are provided (Milner, 1990). Calcium and most essential trace elements (zinc, iron, copper, manganese, and selenium) where found less bioavailable from infant formulas than from human milk (Fomon et al., 1963; Casey et al., 1981; Rudloff and Lönnerdal, 1990). Consequently, in order to ensure optimum intake, those elements are added to infant formulas at higher levels than occur in human milk (Fransson and Lönnerdal, 1982).

Various processes to which foods are subjected may affect the form of minerals and influence their absorption (Clydesdale et al., 1991). Milk proteins may be preheated and demineralized. Ingredients (fats, vitamins, minerals, lactose), approximating human milk composition, are first blended at warm temperatures. This is followed by forewarming the blend at high temperature, adding emulsifiers, stabilizers and heat-stable vitamins, homogenization, and sterilization or spray-drying (McDermott, 1987). Availability of some minerals, such as Ca and Zn may be reduced by becoming less soluble upon heat treatments. For Ca, the extent of precipitation to a colloidal form is proportional to the severity of heat treatments (Pouliot et al., 1989).

Few studies have been conducted to evaluate the effect of heat treatments on availability of minerals. Williamson et al. (1978) reported no significant difference in retention of nitrogen, calcium, phosphorus, and sodium between pasteurized and raw human milk. However, all preterm infants gained weight more rapidly when fed raw human milk. Weeks and King (1985) observed no effects on intestinal absorption and femoral deposition of Ca in rats fed pasteurized milk. No data are published concerning the effect of processing on Zn availability. An evaluation of mineral solubility at each step during processing, up to the final sterilized formulas as purchased, and how it compares with human milk, has not previously been reported.

Solubility of minerals in milk products, determined after an *in vitro* sequential adjustment of pH simulating digestion, is one factor which may influence *in vivo* absorption. This is based on the assumption that for a mineral to be absorbed, it must be soluble or bound to a substance which can be absorbed and then released (Miller et al., 1981; Kim and Zemel, 1986; Keane et al., 1988; Nadeau and Clydesdale, 1991; Schnepf and Madrick, 1991). Factors which determine bioavailability of Ca and Zn are complex, and solubility may only be one predictive factor of potential bioavailability (Brennan et al., 1991).

Ca and Zn in human milk are mainly bound to whey protein and to low-molecular-weight compounds, whereas in cow milk they are bound mainly to casein. Significant proportions of Ca and Zn are in the fat fraction of human milk. Almost none of either is found in the fat fraction of cow milk (Fransson and Lönnerdal, 1983). The amount present in the fat portion of infant formulas is unknown, and the significance of lipid-binding on the bioavailability of Ca and Zn has not been established.

Our objective was to determine, by an *in vitro* digestion method, whether and to what extent each step, during the manufacture of infant milk formulas, affected soluble and lipid-bound Ca and Zn. Specific objectives were (1) to determine the proportions of soluble and lipid-bound Ca and Zn throughout the manufacture of powdered and liquid infant milk formulas, and (2) to compare the proportions of soluble and lipid-bound Ca and Zn in various milk samples to those in human milk.

MATERIALS AND METHODS

Milk samples

Milk samples were taken at each step during the manufacture of infant milk formulas in both liquid and powder forms (Fig. 1): skim milk (S), pasteurized skim milk (P), blend forewarmed at low temperature (70–75°C) (FL), blend forewarmed at high temperature (101°C) (FH), sterilized (121°C) (St) or spray-dried (SD) infant formula, and human milk (H). Liquid and powder forms were manufactured in two batches. A pool of human milk was constituted from the milk of six healthy women who had been breast-feeding for 1 to 5 mo. Human milk samples were extracted by breast pump at different times in the day and immediately frozen. Liquid samples were freeze-dried, and all milk powders were frozen until analyzed.

Enzymes

Pepsin, pancreatin, and lipase were purchased from Sigma Chemical Co. (St. Louis, MO). Enzyme stock solutions were freshly prepared prior to use. Pepsin stock solution was obtained by dissolving 500 mg pepsin (1:10,000 activity from porcine stomach mucosa) in 25 mL 0.1N HCl. Pancreatin stock solution was made up by dissolving 1g pancreatin (4 × activity from porcine pancreas) in 25 mL of 1 M $NaHCO_3$ (Fisher Scientific Co., Montreal). For the lipase stock so-

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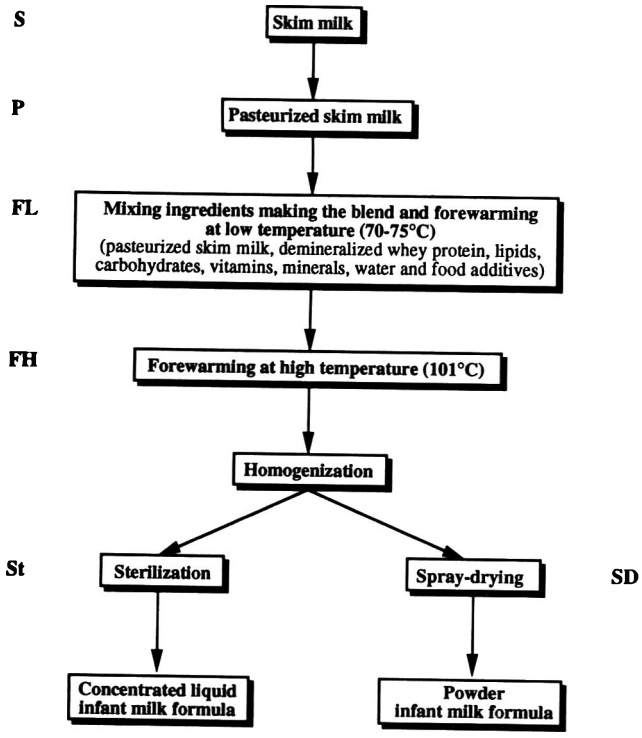

Fig. 1.—Processing scheme for manufacture of liquid and powder infant milk formulas. Letters indicate at which steps samples were taken for analysis (S = skim milk, P = pasteurized skim milk, FL = infant milk formula forewarmed at low temperature, FH = infant milk formula forewarmed at high temperature, ST = sterilized infant milk formula), SD = spray-dried infant milk formula).

lution, 1.5 mg lipase from porcine pancreas (69,700 units activity/mg solids) was dissolved in 25 mL of 1M $NaHCO_3$.

Digestion procedure

All reagents were analytical grade, and solutions were prepared with double-distilled deionized (DDD) water. All glassware and magnetic stir bars were acid-washed with concentrated HCl and thoroughly rinsed with DDD water to remove contaminants. Soluble and lipid-bound Ca and Zinc in milk samples were determined by *in vitro* digestion (Nadeau and Clydesdale, 1991) with some modifications. Each freeze-dried milk sample was reconstituted to 50 mL with DDD water. The pH was lowered to 2.0 by dropwise addition of 6N and 1N HCl under constant magnetic stirring, and 1.25 mL pepsin stock solution, kept at 37°C, was added. Samples were incubated at 37°C for 30 min in a shaking thermostated water bath (Versa Bath, Model 236, Fisher Scientific Co., Pittsburgh, PA). The pH was then raised to 6.0 by addition of 1M $NaHCO_3$ and 1.25 mL pancreatin and 2 mL lipase stock solutions, both kept at 37°C, were added. Samples were incubated an additional 30 min. Aliquots of 40 mL of each digested sample were immediately centrifuged at 30,000 × *g* for 1 hr at 5°C (SS-34 rotor, Sorvall RC-5B, Newtown, CT). Supernatants and floating lipids were separately frozen for subsequent analysis.

Mineral analysis

Sample aliquots were dry-ashed twice at 500°C for 5 hr in a muffle furnace (Thermolyne Sybron, Furnatrol 1, type 18200, Dubuque, IA) for total, soluble (fat-free supernatant after centrifugal separation) and lipid-bound mineral determinations. Ashed samples were dissolved in 2 mL conc. nitric acid, heated at 80°C on a hot plate until dry, and redissolved in 1 mL conc. HCl and 2 mL DDD water. Samples were diluted as needed in 0.1N HCl (Keane et al., 1988). Total, soluble and lipid-bound Ca and Zn were determined by atomic absorption spectrophotometry (Perkin-Elmer Spectrophotometer, Model 603, Norwalk, CT). Standards of 0.5, 1, and 1.5 ppm Zn and 5, 15, and 25 ppm Ca were prepared from 1,000 ppm Zn and Ca reference solutions (Fisher Scientific Co., Montreal). Lanthanum chloride

Table 1—Proximate composition of milks and formula[a] and contribution of skim milk[b] to nutrient content

Components	Skim milk (g/100 mL)	Human milk (g/100 mL)	Formula (g/100 mL as fed)	Contribution of skim milk to nutrient content of formula (%)
Protein	3.7	1.19	1.5	
casein	3.0	0.4	0.6	100
whey	0.7	0.6	0.9	17
Fat	0.01	3.5	3.6	0
Carbohydrate	5.0	6.3	7.2	15.4
Ash	0.8	0.2	0.25	61.8
Water	90.2	87.5	87.5	22.9
Total calcium[c]	0.120	0.0232	0.042	62.5
Total zinc[d]	0.0005	0.00018	0.0005	16.8

[a] Nutrients pertinent to this study.
[b] Contribution of skim milk to nutrients is calculated based on 1.5 g protein/100 mL as fed, 60/40 whey protein/casein ratio and the nutritional composition of skim milk.
[c] Calcium added as Ca citrate and chloride in sterilized formula and Ca hydroxide and chloride in spray-dried formula.
[d] Zinc added as Zn sulfate.

($LaCl_3 \cdot 7H_2O$, Sigma, St. Louis, MO) stock solution (5%) was added to a 0.5% final concentration, in all samples and standards analyzed for Ca (to correct for possible phosphate interferences).

Statistics

Five repetitions were made for each sample taken during the process and for human milk, according to a randomized complete block design (Steel and Torrie, 1980). Statistical analyses on all data were tested for homogeneity of variance by Bartlett's test. The influence of processing on soluble and lipid-bound Ca and Zn in samples was tested by analysis of variance (ANOVA) and significant differences among means were located by the Duncan's new multiple range test (Steel and Torrie, 1980).

RESULTS AND DISCUSSION

THE COMPOSITION of skim milk, liquid infant milk formula and human milk and the contribution of skim milk to the nutrient content of infant formulas were compared (Table 1). Skim milk (used in the production of infant milk formulas), infant milk formulas, and human milk, contained 120, 42, and 23 mg total Ca, and 0.50, 0.50, and 0.18 mg total Zn, respectively, per 100 mL milk as consumed. Both liquid and powder infant milk formulas contained 3.6 g fat/100 mL as consumed, while human milk contained 3.5 g fat/100 mL as determined by the Mojonnier method (Mojonnier Bros. Co., 1925).

The solubility of Ca and Zn was estimated as the ratio of soluble Ca and Zn in the supernatant after centrifugal separation of samples subjected to *in vitro* pepsin, pancreatin, and lipase digestion, to the total content of Ca and Zn in milk samples. Figures 2 (liquid) and 3 (dried) illustrate the effect of various processing steps during manufacture of liquid and powder infant milk formulas on the solubility of Ca, compared to human milk in which the solubility was estimated by the same technique. No reduction in Ca solubility occurred due to pasteurization of skim milk used for production of either liquid or powder formulas. This was expected, since several workers reported no reductions, attributable to pasteurization, on Ca balance, Ca absorption and femoral deposition of Ca, or dialyzable and soluble Ca in different milks and various pasteurization conditions. Ca balance was not affected in preterm infants fed human milk which had been subjected to low-temperature, long-time pasteurization (Williamson et al., 1978). Weeks and King (1985) have shown that intestinal absorption and femoral deposition of Ca were not different in high- and low-calcium-adapted rats consuming high-temperature, short-time and ultra-high-temperature pasteurized milks. Keane et

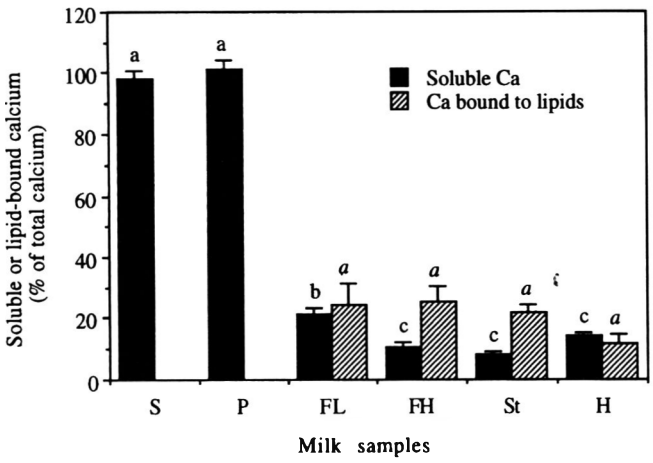

Fig. 2. – (Liquid Milk Formula) Percentage of soluble and lipid-bound calcium fractions in enzymatic digests of skim milk (S), pasteurized skim milk (P), blend forewarmed at low temperature (FL), blend forewarmed at high temperature (FH), sterilized liquid infant milk formula (St) and human milk (H). Each bar is the mean of five repetitions ± SEM. Different letters in the same group of bars are significantly different (p < 0.05).

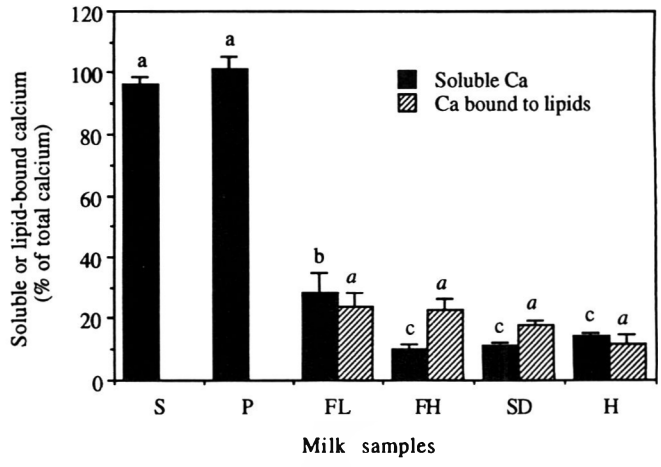

Fig. 3. – (Dried Milk Formula) Percentage of soluble and lipid-bound calcium fractions in enzymatic digests of skim milk (S), pasteurized skim milk (P), blend forewarmed at low temperature (FL), blend forewarmed at high temperature (FH), spray-dried powder infant milk formula (SD), and human milk (H). Each bar is the mean of five repetitions ± SEM. Different letters in the same group of bars are significantly different (p < 0.05).

al. (1988) reported that milk pasteurization did not reduce the percentage of dialyzable Ca *in vitro*. Reykdal and Lee (1991), using an *in vitro* digestion technique with centrifugal separation of digests, obtained 64% (±12.7) and 89% (±1.7) soluble Ca, respectively, for raw and pasteurized skim milk as compared to 98% (±2.9) and 101% (±3.1), respectively, in our study. Thus, pasteurization of skim milk commonly used in the manufacture of infant milk formulas apparently does not affect solubility of Ca in skim milk.

After adding various ingredients making the blend and forewarming at low temperature, the solubility of Ca was lower than in skim milk (Fig. 2 and 3). It cannot be concluded that this decrease was soley due to forewarming, because the composition of the blend differed from that of skim milk. It was not technically feasible to get a non-heated blend to compare. The ingredients added (demineralized whey protein, oils, lactose, vitamins, minerals, emulsifiers, stabilizers) may have influenced the solubility of Ca in specific ways. Corneau et al. (1992) reported that the chemical form of Ca added to a formulation influenced Ca solubility.

The solubility of Ca decreased ($p < 0.05$) upon subsequent forewarming at high temperature but did not decrease further after the final sterilization or spray-drying. The percentage of soluble Ca in liquid and powder infant formulas forewarmed at high temperature was more than two times lower ($p < 0.05$) than for blends forewarmed at low temperature. In a study on calcium phosphate partition and heat stability during manufacture of sterilized concentrated milk, Hardy et al. (1984) reported a gradual reduction of soluble salts with a corresponding increase in Ca and phosphate bound to the colloidal phase throughout the process until the clotting time was reached. They reported a 20% decrease in soluble Ca upon forewarming at 90°C. Our results showed that a higher forewarming temperature (101°C vs 70°C) probably decreased Ca solubility by causing more Ca and phosphate transfer to the colloidal phase which reduced Ca solubility.

Though human milk received no heat treatment, Ca solubility was not different from sterilized or spray-dried infant milk formulas and was lower ($p < 0.05$) than in cow milk (Fig. 2 and 3). Heating is not the only factor involved in Ca solubility. Milk constituents have an important effect. For example, casein binds trace elements and minerals in cow milk but only minor amounts in human milk where Ca is mainly bound to whey protein and low-molecular-weight ligands (Fransson and Lönnerdal, 1983). The ability to keep bound minerals in solution may be different from one protein to another. In our study, 37.5% of Ca in infant milk formulas was provided by Ca salts in the forms of $CaCl_2$ and Ca citrate in the sterilized formula and Ca hydroxide and $CaCl_2$ in the spray-dried formula (Table 1). However, those salts have a low solubility in order to keep protein stability upon heating (Parry, 1974). In enteral formula, 50 mg Ca/100 mL added as $CaCl_2$ was found to be 15% soluble (Corneau et al., 1992). The solubility of minerals may only be one predictive factor of their potential bioavailability. There are physiological factors promoting mineral absorption which cannot be reproduced in vitro. For instance, the Ca content of the diet can affect both active and passive Ca absorption (Bronner, 1987), and active absorption cannot be measured *in vitro*. Whether Ca bound to human whey is more bioavailable than that bound to bovine casein is not known. Nevertheless, higher amounts of Ca than normally present in human milk are purposely added to infant formulas (Santé et Bien-être Social, Canada, 1990), to compensate for the hypocalcemic effect of phosphate load in cow milk-derived formulas (Cruz and Tsang, 1992). Ca retention from human milk was higher than from milk-based infant formulas when fed to weanling rhesus monkeys (Rudloff and Lönnerdal, 1992).

Protein and minerals in milk are partly associated with lipids. The percentage of Ca in the fat fraction is shown in Figures 2 and 3. The fraction of Ca bound to lipids was not different in the blend forewarmed at low temperature, at high temperature, or in sterilized or spray-dried infant milk formulas. The percentage of Ca associated with lipids was not different between sterilized or spray-dried infant milk formulas and human milk. The distribution of several essential trace elements differed considerably in human milk and processed dairy products. The percentage of Ca associated with the fat fraction in human milk was 12% (Fig. 2), close to the value reported by Fransson and Lönnerdal (1983). They performed analyses without enzymes. Nadeau and Clydesdale (1991) reported that the addition of pepsin-pancreatin during sequential pH treatment led to significant increases in mineral solubility with increasing milkfat concentration, similar to analogous systems without enzymes. Addition of lipase negated this relationship. They suggested that hydrolyzed fatty acids may have led to insoluble-Ca soap formation. Infant milk formulas do not contain notable amounts of milk fat globule membranes. Fats are added in the form of oils to provide a fat composition closer to the fatty acid composition of human milk (Packard, 1982).

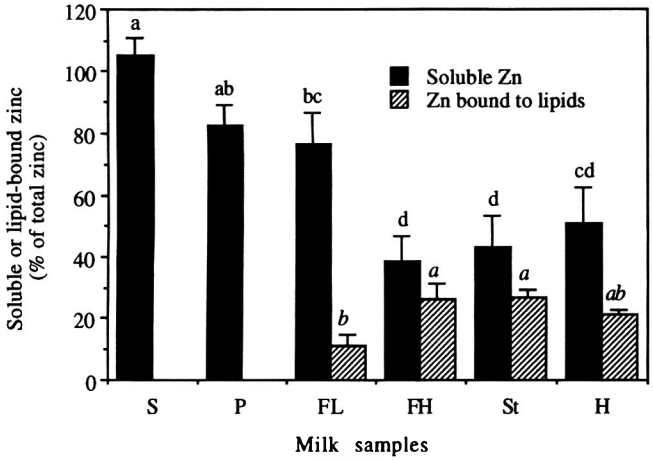

Fig. 4.—(Liquid Milk Formula) Percentage of soluble and lipid-bound zinc fractions in enzymatic digests of skim milk (S), pasteurized skim milk (P), blend forewarmed at low temperature (FL), blend forewarmed at high temperature (FH), sterilized liquid infant milk formula (St), and humanmilk (H). Each bar is the mean of five repetitions ± SEM. Different letters in the same group of bars are significantly different ($p < 0.05$).

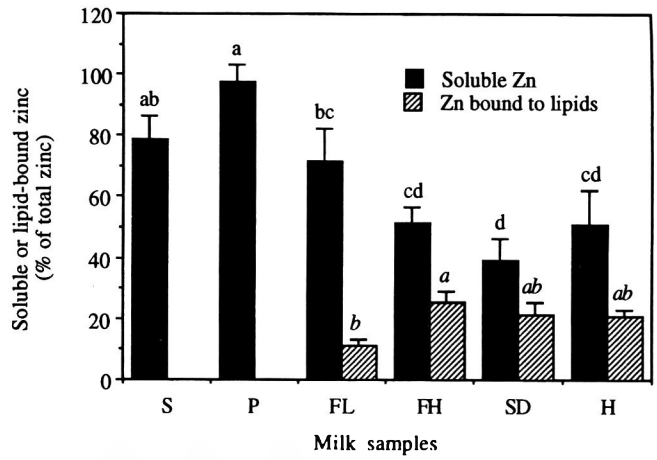

Fig. 5.—(Dried Milk Formula) Percentage of soluble and lipid-bound zinc fractions in enzymatic digests of skim milk (S), pasteurized skim milk (P), blend forewarmed at low temperature (FL), blend forewarmed at high temperature (FH), spray-dried powder infant milk formula (SD), and human milk (H). Each bar is the mean of five repetitions ± SEM. Different letters in the same group of bars are significantly different ($p < 0.05$).

From our results in the preparation of infant milk formulas, where oils were used, the distribution of Ca in the fat fraction of liquid and spray-dried infant milk formulas was not significantly higher, than in human milk.

The markedly lower Ca solubility of infant milk formulas compared to skim milk is noteworthy. According to Ashmead et al. (1985), some unsaturated fatty acids can form Ca salts which are poorly absorbed. Thus, the absorption of Ca can be depressed in the presence of high levels of certain fats. Reykdal and Lee (1991) reported high Ca solubility in skim milk as compared to whole milk. They attributed this effect to the formation of Ca soaps and to interactions with digestion products. According to Irving (1973), however, Ca soaps may be solubilized by the bile in the intestine, thus facilitating Ca absorption. Therefore, the significance of lipids on the bioavailability of Ca to infants cannot be predicted.

Figures 4 (liquid) and 5 (powder) summarize the effect of heat treatments on *in vitro* Zn solubility in skim milk, liquid and powder infant formulas, and human milk samples. Pasteurization of skim milk used for production of both liquid and powder infant formulas did not affect Zn solubility. After adding various ingredients making the blend and forewarming at low temperature, the percentage of soluble Zn was not lower than in the skim milk used for the formulation of infant milk formulas. The percentage of soluble Zn decreased by 33–50% in liquid infant formulas upon subsequent forewarming at high temperature. Final sterilization or spray-drying did not reduce Zn solubility as compared to infant formulas forewarmed at high temperature. However, Zn solubility in the spray-dried formula was lower ($p < 0.05$) than for the blend forewarmed at low temperature. Although Zn concentration was almost 3 times higher in sterilized or spray-dried infant milk formulas than in human milk (0.50 mg vs 0.18 mg), the percentage of soluble Zn was not different from human milk.

A severe heat treatment, such as forewarming at high temperature, seemed to alter Zn solubility. Protein solubility decreases upon severe heating. Probably Zn, attached mainly to casein in cow milk, would also become less soluble after forewarming at high temperature. The major factor affecting Zn solubility in infant milk (compared to human milk) would again be the fractions to which it is bound. Zinc in cow milk is primarily bound to casein (84%), and only small amounts are bound to whey protein (13%), citrate (2%) and fat (1%). In human milk, it is predominantly bound to whey protein (50%), citrate (20%), and milk fat (18%) (Fransson and Lönnerdal, 1983). Also, not only is the casein content different between human milk and cow milk, but so is the composition of casein. This can affect its ability to bind metals (Lönnerdal, 1985). The amount of Ca and Fe, and the presence of Zn-binding compounds in milk, such as casein and citrate, can affect Zn absorption (Rudloff and Lönnerdal, 1992).

The percentage of Zn in the fat fraction is shown in Figures 4 and 5. Notable amounts of Zn were found in the lipid fraction at all steps in liquid and powder infant milk formulas. Forewarming the blend at high temperature increased the percentage of lipid-bound Zn by 59 and 56% ($p < 0.05$) for liquid and powder infant milk formulas, respectively, as compared to infant formulas forewarmed at low temperature. The percentage of Zn in the fat fraction was not different between sterilized or spray-dried formulas and human milk. In our study, the percentage of Zn associated with lipids in human milk (21%) was similar to the value (18%) reported by Fransson and Lönnerdal (1983). They performed Zn determinations after solubilizing milk fat globule membranes with detergent. In our study, Zn was determined in enzymatic digests. According to Fransson and Lönnerdal (1983), Zn in the membrane proteins of the outer membrane surrounding the fat globule is bound to alkaline phosphatase, a metalloenzyme also present in human milk fat. Cunnane (1982) proposed that the higher amount of essential fatty acids in human milk, (compared to cow milk) could partly explain the greater Zn absorption by infants fed human milk, by affecting the intestinal brush-border lipid composition, thereby altering permeability of the membrane. According to Singh et al. (1989), the lower bioavailability of Zn from cow milk may be caused by high casein content, since bovine casein is poorly digested by infants. Further disturbances of Zn absorption could also be caused by phosphopeptides formed by the action of trypsin and chymotrypsin which retain their high zinc-binding capacity. The apparent higher absorption from formula, compared to cow milk, may be due to the adjustment in protein ratio, which is ≈ one-half that of cow milk (Harzer and Kauer, 1982). However, it would most likely be due to the fatty acid profile differences since infant milk formulas are made to resemble, as closely as possible, the fatty acid profile of human milk.

In considering our results one must distinguish between solubility and bioavailability of Ca and Zn. Note that the purpose of our study was not to determine the bioavailability of Ca and Zn in infant milk formulas and in human milk. It was to examine in which fractions (hydrosoluble phase, liposoluble phase,

or precipitate) Ca and Zn were mostly distributed after various processing steps used in production of infant formulas, and to compare with human milk. All Ca and Zn present in the hydrosoluble phase is not necessarily bioavailable and the bioavailability of Ca and Zn in the liposoluble phase is not yet known.

CONCLUSION

CALCIUM solubility in both liquid and powder infant milk formulas was reduced upon forewarming the blend at high temperature. Zinc solubility was lower in sterilized or spray-dried infant formulas as compared to blends forewarmed at low temperature. Notable amounts of Ca and Zn were bound to the lipid fraction in infant milk formulas and in human milk. Although formulas were supplemented with Ca and Zn at levels higher than those found in human milk, the solubility of Ca and Zn after *in vitro* digestion was similar in infant milk formulas and in human milk. These results suggest research avenues which should help identify factors affecting bioavailability of minerals and better understanding of nutrient interactions.

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GELATION OF TURKEY MYOFIBRILS. . .From page 730

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Carbohydrate or Protein Based Fat Mimicker Effects on Ice Milk Properties

K. SCHMIDT, A. LUNDY, J. REYNOLDS, and L. N. YEE

ABSTRACT

Batches of ice milks (2 – 5% milk fat) made with a carbohydrate- or a protein-based fat mimicker were compared to conventional ice milk (4.8% milk fat). Rheological, freezing, and melting properties were evaluated. The carbohydrate-based fat mimicker changed rheological properties, resulting in higher viscosities, greater deviations from Newtonian flow, and higher consistency indices. Less air was incorporated in the mix containing the carbohydrate-based fat mimicker than in the control or the mix with protein-based fat mimicker. The protein-based fat mimicker mix had rheological and melting properties similar to those of the control but achieved higher air incorporation than did the control.

Key Words: ice milk, dairy, fat replacers, texture, flavor

INTRODUCTION

ICE CREAM and other frozen desserts rely on emulsion stabilization followed by an emulsion destabilization process to produce an optimal product (Thomas, 1981; Keeney, 1982). The emulsion stabilization process occurs when the mix is homogenized, resulting in a decrease in milk fat globule sizes as well as redistribution of milk proteins within the system (Berger, 1990). Many manufacturers use commercial emulsifiers to aid in this stabilization. To induce emulsion destabilization, two mechanisms are usually used. While the mix is being frozen, vigorous agitation occurs to destabilize the emulsion. Also mixes containing emulsifiers show enhanced fat destabilization rates (Keeney and Josephson, 1958). Optimal fat destabilization during freezing results in higher quality frozen dairy desserts. Those with too much or too little fat destabilization often do not have acceptable freezing, melting, or stand-up qualities (Kloser and Keeney, 1958; Govin and Leeder, 1971; Goff and Jordon, 1989). The frozen dairy dessert industry has optimized ingredient levels to produce the best products.

Fat mimickers (fat substitutes or fat replacers) are an important growing market for food ingredient manufacturers. They can be used to produce low-fat dairy products, mimicking the function of fat in the product (Mann, 1991; National Starch and Chemical Company, 1992; NutraSweet Company, 1992). Such fat mimickers may be carbohydrate- or protein-based products. However, some are lipid-based and have been used successfully as emulsifiers in frozen dairy products (Buck et al., 1986).

Two fat mimickers are Simplesse®, a protein-based product made by NutraSweet Company, and N-Lite™ D, a maltodextrin product developed by National Starch and Chemical Company. Replacing fat with protein or carbohydrate alters physical properties. This is of particular concern in frozen dairy desserts. In such systems, the balance of fat and serum solids helps promote emulsion stability during mix processing and allows fat destabilization during ice cream freezing. Replacing the fat alters this balance, which then affects whipping and melting properties (Arbuckle, 1977; Thomas, 1981). Both carbohydrates and protein may help stabilize emulsions by different mechanisms. Carbohydrates increase viscosity of the continuous phase, whereas proteins act at the oil/water interphase generally decreasing interfacial tension (Powrie and Tung, 1976).

Frozen dairy dessert processing has been optimized to produce emulsions with conventional levels of fat, protein, and carbohydrate. Our objective was to investigate the effect of two fat mimickers (a carbohydrate-base and a protein-base product) on the rheological, freezing, and melting characteristics of ice milk mix.

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MATERIALS & METHODS

Mix preparation

Three ice milks were investigated: a control (4.8% milk fat), a 2.1% milk fat product with Simplesse® D100 (whey protein concentrate) (designated as fat replacer protein, FR-P), and a 2.1% milk fat product with N-Lite™ D (designated as fat replacer carbohydrate FR-C) (Table 1). Usage levels were selected based on recommendations by fat mimicker manufacturers. Liquid ingredients were blended, and dry ingredients were added to make a total volume of 5.83L. The FR-P was hydrated overnight in water (NutraSweet Company, 1992) and added the next day.

All mixes were pasteurized on a BacTherm IV Unitherm (APV, Chicago, IL) for 4 sec at 116°C and homogenized (60°C) at 135 and 35.2 kg/cm^2. Mixes were collected in stainless steel containers at 18°C, covered with aluminum foil, and cooled to ≤ 7°C for ≥ 3.5 hr.

All mixes were frozen for ≤20 min in a gravity-fed Freez King soft-serve ice cream freezer (Tastee Freez, Chicago, IL). When ice milk overruns reached 75% (or maximum overrun), four 100-mL plastic sample cups were filled and placed in a hardening room at −32°C for ≥24 hr.

Chemical and microbiological analyses

The chemical composition of the mixes were determined, and changes in composition and microbiological quality were monitored. Standard methods were used to determine moisture, fat, protein, and ash (AOAC, 1990). Carbohydrate content was calculated by difference. Total plate,

Table 1—Formulations for ice milks

Ingredients	Percentage (wt/wt basis) Control	FR-C[a]	FR-P[b]
Milk fat[c]	4.8	2.1	2.1
MSNF[d]	11	11	11
Sucrose[e]	15	15	15
Maltodextrin[f]	2	2	2
Stabilizer[g]	0.8	0.8	0.8
Simplesse®[h]			3.4
N-Lite™ D[i]		4.8	
Total solids	33.6	35.7	34.3

[a] FR-C represents ice milk made with N-Lite™ D (carbohydrate-base).
[b] FR-P represents ice milk made with Simplesse® (protein-base).
[c] Cream (36% milk fat), University of Georgia Creamery, Athens, GA.
[d] Low-heat spray process nonfat dry milk, Maryland Virginia Dairy Cooperative, Laurel, MD.
[e] 100% granulated sugar, Kraft General Foods, Glenview, IL.
[f] Maltodextrin 100, Grain Processing Corporation, Muscatine, IA.
[g] Summit, a proprietary blend of stabilizers and emulsifiers, Germantown Manufacturing Co., Broomall, PA.
[h] The NutraSweet Company, Deerfield, IL.
[i] National Starch and Chemical Co., Bridgewater, NJ.

Table 2—Chemical and microbiological analyses of ice milks

	Control	FR-C[c]	FR-P[d]
Fat	4.87%[a]	2.01%[b]	2.07%[b]
Protein	3.79%[b]	3.65%[b]	5.85%[a]
Carbohydrate[e]	24.57%[b]	26.95%[a]	24.87%[b]
Ash	0.78%[a]	1.08%[b]	0.95%[a,b]
Total solids	34.01%[a]	33.69%[a]	33.74%[a]
pH	6.57[a]	6.44[b]	6.41[b]
Titratable acidity[f]	0.157[b]	0.147[b]	0.283[a]
SPC[g]	31/mL	31/mL	10/mL
Yeast and mold count	0/mL	0/mL	2/mL
Coliform count	0/mL	0/mL	0/mL

[a,b] Means of three trials. Numbers with the same letter in a row are not significantly different from each other at $p \leq 0.05$.
[c] FR-C represents ice milk made with N-Lite™ D.
[d] FR-P represents ice milk made with Simplesse®.
[e] Calculated by difference.
[f] Expressed as percent lactic acid.
[g] Standard plate count.

Table 3—Means of rheological values, whipping properties, and melt characteristics of ice milks made with milkfat or fat mimickers

	Control	FR-C[d]	FR-P[e]
Viscosity (mP.s)	41.4[b]	175.1[a]	43.6[b]
Flow behavior index	0.748[b]	0.566[c]	0.849[a]
Consistency index	0.154[b]	1.69[a]	0.093[b]
Fat destabilization	72.5[a]	70.4[a]	63.0[a]
Maximum overrun (%)	78.3[b]	45.0[c]	111.5[a]
Time to maximum overrun (min)	8.0[a]	7.3[a]	17.5[b]
Fat destabilization (%)	72.5[a]	70.38[a]	63.0[a]
Start of melt (min)	29.8[b]	47.0[a]	20.2[b]
Rate of melt (mL/min)	0.54[a]	1.43[a]	0.93[a]

[a-c] n = 3. Numbers with the same letter in a row are not significantly different from each other at $p \leq 0.05$.
[d] FR-C represents ice milk made with N-Lite™ D.
[e] FR-P represents ice milk made with Simplesse®.

psychrotrophic, and yeast and mold counts were done as described by Marshall et al. (1985). These tests were done to ensure that legal and safe products were produced. The pH of the mix was measured using a Markson pH meter (model 41064 Markson Science Inc., Houston, TX), and titratable acidity was determined by the method of Marshall et al. (1985).

Rheological measurements

Aliquots of mixes from each of the formulas were kept at 4°C, and rheological properties were measured between 22-28 hr after production as described by Schmidt and Smith (1989) with slight modifications. A Haake Rotovisco Viscometer (HBI, Paramus, NJ) equipped with MVI sensor was used to measure viscosity of the mixes at 4°C over a shear rate range of 0 to 1000 sec^{-1}. A Haake refrigerated water bath was used to maintain temperatures throughout measurement. Apparent viscosities were compared at a shear rate of 105 sec^{-1}. Consistency and flow behavior indices were calculated using the Ostwald equation (Holdsworth, 1971). Duplicate measurements were obtained on all samples, and values were averaged.

Freezing properties

Mixes were evaluated for freezing properties. Fat destabilization tests were done as described by Keeney and Josephson (1958). Ice milk or mix (1g) was diluted 500 times with distilled, deionized water. Samples were centrifuged at 1,000 × *g* for 10 min. Absorbance was measured 10 min later at 540 nm on a spectrophotometer (model 20D, Milton Roy, Inc., Rochester, NY). Distilled, deionized water was used as the blank (zero absorbance). Fat destabilization was calculated as $(A_{mix} - A_{frozen})/A_{mix} \times 100$. Duplicate readings were made on the mix and frozen product and averaged separately, then fat destabilization was calculated.

Mixes were also evaluated for whipping ability. These data generated three values: (1) the maximum amount of air incorporated during 20-min freezing, (2) time to maximum air incorporation, and (3) time to reach 80% overrun (Leighton and Leviton, 1939). At the freezer, samples were removed every minute, weighed in a 100-mL cup, and the temperature determined. When product temperature reached −5°C, the freezer compressor was switched on and off for 1-min intervals to maintain product temperature (−5°C ± 1°C). Weights were used to calculate product overrun. Whipping curves were generated by plotting overrun vs. time to determine time to reach 80% overrun, time to reach maximum overrun, and maximum overrun.

Melt properties

Melt properties were evaluated using the method of Lee and White (1991). Product (140 g) was suspended on a wire screen attached to a funnel and graduated cylinder. Room temperature was kept at 16 ± 1°C. Volume of drainage was determined at 10-min intervals. Time vs. volume of drainage was plotted. Data were regressed to determine the rate of drainage (slope) as well as initial time of drainage (× intercept). Duplicate determinations were done on all samples.

Experimental design

On three separate days, all three mixes were made and frozen. In all cases duplicate measurements were made and averaged, except for freezing curves. Data were analyzed using PC-SAS (SAS, 1988) by one-way analysis of variance or simple linear regression. An effect (treatment) was considered significant at $p \leq 0.05$. When significant differences were found among treatments (formulas), Fisher's protected LSD tests were applied to determine what differences existed (Montgomery, 1984).

RESULTS & DISCUSSION

SIGNIFICANT DIFFERENCES were shown for fat, protein, carbohydrate, pH, and titratable acidity among the three mixes (Table 2). Due to mix formulations, such differences were expected. The control mix had higher fat and pH than did FR-P and FR-C. The protein content of the FR-P was higher than that of the other two mixes. The carbohydrate content of the FR-C mix was higher than that of the other two mixes. The FR-P ice milk had a significantly higher titratable acidity value than that of the control or FR-C mix. This was attributed to the larger amount of whey protein in the FR-P mix. In fresh milk, titratable acidity (as lactic acid) is partially attributed to proteins and minerals. The additional whey protein in the FR-P apparently contributed to higher titratable acidity values. These values may be of concern for acidic frozen desserts where additional buffering capacity would resist lowering the mix pH.

Rheological properties were measured on the mixes. Viscosities were compared at a shear rate of 105 sec^{-1}. Consistency and flow behavior indices were also calculated and compared (Table 3). Significant differences among the mixes for all three rheological parameters were found. FR-C had a greater viscosity and consistency index and a smaller flow behavior index than those of the control or FR-P. The control and FR-P were considered to have the same viscosity and consistency index, but FR-P had a larger flow behavior index. Thus FR-C had the greatest deviation from Newtonian flow and was much more viscous than the other two mixes. The FR-P was considered to be the same as the control but exhibited more Newtonian-like flow behavior than did the control. These changes in rheological behavior could affect pumping rates, as suggested by Stepp and Smith (1991), as well as freezing properties. Fat destabilization data were similar for the three products (Table 3). All formulas contained sufficient milk fat, milk proteins, and emulsifiers to produce similar fat destabilization values (Govin and Leeder, 1971).

Whipping curves (Fig. 1, 2, 3) show the control mix peaked the quickest, while the FR-C mix did not incorporate much air. The FR-P mix continued to incorporate air throughout freezing. Maximum overrun and time to reach it were different between treatments (Table 3). The FR-C ice milk did not incorporate as much air as did the control, which had significantly less air incorporation than did the FR-P. Times to maximum overrun also differed between treatments. The FR-C and control incorporated air at a similar rate, significantly faster than that of the FR-P. The FR-P incorporated significantly more air than did the other two mixes. The FR-P mix

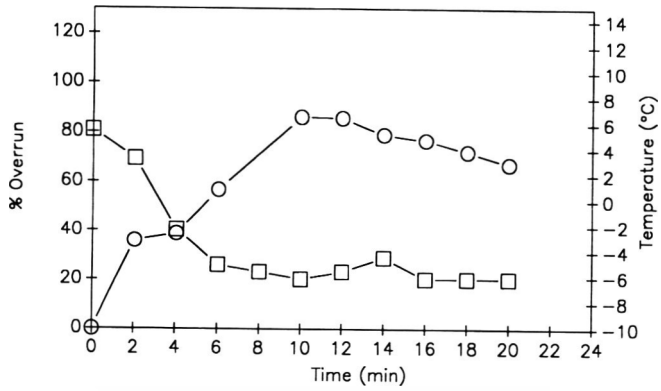

Fig. 1—Representative whipping curve for control ice milk (○ air incorporation; □ temperature).

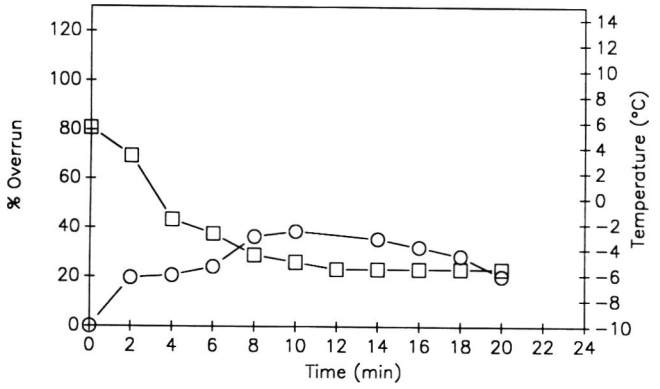

Fig. 2—Representative whipping curve for ice milk made with carbohydrate-based fat mimicker N-Lite™ D (○ air incorporation; □ temperature).

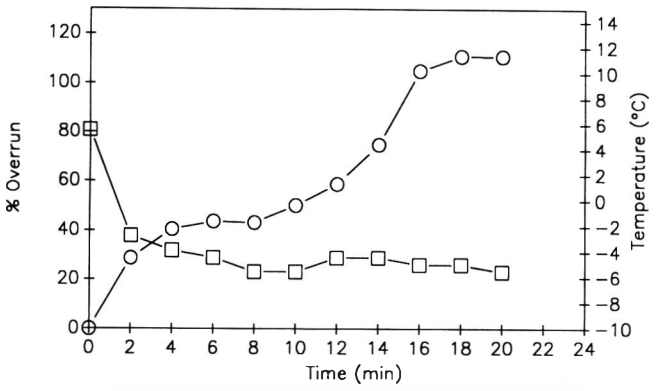

Fig. 3—Representative whipping curve for ice milk made with protein-based fat mimicker, Simplesse® (○ air incorporation; □ temperature).

initially incorporated air more slowly but gradually the rate increased resulting in significantly higher air incorporation. The FR-C product never achieved overruns greater than 60%. Probably the most important reason for this was the high viscosity of the mix, which may have prevented vigorous agitation. It was once hypothesized that increased mix viscosity aided air incorporation, but it is now thought that high viscosity accompanies good whipping, melt resistance, and smoothness in ice cream (Arbuckle, 1977). That the FR-C mix had such high viscosity may imply an optimum viscosity range for good whipping qualities. Going above or below that range may produce mixes with less effective whipping qualities. In addition, high viscosity might affect the rate of temperature change in the freezer. The FR-C mix apparently never achieved the same

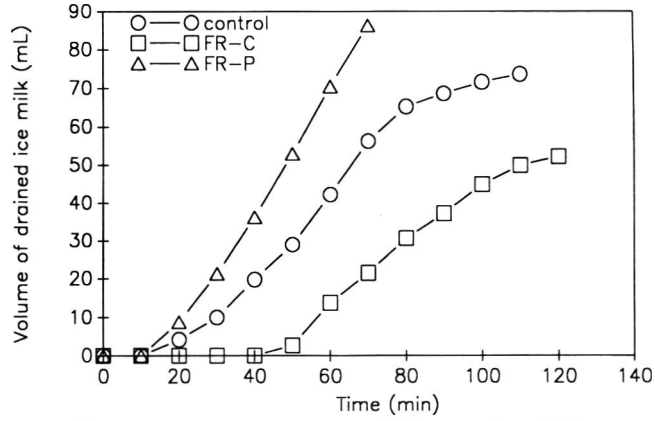

Fig. 4—Change in volume (mL) of drained ice milk with time (○ control made with 4.8% milk fat; □ FR-C, ice milk made with N-Lite™ D; △ FR-P, ice milk made with Simplesse®).

structure as did the control or the FR-P. All mixes were frozen in a soft-serve freezer. Such lack of air incorporation for the FR-C mix may not be as pronounced in a continuous freezer. Differences in whipping also attest to the fact that some proteins and fats are known for their abilities to aid in air incorporation, whereas carbohydrates are not well noted for this effect (Thomas, 1981; Keeney, 1982).

Hardened ice milks were subjected to a melt test to determine the effect of treatment on melt quality. As can be seen (Fig. 4) both the control and FR-C stopped draining towards the end of the testing period. This confirmed findings of other researchers (Lee and White, 1991). However, the FR-P continued to drain throughout the testing period. To analyze, data were selected prior to the leveling off period and used for linear regression to determine drain rate (slope value) and initial time of drainage (x intercept). Regression lines showed r^2 values of ≥ 0.95. All formulas drained at a similar rate (Table 3). However, differences were found among treatments for the time to start draining. The FR-C ice milk required a longer time to initiate drainage than did the control or FR-P. These differences were probably due to structure of the ice milk during freezing.

Overall, the FR-P products were more similar to the control than were the FR-C. This was somewhat expected since a function of milk fat in frozen desserts is emulsion stabilization and destabilization (Goff and Jordon, 1989). Proteins, due to their amphoteric nature, are much more functional in emulsions than are carbohydrates. The higher viscosity of the FR-C mix might be useful in creating new types of frozen dairy desserts.

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—Continued on page 779

Rheological Behavior of Frozen and Thawed Low-Moisture, Part-Skim Mozzarella Cheese

H. A. DIEFES, S. S. H. RIZVI, and J. A. BARTSCH

ABSTRACT

Stress relaxation and dynamic profiles of low-moisture, part-skim (LMPS) Mozzarella cheese cylinders refrigerated 14 days (control), frozen and thawed, and stored frozen and refrigerated up to 90 days were compared. Samples were frozen at −30°C and stored at −20°C. Thawing and refrigerated storage were at 5°C. Stress relaxation tests were conducted at 20°C and dynamic spectrometry at 20°C and 60°C. The frozen and thawed Mozzarella cheese tested at 20°C became harder and more elastic with storage time, while refrigerated stored samples became softer and more elasticoviscous with time. Upon melting, both 90-day-frozen and 90-day-refrigerated cheeses were less elastic and less viscous than 14-day-refrigerated samples.

Key Words: freezing, Mozzarella, cheese, stress relaxation, dynamic mechanical spectrometry, viscosity, rheology

INTRODUCTION

Proper freezing has been cited as an effective way of preserving cheese color, flavor, and nutritive value. However, freezing has been reported as only moderately effective for preserving cheese texture (Fennema, 1972). Freezing of cheese is usually avoided, because it tends to produce breakdown of body and structural characteristics. However, much commercially produced Mozzarella cheese is frozen to stop ripening and prolong shelf-life during marketing (Webb and Arbuckle, 1977; Alvarez, 1986). According to the Refrigeration Research Foundation (Anon., 1986), "current information for recommended procedures for freezer storage of cheese is inadequate." Further, it is "advisable that owners [of the product] be required to assume total responsibility for conditions for storage and thawing and for the quality of the final product."

The reported extent to which Mozzarella cheese is altered or damaged by freezing varies greatly with differences in freezing methodology and timing of post-thawing tests. Dahlstrom (1978) froze Mozzarella cheese at a very slow rate such that freezing through the critical temperature zone (−1.1 to −6.7°C) required 5 to 131 hr. Immediately after thawing through that temperature range for 12, 36, and 60 hr, the cheese was examined using Texture Profile Analysis (TPA). Mozzarella cheese frozen for 48 wk exhibited poor melt-down, high fat leakage, excessive free-surface moisture, low cohesiveness and bleached discoloration upon thawing. The frozen Mozzarella cheese recovered to its optimum values of cohesiveness and meltability upon tempering at 4.4°C for 21 days after frozen storage.

Cervantes et al. (1983) froze 2.5 kg Mozzarella bricks (30 × 8 × 8 cm) of various salt contents through the critical zone in 6 hr. After storage up to 1 wk at −15°C, samples were thawed through the critical zone in 5 hr and analyzed using compression and beam bending. No changes in firmness, cohesiveness, and fibrousness were observed. Mozzarella samples previously frozen and tempered for 21 days at 5.6°C softened in a similar manner to unfrozen samples. The texture of cheese was concluded to be controlled to some extent by a proper combination of salt content and aging time.

Tunick et al. (1991) also utilized TPA to study Mozzarella cheese samples (224 mL in volume) of varying fat and moisture contents. Samples were frozen 8 wk at −20°C and tempered at 4°C for 3 wk. Frozen, tempered samples comparable to FDA standards for LMPS Mozzarella cheese (Kosikowski, 1977) had lower hardness, gumminess, and springiness and higher cohesiveness and meltability. Oberg et al. (1992) froze LMPS Mozzarella cheese in blocks (5 × 10 × 7 cm) and in shredded form at −196°C and stored at −70°C or froze and stored at −20°C up to 42 days. Using an empirical helical viscometric test and a melt test, frozen cheese had greater stretch but melted less than refrigerated controls.

For empirical methods in which stress and strain are not known, results cannot be expressed in terms of well defined parameters such as viscosity (Mitchell, 1984). Results of empirical studies are useful in comparing quality of one cheese to another. However, they do not facilitate understanding structure-function relationships. In fundamental methods stress and strain are known, and results are independent of equipment and expressible in terms of rheological parameters.

Two fundamental tests applied to cheese rheology are stress relaxation and dynamic mechanical spectrometry. Masi and Addeo (1986) performed stress relaxation experiments on fresh Italian Mozzarella cheese and reported it exhibited a viscoelastic behavior characterized by rapid decay of applied force to a low equilibrium stress. The viscoelastic behavior was attributed to breaking and reformation of labile bonds in the casein network, such as salt linkages and hydrogen bonds. Nolan (1987) used a power law fit on normalized stress relaxation data to compare Cheddar cheese curd of different ages. A decrease in the power term (exponential value) indicated irreversible changes in protein structure with storage time. Much research has developed stress relaxation methods for cheese that minimize artifacts (Culioli and Sherman, 1976; Peleg and Calzada, 1976; Peleg, 1979; Atkin and Sherman, 1984; Chu and Peleg, 1985; Goh and Sherman, 1987; Nolan, 1987; Masi, 1989).

A dynamic viscosity approach was applied by Taneya et al. (1979) to quantitatively demonstrate significant differences between Gouda and Cheddar cheeses of varying maturity over −5 to 90°C. The elastic modulus (G′), the loss modulus (G″), and loss tangent were reported. Nolan et al. (1989) utilized dynamic mechanical spectrometry frequency sweeps to determine dynamic shear moduli and viscosities of natural LMPS Mozzarella and imitation Mozzarella. Relationships between G′ and frequency (ω) and G″ and frequency (ω) were reported for natural Mozzarella cheese. They also reported solutions to prevent artifacts in measurements. Tunick et al. (1990) employed dynamic strain sweeps to distinguish between Cheddar and Cheshire cheeses. They determined the two cheeses could be distinguished by dynamic rheological profiles.

The objective of our study was to utilize selected fundamental rheological techniques to quantify rheological changes by LMPS Mozzarella cheese during a commercially usable freeze-thaw protocol. To simulate behavior of stored samples under practical use, some measurements were conducted at 60°C.

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MATERIALS & METHODS

Samples and chemical analysis

One batch of LMPS Mozzarella cheese was manufactured using the method developed by Yun et al. (1993). It yielded 12 LMPS Mozzarella cheese cylinders (7 × 30 cm). Fat content was determined using a Babcock test (Richardson, 1985). Percent total nitrogen was determined by Kjeldahl (AOAC, 1990; Barbano et al., 1990). Cheese moisture was determined gravimetrically by drying 2g at 100°C in a forced-air oven (Model OV-490A-2, Blue M, Blue Island, IL) for 24 hr. All composition analyses were done in duplicate, except moisture analysis which was in quadruplicate.

Freezing and thawing

The freezing and thawing temperature profiles for the LMPS Mozzarella cheese cylinders were determined using 5 type K thermocouples (1.3 mm diam.) connected to a portable recorder (Model 3087, Yokogawa Hokushin Electric, Inc., Shenandoah, GA). Thermocouples were inserted radially into a cylinder to depths of 35 mm (center), 25 mm, and 13 mm. One thermocouple was placed on the surface of the cheese inside the packaging, and a final thermocouple was used to monitor ambient temperature. The sample cylinder was placed in a −30°C blast freezer, and the temperature profile was monitored until the center of the sample reached −20°C. The sample was then placed in a storage freezer (−20°C) to equilibrate for 1 wk. Following the period of equilibration, the sample was placed in a 5°C refrigerator where the thawing profile was monitored.

Storage treatments

To simulate commercial handling, LMPS Mozzarella samples were held in refrigerated storage (5°C) for a pretreatment period of 14 days prior to freezing (Kosikowski, 1991). Subsequently, the 12 cylinders were randomly assigned to treatments. Two cylinders were studied immediately following pretreatment (control). Eight cylinders were frozen (−20°C) and two cylinders remained refrigerated (5°C). All samples to be frozen were initially placed in the blast freezer 4.5 hr, and then placed in the storage freezer. Duplicate samples were removed from the freezer after 16 hr, and 30, 60, and 90 days frozen storage. Samples were then placed in refrigerated storage (5°C) to thaw 24 hr before analysis. One of the 90-day-frozen samples was tempered at 5°C for 21 days. Refrigerated samples were analyzed after 30 and 90 days storage post-pretreatment period. Cylinders were divided into 4 segments for conducting stress relaxation and dynamic mechanical spectrometry, and determining free oil and salt content. Segments for rheological analysis were always removed from the center of cylinders.

Stress relaxation

From each LMPS cylinder, 10 cylindrical specimens of Mozzarella cheese 15 mm in diameter and 10 mm in length were cut from specified 20-mm-thick slabs with a #11 cork borer and trimmed to length by twin parallel violin E strings (Peleg, 1979). This sample size produced stress relaxation data within the range of linear viscoelastic behavior (Chu and Peleg, 1985). Samples were coated with mineral oil to minimize dehydration and friction. Each sample was compressed to 20% deformation by an Instron Universal Testing Machine (Model 1102, Instron Inc., Canton, MA) fitted with a 2-kg load cell. The compression of 20% had been reported as the largest deformation that demonstrated no internal breakdown or barreling effects (Culioli and Sherman, 1976; Goh and Sherman, 1987). The deformation crosshead speed was 50 mm/min (Nolan, 1987). This relatively high speed was used to ensure minimal relaxation during compression (Masi, 1989). At that speed, the deformation of 20% was achieved in 2.4. sec. At the preset deformation, the crosshead was stopped, and the force was recorded for 8 min (Peleg, 1979; Nolan, 1987). A chart speed of 10 mm/min was maintained throughout the test cycle.

Data analysis for stress relaxation profiles

Force-time data were read directly from the strip chart at 0.2-sec intervals for the first 3.0 min and at 0.4-sec intervals for the remaining time. The relaxation data were normalized with respect to the initial compressive force F_o by calculating the dimensionless decaying parameter Y(t) (Eq. 1) which represents the decay of force, stress, or modulus (Peleg and Calzada, 1976; Peleg, 1979; Nolan, 1987):

$$Y(t) = F_0 - \frac{F(t)}{F_0} \quad (1)$$

where F(t) = forced recorded at time t, and F_o = initial force to compress to 20% strain. In order to examine rates of relaxation (n) and fractional loss of the initial compressive force at t = 1 unit (c), the normalized stress-time data for LMPS Mozzarella cheese were fitted to a power law model; (Nolan, 1987):

$$Y(t) = ct^n \quad (2)$$

The values of c, n, and F_o were used to compare Mozzarella cheese samples subjected to different storage treatments. On each stress relaxation parameter, an analysis of variance and an F-test ($\alpha = 0.05$) were performed to determine whether differences were significant.

Dynamic mechanical spectrometry at 20°C

LMPS Mozzarella cheese samples were cut into 4-mm-thick slices with a U.S. Berkel deli meat slicer. Disks (4 × 25 mm) were cut with a cork borer (Nolan et al., 1989) and analyzed on a Rheometrics Dynamic Spectrometer (Model RDS - 7700 Rheometrics Inc., Piscataway, NJ) fitted with a 1-kg load cell in a parallel plate configuration. Dehydration was minimized by coating the exposed edge of each sample with mineral oil and taking measurements at 20°C in a controlled environment chamber (Rheometrics Inc.). The temperature was maintained by attaching the jacketed chamber to a refrigerated circulating bath (Lauda model RMS-6, Brinkmann). The water activity (a_w) of Mozzarella cheese was assumed to be near that of Gouda or Cheddar cheese (a_w value 0.950, Labuza, 1984). Therefore, the inside of the chamber was humidified to 94.62 ± 0.66% RH by bubbling air through a saturated solution of potassium nitrate at 20°C (Rüegg and Blanc, 1981). Cyanoacrylate resin was used to attach the cheese disks to 25-mm plates. An initial normal force of 100g was supplied and allowed to relax to zero (Nolan et al., 1989). The maximum shear strain used in each test was 0.5%. Since the smallest deformation possible on the instrument was 0.5%, this value was considered to be within the range of linear behavior (Nolan et al. 1989). Sinusoidal cyclical oscillatory frequency sweeps were run from 15.9 mHz to 15.9 Hz with 10 steps/log cycle. Each sweep required about 12 min. The measurements obtained through the data processor unit (Rheophaser, Rheometrics Inc.) were shear storage modulus G′ (Pa), shear loss modulus G″ (Pa), and complex dynamic viscosity η^* (Pa-s).

Dynamic mechanical spectrometry at 60°C

LMPS Mozzarella cheese samples refrigerated 14 and 90 days and frozen 90 days were analyzed on a Bohlin Rheometer (Bohlin Instruments Inc., Cranbury, NJ) fitted with a 90.654 g cm torque element. Mozzarella cheese disks (2 × 25 mm) were placed in contact with 25-mm parallel-serrated plates and brought to 60°C. The plates were set at 1.5 mm gap width, disks were trimmed, the edge coated with mineral oil, and the sample allowed to equilibrate 2 min. The ability to monitor the normal force and to establish a controlled environment around the sample was not available with this equipment. Frequency sweeps were run from 0.1 to 20 Hz at a maximum strain of 2.5% with 5 steps per log cycle. Each sweep lasted approximately 2 min. Shear storage modulus G′ (Pa), shear loss modulus G″ (Pa), and complex dynamic viscosity η^* (Pa-s) were measured.

Data analysis for dynamic mechanical spectrometry

Power law fits relating each of G′, G″, and η^* to frequency (ω) were determined as in Equations 3, 4 and 5 (Nolan et al., 1989):

$$G' = a\omega^x \quad (3)$$

$$G'' = b\omega^y \quad (4)$$

$$\eta^* = c\omega^z \quad (5)$$

The values a, b and c and x, y and z were used to compare LMPS Mozzarella cheese storage treatments. Analysis of variance and an F-test ($\alpha = 0.05$) were performed on each dynamic mechanical spectrometry parameter to determine whether differences were significant.

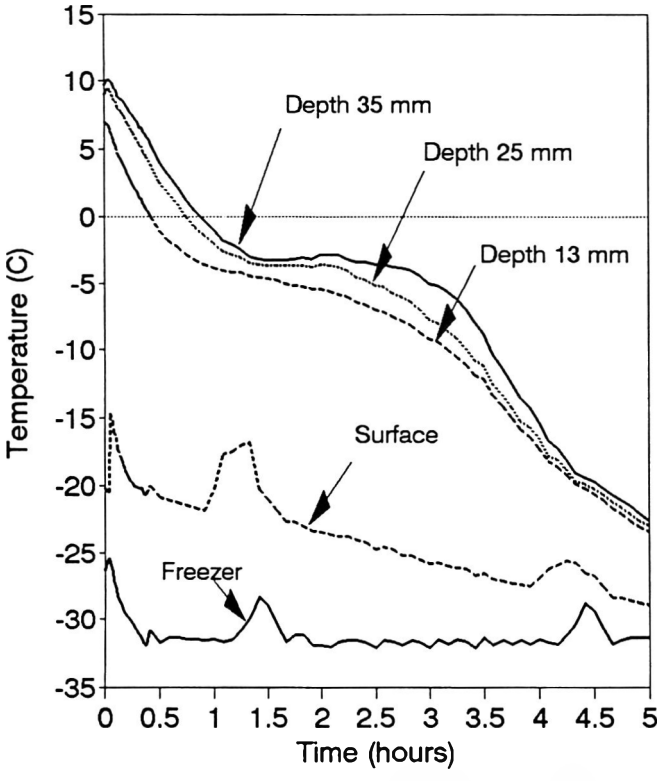

Fig. 1—Temperature profile for freezing LMPS Mozzarella cylinder (length 150 mm and diameter 70 mm) in a −30°C blast freezer.

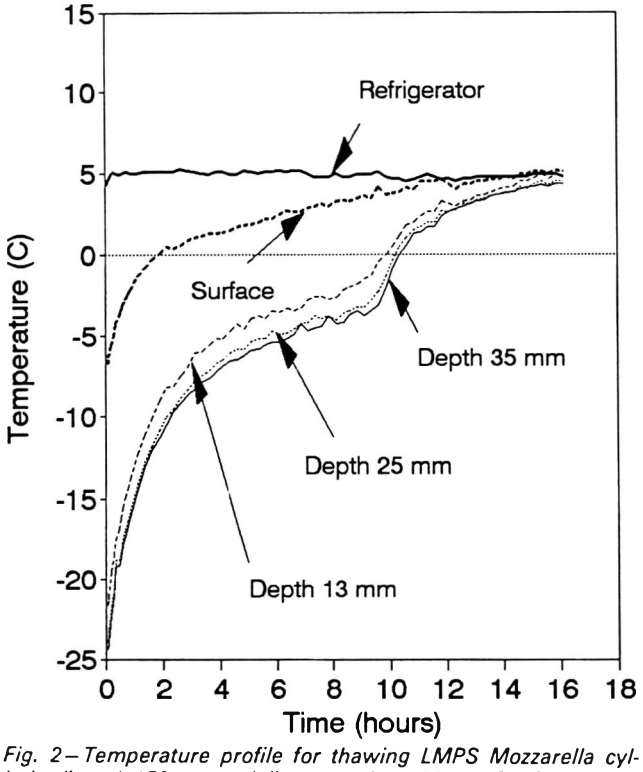

Fig. 2—Temperature profile for thawing LMPS Mozzarella cylinder (length 150 mm and diameter 70 mm) in a 5°C refrigerator.

Free oil and salt tests

A free oil test was performed as described by Kindstedt and Rippe (1990). Five samples were taken from each cylinder. Free oil (FO) as percent weight of cheese was used to compare storage treatments. Salt content was determined by the Volhard procedure (Richardson, 1985). Salt testing was done in duplicate on four concentric sections (0–9, 9–17, 17–26, and 26–35 mm) to determine whether a salt gradient formed during manufacture.

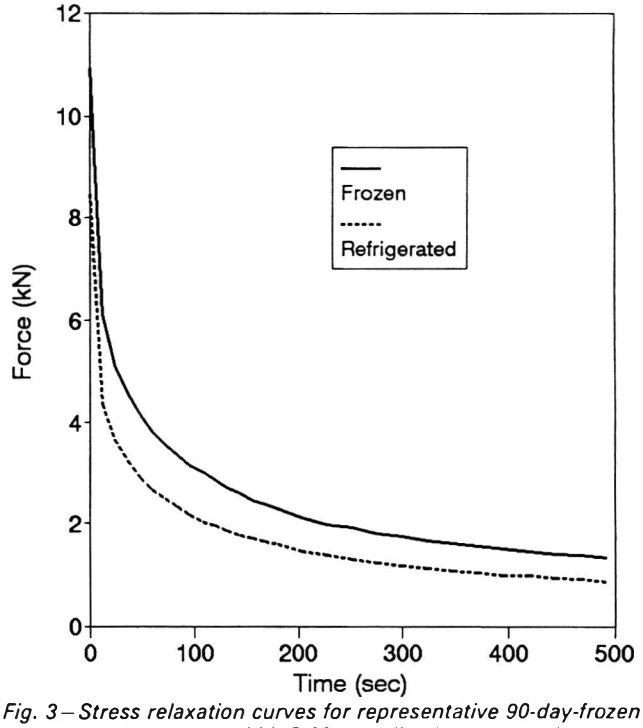

Fig. 3—Stress relaxation curves for representative 90-day-frozen and 90-day-refrigerated LMPS Mozzarella cheese samples.

RESULTS & DISCUSSION

Chemical composition

The single batch of LMPS Mozzarella cheese had 44.6% moisture, 23.0% fat, 27.09% total N, and 1.33% salt. The moisture content was below the legal minimum of 45% for LMPS Mozzarella. There was no significant difference in composition among cylinders. Cheese characteristics vary considerably from batch to batch, thus conclusions based on this single cheese making experiment may not be generally representative.

Instead of conventional brining the salt was partially incorporated by hand after milling. The remainder was incorporated into the cheese through a hot salt water solution used in the stretching machine. There was no significant salt gradient in the cylinder and analyzed samples were homogeneous.

Temperature profiles

The time to freeze the cheese cylinders in the −30°C blast freezer was 4.5 hr (Fig. 1). The time to pass through the critical freeze-thaw zone (−1.1 to −6.7°C) was 2.3 hr. This short freezing time was expected to minimize the extent of damage associated with prolonged freezing by reducing ice crystal growth and enzymatic and chemical activity. The freezing point of the cheese, where the temperature profile remained constant or had an inflection point, was within the range of −3 to −5°C. The 5°C increase of the blast freezer and at the surface of the cheese after 1 and 4 hr was caused by the defrost cycling. The time to defrost the samples was 16 hr (Fig. 2) and the time for center temperature to change from −6.7 to −1.1 was 5.7 hr.

Stress relaxation

A pair of stress relaxation curves for samples stored at 5°C and −20°C for 90 days (Fig. 3) showed that as time (t) increased, the force relaxed asymptotically to a positive value as expected for a viscoelastic solid (Peleg, 1979). The initial

Table 1 – Stress relaxation parameters at 20°C for LMPS Mozzarella cheese

Storage treatment	F_o (kN)	c	n
Control (5°C)	9.452[a]	0.3422[a]	0.1681[a]
Frozen (−20°C)			
16 hours	9.863[a]	0.3308[b]	0.1702[a,b]
30 days	9.709[a]	0.3118[c]	0.1757[b,c]
60 days	11.054[b,c]	0.3145[c]	0.1778[c]
90 days	11.800[b]	0.3113[c]	0.1722[a,b]
Tempered 21 days	10.056[a,c]	0.3290[b]	0.1655[a,d]
Refrigerated (5°C)			
30 days	7.270[d]	0.3458[a]	0.1586[d,e]
90 days	8.886[a]	0.3545[a]	0.1558[a]

[a-e] Treatments with different superscripts in a column are significantly different at α = 0.05.

force (F_o) and the force F(t) required to compress and maintain samples at a strain of 20% was greater for frozen and thawed samples than for refrigerated samples. As compared to the maximum force to compress control samples, the maximum force to compress frozen and thawed samples showed a significant increase at 60 days storage (Table 1). Over the 90-day frozen storage period, the maximum force to compress the thawed samples increased 24.9%. Refrigerated, nonfrozen samples demonstrated a significant decrease in F_o during the first 30 days storage (Table 1). This was followed by an increase at 90 days. The F_o required to compress samples from cylinders frozen and thawed and refrigerated for 30 and 90 days differed. The 90-day frozen and thawed cheese samples required 32.7% more force than the 90-day-refrigerated sample to achieve a strain of 20%.

Normalized stress relaxation curves for samples frozen and thawed and refrigerated for 90 days were compared (Fig. 4). Another indication that Mozzarella cheese is a viscoelastic solid is that the normalized curves asymptotically approached a value < 1 (Peleg, 1979). The r^2 value for all curves fit to the power law model was 0.959 ± 0.012. At any given time (t), the refrigerated samples had lost a greater percentage of initial compressive force than the frozen samples.

In the power law model (Eq. 2), the c value can be interpreted as the magnitude of the initial force or the fraction of initial force lost at 1 sec. An increase in c is indicative of material softening since a greater loss of force occurs during the first second of constant strain. Conversely, a decrease in c indicates product hardening as there is less loss of force during the first second of applied constant strain. The value of c for frozen and thawed Mozzarella cylinders decreased during 16 hr freezing and continued to decrease up to 30 days frozen storage. The c value of refrigerated samples did not significantly increase over the 90-day storage period.

Nolan (1987) reported that relatively low values of n indicated the occurrence of internal irreversible changes in structure. The value of n for frozen and thawed LMPS Mozzarella cheese steadily increased up to 60 days and then declined. The drop in rate of stress relaxation in the third month of frozen storage may indicate a maximum time of frozen storage at these conditions before structural degradation was evident. Refrigerated samples demonstrated a decrease in n value over the 90-day storage. The value of n for refrigerated samples was significantly lower than that of the frozen sample. Refrigerated samples were evidently undergoing more and faster structural change than the frozen samples.

The LMPS Mozzarella cylinder that was frozen and tempered recovered some original rheological properties of the controls. F_o decreased by 15%, c increased by 5.8%, and n decreased by 4.1% compared to the 90-day frozen and thawed samples. All changes in stress relaxation measurements indicated that the tempering of frozen LMPS Mozzarella cheese allowed softening to occur. F_o and n were not significantly different from controls, although the lower c value indicated that the tempered sample was harder than the control.

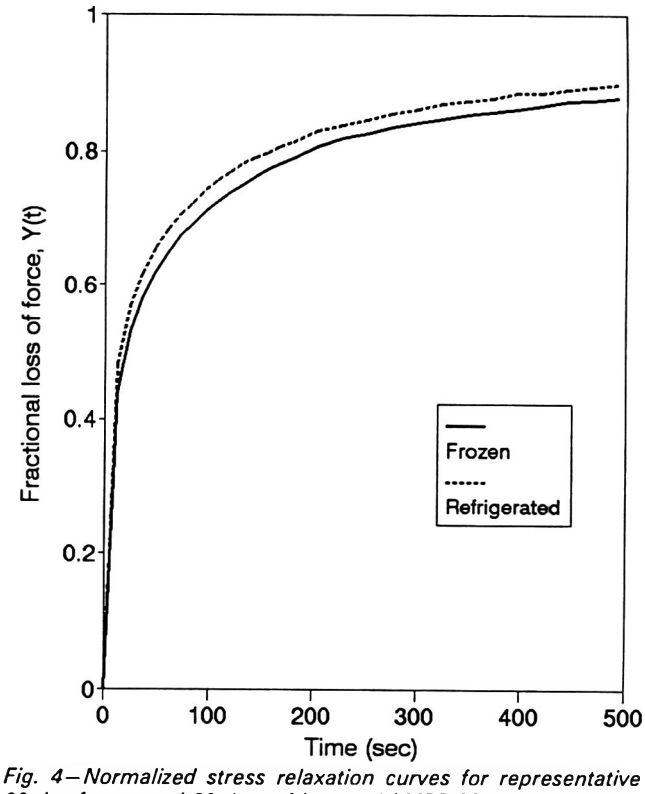

Fig. 4—Normalized stress relaxation curves for representative 90-day-frozen and 90-day-refrigerated LMPS Mozzarella cheese samples where $Y(t) = (F_o - F(t))/F_o$

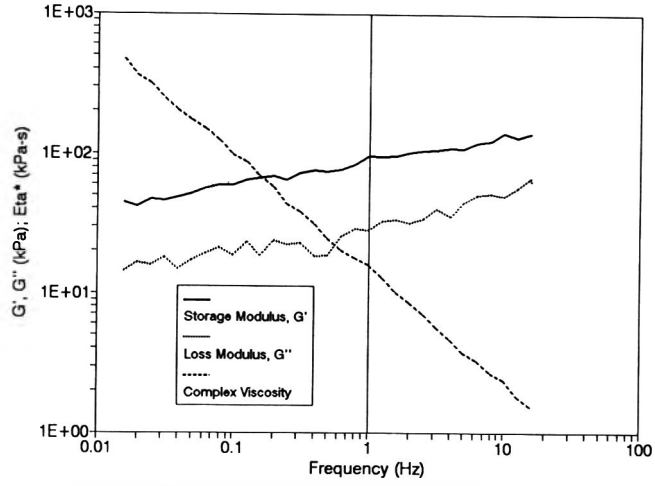

Fig. 5—Dynamic mechanical spectrometry frequency sweep profile for a representative control LMPS Mozzarella sample at 20°C with 0.5% strain. Power law values: a = 87.82 kPa, x = 0.1724, r^2 = 0.99; b = 30.88 kPa, y = 0.2028, r^2 = 0.91; c = 14.84 kPa-s, z = −0.8238, r^2 = 1.00.

Dynamic mechanical spectrometry at 20°C

A representative frequency sweep profile of control cheese (Fig. 5) showed that the storage modulus (G') was greater than loss modulus (G") at any given frequency which indicated that the elastic component contributes more to the complex dynamic viscosity than does the viscous component. This type behavior is expected for a viscoelastic solid. Power law fits for G', G", and η* for all samples had r^2 values of 0.985 ± 0.011, 0.925 ± 0.034, and 0.999 ± 0.003, respectively. The magnitudes of the a, b, x, and y values (Table 2) were similar to those reported by Nolan et al. (1989).

A comparison of control, 90-day-frozen and 90-day-refrigerated samples showed that the a value significantly increased

Table 2—Power law parameters for dynamic mechanical spectrometry frequency sweeps at 20°C for LMPS Mozzarella cheese

Power law parameters		Storage treatment			
				Frozen 90 days	
		Control	Refrig. 90 days	Tempered 1 day	Tempered 21 days
G'	a (kPa)	88.34[a]	83.40[a]	111.82[b]	111.36[b]
	x	0.1576	0.1718	0.1725	0.1709
G"	b (kPa)	30.91[a,b]	27.72[b]	37.25[a]	37.17[a]
	y	0.2100[a]	0.2249[b]	0.2171[a,b]	0.2138[a,b]
η*	c (kPa-s)	14.93[a]	14.03[a]	18.80[b]	18.72[b]
	z	−0.8273	−0.8224	−0.8226	−0.8244

[a-b] Values with different superscripts in a row are significantly different at $\alpha = 0.05$.

Table 3—Power law parameters for dynamic mechanical spectrometry frequency sweeps at 60°C for LMPS Mozzarella cheese

Power law parameters		Storage treatment		
		Control	Refrig. 90 days	Froz. 90 days Temp. 1 day
G'	a (kPa)	2.107[a]	0.697[c]	1.561[b]
	x	0.668[a]	0.777[b]	0.633[a]
G"	b (kPa)	2.690[a]	1.250[b]	2.334[a]
	y	0.399[a]	0.604[b]	0.437[a]
η*	c (kPa-s)	0.576[a]	0.230[b]	0.456[a]
	z	−0.486[a]	−0.347[b]	−0.492[a]

[a-c] Values with different superscripts in a row are significantly different at $\alpha = 0.05$.

during 90 days frozen storage, while the refrigerated sample showed no difference after 90 days storage (Table 2). Since the a value refers to elastic component (G') at a frequency (ω) of 1 Hz, the 90-day-frozen samples evidently behaved more elastically over small deformations than the 90-day-refrigerated samples.

Similar results were observed for b and c. The b value (viscous component at 1 Hz) for 90-day frozen and thawed samples was higher than that of 90-day-refrigerated samples. There was no difference between 90-day-frozen and control or 90-day-refrigerated and controls. The c value (complex dynamic viscosity at 1 Hz) increased for 90 days of frozen storage; the refrigerated sample did not show a difference between the controls and samples stored frozen for 90 days. The rise in c over the duration of frozen storage were attributed more to an increase in the elastic component than the viscous component; the Mozzarella cheese was behaving increasingly as a solid. This result confirmed observations from the stress relaxation study. That is, the frozen and thawed cheese samples had a greater viscous component and complex viscosity at a frequency of 1 Hz than the refrigerated samples. The values x, y, and z denote the rate of change of G', G", and η* with increasing in ω. There was no significant difference in x and z over storage. The y value for refrigerated 90-day samples was higher than that for control s. When comparing frozen and thawed and frozen-tempered Mozzarella tempering evidently did not significantly alter dynamic values. Overall, the dynamic mechanical spectrometry frequency sweep study at 20°C indicated that the LMPS Mozzarella cheese became increasingly hard and elastic-solid with longer freezing. Refrigeration storage caused it to become softer and more elasticoviscous.

Dynamic mechanical spectrometry at 60°C

The behavior of the cheese when melted ultimately determines consumer acceptability. Tests were conducted at 60°C to simulate commercial use of Mozzarella cheese in pizza preparation. As compared to dynamic power law model value at 20°C, all intercept values (a, b, and c) for 60°C melted samples were an order of magnitude less (Table 3). Power law fits for G', G", and η* for all samples had r^2 values of 0.989 ± 0.011, 0.963 ± 0.020, and 0.979 ± 0.007, respectively. The 90-day frozen and thawed samples showed a significant drop in a and c while b did not change. That is, the elastic component and the complex viscosity at 1 Hz decreased over 90 days. However, the decline in those values was not as great as those for 90-day-refrigerated samples; the 90-day-refrigerated samples were much less elastic and viscous.

Typical profiles of G' for various storage treatments were compared (Fig. 6). The elastic component (G') for 90-day frozen and thawed Mozzarella cheese was consistently lower than that of 14-day-refrigerated cheese over the frequency range. The G' values for the 90-day-refrigerated samples were significantly lower than both 14-day-refrigerated and 90-day frozen and thawed samples. The x, y, and z values were not significantly different for control and frozen LMPS Mozzarella

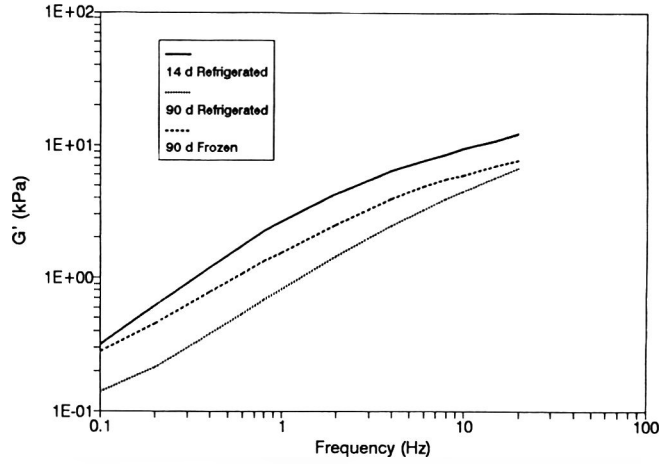

Fig. 6—Typical G' profiles for dynamic mechanical frequency sweeps with 2.5% strain for LMPS Mozzarella cheese at 60°C.

samples. For refrigerated samples, elastic and viscous components were more sensitive to changes in frequency as indicated by an increase in x and y.

Free oil test

The weighted average of free oil as percent weight of cheese in 14-day-refrigerated samples was 6.48 ± 0.47%. This value fell within the range reported by Kindstedt and Rippe (1990) for LMPS Mozzarella cheese. Compared to controls, the frozen and thawed samples experienced a 14.7% decrease in percent free oil (w/w) while refrigerated samples decreased 12.9%. There was no statistical difference ($\alpha = 0.05$) in free oil content of frozen and thawed and refrigerated Mozzarella samples at 30 or 90 days storage.

At the end of the free oil test performed on 90-day frozen and thawed and refrigerated Mozzarella samples, an outstanding difference in quality of the melted cheese mass was observed. The 90-day-refrigerated samples were very soft and translucent yellow. Upon stretching by hand, the samples flowed with little force and broke only with large deformations. The 90-day frozen and thawed samples were hard and rubbery and white. The melted mass required much force to stretch but broke with moderate deformation. Mozzarella cheese samples, having undergone the free oil test following pretreatment and early stages of frozen storage, were moderately hard and white. These samples could be stretched with moderate force, and behaved elastically up to relatively large deformations.

Rheological changes

Mozzarella cheese softens at refrigeration storage temperatures as a result of residual coagulant and milk plasmin breakdown of α_{s1}-casein and β-casein (Fox and Guinee, 1987; Farkye et al., 1991). Many breakdown products of casein are water-soluble and cannot contribute to the framework provided by

the protein matrix. Softening of the refrigerated cheese was evident in both stress relaxation and dynamic mechanical spectrometry analysis. The increased force required to compress refrigerated samples after 30 days storage at 5°C may have been due to moisture loss through imperfect heat seals or an increasing effect of cold-induced fat granule formation (Walstra, 1984). Since stress relaxation tests were performed at 20°C, fat globules would not be completely melted, and the formation of fat granules could contribute to harder texture.

The effect of freezing on Mozzarella cheese is not clear. One theory concerning the effect of freezing on proteins in food and biological systems is that proteins undergo local dehydration. This causes the proteins to either become more compact as a result of exposure to supersaturated solutes, or interact irreversibly by forming disulfide bonds (Fennema et al., 1973). The proteins thus become more elastic over small deformations. The increase in hardness of frozen LMPS Mozzarella cheese samples could also be attributed to the increasing effect of fat crystallization and granule formation (Walstra, 1984). Upon melting, granules formed during freezing coalesce into larger fat globules. The size of such globules would be a limiting factor in their ability to separate from the protein mesh and contribute to free oil. This may explain the significantly greater decrease in free oil of frozen samples as compared to refrigerated samples.

A combination of the theories seems plausible. As Mozzarella cheese undergoes freezing, local dehydration of proteins causes breaks in protein structures that allow small fat globules to contact each other and form granules. The proteins become more compact or interact to form disulfide bridges around new fat granules. Upon thawing, the proteins are unable to fully rebind water. This leads to a harder and more elastic-solid cheese structure with less free oil.

The softening of frozen-tempered Mozzarella cheese may be due to increased coagulant and plasmin activity, partial rehydration of the protein matrix, and partial melting of fat crystals and granules at refrigeration temperatures. We may assume that hardness of frozen and thawed samples is due primarily to granule formation. It is doubtful that tempering Mozzarella cheese for a longer period of time would cause full recovery since a higher energy would be required to break fat granules than can be achieved at 5°C. Continued storage at refrigeration temperatures would cause Mozzarella cheese to undergo protein degradation and off-flavors development.

CONCLUSIONS

LMPS MOZZARELLA cheese changed in rheological behavior over 90-days frozen storage. While Mozzarella cheese stored at refrigeration temperatures became softer and more elasticoviscous, that stored frozen became harder and more elastic solid. Upon melting, both frozen and thawed and refrigerated samples underwent a decrease in elasticity and viscosity. However, refrigerated samples were less elastic and viscous than frozen and thawed samples of equal age. Upon tempering, the rheological profiles of frozen and thawed LMPS Mozzarella cheese approached that of control Mozzarella cheese. Stress relaxation tests proved valuable for detecting changes in unmelted Mozzarella cheese rheology. Dynamic mechanical spectrometry was more suited to assessing changes in melted cheese samples. The power law models adequately described both stress relaxation and dynamic mechanical properties. Since rheological changes incurred by frozen and thawed Mozzarella cheese were opposite those of refrigerated cheese, we could reasonably conclude that the governing mode of deterioration was different for freezing and refrigeration. For refrigeration of cheese, proteolysis by plasmin and residual coagulant causes progressive cheese softening. Several theories explain the change in rheology of frozen and thawed cheese. During freezing and frozen storage, local dehydration causes structural changes in proteins. Upon thawing, these proteins are unable to rebind water and, thus, elasticity is lost. Also, the formation of fat granules at freezing temperatures causes hardening.

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Flesh Quality in Snapper, *Pagrus auratus*, Affected by Capture Stress

T.E. LOWE, J.M. RYDER, J.F. CARRAGHER and R.M.G. WELLS

ABSTRACT

Muscle metabolites in resting, tank acclimated snapper, *Pagrus auratus*, were monitored for 72 hr postmortem and compared with values from exercised or commercially caught fish. The physiological status of the live animal was quantified by plasma cortisol and blood chemistry. Cortisol levels were lowest in unstressed controls (6.8 ± 2.1 ng mL^{-1}) while exercised laboratory fish had highest levels (67.7 ± 11.2 ng mL^{-1}). Control fish maintained a constant K-value for 72 hr in chilled storage; all other groups had significant increases. Onset of rigor development and muscle ATP depletion was delayed in unstressed fish and was more rapid in line-captured than exercised fish. Commercial users minimizing stress would maintain high flesh quality.

Key Words: snapper, stress, nucleotides, lactate, flesh quality

INTRODUCTION

THE PHYSIOLOGICAL EFFECTS of fish capture and live handling influence postmortem changes, yet their effects on flesh quality have not received the attention given to postharvest handling procedures (Wells, 1987). The progression of rigor mortis is used as an index of flesh quality and is determined primarily by nucleotide triphosphate metabolism (Saito et al., 1959; Harada, 1988). Onset of rigor may be delayed if, at the point of live capture, the brain is spiked (also known as "iki jime"), which maintains high muscle ATP levels and hence flesh quality (Boyd et al., 1984). Further improvements have been found when fish were chilled postmortem until onset of rigor, whereupon storage on ice optimised flesh quality (Iwamoto et al., 1985; Iwamoto and Yamanaka, 1986).

The snapper *Pagrus auratus*, is an Eastern Pacific sea bream of the Sparidae family (Paulin, 1990), of high commercial value. The requirement for high flesh quality in raw fish products for commercial markets has stimulated research into the effects of stress physiology on postmortem changes in muscle tissues in the snapper. In particular, freshness of fish flesh may be monitored analytically by determining products of nucleotide degradation using the "K-value" Saito et al. (1959).

Our objective was to follow postmortem nucleotide degradation and lactate accumulation together with rigor index in laboratory acclimated "stress free" snapper, and compare data with those from fish captured by two commercial longlining procedures. The physiological status of experimentally acclimated and wild-caught fish was evaluated using blood samples assayed for plasma cortisol, lactate, and hemoglobin-oxygen transport potential as sensitive primary and secondary indicators of stress (Pickering, 1981; Weber and Wells, 1989; Pankhurst et al., 1992). We tested relationships of capture and handling methods to flesh quality.

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MATERIALS & METHODS

Fish capture and handling

Live snapper, *P. auratus*, (0.6-1.0 kg) were captured from the Hauraki Gulf, New Zealand (36° 17'S, 174° 49'E), using longlines with 50 baited hooks. Live fish were placed in onboard aquaria supplied with running seawater at 17°C. To minimize possible effects of barotrauma, the maximum depth fished was 20m and swimbladders were vented with a 16-gauge needle to relieve excess pressure. Fish were transported ≈ 10 km to the Leigh Marine Laboratory, and were transferred to 565 L indoor flow-through system seawater tanks at a stocking rate of 9–10 fish per tank. They had a natural light photoperiod and ambient water temperature (17°C). Fish were left undisturbed for 5 days to recover from capture and transport trauma, and were not fed during that time.

Resting fish (N = 8) were removed quickly from holding tanks and killed by "iki jime" within 30 sec. A second group of captive fish were vigorously exercised (N = 9) using blunt poles for 1 hr before re-capture and "iki jime." Other live fish were obtained from longline sets of 2 hr (N = 11) and 12 hr (N = 7) duration (overnight), in accordance with commercial practice and brain ablated by "iki jime."

Tissue sampling and rigor index

A 1-mL heparinized blood sample was taken by caudal venepuncture at time 0 hr postmortem. Following this, the dorsal epaxial musculature was sampled from one side of the fish only, starting midway and progressing anteriorly just above the lateral line at time intervals of 0, 4, 8, 16, 28, 40, and 72 hr. Initial tissue samples of ≈ 0.25g, were taken using a 7 mm internal diameter stainless steel coring tool pre-cooled in liquid nitrogen. The resulting frozen pellet of tissue was placed immediately in liquid nitrogen. Subsequent tissue samples were dissected from the epaxial musculature at specified intervals.

Rigor Index was measured by the horizontal displacement method described by Iwamoto et al. (1987), and measurements averaged from left and right aspects.

Fish were stored at 3°C in an ice-seawater slurry for 2 hr postmortem, then removed and placed on a bed of ice in a fish bin (370 × 600 × 230 mm), with the ventral side of the fish resting on the ice, following commercial fishery practice. The fish were stored at 2.5 ± 0.5°C. The rectal temperature at equilibrium was 1.0 ± 0.5°C and this remained constant for the duration of the experiments. The fish were serially sampled so that the progress of the muscle metabolites and rigor index were monitored for each fish over time. A pilot experiment showed no effect of tissue sampling on rigor index.

Blood analysis

Cortisol was measured by radioimmunoassay (RIA) using the method of Pankhurst et al. (1992). Steroids were extracted with ethyl acetate and assays were performed using 1,2, 6, 7^{3}H Cortisol (Amersham International), antibody (Bioanalysis Ltd., Cardiff, U.K.) and phosphate gelatine buffer. Four assays corresponding to each treatment were performed where the coefficient of variation was 17%. Assay detection limit was 0.5 ng mL^{-1} plasma.

Hematocrit and hemoglobin were determined using standard hematological procedures (Dacie and Lewis 1984) and plasma lactate using a Sigma enzymatic test kit (Sigma 826-UV, St Louis, MO). Erythrocyte nucleoside triphosphate (NTP) was determined using a Sigma enzymatic kit for ATP which also measures other nucleoside triphosphates (Sigma 366-UV).

Muscle nucleotides and lactate analysis

Weighed frozen muscle (≈0.25g) was homogenized in 1.5 mL chilled 0.6 M perchloric acid (PCA) at 0°C for 30 sec using an Ultra Turrax

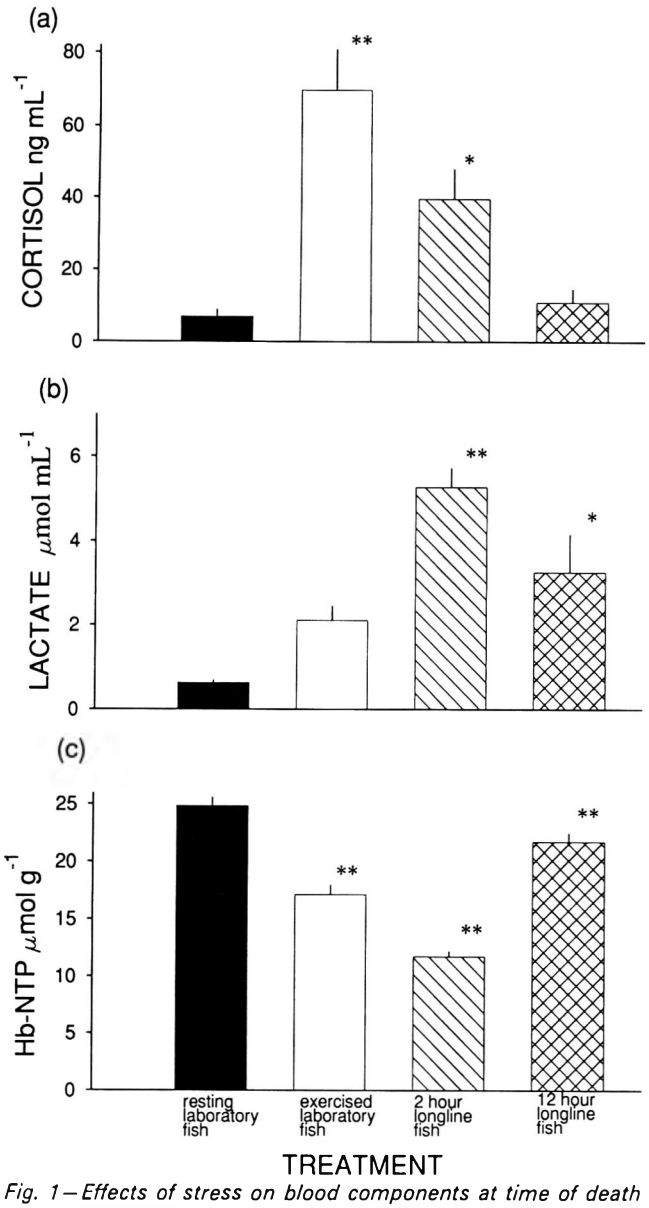

Fig. 1—Effects of stress on blood components at time of death (mean ± S.E.): (a) plasma cortisol, (b) plasma lactate, and (c) hemoglobin nucleotide triphosphate (Hb-NTP). •Different from resting laboratory fish ($p<0.05$). ••Different from all other groups of fish ($p<0.05$).

T25 homogenizer, with an 8 mm diameter shaft (Janke and Kunkel GmbH., Staton, Germany). After standing 15-45 min in ice the homogenate was centrifuged at 1000 × *g* for 5 min to remove precipitated protein. The supernatant was neutralized to pH 6.8-7.0 with 5 M K_2CO_3 and stored frozen at −20°C until analysis.

Using a Waters High Performance Liquid Chromatography (HPLC) system, ATP and its catabolites were analysed as described by Ryder (1985). A Merck Hibar pre-packed column (LiChrosorb RP C-18, 250 × 4 mm) was used with an isocratic mobile phase of 0.04 M KH_2PO_4 and 0.06 M K_2HPO_4 with a flow rate of 1 mL min^{-1}. Peaks were monitored by u.v. optical detection of 20 µL injections of filtered extract at 254 nm. K-values were calculated as described by Saito et al. (1959). Muscle lactate was determined using the Sigma enzymatic test kit.

Statistical analysis

Data were analyzed using analysis of variance (ANOVA) to establish treatment effects, and where differences were significantly different ($p<0.05$), means were compared using Tukey's test. Correlation coefficients for relationships between rigor index, muscle lactate and muscle ATP were performed using pooled within-class correlation coefficients.

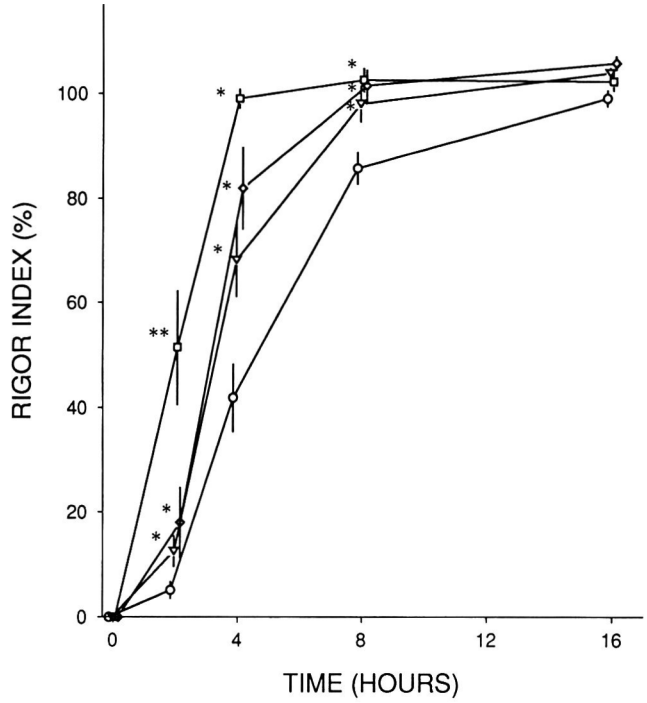

Fig. 2—Development of rigor mortis (mean ± S.E.). Resting fish (○), exercised fish (▽), 2 hr longline fish (□) and 12 hr longline fish (◇). •Different from resting fish ($p<0.05$). ••Different from all other groups of fish ($p<0.05$).

RESULTS

Blood parameters

Plasma cortisol levels, at time of death, were lowest in resting laboratory acclimated fish (6.8 ± 2.1 ng mL^{-1}) and were similar to levels found in 12 hr longline fish (10.8 ± 3.9 ng mL^{-1}). Exercised laboratory fish had high levels of cortisol (69.7 ± 11.2 ng mL^{-1}) as did fish from the 2 hr longline set (39.8 ± 8.4 ng mL^{-1}) (Fig. 1a). Plasma lactate levels were lowest, and erythrocyte NTP highest in resting laboratory fish (Fig. 1b,c). Those from the 2 hr longline set had the highest plasma lactate levels ($p<0.05$) (Fig. 1b) and lowest levels of erythrocyte NTP ($P<0.05$) (Fig. 1c).

Rigor index

Resting laboratory fish remained the longest time before rigor mortis, and full rigor was not reached until 16 hr postmortem (Fig. 2). Rigor developed rapidly in 2 hr longline fish, and this was different from 2 hr postmortem. Rigor development was intermediate in fish removed from the longline after 12 hr and in laboratory exercised fish.

Muscle ATP and lactate

Muscle ATP was maintained 8 hr postmortem in rested laboratory fish (Fig. 3a), and 4 hr in exercised laboratory fish (Fig. 3b). In longlined fish, muscle ATP levels decreased immediately after death, dropping more rapidly in 2 hr longlined fish than the 12 hr fish (Figs. 3c,d). By 16 hr postmortem, fish from all treatments had low levels of ATP. In all cases, development of rigor mortis correlated significantly with decreasing levels of ATP ($p<0.05$).

Rising muscle lactate concentrations negatively correlated with decreasing ATP levels for all groups except the 2 hr longline fish ($p<0.05$). Two fish (from the seven sampled) from the 12 hr longline set had unexpected low maximal muscle lactate concentrations (26.6 and 20.4 µmol g^{-1}). Compa-

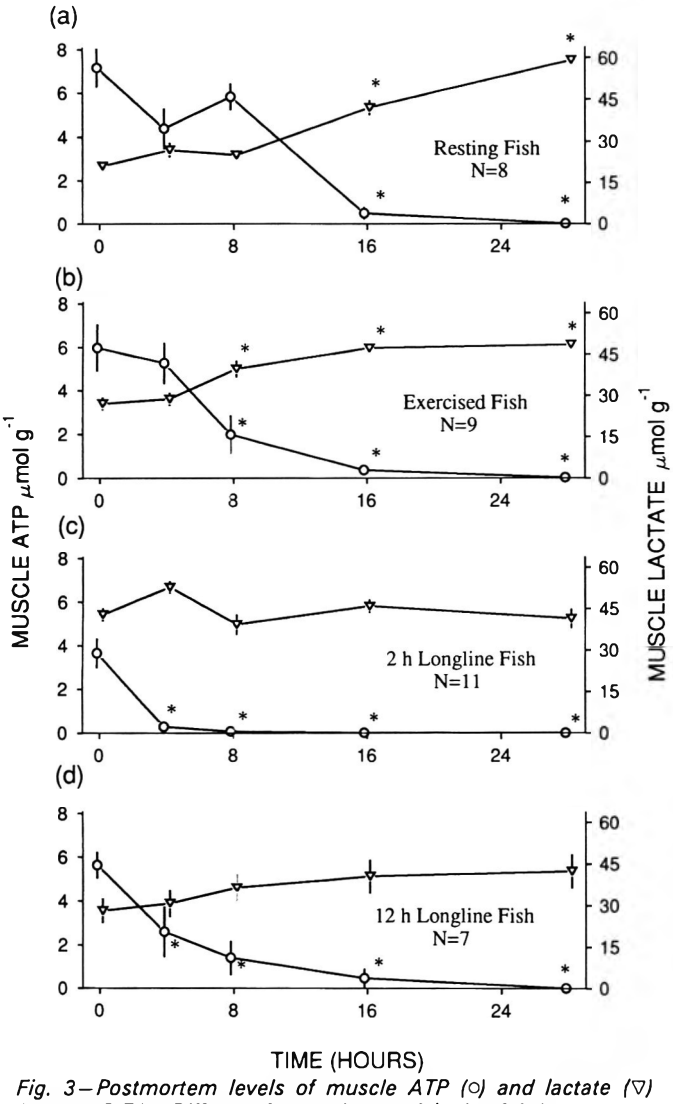

*Fig. 3—Postmortem levels of muscle ATP (○) and lactate (▽) (mean ± S.E.). *Different from values at 0 hr ($p < 0.05$).*

rable levels for all other fish sampled, regardless of treatment, ranged from 39.6–70.1 μmol g^{-1}.

K-values

K-values were generally low for all treatments after 72 hr (Fig. 4). The two exceptional fish with low postmortem lactate were also plotted as these fish showed higher K-values than the other fish. Plotted separately, K-values for the other fish in the 12 hr group were very similar to the 2 hr longline fish (Fig. 4). ANOVA indicated a significant treatment and time effect for K-values ($p < 0.05$). The resting laboratory fish were the only group to show no significant increase in K-value from 16–72 hr postmortem (Fig. 4).

DISCUSSION

PLASMA CORTISOL is widely recognized as a sensitive indicator of stress in fish (Mazeaud et al., 1977; Pickering, 1981; Pankhurst et al., 1992) and is thought to exert endocrine control over mobilization and distribution of metabolic energy sources (Mazeaud et al., 1977; Vijayan and Leatherland, 1989). The potential for cortisol to inhibit tissue glucose oxidation and promote catabolic effects on glycogen reserves was reported (Van der Boon, 1991). Severity, or type of stress are known to influence the rate of plasma cortisol elevation. Capture of snapper by rod and line indicated a significant increase

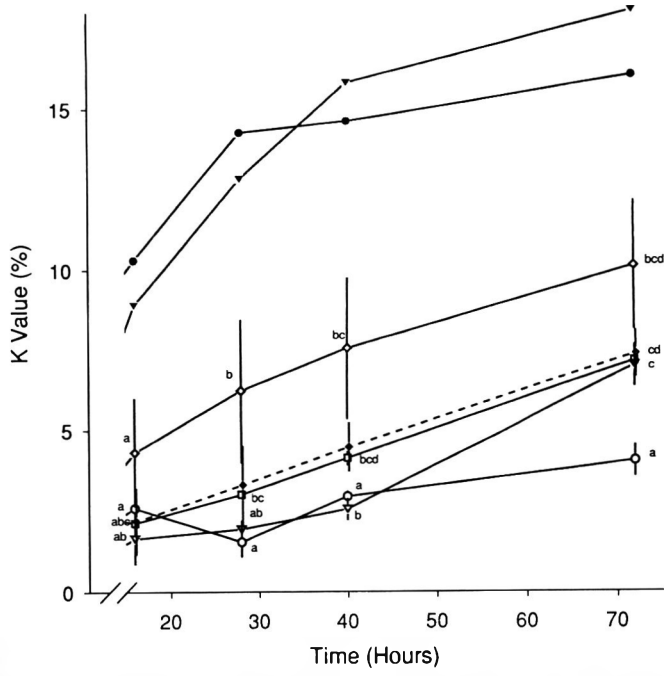

*Fig. 4—K-values of muscle tissue postmortem (mean ± S.E.). Resting fish (○), Exercised fish (▽), 2 hr longline fish (□) and 12 hr longline fish (◇). The K-values of two outlying fish from the 12 hr longline set are represented by solid symbols. A separate plot of the 12 hr longline fish minus the two outlying fish is grepresented by a dashed line. *Different from values at 16 hr ($p > 0.05$).*

in plasma cortisol occurred after about 30 min confinement stress (Pankhurst and Sharples, 1992).

In our study, plasma cortisol levels from resting snapper in holding tanks were very low and close to values considered typical of wild, unstressed snapper (<10 ng mL^{-1}; Pankhurst and Sharples, 1992). Their low plasma lactate and high erythrocyte NTP concentrations clearly indicated the predominance of oxidative metabolism over minimal glycolytic activity. This combined with the low cortisol levels suggested that these fish were unstressed.

Conversely, severe exercise evoked a stress response through elevated cortisol (67.7 ± 11.2 ng mL^{-1}) and reduced erythrocyte NTP (17.1 ± 0.9 μM g^{-1}) although plasma lactate was not significantly elevated (Fig. 1b). Reduction in erythrocyte NTP improves the efficiency of hemoglobin-oxygen binding in the gills, especially during lactate acidosis, and is thus an important adaptive response to stress in fish (Weber and Wells, 1989; Morris, 1990). The appearance of lactic acid in the blood following exercise or stress reflects the abandonment of a steady aerobic state and has been linked to fish death during capture of severe exercise (Wood et al., 1983; Wieser et al., 1986). Plasma lactate levels do not directly reflect muscle lactate concentrations. In contrast to mammals, fish appear to retain most of the lactate produced in the muscle tissue (Wardle, 1978; Wood et al., 1983). Following exercise, blood lactate levels required 2–4 hr to peak in plaice, *Pleuronectes platessa* L. (Batty and Wardle, 1979). Thus the exercised laboratory fish demonstrated a significant stress response in terms of plasma cortisol and erythrocyte NTP levels. Although there was no statistically significant rise in plasma lactate, likely plasma lactate had not reached peak levels.

The blood chemistry results for the 2 hr longline fish were somewhat unexpected. Blood lactate and erythrocyte NTP levels suggest that the fish from the 2 hr longline set were hypoxic. They indicate that the stress of capture and holding on the longline leads to more severe hypoxia than does forced exercise in the tanks. However, the plasma cortisol levels of

the 2 hr longline fish were lower than those in fish exercised in holding tanks (Fig. 1). This could be explained by quantitative or qualitative stress responses. The exercised fish in the laboratory were being stimulated for 1 hr, and although this did not give rise to severe hypoxia, it obviously produced a chronic endocrine stress situation. On the other hand, the stress response to capture for 2 hr longline fish resulted in less elevated cortisol, but led to severe hypoxia. This was probably due to a more rapid and urgent need for ATP as a result of escape effort mediated by adrenaline release.

The hypothesis that activation of the endocrine stress response is not a necessary consequence of sustained high muscular activity gains support from the data of the 12 hr longline fish. In that case, the fish had cortisol levels close to those of resting fish, though plasma lactate levels were raised and erythrocyte NTP levels decreased. Fish from the overnight longline set appeared to have been captured early in the set as judged by the extent of tissue damage at the site of hook penetration. Possibly recovery occurred on the longline in the 12 hr fish. This is consistent with lower plasma lactate and higher erythrocyte NTP levels than the 2 hr longline fish.

What then, were the effects of ante-mortem handling on postmortem quality? The unstressed tank fish remained in the lag phase before the onset of rigor mortis for the longest period, followed by the tank-exercised, 12 hr longline fish, and the 2 hr longline fish. The same pattern was seen for the time to reach maximal rigor (Fig. 2) with a 12 hr difference between the 2 hr longline fish and the resting laboratory fish. As expected, our data correlated development of rigor mortis with muscle ATP depletion for all treatments, and confirmed results from other species (Iwamoto et al., 1985; 1987; Iwamoto and Yamanaka, 1986; Watabe et al., 1991).

Similar patterns of muscle ATP depletion and lactate accumulation were shown by both groups of fish in holding tanks, though rates of reaction were more rapid in exercised fish (Figs. 3a,b). During anaerobic metabolism in fish white muscle, glycogen, as the main source for generation of ATP through glycolysis, is depleted as lactate accumulates (Driedzic and Hochachka, 1978; Schwalme and Mackay, 1991). In our study, production of lactate was initially low in both groups but increased rapidly once ATP levels began to fall. Presumably this was due to increased glycolysis, with lactate levels reaching a plateau as substrate was depleted (Iwamoto et al., 1988; Watabe et al., 1991).

However, the patterns of change in muscle ATP and lactate were different for the 2 hr longline fish. Initial muscle ATP levels in 2 hr longline fish were $\approx$ 60% of those in tank fish, and declined rapidly, with correspondingly rapid onset of rigor mortis. Initial lactate levels were high and did not change. Lactate takes a long time to clear from fish muscle tissue (Wardle, 1978), and thus had already accumulated during the time the fish were hooked.

In 12 hr longline fish, initial muscle ATP levels were similar to those of both groups of tank fish, and decreased in a similar pattern to the exercised tank fish. This reflected the similar curves obtained for rigor mortis for these two groups of fish. These results were consistent with recovery while on the longline, such that adequate glycogen reserves remained at time of death.

Fasting has minimal effects on muscle glycogen in fish. Stress situations that require rapid energy sources are the most common cause for glycogen utilization (Baanante et al., 1991). The resting tank fish were fasted for 5 days prior to experimentation, yet showed the largest increase in muscle lactate postmortem. Thus, it is unlikely that fasting for 12 hr on a longline was the major cause for glycogen depletion in those fish.

The low K-values (<20%) indicated that all fish remained of high quality after 72 hr storage. Nevertheless, different trends were evident from the different treatments. The resting fish maintaining lowest K-values, showed no increase during the first 72 hr. The other treatments showed varying increases, albeit slow, during the 72 hr. These data suggest that resting fish would maintain high quality for longer periods.

Two fish from the 12 hr longline set with low maximal muscle lactate concentrations had considerably higher K-values than those from other fish (Fig. 4), leading to more rapid decline in flesh quality. Changes of lactic acid concentration reflected changes of pH postmortem in kahawai (*Arripis trutta*) (Boyd et al., 1984), high concentrations of lactate accompanying low pH. Furthermore, dephosphorylation of IMP would be increased at higher pH (Dingle and Hines, 1971). Thus, the exceptionally low lactate concentrations reached postmortem may result in faster breakdown of IMP and hence increase K-values.

The stress imposed by fish capture and handling protocols impacted postmortem flesh quality when evaluated by rigor measurement and muscle biochemistry. Plasma lactate and blood NTP concentrations measured immediately following iki jime were useful indices of metabolic stress. Cortisol was also a useful stress indicator. We did not correlate the dynamics of this hormone with postmortem flesh quality. Resting tank fish clearly showed that minimizing stress enhanced flesh quality. Thus, while killing fish by spiking the brain enhances keeping qualities further improvement is possible if fish are resting when killed. Commercial fishing practices minimizing physiological stress responses that utilize endogenous energy reserves in muscle would likely provide better flesh quality. Recovery from stress in fish holding tanks is one option.

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Glucose Oxidase/Catalase Improves Preservation of Shrimp (*Heterocarpus reedi*)

MARTA DONDERO, WLADIMIR EGAÑA, WASHINGTON TARKY, ANTONIO CIFUENTES, and J. ANTONIO TORRES

ABSTRACT

The preservative effect of glucose oxidase (GOX) and catalase (CAT) in a 4% glucose aqueous solution were evaluated on shrimp (*Heterocarpus reedi*) stored at 0–2°C. On-board and immediately after catch, shrimp were washed with tap water at 10°C, and dipped or kept in the enzyme solution. Microbial (total psychrotrophs and *Pseudomonas* spp. counts), chemical (total volatile bases, ammonia and pH) and sensory tests (whole-raw and cooked-peeled samples) showed that holding shrimp in the GOX/CAT/glucose solution retarded microbial spoilage and preserved quality more effectively than dipping shrimp in the same solution. The importance of treating shrimp as early as possible was demonstrated by comparing shrimp treated 4 and 82 hr after catch.

Key words: shrimp, glucose oxidase, catalase, enzymatic preservation

INTRODUCTION

FISHERY PRODUCTS, particularly shrimp are inherently perishable, and their quality depends upon many factors, including storage time and temperature. Deterioration of shrimp quality is caused by microorganisms (Cobb et al., 1976), endogenous enzyme activity including melanogenesis or shrimp blackening (Bailey and Fieger, 1954; Bailey et al., 1960), and physical handling. Loss of quality during early storage is mainly caused by autolysis, whereas long-term deterioration is the result of bacterial action (Fatima et al., 1988). The usual method of preserving shrimp on the trawler is to store the catch in crushed ice for 1 to 4 days (Fatima et al., 1988; Toledo, 1982). Inadequate handling and ice preservation on board decrease quality of the final product.

Different methods have been investigated to increase shrimp shelf life and include chilling and dipping (10 min) in a solution containing 10% condensed phosphate, 5% sodium sorbate and 0.9% citric acid (Toledo, 1982); controlled atmosphere storage (Matches and Layrisse, 1985); and superchilling (−3°C) (Fatima et al., 1988). Field et al. (1986) reported that the use of the enzymes glucose oxidase (GOX) and catalase (CAT) in a glucose solution extended the shelf life of refrigerated fish by 67%. This system generates gluconic acid and lowers surface pH, which they suggested may cause the preservative effect. GOX/CAT treatments were also evaluated by Shaw et al. (1986) using cod fillets and Dondero et al. (1989) using hake and jack mackerel fillets, but few, if any, benefits were apparent in chemical, microbial or sensory assessment of the fillets. Frels et al. (1984) evaluated the use of GOX/CAT on poultry meat and found no significant inhibition of Salmonella spp. growth but a significant effect on total microbial counts on fresh broiler legs stored at 4°C. In another study (Kantt and Torres, 1993), the effect of glucose oxidase (GOX)-catalase (CAT) with different GOX/CAT ratios and of chemicals produced by GOX, gluconic acid and hydrogen peroxide, were evaluated on four microorganisms usually present in shrimp (*Pandalus jordani*). Growth inhibition was independent of enzyme ratio. Hydrogen peroxide inhibited *Pseudomonas fluorescens*, *Hansenula polymorpha* and *Acinetobacter calcoaceticum*. Only *Corynebacter aquaticum* was inhibited by gluconic acid but was not affected by hydrogen peroxide. In a subsequent study (Kantt et al., 1993), glucose oxidase (GOX, 1 unit/mL) and catalase (CAT) was added to 4% (w/v) glucose in artificial seawater to determine the effectiveness of this treatment in trawlers using refrigerated seawater for on-board shrimp preservation. Fresh shrimp were frozen, radiation-sterilized, thawed by adding artificial seawater and inoculated with *Pseu-*

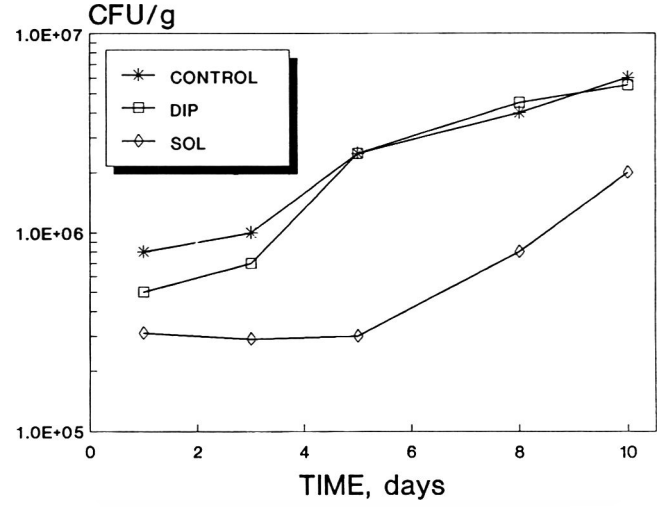

Fig. 1—Effect of GOX/CAT/glucose application method on psychrotrophs counts.

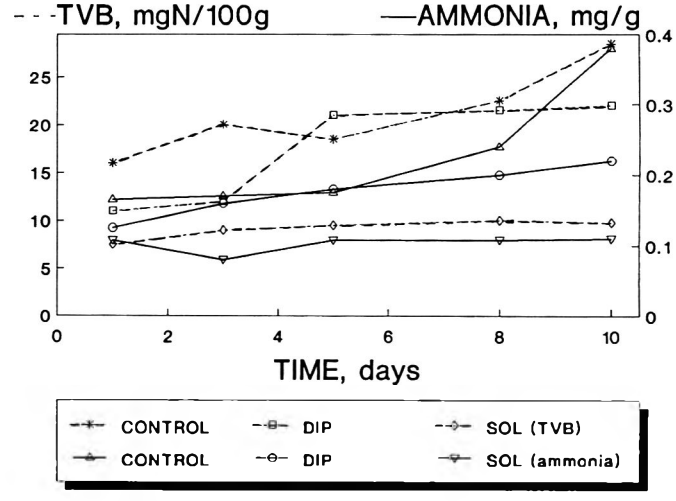

Fig. 2—Effect of GOX/CAT/glucose application method on total volatile bases (TVB) and ammonia concentration.

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domonas fluorescens (10^4 CFU/g shrimp). Samples were stored at 1°C and treated after 24 hr. Bacterial and chemical changes were monitored by total plate counts, ammonia and total volatile nitrogen. Microbial lag phase was extended ≈5 days and after 14 days, GOX/CAT had reduced solution discoloration by ≈80% and inhibited ammonia and total volatile nitrogen production. Kantt et al. (1993) estimated that the enzyme solution would need to be replaced after 14 d on-board use due to a build-up of basic nitrogen compounds in the solution.

Our objective was to determine the preservative effect of glucose oxidase/catalase in 4% aqueous glucose solution (GOX/CAT/glucose) treatments on refrigerated shrimp (*Heterocarpus reedi*) using subjective and objective tests and to evaluate application alternatives. The effect of post-catch age on GOX/CAT/glucose effectiveness was of particular interest.

MATERIALS & METHODS

SHRIMP (*Heterocarpus reedi*) were caught in March and April, 1986 by the research vessel *Tiberíades* in Valparaíso, Chile. Immediately after catch and while still on board, shrimp were washed with tap water at 10°C. Samples were left as is (controls) or immersed for 2 min (DIP samples) in an agitated tap water solution containing 4% (w/v) glucose and 1U/mL of GOX (DEE-0, GOX activity = 1,270 U/g enzyme preparation, source = *Aspergillus niger*, Miles Laboratories, Inc.) or held in enzyme solution for the duration of the study (SOL samples). CAT activity in the DEE-0 enzyme was not measured, however, GOX preparations with CAT activities in the range found in commercial preparations have been found equally effective (Kantt and Torres, 1993). Treated and control samples were stored in closed plastic containers at 0–2°C up to 10 d. In all samples (DIP and SOL) the shrimp:solution ratio was 1:1.5. At preestablished times post-capture, samples were removed from storage for quality evaluation.

Effect of shrimp catch age

In a second experiment, the effectiveness of the GOX/CAT/glucose enzyme solution was tested using shrimp with different degrees of freshness. Samples were not washed with tap water and before landing, shrimp were kept in wood boxes with crushed ice. Samples received at landing 4 and 82 hr after-catch (4H and 82H samples) were held at 0–2°C for 13 days in the enzyme solution.

Quality measurements

Objective tests included total volatile bases (Conway, 1962), ammonia (Field, 1981) and pH measured on ground shrimp using a flat surface electrode (Model 95-12, Orion Research, Inc., Cambridge, MA) connected to an Orion pH meter (Model 701-A). Microbial assays included *Pseudomonas* spp. and total psychrotrophs counts on about 25g shrimp samples (Anonymous, 1976). Subjective tests were done by a 6-member experienced panel who evaluated 3 whole, raw or cooked-peeled shrimp. Cooking was done in an oven at 250°C for 15 min. Panelists were asked to evaluate samples for color, odor, texture and flavor (cooked samples only), using a 0 to 7 scale, 7 was the maximum desirability value of each attribute and <3.5 was considered unacceptable quality.

Statistical analysis

The effect of the GOX/CAT/glucose application method was analyzed using Fisher protected least significant differences (FPLSD) in a randomized block design with days as blocks. Contrasts tested to determine the effect of shrimp catch age using LSD values were selected in advance.

RESULTS & DISCUSSION

Effect of GOX/CAT/glucose application method

Total psychrotroph counts for control samples were not statistically different than those for DIP. For SOL samples, a lower count ($p < 0.05$) was observed during storage (Fig. 1). Keeping shrimp in the GOX/CAT/glucose solution resulted in no increase in bacterial numbers up to 5 days. The antimicrobial action of the SOL treatment extended the time to reach 10^6 CFU/g by 5 days over controls. Toledo (1982) used chilling (2–4°C) and immersion for 10 min in a solution containing 10% condensed phosphate, 5% potassium sorbate and 0.9% citric acid to control microbial spoilage of shrimp. Our treatment extended the time to reach 10^6 CFU/g by 4.5 days over shrimp held on ice. A recommended maximum microbial count of 10^7 CFU/g has been suggested by Farooqui et al. (1978) for acceptable shrimp quality. The antimicrobial action of the GOX/CAT/glucose solution was consistent with results reported by Field et al. (1986). However, the effect of GOX/CAT/glucose on cod (*Gadus morhua*), hake (*Merluccius gayi gayi*) and jack mackerel (*Trachurus murphyi*) (Shaw et al., 1986; Dondero et al., 1989) demonstrated little benefit toward retarding spoilage. Note that in all previous studies the enzyme was applied as a dip.

Total volatile bases (TVB) for controls were not different ($P > 0.05$) than those for DIP. For SOL samples, a significantly lower ($p < 0.05$) TVB (Fig. 2) confirmed a difference

Table 1 – Effects of the GOX/CAT/glucose application method

	Contrasts			
	Control: DIP	Control: SOL	DIP: SOL	LSD
Raw shrimp				
color	0.2	2.9*	2.7*	0.7
odor	1.0*	0.1	0.9*	0.6
texture	0.2	2.4*	2.2*	0.8
Cooked shrimp				
color	0.4	2.4*	2.0*	0.7
odor	0.3	1.7*	1.4*	0.7
texture	0.2	1.3*	1.1*	0.6
flavor	0.1	1.7*	1.6*	0.8

* Statistically different at the 5% level

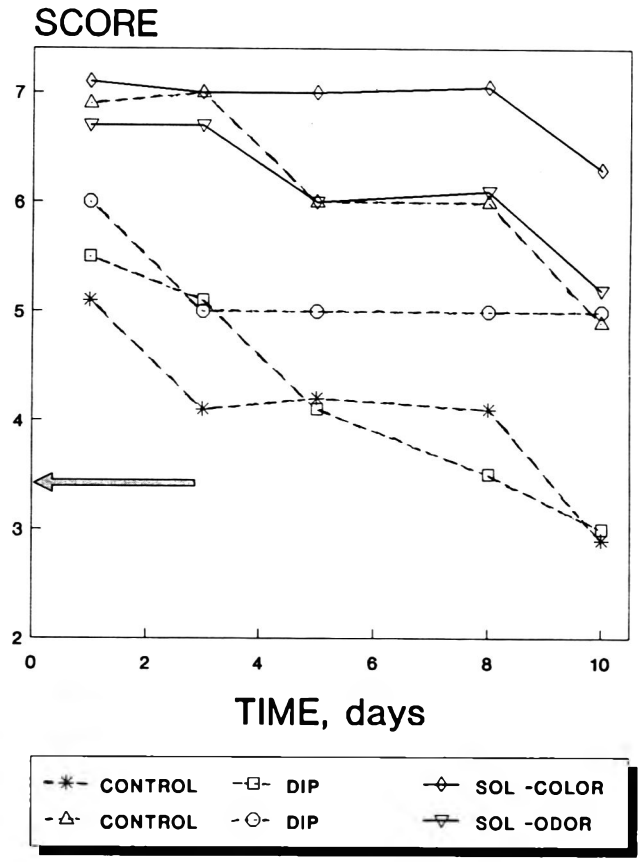

Fig. 3 – Effect of GOX/CAT/glucose application method on color and odor sensory scores for whole-raw shrimp stored at 0–2°C. Arrow indicates the minimum acceptable score on a 0 to 7 scale.

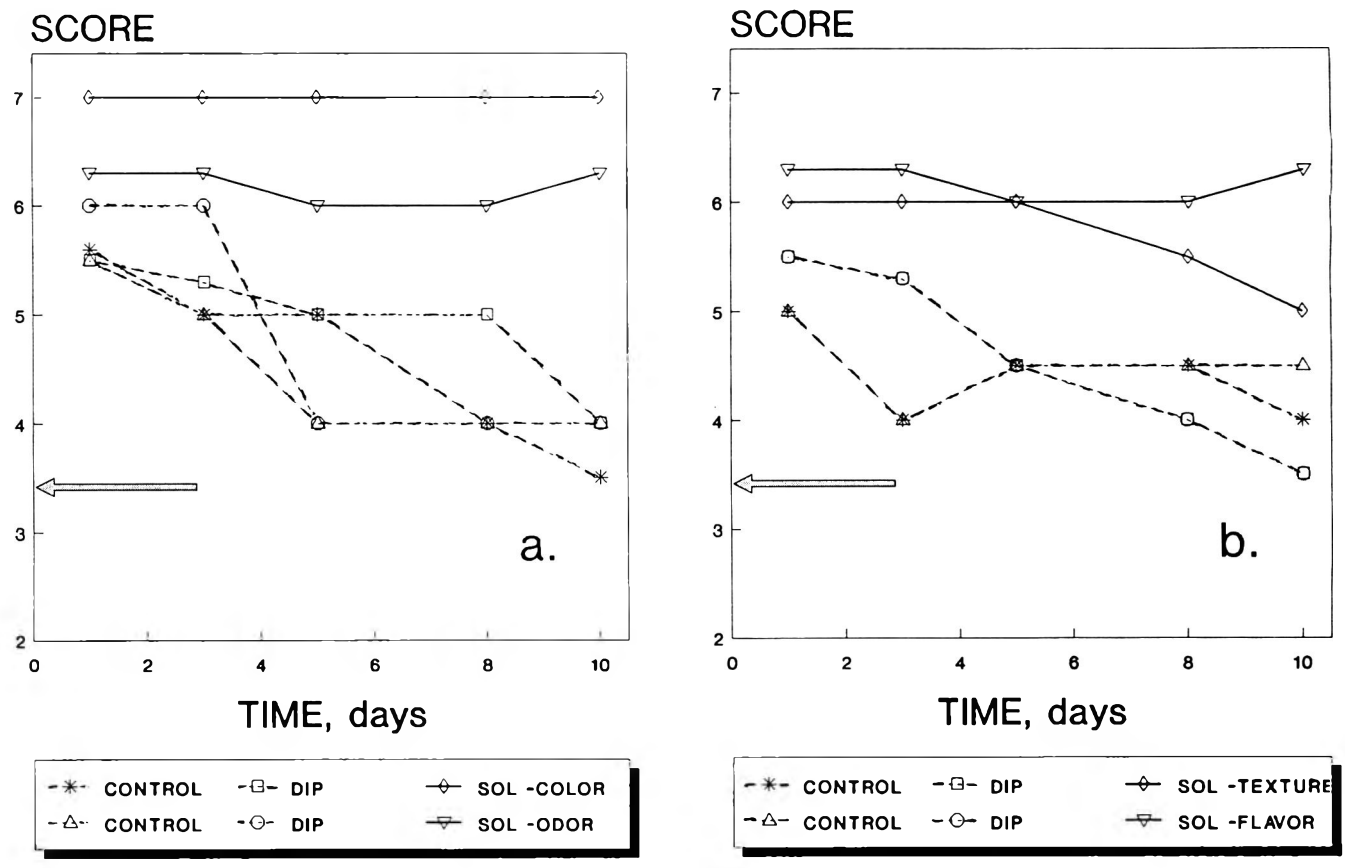

Fig. 4—Effect of GOX/CAT/glucose application method on sensory scores for cooked-peeled shrimp stored at 0–2°C. (a) color and odor; (b) texture and flavor. Arrow indicates the minimum acceptable score on a 0 to 7 scale.

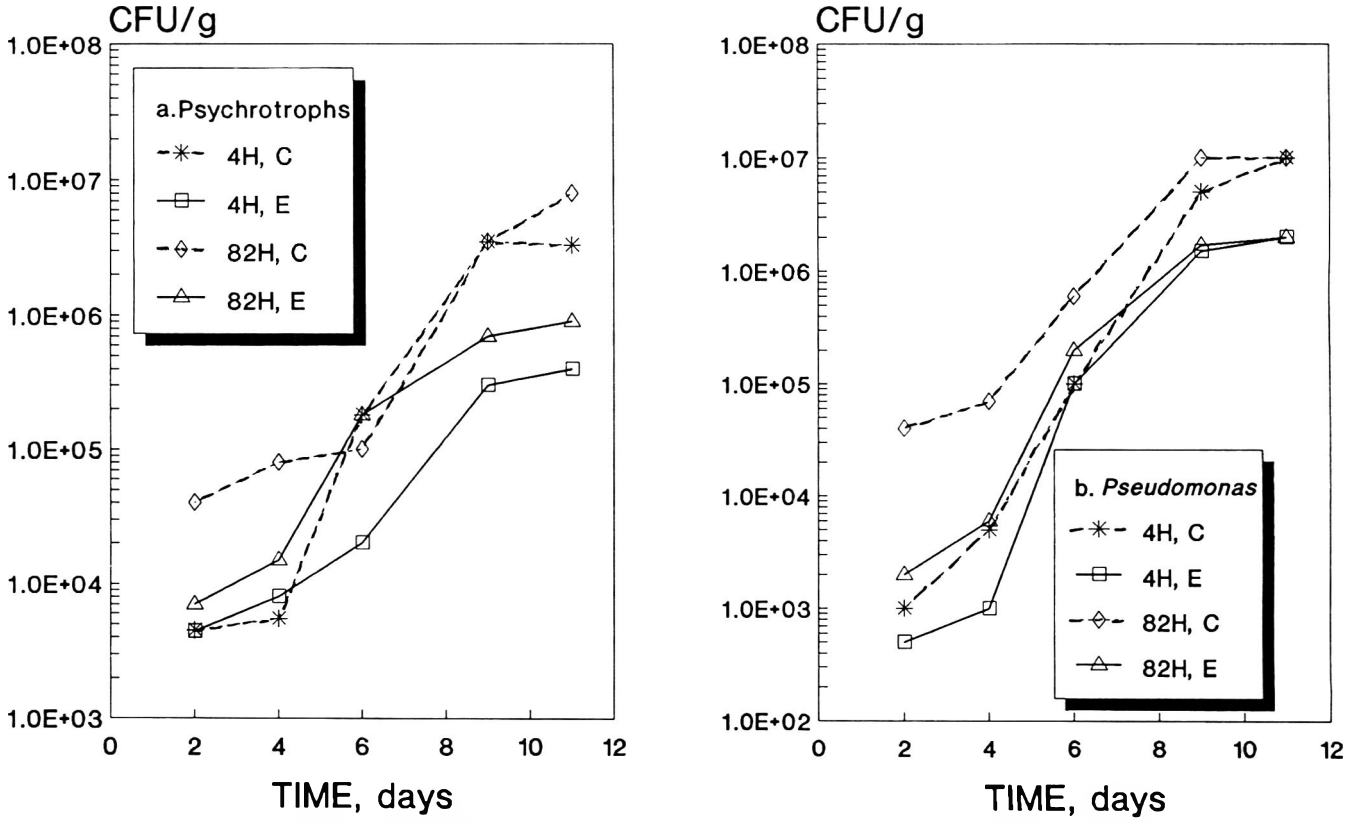

Fig. 5—Effect of catch age on the effectiveness of the enzyme treatment. C and E denote control and enzyme treated shrimp, respectively, received 4 (4H) and 82 hr (82H) after catch. (a) psychrotrophs counts; (b) Pseudomonas counts.

Table 2—Effects of enzyme application time

	CONTRASTS			
	Control: SOL (4H)	Control: SOL (82H)	4H SOL: 82H SOL	LSD
Microbial analysis				
psychrotrophs	$1x10^6$	$2x10^{6*}$	$3x10^5$	$2x10^6$
Pseudomonas	$2x10^6$	$3x10^{6*}$	$2x10^5$	$3x10^6$
Chemical assays				
TVB	18*	28*	11	15
ammonia	0.37*	0.64*	230	270
Sensory (raw shrimp)				
color	4.6*	2.9*	1.9*	0.8
odor	3.3*	3.0*	1.4*	0.9
texture	1.9*	0.7*	1.5*	0.7
Sensory (cooked shrimp)				
color	4.0*	2.1*	1.5*	1.0
odor	3.0*	0.02	3.0*	0.7
texture	0.9	1.4*	0.2	1.3
flavor	1.5*	0.3	0.7	0.4

* Statistically different at the 5% level

in microbial activity between treated and control samples. After 10 d, treated samples had TVB well below the commercially acceptable limit of 30 mg N/100g (Cobb and Vanderzant, 1975) while controls had almost reached that level. Ammonia levels were analogous to TVB values (Fig. 2). Ammonia concentrations for controls increased rapidly after 6–8 days. The process was retarded by holding shrimp in the enzyme solution. Similar findings were reported for winter flounder (Field et al., 1986), but not for cod, hake and jack mackerel dipped in GOX/CAT/glucose solutions (Shaw et al., 1986; Dondero et al., 1989). Low TVB and ammonia levels in SOL samples might also reflect diffusion of reaction products into the enzymatic solution.

Statistical analysis of pH values showed some indication of differences between DIP and SOL samples ($p = 0.07$) and between control and SOL samples ($p = 0.16$). At the end of 10 days, pH values for SOL samples increased from 6.7 to 7.6 vs 7.4 and 7.1 for control and DIP samples, respectively. Field et al. (1986) had observed in enzyme-dipped flounder a pH decrease presumably caused by gluconic acid formation.

Statistical analysis of the effects of the GOX/CAT/glucose treatment on sensory evaluation of whole and cooked-peeled shrimp showed that panelists consistently gave highest scores to the SOL samples (Table 1). Untreated and DIP whole shrimp were rejected at 10 d on the basis of color, but the SOL samples still showed the maximum score for color (Fig. 3). Untreated cooked-peeled samples were rejected by 10 d on the basis of color (Fig. 4a) and flavor (Fig. 4b). However, at that time, SOL samples still showed maximum score for color and a slight decrease in odor (Fig. 4a), texture and flavor acceptability (Fig. 4b).

Effect of shrimp catch age

Statistical analysis confirmed the effectiveness of holding shrimp in an enzymatic solution (Table 2). Total psychrotrophs and *Pseudomonas* spp. counts showed large differences between treated and controls both for 4H and 82H samples (Fig. 5). Control of *Pseudomonas* spp. is important because they constitute the largest group of spoilage microflora in shrimp (Smith, 1983; Jay, 1986; Kantt and Torres, 1993; Kantt et al., 1993). As expected, the degree of freshness had a large effect on initial microbial counts and highlights the importance of early enzyme treatment.

Higher TVB and ammonia levels (Fig. 6) were found in 82H samples. TVB and ammonia levels increased rapidly for control samples, but were reduced by the GOX/CAT/glucose system. Treated samples reached the commercially acceptable TVB limit of 30 mg N/100g (Cobb and Vanderzant, 1975)

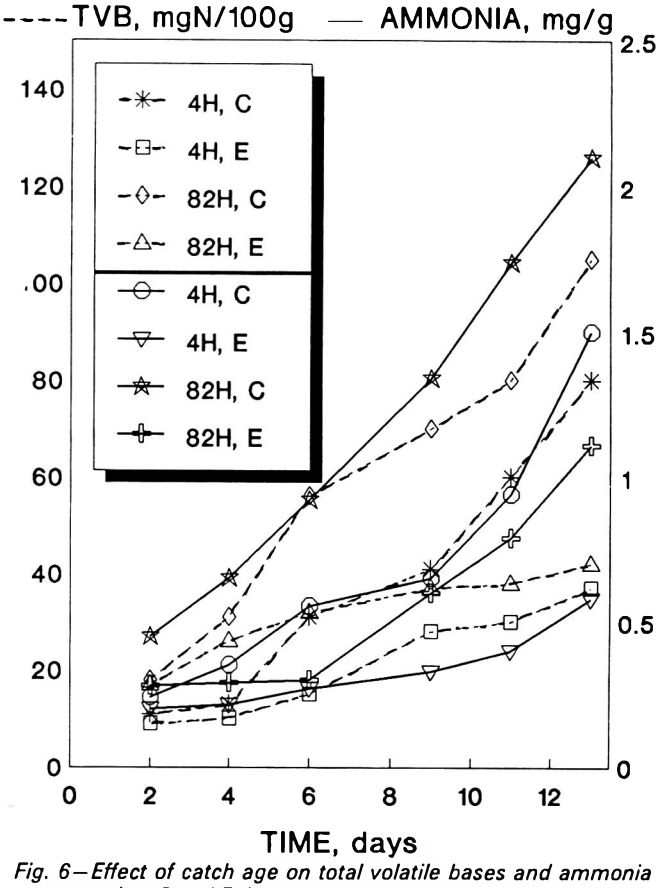

Fig. 6—Effect of catch age on total volatile bases and ammonia concentration. C and E denote control and enzyme treated shrimp, respectively, received 4 (4H) and 82 hr (82H) after catch.

after 6 days and 11 days for 82H and 4H samples, respectively, while controls reached that value after 4 days and 6 days for 82H and 4H samples, respectively.

Overall acceptability of the enzyme-treated samples was always superior to that of untreated samples (Fig. 7). Untreated 82H whole shrimp samples were rejected at 2 days on the basis of odor (Fig. 7a) and around 3 days and 8 days on the basis of color (Fig. 7a) and texture (Fig. 7b), respectively. Treated 82H whole shrimp was rejected after 8–9 d on the basis of color, odor and texture (Fig. 7). Treated 4H whole shrimp remained acceptable after 13 d while control samples reached the limits of color, odor and texture acceptability in less time (Fig. 7).

Cooked shrimp samples treated more than 3 days after catch (82H samples) were rejected after 4 days storage at 0–2°C because of odor (Fig. 8a) and after 8–10 days because of texture and flavor (Fig. 8b) degradation, but color (Fig. 8a) remained acceptable after 13 days. Controls were rejected after 4–6 d while 4H cooked enzyme-treated samples reached the flavor acceptability limit after 8 days but retained acceptable color, odor and texture for the duration of the study (Fig. 8). The flavor of controls reached the acceptability limit after 6 d and the acceptability limit for color, odor and texture at 4 days.

CONCLUSIONS

KEEPING SHRIMP on board in a GOX/CAT/glucose solution at 0–2°C would increase shelf life. Tests showed that an effective treatment was to hold shrimp in the enzyme solution (at 0–2°C). Differences between 4H and 82H samples stressed the importance of treating shrimp as early as possible, preferably immediately after catch. This method could benefit the seafood industry by allowing longer catching operations while retaining product quality.

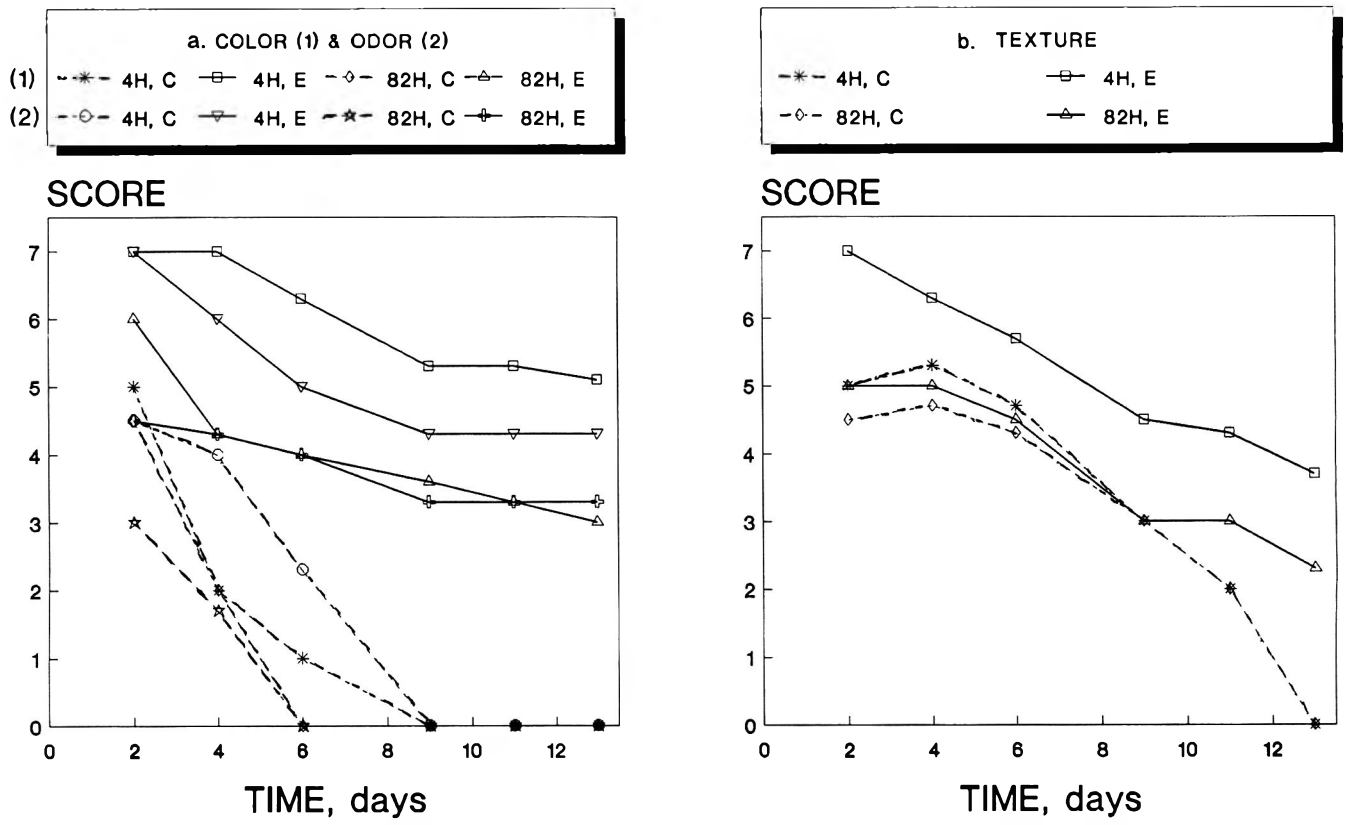

Fig. 7—Effect of catch age on sensory scores for raw shrimp stored at 0–2°C. C and E denote control and enzyme treated shrimp, respectively, received 4 (4H) and 82 hr (82H) after catch. (a) Color and odor; (b) texture.

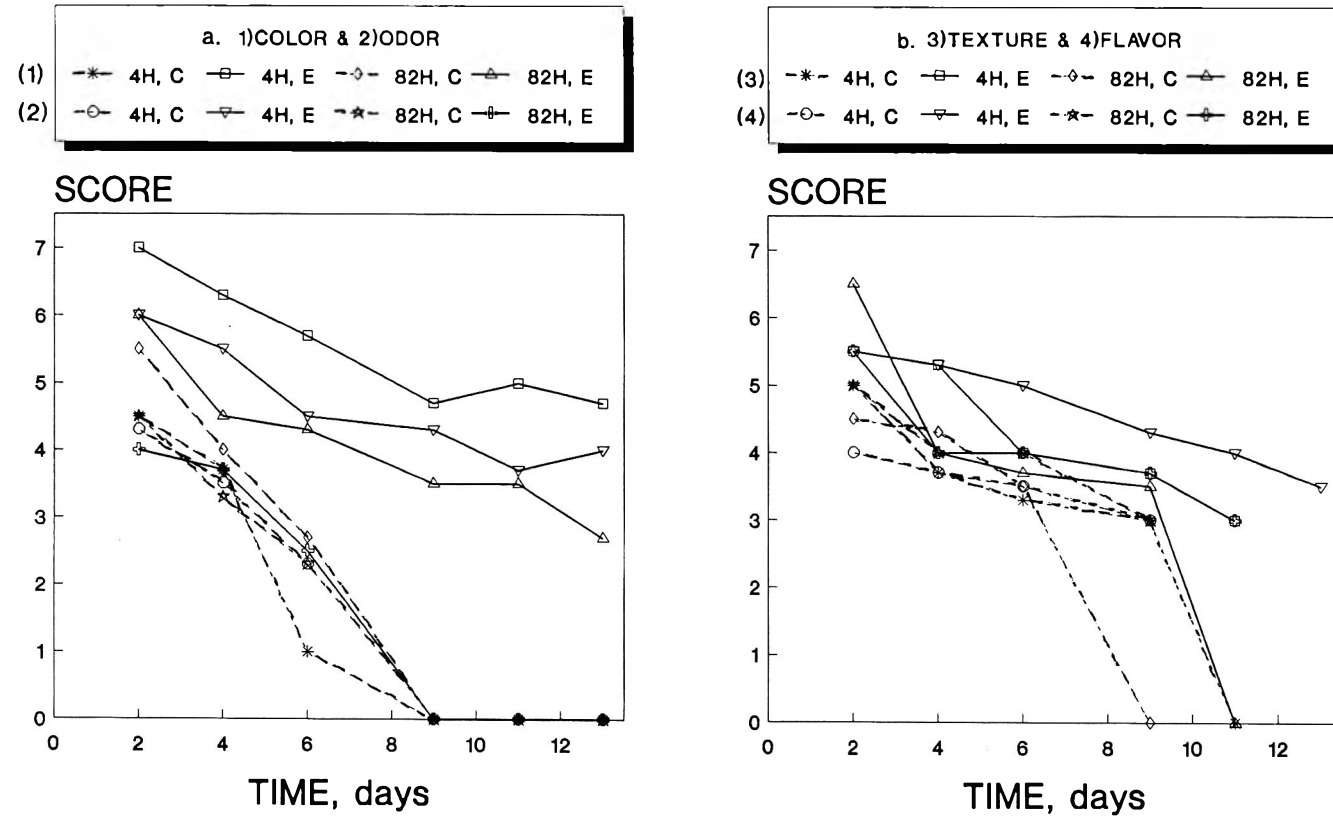

Fig. 8—Effect of catch age on sensory scores for cooked-peeled shrimp stored at 0–2°C. C and E denote control and enzyme treated shrimp, respectively, received 4 (4H) and 82 hr (82H) after catch. (a) Color and odor; (b) texture and flavor.

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Trypsin-like Enzyme from Sand Crab (Portunus pelagicus): Purification and characterization

DAVID A. DIONYSIUS, KEITH S. HOEK, JOANNE M. MILNE, and STEVEN L. SLATTERY

ABSTRACT

Studies with synthetic substrates and specific inhibitors indicated that a proteinase from the hepatopancreas of the sand crab (*Portunus pelagicus*) was a trypsin-like serine proteinase. The molecular weight of the enzyme estimated by gel filtration and mass spectrometry was ≈ 25,000, whereas SDS-PAGE indicated a molecular weight of 34,800. The optimum temperature for hydrolysis of azocasein was 60°C, while inactivation of 50% enzymic activity occurred at 68°C. The enzyme, optimally active at pH 8.0 towards *p*-tosyl-L-arginine methyl ester and unstable at acid pH, was high in acidic amino acid residues. Under some conditions the enzyme readily autodigested. Our results can help understand and avoid problems of meat softening during storage of seafood products.

Key Words: trypsin, sand crab, hepatopancreas, proteinase

INTRODUCTION

PROTEINASES from the tissue and viscera of aquatic food sources are important in development of undesirable textural changes during storage and processing. Such enzymes may also prove useful as processing aids in the food industry (Haard, 1992). Consequently, studies aimed at characterizing proteinases responsible for flesh degradation have been undertaken in many commercially important species, including mackerel (Matsumiya et al., 1989), cod (Raa and Gildberg, 1976; Simpson et al., 1989), salmon (Konagaya, 1985), krill (Kimoto et al., 1983) and prawns (Jiang et al., 1991; Lindner et al., 1988).

Several studies have investigated the effects of digestive enzymes on degradation of the flesh in crustacea. Shibata and Ozaki (1983) and Kawamura et al. (1981) reported that contamination of the body meat of Antarctic krill, *Euphausia superba,* with hepatopancreas led to autolysis and loss of texture. Others implicated enzymes of hepatopancreatic origin in textural defects in flesh of rock lobster, *Jasus lalandii* (Wessels and Olley, 1973), freshwater crawfish, *Procambarus clarkii* (Marshall et al., 1987), mud crab, *Scylla serrata* (Gillespie et al., 1983) and freshwater prawn, *Macrobrachium rosenbergii* (Nip et al., 1985).

We reported processing conditions which resulted in damage to the hepatopancreas of the sand crab, *Portunus pelagicus*, led to development of mushiness in the flesh during subsequent storage (Slattery et al., 1989). The objective of our current study was to purify and characterize one of the major proteinases responsible for this tissue softening in *P. pelagicus,* an important seafood.

MATERIALS & METHODS

Extraction and purification of trypsin-like enzyme

Live sand crabs (*P. pelagicus*) were stored on ice immediately after capture and brought to the laboratory within several hours. After removal of the carapace, the hepatopancreas was excised and an homogenate prepared by blending the tissue with two volumes of 50 mM Tris-HCl buffer, pH 7.0 in a Panasonic Super Blender using the pulse setting for 40 sec. The homogenate was centrifuged at 20,000×*g* for 45 min in a GSA rotor of a Sorvall RC5C refrigerated centrifuge, and the supernatant filtered through Whatman 541 filter paper. The filtered extract was the source of enzyme for further purification. All steps of enzyme extraction and purification were carried out at 0–4°C. Protein concentration was determined by the method of Lowry et al. (1951) using bovine serum albumin as standard.

Enzyme extract was dialyzed overnight against 20 mM Tris-HCl buffer, pH 7.0 (Buffer A) and then centrifuged at 20,000×*g* for 30 min to remove precipitates. The supernatant was filtered through a Whatman GF/A filter, and 390 mL of dialyzed, filtered extract was mixed with 150 mL of DEAE-Sepharose CL-6B equilibrated in Buffer A. Resin and extract were shaken continuously for 40 min, and the resin then poured into a sintered glass funnel and washed with 250 mL of Buffer A, followed by 250 mL of Buffer A containing 0.35 M NaCl. Enzyme was subsequently eluted with 500 mL of Buffer A containing 1 M NaCl, subjected to ultrafiltration and diafiltration using an Amicon ultrafiltration cell fitted with a PM-30 membrane, and loaded onto a DEAE-Sepharose CL-6B column (2.5 × 28 cm) equilibrated in Buffer A containing 0.3M NaCl. The column was washed with 200 mL of equilibration buffer, and eluted with a linear gradient of 0.3–1.0M NaCl in Buffer A (750 mL total volume) at 50 mL/hr. Fractions (10.0 mL) with trypsin-like activity were pooled and dialyzed against 50 mM Tris-HCl buffer, pH 7.0 (Buffer B).

The dialyzed enzyme was applied to an Arginine Sepharose 4B column (1 × 24 cm) previously equilibrated with Buffer B. Following sequential washes with Buffer B (30 mL), a linear 0-0.5 M NaCl gradient in Buffer B (100 mL), Buffer B containing 1M NaCl (30 mL) and Buffer B (30 mL), enzyme was eluted with a linear gradient of 0–0.3M arginine in Buffer B (100 mL total volume). Fractions of 3.0 mL were collected at 60 mL/hr, and those with trypsin-like activity were pooled and concentrated using an Amicon cell fitted with a PM-10 membrane. Concentrated enzyme was applied to a Sephadex G-50 column (2.6 × 94 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0 containing 0.2M NaCl, and eluted in the same buffer at 16 mL/hr with 5 mL fractions.

Enzyme assays

The proteinase activity was determined using azocasein as substrate in a modification of the method of Jensen et al. (1980). The reaction mixture contained 1.0 mL 0.5% azocasein in 100 mM Tris-HCl buffer, pH 7.5, 0.1 mL enzyme and buffer in a final volume of 3.0 mL. The reaction was stopped after 30 min by addition of 3 mL 10% trichloroacetic acid (TCA) and after standing at 60°C for 30 min, the precipitate was removed by filtration. Control samples containing TCA added prior to enzyme addition were included in each assay. One unit of activity was defined as an increase in absorbance at 366 nm of 0.10 in 60 min at 37°C.

Trypsin-like esterase activity was tested using two substrates: *p*-tosyl-L-arginine methyl ester (TAME; Walsh, 1970) and *N*-benzoyl-L-arginine ethyl ester (BAEE; Rick, 1965). One unit of TAME activity was defined as the amount of enzyme which catalyzed the hydrolysis of 1 μmole of TAME/min at 25°C, while one unit of BAEE activity was defined as the amount of enzyme which produced an increase in absorbance at 253 nm of 0.10/min at 25°C. Chymotrypsin-like esterase activity was determined with *N*-benzoyl-L-tyrosine ethyl ester (BTEE) according to the method of Walsh and Wilcox (1970). One unit of BTEE activity was defined as the amount of enzyme which produced an increase in absorbance at 256 nm of 0.10/min at 25°C.

Carboxypeptidase A activity of samples was determined using furylacryloyl-L-phenylalanine-L-phenylalanine (FAPP) as substrate according to Riordan and Holmquist (1984). One unit of carboxypeptidase A activity was defined as the amount of enzyme which produced a decrease in absorbance at 350 nm of 0.10/min at 25°C. Amino acid

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Table 1—Purification of trypsin-like enzyme from crab hepatopancreas

Sample	Total protein (mg)	Total activity[a]	Specific activity (units/mg)	Recovery (%)	Purification (fold)
Extract	4640	25300	5.4	100	1
DEAE-Sepharose (batch)	859	15900	18.5	63	3.4
DEAE-Sepharose (column)	37	13100	356.7	52	66.1
Arginine Sepharose	28	13200	477.5	52	88.4
Sephadex G-50	23	12400	546.2	49	101.1

[a] *p*-Tosyl-L-arginine methyl ester (TAME) used as substrate.

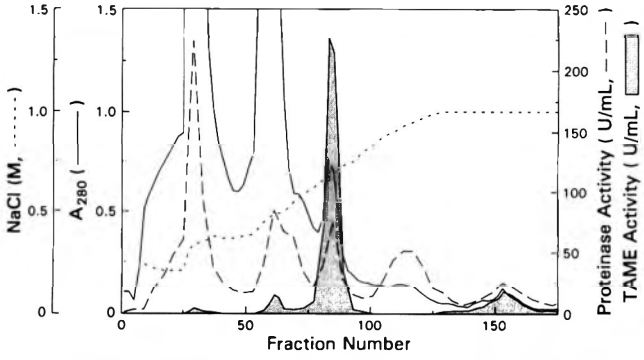

Fig. 1—DEAE-Sepharose CL-6B column chromatography of trypsin-like enzyme from crab hepatopancreas in 20 mM Tris-HCl buffer, pH 7.0.

arylamidase activity was determined using L-alanine *p*-nitroanilide as substrate according to Hafkenscheid (1984). One unit of amino acid arylamidase activity was defined as the amount of enzyme which produced an increase in absorbance at 405 nm of 0.10/min at 25°C.

Elastase activity assays employed a modification of the Geiger method (1984). To a 3 mL cuvette was added 1.90 mL 0.2 M ethanolamine buffer, pH 7.8 containing 0.05% (w/v) Triton X-100, 1.0 mL 3 mM *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide as substrate and 0.1 mL enzyme sample to start the reaction. The absorbance at 405 nm was recorded for 5 min. One unit of elastase activity was defined as the amount of enzyme which produced an increase in absorbance at 405 nm of 0.10/min at 25°C.

Determination of molecular weight

The molecular weight of trypsin-like enzyme was determined by gel filtration with a Sephadex G-75 column (1.6 × 66 cm) equilibrated with 50 mM Tris-HCl buffer, pH 7.0 containing 0.2M NaCl. Blue Dextran 2000 was used to determine the void volume, and calibration was achieved using bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), chymotrypsinogen A (M_r = 25,000) and ribonuclease A (M_r = 13,700). Flow rate was 16 mL/hr and 1.6 mL fractions were collected. Samples of active enzyme, and enzyme inactivated with 1 mM *p*-tosyl-L-lysine chloromethyl ketone (TLCK) were chromatographed separately in the absence or presence of 0.1 mM TLCK, respectively, and the eluted protein peak assayed for trypsin-like activity.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in a vertical slab gel apparatus using the method of Laemmli (1970). Separation was performed in a 15% gel and protein bands were stained with Coomassie Blue R-250. Molecular weight marker proteins included phosphorylase b (M_r = 94,000), bovine serum albumin (M_r = 67,000), ovalbumin (M_r = 43,000), carbonic anhydrase (M_r = 30,000), soybean trypsin inhibitor (M_r = 20,100) and α-lactalbumin (M_r = 14,400).

The molecular mass of the trypsin-like enzyme was determined by matrix-assisted laser desorption mass spectrometry (Lasermat; Finnigan Mat). The enzyme was dialyzed against deionized water at 4°C, lyophilized, and a sample (1 mg/mL) prepared in 0.1% trifluoroacetic acid (TFA) and diluted 6-fold with matrix solution (10 mg/mL sinapinic acid in 70% acetonitrile containing 0.1% TFA). Following this treatment the enzyme was fully inactivated. The sample/matrix solution (0.6 μL) was applied to the Lasermat target and dried at room temperature. The sample was then analyzed using the positive polarity mode for the instrument, a laser power of 55 and a gain setting of 62 mV. The resulting spectrum was obtained from 20 laser shots, and mass data were analyzed using equine myoglobin (M_r = 16,952) as an external calibrating standard. Calculation of peak centroids was performed by computer (Finnigan Mat).

Enzyme characterization

The optimum temperature for activity of the trypsin-like enzyme was determined using the proteinase assay at pH 7.5 with azocasein as substrate. Samples containing 50 μg enzyme were tested in a 30-min assay from 20°C to 70°C. Thermostability of the enzyme was studied by heating samples (0.02 mg/mL in 46 mM Tris-HCl buffer, pH 8.1 containing 11.5 mM $CaCl_2$) at different temperatures for 30 min, followed by cooling and assay for trypsin-like activity using TAME as substrate.

The optimum pH for activity of the trypsin-like enzyme was determined by assaying at different pH values using TAME as substrate. The buffers were prepared as 0.1M solutions, and included sodium citrate (pH 3.0, 4.0, and 5.0), sodium acetate (pH 5.0 and 6.0), sodium phosphate (pH 6.0, 7.0 and 8.0) and sodium borate (pH 8.0, 9.0 and 10.0). The stability of trypsin-like activity at different pH values was studied by incubating samples in different buffers for 30 min at 25°C. Enzymic activity was then determined with TAME as substrate. The buffers were prepared as 0.1 M solutions, and included sodium citrate (pH 3.0, 4.0 and 5.0), sodium acetate (pH 6.0), Tris-HCl (pH 7.0 and 8.0) and sodium carbonate (pH 9.0, 10.0 and 11.0).

The effects of various proteinase inhibitors, reducing agents and metal ions on trypsin-like activity were tested using TAME as substrate. Enzyme was incubated with each one for 15 min at 25°C prior to addition of substrate to start the reaction. In studies on the effect of EDTA or metal ions, $CaCl_2$ was excluded from the assay buffer. The amino acid composition of trypsin-like enzyme was determined following sample hydrolysis in the gas phase for 1 hr at 150°C with 6N HCl containing 1% (v/v) phenol. Hydrolysates were dried, then derivatized with phenylisothiocyanate to produce phenylthiocarbamyl amino acid derivatives. These were analyzed by the Pico-Tag method (Waters Associates). The N-terminal amino acid sequence of the enzyme was determined by Edman degradation with an Applied Biosystems 470A automatic gas phase protein sequencer coupled to a 120A PTH Analyzer using a standard PTH program.

RESULTS

Purification of trypsin-like enzyme

The initial steps in the purification of trypsin-like enzyme from crab hepatopancreas were based on the acidic nature of the enzyme. Anion exchange chromatography employing batch and column DEAE-Sepharose chromatographic procedures resulted in a 66-fold purification of the enzyme (Table 1). Separation on the DEAE-Sepharose column revealed several peaks (Fig. 1) with activity towards TAME, a synthetic substrate for trypsin-like enzymes. Only the major peak of TAME activity was collected for further purification. Subsequent chromatography on Arginine Sepharose, an affinity resin specific for serine proteinases, and gel filtration on Sephadex G-50 resulted in a purified preparation. Overall, 23 mg of enzyme was obtained from 150 g of hepatopancreas, recovery 49%, 101-fold purification (Table 1).

The purity of the enzyme was determined using SDS-PAGE (Fig. 2). In addition to the major protein band migrating at M_r = 34,800, several contaminating bands were observed (Fig. 2, lane 3). However, upon addition of 1 mM TLCK to the sample prior to boiling in electrophoresis sample buffer, the minor bands disappeared (Fig. 2, lane 1). As TLCK is an inhibitor of trypsin-like enzymes, this result suggested that the contaminants were products of autolysis of the enzyme during sample preparation, and that the enzyme was essentially homogeneous following purification.

Enzyme characterization

Molecular weight. Gel filtration of the purified trypsin-like enzyme on Sephadex G-75 indicated a molecular weight of

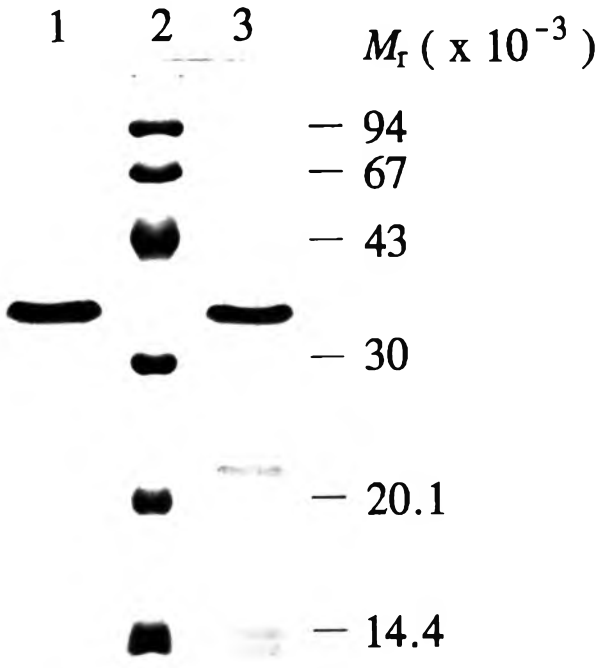

Fig. 2—SDS-PAGE of trypsin-like enzyme from crab hepatopancreas. Lane 1, purified enzyme (12 μg) treated with 1 mM p-tosyl-L-lysine chloromethyl ketone; lane 2, molecular weight marker proteins; lane 3, purified enzyme (12.5 μg).

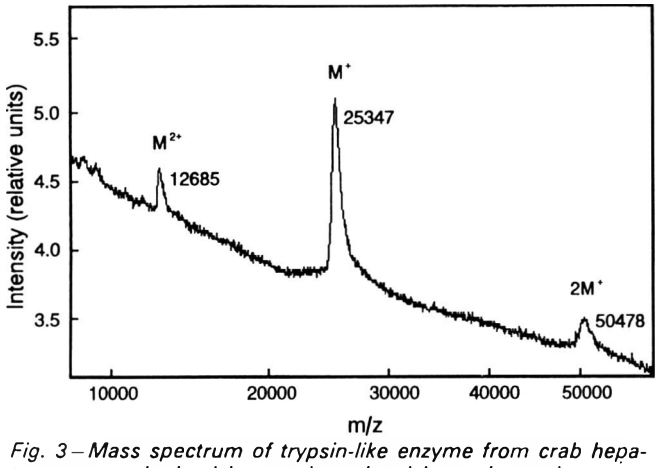

Fig. 3—Mass spectrum of trypsin-like enzyme from crab hepatopancreas obtained by matrix-assisted laser desorption mass spectrometry.

23,400, with enzymatic activity coeluting with one protein peak. The same estimate of molecular weight was obtained for TLCK-inactivated enzyme, indicating autolysis did not occur during gel filtration of the active enzyme. However on SDS-PAGE the protein migrated as a single band, apparent molecular weight 34,800 (Fig. 2, lane 1), a result achieved using TLCK-inactivated enzyme prepared for gel filtration studies. Analysis of the molecular mass of the enzyme by laser desorption mass spectrometry indicated a mass of 25,346 (Fig. 3). A doubly charged monomeric species (m/z = 12,685) and a singly charged dimer (m/z = 50,478) were also detected (Fig. 3) but no other protein peaks were observed. Inactivation of the enzyme during sample preparation prevented autolysis during mass spectrometry analysis.

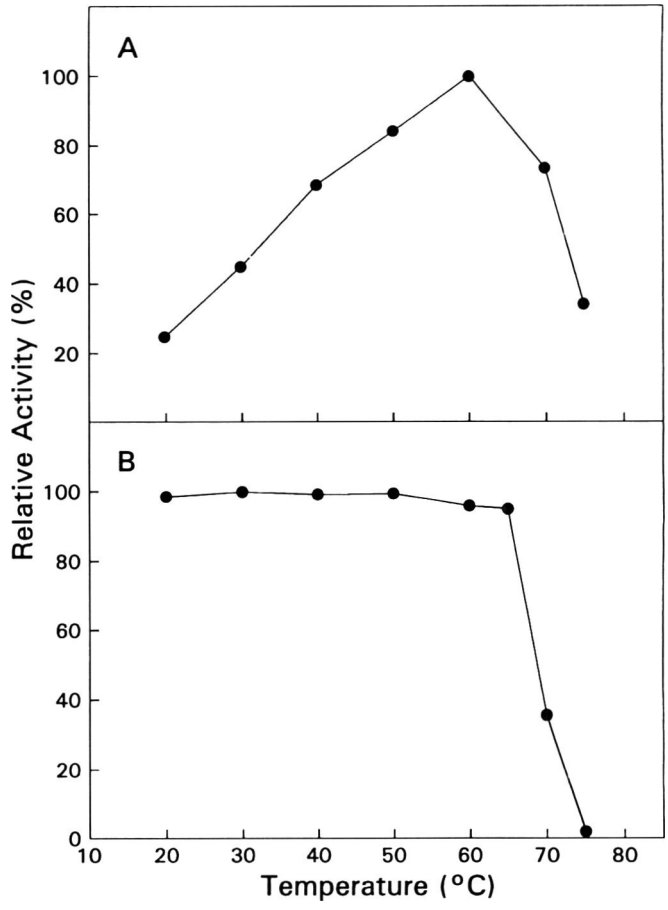

Fig. 4—Effect of temperature on activity and stability of trypsin-like enzyme from crab hepatopancreas. (A) profile using 30-min proteinase assay. (B) activity following 30-min incubation. TAME substrate at 25°C.

Temperature and pH studies. The enzyme displayed optimum activity at 60°C, Fig. 4(A), when azocasein was substrate. The enzyme was remarkably thermostable, with 36% of original activity remaining after heating at 70°C for 30 min (Fig. 4(B)). Half the TAME activity was lost under experimental conditions at 68°C. Trypsin-like enzyme exhibited optimum activity for the hydrolysis of TAME at pH 8.0 (Fig. 5(A)). Studies on effects of pH showed the enzyme was stable in the alkaline range (pH 7.0–11.0), but unstable in the acidic region, with total loss of activity following incubation at pH 4.0 for 30 min (Fig. 5(B)).

Substrate specificity and Inhibitors. The only synthetic substrates hydrolyzed by the enzyme were TAME and BAEE (Table 2). These results indicate the enzyme had trypsin-like properties. The protein substrate azocasein was also hydrolyzed by the enzyme. Various inhibitors of proteinase activity affected the trypsin-like enzyme (Table 3). The serine proteinase inhibitor PMSF affected activity to some extent, while TLCK and STI, specific inhibitors of trypsin-like enzymes, and leupeptin, an inhibitor of serine and cysteine proteinases with trypsin-like activity were highly inhibitory. Inhibitors of cysteine- and metalloproteinases did not markedly affect activity, and reducing agents including cysteine, β-mercaptoethanol and glutathione at 1 mM also had little effect. An inhibitor of proteinases with chymotrypsin-like activity (TPCK) had no effect. These results, together with data from studies on substrate specificity (Table 2), strongly supported classification of the enzyme as a trypsin-like proteinase.

Amino acid composition. The amino acid composition of the trypsin-like enzyme (Table 4) was compared with that of similar enzymes from two species of shrimp (Gates and Travis,

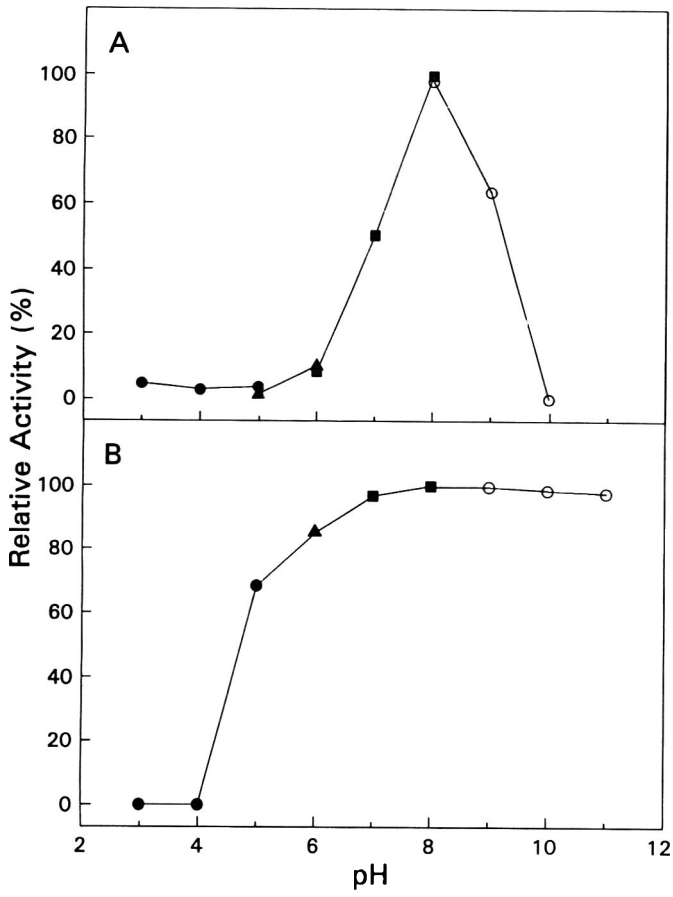

Fig. 5—Effect of pH on activity and stability of trypsin-like enzyme from crab hepatopancreas. (A) Profile using TAME as substrate. ●, sodium citrate buffer; ▲, sodium acetate buffer; ■, sodium phosphate buffer; ○, sodium borate buffer. (B) Activity following 30-min incubation at various pH values. ●, sodium citrate buffer; ▲, sodium acetate buffer; ■, Tris-HCl buffer; ○, sodium carbonate buffer. TAME was used as substrate.

Table 2—Substrate specificity of trypsin-like enzyme from crab hepatopancreas

Substrate[a]	Specific activity (units/mg)
TAME	546.2
BAEE	189.8
BTEE	0
FAPP	0
Ala-NA	0
Succ-(ala)$_3$-NA	0
Azocasein	138.0

[a] TAME: *p*-tosyl-L-arginine methyl ester; BAEE: *N*-benzoyl-L-arginine ethyl ester; BTEE: *N*-benzoyl-L-tyrosine ethyl ester; FAPP: furylacryloyl-L-phenylalanine-L-phenylalanine; Ala-NA: L-alanine *p*-nitroanilide; Succ-(ala)$_3$-NA: *N*-succinyl-L-alanyl-L-alanyl-L-alanine *p*-nitroanilide.

1969; Honjo et al., 1990). Of particular interest was the predominance of acidic residues (asp and glu), and the low content of basic residues (lys and arg) in the protein. A trypsin-like enzyme from krill also had a similar amino acid composition (Osnes and Mohr, 1985). The N-terminal amino acid sequence of crab trypsin-like enzyme (IVGGQEATPG) was similar to sequences of two proteases from the shrimp, *Penaeus indicus* (Honjo et al., 1990), with six (shown underlined) of the first ten residues identical in the three proteins.

Studies on autolysis of trypsin-like enzyme

The results of SDS-PAGE using TLCK-treated and untreated enzyme indicated that the enzyme autolyzed at elevated

Table 3—Effect of various compounds on activity of trypsin-like enzyme from crab hepatopancreas

Compound[a]	Final conc (mM)	Relative activity[b] (%)
TLCK	0.1	0
TPCK	0.1	100
STI	0.0005[c]	0
PMSF	1.0	61
Leupeptin	0.001	0
E-64	0.1	84
PCMB	0.1	105
Iodoacetic acid	0.1	100
EDTA	1.0	92
L-Cysteine	1.0	88
β-Mercaptoethanol	1.0	100
Glutathione	1.0	93
Zn^{2+}	1.0	103
Mg^{2+}	1.0	105
Ca^{2+}	1.0	94

[a] TLCK: *p*-tosyl-L-lysine chloromethyl ketone; TPCK: L-1-tosylamide-2-phenylethyl-chloromethyl ketone; STI: soybean trypsin inhibitor; PMSF: phenylmethylsulfonyl fluoride; E-64: trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane; PCMB: *p*-chloromercuribenzoic acid.

[b] Enzyme (0.8 μg) incubated with compound 15 min at 25°C prior to assay with *p*-tosyl-L-arginine methyl ester. Results relative to appropriate controls.

[c] Based on a molecular weight 20,100.

Table 4—Amino acid composition of trypsin-like enzymes from crab hepatopancreas and shrimp

Amino acid	Residues/100 residues		
	Crab[a]	Shrimp[b]	Shrimp[c]
Asp/Asn (D/N)	10.9	10.7	13.2
Glu/Gln (E/Q)	12.7	10.8	10.6
Ser (S)	8.9	9.6	10.6
Gly (G)	15.9	14.0	12.3
His (H)	4.9	2.6	2.2
Arg (R)	1.3	1.0	1.3
Thr (T)	4.5	3.9	4.4
Ala (A)	6.1	5.9	7.0
Pro (P)	4.6	6.6	4.8
Tyr (Y)	4.2	4.2	4.4
Val (V)	6.5	8.2	7.9
Met (M)	5.6	1.3	0.9
Cys (C)	nd[d]	2.8	3.5
Ile (I)	2.9	7.8	6.2
Leu (L)	4.6	4.6	4.4
Phe (F)	3.9	3.8	2.6
Lys (K)	2.7	2.4	2.2
Trp (W)	nd[d]	—	1.3

[a] This study

[b] Honjo et al. (1990)

[c] Gates and Travis (1969)

[d] Not determined

temperatures (Fig. 2). The enzyme also had a high optimum temperature for activity (60°C). Consequently, an experiment was carried out to determine the propensity of the enzyme to autolyze at 70°C. Samples of enzyme (0.5 mg/mL) in 50 mM Tris-HCl buffer, pH 7.0 containing 0.2M NaCl were incubated at 70°C for periods up to 5 min, prior to rapid cooling on ice. Samples were then prepared for SDS-PAGE by boiling in electrophoresis sample buffer (5 min) and analyzed on 15% gels. The protein was completely degraded after 5 min at 70°C (Fig. 6) and polypeptides with molecular weights about 21,000 to 23,000, 14,000 to 16,000, and 11,500 were generated. Loss of enzyme activity was concomitant with a decrease in the protein band at 34,800, with no activity remaining after heating 5 min. Inactivation of the enzyme with 1 mM TLCK prior to incubation kept the protein intact (Fig. 6, lane 6).

DISCUSSION

TRYPSIN-LIKE enzymes are widely distributed in crustacea. DeVillez and Buschlen (1967) reported them in over 20 species of amphipods, isopods and decapods. Enzymes from krill (Chen et al., 1978; Kimoto et al., 1983; Osnes and Mohr, 1985) and several shrimp species (Gates and Travis, 1969; Honjo et al.,

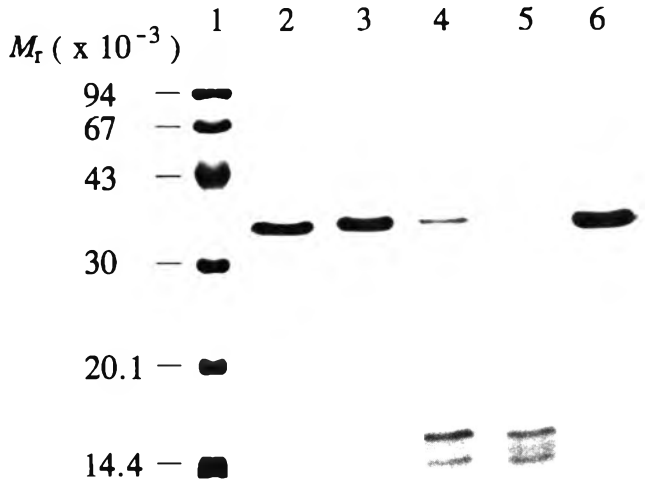

Fig. 6—SDS-PAGE of trypsin-like enzyme from crab hepatopancreas following incubation at 70°C for various times. Lane 1, molecular weight marker proteins; lane 2, enzyme control (0-sec treatment); lane 3, 30-sec treatment; lane 4, 60-sec treatment; lane 5, 5-min treatment; lane 6, enzyme treated with 1 mM p-tosyl-L-lysine chloromethyl ketone prior to 5-min incubation at 70°C. All enzyme loads contained 12.5 μg protein.

1990; Jiang et al., 1991; Olalla et al., 1978) have been purified and characterized. Our present study reports a trypsin-like enzyme from a crab, *Portunus pelagicus* with classification as trypsin-like based on substrate specificity (Table 2) and inactivation by specific inhibitors (Table 3).

Trypsin-like enzymes of mammalian origin are generally characterized by molecular weights around 25,000, inhibition by specific compounds such as those we used, stability at acid pH and high isoelectric point (Shaw et al., 1965; Walsh, 1970). Our crab trypsin-like enzyme displayed the first two properties, but was unstable at low pH, and had a very low isoelectric point, evidenced by its amino acid composition (Table 4) and its strong binding to DEAE-Sepharose at pH 7.0 (Fig. 1). Gates and Travis (1969) noted similar properties for a trypsin-like enzyme from the hepatopancreas of white shrimp, *Penaeus setiferus*. Acidic trypsin-like proteinases have also been reported from the gastric juices of American lobster, *Homarus americanus* (Brockerhoff et al., 1970) and crayfish *Orconectes virilis* (DeVillez, 1965), and the hepatopancreas of *Euphausia superba* (Kimoto et al., 1983; Osnes and Mohr, 1985).

Estimations of the molecular weight of the enzyme varied with the method employed. Data by mass spectrometry and gel filtration were in close agreement (25,346 and 23,400, respectively). SDS-PAGE analysis however, gave a significantly higher result (34,800). Honjo et al. (1990) observed a similar effect when shrimp trypsin-like enzyme was analyzed by gel filtration and SDS-PAGE (18,000 and 36,000, respectively). Of the methods used, mass spectrometry is most reliable, with accuracies typically ± 1% for biomolecules of this molecular mass (Carr et al., 1991). The anomalous result for SDS-PAGE cannot be explained, but the results showed conclusively that the enzyme is homogeneous. A molecular weight of about 25,000 is similar to that reported for bovine trypsin and several purified crustacean trypsin-like enzymes (Gates and Travis, 1969; Jiang et al., 1991; Kimoto et al., 1983).

Crab trypsin-like enzyme was remarkably stable at elevated temperatures (Fig. 4(B)), but was susceptible to autolysis at 70°C (Fig. 6). This apparent anomaly could be explained by differences in experimental conditions. Thermal stability studies were done in the presence of calcium, whereas autolysis studies were done in its absence. The inclusion of 10 mM calcium chloride in the buffer for the autolysis trials resulted in a mostly intact protein on SDS-PAGE, and the retention of over 90% of the enzymic activity following incubation at 70°C for 5 min (data not shown). Hence, calcium increased the stability of the enzyme at elevated temperatures, and interestingly resulted in no autodigestion. However, calcium was not required for activity of the enzyme (Table 3), in agreement with the results of Gates and Travis (1969) for shrimp trypsin.

The stability of the enzyme at high temperatures has implications for processing of crabs. If they are undercooked, the internal temperature may not reach 70°C by the end of cooking (Slattery and Dionysius, 1988). In such circumstances, trypsin-like enzyme would retain enough proteolytic activity to, under sub-optimal storage conditions (e.g. elevated temperature), possibly contribute to flesh degradation and mushiness (Slattery et al., 1989). However, despite such effects, the enzyme could prove useful in food applications requiring a heat-stable proteinase. For example, the ability to withstand elevated temperatures may be advantageous in processing food products where enzymes producing off-flavors, or product instability need to be inactivated following heat treatments (Haard, 1992).

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ε-(γ-Glutamyl)lysine Crosslink Formation in Sardine Myofibril Sol during Setting at 25°C

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ABSTRACT

The quantitative change of ε-(γ-glutamyl)lysine (EGL) crosslink and relationship between crosslink content and gel-strength were examined on salt-ground myofibril sol from sardine (*Sardinops melanostictus*) during incubation at 25°C. In the presence of EGTA, no EGL crosslinks were detected in myofibril sol and gelation did not occur. The EGL crosslink content and breaking strength of gels increased in proportion to incubation time. High correlation was observed between the logarithm of breaking strength and logarithm of EGL crosslink content ($r = 0.987$). The EGL crosslinks formed by transglutaminase are important in the setting of sardine meat sol at < 30°C.

Key Words: sardines, fish, lysine, myofibrils, transglutaminase, texture

INTRODUCTION

SALT-GROUND MEAT sols from Alaska pollack (*Theragra chalcogramma*), hoki (*Macruronus novaezelanäiae*) and sardine (*Sardinops melanostictus*) reportedly have a property of setting into gels at temperatures <30°C, where meat sols from most other species of fish do not set. This setting phenomenon always occurred with polymerization of myosin heavy chain (MHC) (Numakura et al., 1985; Ree et al., 1990; Tsukamasa and Shimizu, 1989). Furthermore, calcium ion was found essential for such low-temperature setting (Tsukamasa and Shimizu, 1990). Endogeneous transglutaminase (TGase), which catalyzes formation of ε-(γ-glutamyl)lysine (EGL) crosslinks, might be involved in such low-temperature setting in Alaska pollack meat sol (Seki et al., 1990). Tsukamasa and Shimizu (1991) demonstrated that TGase also was important in low-temperature setting of sardine meat sol. To confirm the contribution of TGase in setting, Kimura et al. (1991) attempted to detect crosslinks in set gel from hoki surimi by the method of Griffin et al. (1982). However, the EGL peak on their chromatogram was not clearly resolved from other peaks. They did not examine the quantitative change of crosslinks in the low-temperature setting process.

We described an improved method (Sato et al., 1992) for identification and determination of EGL crosslink formed in set fish gel. Our current objective was to use that improved method to examine the quantitative change of EGL crosslinks in sardine myofibril sols during incubation at 25°C and relationships between EGL crosslink content and gel-strength of set gels.

MATERIALS & METHODS

THIS STUDY consisted of two experiments. In the first, we examined the relationship between changes in SDS-PAGE pattern and the formation of EGL crosslinks in sols during incubation at 25°C using a myofibrillar protein solution containing 1% protein. In the second experiment, we examined relationships between crosslinks and gel-strength of set gels using myofibril sols containing 89.4% water.

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Myofibrillar protein

Sardine was purchased from a local fish supplier. The fish was used within 12-hr from time of death. The dorsal white muscle of the sardine was minced by passing through a 10-mesh screen. The minced muscle was homogenized with 5 volumes of sodium phosphate buffer; ionic strength was adjusted to 0.05 and pH to 7.5. The homogenate was centrifuged at 3,000 × g for 10 min. The same procedure was repeated twice. The final precipitate was used as myofibrillar protein.

Preparation of myofibril gels

Low and high protein (LP and HP) gels were prepared for SDS-PAGE analysis and puncture tests. For preparation of the LP gel, myofibrillar protein was solubilized in 20mM Tris-malcate buffer, pH 7.5, containing 0.5M KCl, to produce a final protein concentration of 1% (w/v). The prepared myofibril sol was pippeted into polypropylene tubes, into which $CaCl_2$ was added to 10mM, and then incubated at 25°C for 0.5, 1, 1.5, 2, 3, 4, 6 and 24 hr to form gels. Since most endogenous calcium ion was removed during preparation of myofibrillar protein, gelation did not occur in LP sols. Therefore, 10mM of $CaCl_2$ was added. The gels were immediately heated at 100°C for 3 min. As a control, the myofibril sol was heated to 100°C without incubation at 25°C in the absence of $CaCl_2$ to provide initial data, and then $CaCl_2$ was added to the coagulated protein to adjust concentration.

For preparation of HP gels, the myofibrillar protein was centrifuged at 10,000 × *g* for 20 min to lower water content. $CaCl_2$ (10 mmol/kg) was added to one portion and EGTA (5 mmol/kg) to the other to

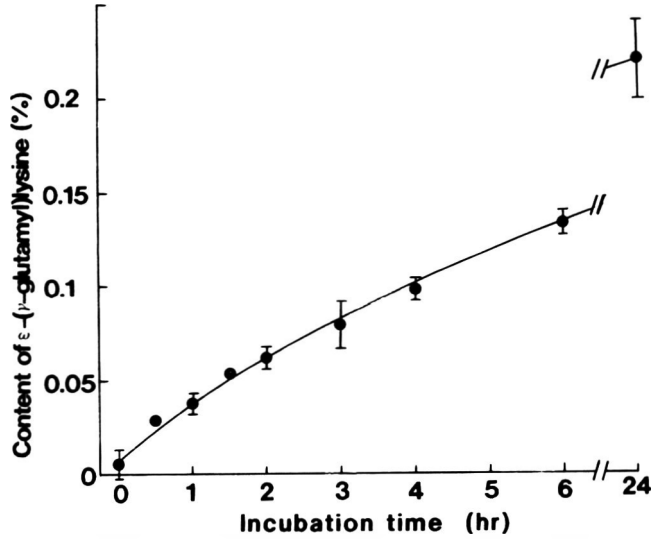

Fig. 1—Formation of ε-(γ-glutamyl)lysine crosslink (mol/mol) in LP sol during incubation at 25°C in the presence of 10 mM $CaCl_2$ and 0.5M KCl. Each value represents the mean of 3 determinations. Vertical bars represent ± standard deviation.

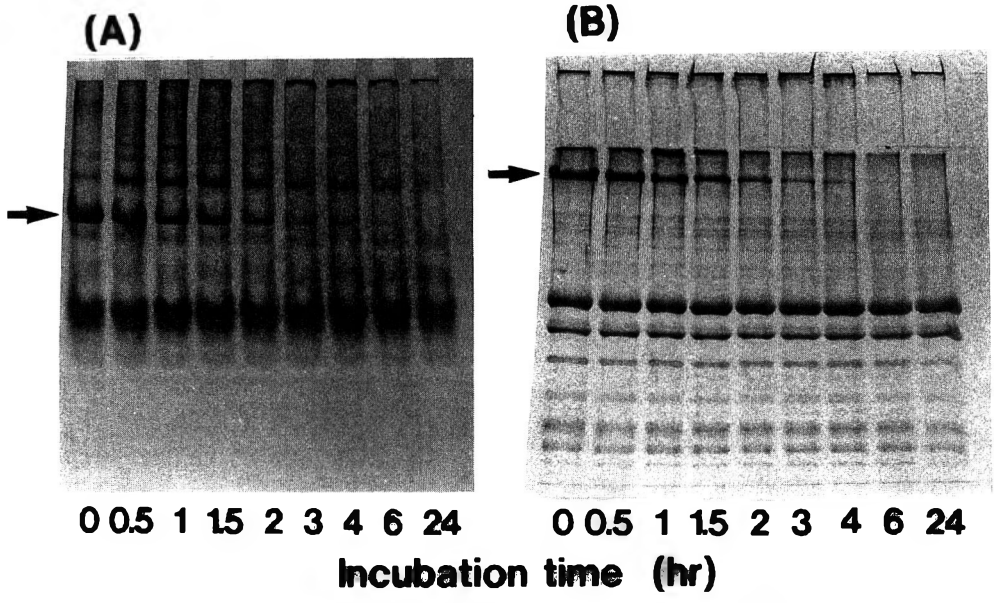

Fig. 2—SDS-PAGE patterns of proteins in LP sol incubated at 25°C on 2.5% acrylamide resolving gel containing 0.5% agarose (A) and 10% acrylamide resolving gel (B). Arrows indicate myosin heavy chain.

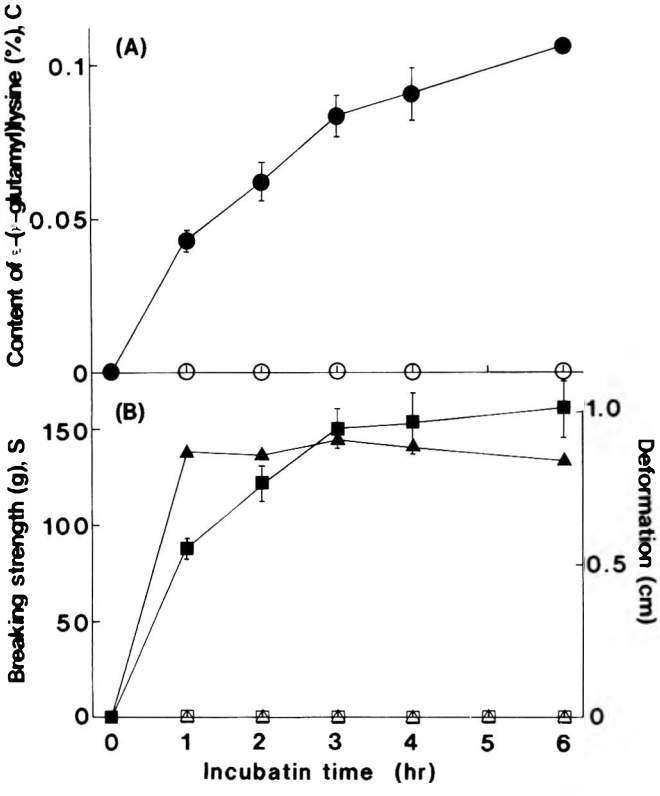

Fig. 3—Formation of ε-(γ-glutamyl)lysine crosslink (mol/mol) and changes in breaking strength (square) and deformation (triangle) of HP sol during incubation at 25°C in the presence of $CaCl_2$ (closed symbols) and EGTA (open symbols). Each value represents the mean of 3 determinations. Vertical bars represent ± standard deviation.

exclude effects of residual calcium. The final concentrations were water 89.4% and NaCl 2.73%. They were then ground for 10 min. A portion of each sol was packed into glass tubes (10 mm diameter and 15 mm high) for puncture test, and another portion was wrapped with plastic film and pressed flatly for determination of EGL. Both samples were incubated at 25°C for 1, 2, 3, 4 and 6 hr. After incubation, the gels packed in glass tubes were cooled immediately and stored in ice water. Gels wrapped in film were immediately heated at 100°C for 3 min to inactivate endogenous TGase and perform the protease digestion at 37°C. Protein concentration was estimated using the microbiuret method. Bovine serum albumin was used as standard.

Gel-strength evaluation

The HP gels were removed from the glass tubes and subjected to puncture tests with a Rheoner RE-3305 (Yamaden Co. Ltd., Tokyo, circular cylinder type plunger with flat end, diameter 4 mm, stage speed 1mm/sec). Gel-strength was evaluated by breaking strength (g) and deformation (cm).

Determination of ε-(γ-glutamyl)lysine

Determination of EGL was performed according to the method of Sato et al. (1992). Proteolytic digestion with pronase E was done for 24 hr for LP gels and 48 hr for HP gels. The reliability of determination of EGL crosslinks in HP sols was confirmed by comparison with that in LP sols.

SDS-PAGE analysis

Samples were dissolved in 20 mM Tris-HCl buffer, pH 6.8, containing 2% SDS, 2% 2-mercaptethanol and 8 M urea. SDS-PAGE was performed using the Tris-glycine buffer system described by Laemmli (1970) with 10% acrylamide gel. The Tris-sodium acetate buffer system described by Hattori and Tatsumi (1989) was used with 2.5% acrylamide gel containing 0.5% (w/v) agarose.

RESULTS & DISCUSSION

THE FORMATION of EGL crosslinks in LP sols during incubation at 25°C (Fig. 1) increased linearly up to 6 hr. After that, the increase became slower from 6 to 24 hr. After 24 hr the EGL content attained 0.22% (mol/mol).

SDS-PAGE analysis of LP sols using 2.5% acrylamide gel, (Fig. 2A) showed density of MHC band decreased and new bands, which might be due to polymerized MHC, appeared in proportion to incubation time. Changes in protein bands of lower molecular weight than MHC in the LP sols at 25°C using 10% acrylamide gel (Fig. 2B) showed the pattern of protein bands did not change during incubation. This suggested that the crosslinks might be formed predominantly between MHC's, the most important protein for gelation of meat, but not between other myofibrillar proteins. Similar results have been

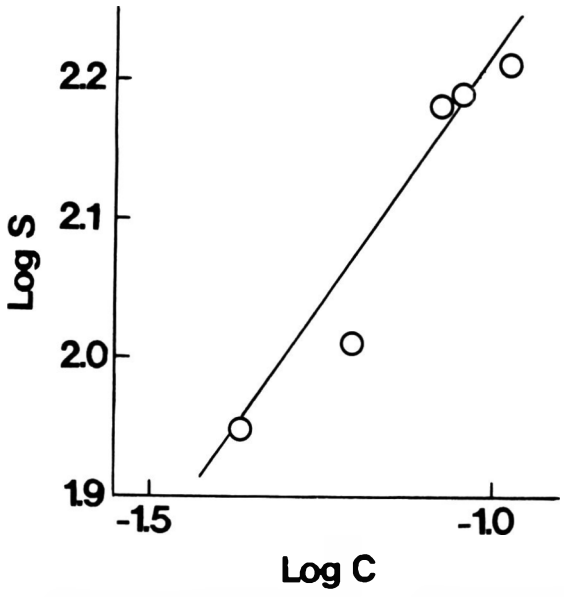

Fig. 4—Relationship between breaking strength (S) and ε-(γ-glutamyl)lysine crosslink content (C) in HP gel produced at 25°C in the presence of $CaCl_2$.

reported on Alaska pollack meat sol by Numakura et al. (1985). To clarify the effect of the EGL crosslink formation upon gel-strength of set gels, HP sol, the protein concentration of which was almost as high as fish cake, was incubated.

Activity of TGase is dependent on calcium ion. When sufficient amount of EGTA, e.g. 5 mmol/kg, is added, myofibril sol will not set to gel at < 30°C. So, the amount of EGL crosslinks and gel-strength (breaking strength × deformation) value of set gels at 25°C were measured in the presence of EGTA and $CaCl_2$, respectively. Results show (Fig. 3A and 3B) that in presence of $CaCl_2$, EGL content increased almost linearly in proportion to incubation time up to 6 hr. Such a pattern for HP sols closely conformed to that for LP sols (Fig. 1) although HP sols were about 10-fold higher in protein concentration. Therefore, LP sol, which is easy to use due to its low viscosity, could be used in place of HP sol, for studies of low-temperature setting.

Changes in breaking strength and deformation of HP gels produced at 25°C in $CaCl_2$ (Fig. 3B) showed the HP sol did not set in the presence of EGTA for 6 hr. In the presence of $CaCl_2$, however, breaking strength of the gel increased linearly up to 3 hr, and then a gradual increase was observed. The increase in breaking strength closely conformed to formation of EGL crosslinks in the presence of $CaCl_2$.

Deformation of the gels increased rapidly within the first hour of incubation, but after that it varied slightly in presence of $CaCl_2$. Other interactions such as hydrophobic ones could possibly affect the deformation. However, the crosslinks likely are important in the deformation, because gelation and deformation could be measured only with the occurrence of crosslinks.

The logarithm of breaking strength, S, of the set gels (Fig. 3B) was plotted against the logarithm of the corresponding EGL crosslink content, C (Fig. 4). A high correlation was observed between the two values in the range from 0.043% to 0.106% of crosslinks content (coefficient of correlation 0.987). This was in agreement with the formula presented by Hirano (1942) regarding the relation between rigidity and protein concentration in set gel; $S = kC^n$, where k and n are constants.

Gelation and formation of EGL crosslinks did not occur when EGTA was added. Further, we (Tsukamasa and Shimizu, 1991) reported that addition of TGase, independent of calcium, induced gelation of sardine meat by heating at 30°C in the presence of EGTA. The setting of sardine meat sol at 30°C was suppressed by TGase inhibitor such as methylamine in the presence of sufficient calcium to activate TGase (Tsukamasa and Shimizu, 1990). These facts indicated that formation of EGL crosslinks was essential for gelation rather than other protein-protein interactions under these conditions.

The EGL crosslinks formed by TGase are important in the low-temperature setting of sardine meat sol. This is the first report demonstrating the relationship between formation of EGL and gel-strength in low-temperature set gels.

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Formation of Anthocyanins from Cells Cultured from Different Parts of Strawberry Plants

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ABSTRACT

Anthocyanin formation and structural identification were investigated in the cultured strawberry, *Fragaria ananassa* cv Shikinari. Friable callus tissues were obtained from apical meristems, leafs, and petioles grown on LS agar medium with 2,4-dichlorophenoxyacetic acid and benzyladenine. Using these calli, effects of auxins and cell inoculum size on pigment production were studied in suspension culture. Anthocyanin was produced under 8000 lux after preculture for 3 wk under 800 lux in suspension culture. The main extracted pigment was identified as a peonidin-3-glucoside and the second major pigment was a cyanidin-3-glucoside by FABMS and NMR. Anthocyanin production was stimulated by 2,4-dichlorophenoxyacetic acid and its yield was about three times that of indole-3-acetic acid. An inoculum rate of 2g (fresh weight)/100 mL LS medium resulted in highest pigment production. The callus derived from the leaf was much higher in anthocyanin production than the others tissues of strawberry.

Key Words: strawberries, callus, color, anthocyanins, cell culture

INTRODUCTION

MANY synthetic food colorants, particularly red, have been banned for use in food products due to toxicity (Timberlake and Henry, 1986). This has promoted research on food colorants from natural sources. Production of naturally occurring anthocyanin pigments is a potential alternative to synthetic red coloring agents. With recent advancement in plant biotechnology, several secondary metabolites, including anthocyanins, can be produced in cell cultures. Anthocyanin production has been reported in callus cultures of wild carrot (Kinnersley and Dougall, 1980; Dougall and Weyrauch, 1980), *Strobilanthes dyeriana* (Smith et al., 1981), *Vitis hybrida* (Yamakawa et al., 1983), *Hibiscus sabdariffa* (Mizukami et al., 1988), *Populus nigra* × *P. maximiwiczii* (Matsumoto et al., 1973), sweet potato (Nozue et al., 1987), carrot (Ozeki and Komamine, 1985), *Vitis* sp (Tamura et al., 1989), and strawberry (Hong et al., 1989).

The main anthocyanin pigment in strawberries was identified as pelargonidin-3-glucoside (Robinson and Robinson, 1932). Lukton et al. (1955) identified a second minor pigment in cultivated strawberries as cyanidin-3-glucoside. Although strawberry cultures have been studied by several researchers (Nitsch, 1950, 1955; Adams, 1972; Boxus, 1974; Lee and de Fossad, 1977), those studies were mostly concerned with micropropagation of meristem culture for regeneration of strawberry plants. Recently, Hong et al. (1989) reported anthocyanin production in suspended cultures of immature strawberry fruits. They revealed that cultivars were different in their ability to initiate callus formation and to produce anthocyanins, and that inoculum size influenced cell growth in suspension cultures. In spite of these findings, the identification and production of anthocyanin in callus cultures from various parts of the strawberry has not been reported.

The objectives of our study were to establish a method of tissue culture to obtain friable calli from different parts of the strawberry and to examine the influence of auxins and cell inoculum size on anthocyanin production.

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MATERIALS & METHODS

Plant materials and callus formation

Callus tissues were induced from the apical meristem, leaf, and petiole of *Fragaria ananassa* cv Shikinari. The excised runners (30–50 mm in length) were obtained from a greenhouse-grown strawberry. They were sonically cleaned in 70% ethanol and sterilized with a 5% sodium hypochlorite solution, and then rinsed with sterile water three times. The apical meristems (0.2 mm in length) were excised under a dissecting microscope from the runners. Leaves and stems were obtained from aseptically regenerated plants. They were placed on an LS (Linsmaier and Skoog, 1965) medium containing 3% sucrose, 0.2% Gellangum (Wako Chemical), benzyladenine (BA), and 2,4-dichlorophenoxy-acetic acid (2,4-D). The tissues were incubated at 25°C under a 16 hr-light, 8 hr-dark cycle with light intensity 800 lux. Callus tissues were transferred every 3 wk to a freshly prepared LS medium. After these calli were placed under 8000 lux for 3 wk, anthocyanin accumulation was found on the surface of the calli.

Cell suspension cultures

Cell suspension 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, and by incubating on a rotary shaker (80 rpm) under 800 lux at 25°C. Suspension cultures were grown for 3 wk, during which the medium was changed every week. The inoculation rate was about 10%. The resulting cell suspension (2g fresh) was transferred to the fresh liquid LS medium and then incubated under light intensity 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. Inoculation size (initial concentration at 1 to 10g fresh cell weight/100 mL) was also changed and the cells were cultured for 20 days under 8000 lux. To examine the influence of auxins on cell growth and anthocyanin contents, cells derived from apical meristems and precultured for 3 wk with 2,4-D (1 mg/L) and BA (0.1 mg/L) were cultured in the liquid LS medium containing naphthaleneacetic acid (NAA), indole-3-acetic acid (IAA), indolebutyric acid (IBA), or 2,4-D for 2 wk under 8000 lux.

HPLC analysis

For HPLC, fresh callus tissues from the leaf, petiole, and apical meristem were extracted for anthocyanins using the 35% solution A (acetic acid : acetonitrile:water–20:25:55) containing 0.1% trifluoroacetic acid (TFA) at 4°C. After filtration, analytical HPLC (Waters 600E) was carried out using octadecyl silica (ODS) column (Develosil ODS-5 4.6 mm ϕ × 250 mm, Nomura Chemical) eluted with a 35% solution A diluted with H_2O containing 0.1% TFA at 40°C. Anthocyanins were analyzed using a pair of JASCO 880-PU pumps equipped with a MULTI-340 photo-diode array detector. HPLC using an Asahipak-ODP column (4.6 × 250 mm) was used and eluted with a linear gradient elution from 10% to 30% aq acetonitrile (CH_3CN) containing 0.5% TFA in 30 min at 40°C.

Table 1—Influence of 2,4-D and BA on the callus formation of the strawberry leaf tissue cultured under 800 lux[a]

BA (mg/L)	2,4-D (mg / L) 0	0.1	0.5	1	2	5
0	0/5	0/5	4/5	4/5	4/5	5/5
0.1	0/5	0/5	5/5	5/5	5/5	5/5
0.5	0/5	0/5	5/5	5/5	5/5	4/5
1.0	0/5	0/5	4/5	4/5	5/5	2/5
2.0	0/5	0/5	5/5	4/5	4/5	1/5
5.0	0/5	0/5	1/5	0/5	1/5	0/5

[a] Each value represents the number of white friable calli formed out of five explants.

Table 2—Influence of light irradiation on anthocyanin accumulation on the surface of white friable calli derived from the strawberry leaf tissue under a light of 8000 lux[a]

BA (mg/L)	2,4-D (mg / L) 0.5	1	2	5
0	1/5	2/5	2/5	2/5
0.1	2/5	5/5	1/5	2/5
0.5	2/5	4/5	3/5	2/5
1.0	2/5	3/5	3/5	1/5
2.0	1/5	2/5	2/5	1/5
5.0	1/5	0/5	1/5	0/5

[a] Each value represents the number of red pigmented calli formed out of five explants.

Extraction and isolation of anthocyanin

Fresh cells (500g) were extracted overnight for anthocyanin using a solution of 0.1% TFA-methanol (MeOH) at 4°C. Cells were filtered, then the filtrate was condensed to about 20% of volume in diminished pressure and poured into an Amberlite XAD-7 column (50 × 250 mm). Pigments were eluted stepwise from H_2O to 50% aq CH_3CN containing 1% TFA. They were concentrated in the 30% fraction of CH_3CN. The solution was evaporated below 35°C, and the concentrate was purified using a preparative ODS (glass column, 20 × 1000 mm) and a methanol solution (water:acetic acid:methanol-80:15:5). Further purification was carried out using a preparative ODS stainless-steel column (20 × 250 mm, Develosil ODS-5, Nomura Chemical) with solution A diluted to 30% and containing 0.1% TFA. After freeze-drying, the pigments were used for structural identification of anthocyanin.

Acid hydrolysis of anthocyanins

The anthocyanidin nucleus was identified with acid hydrolysis of anthocyanins followed by HPLC analysis comparing with authentic samples. Anthocyanin (1mg) was dissolved in 0.5 mL of 6 N HCl–MeOH (1:1) and heated at 80°C for 6 hr. The reaction mixture was condensed *in vacuo,* filtered, and then analyzed by HPLC. The sample solution was co-injected into the HPLC column with authentic cyanidin produced by acid hydrolysis from commercially available cyanin and authentic peonidin obtained from acid hydrolysis of peonin isolated from red sepals of *Fuccia hybrida,* respectively.

Spectroscopic analysis

Purified anthocyanin from the leaf callus was dissolved in 0.01% HCl–MeOH, and its UV and visible spectra were recorded on a Hitachi UV-228 spectrometer. The ^{1}H-NMR (500 MHz) spectra were obtained on a JEOL GX-500 spectrometer in a 5 mm ϕ tube at variable temperatures using a 10% TFA*d*-CD_3OD solution as a solvent. Chemical shifts were recorded in parts per million downfield from internal tetramethylsilane (TMS) as a standard. The FAB-mass spectrum was recorded using JEOL DX304/DA5000 systems and a 1N HCl-glycerol matrix.

Determination of anthocyanin and cell growth

Fresh callus tissues were extracted overnight using a solution containing 0.1% HCl–MeOH at 4°C. After centrifugation at 1000 × *g* for 5 min, the absorbance of the clear supernatant was measured at 528 nm. Anthocyanin content was calculated with the extinction coefficient ($E^{1\%}_{1cm}$ = 680 at 528 nm) which was obtained by using purified peonidin-3-glucoside from cultured strawberry cells as a standard.

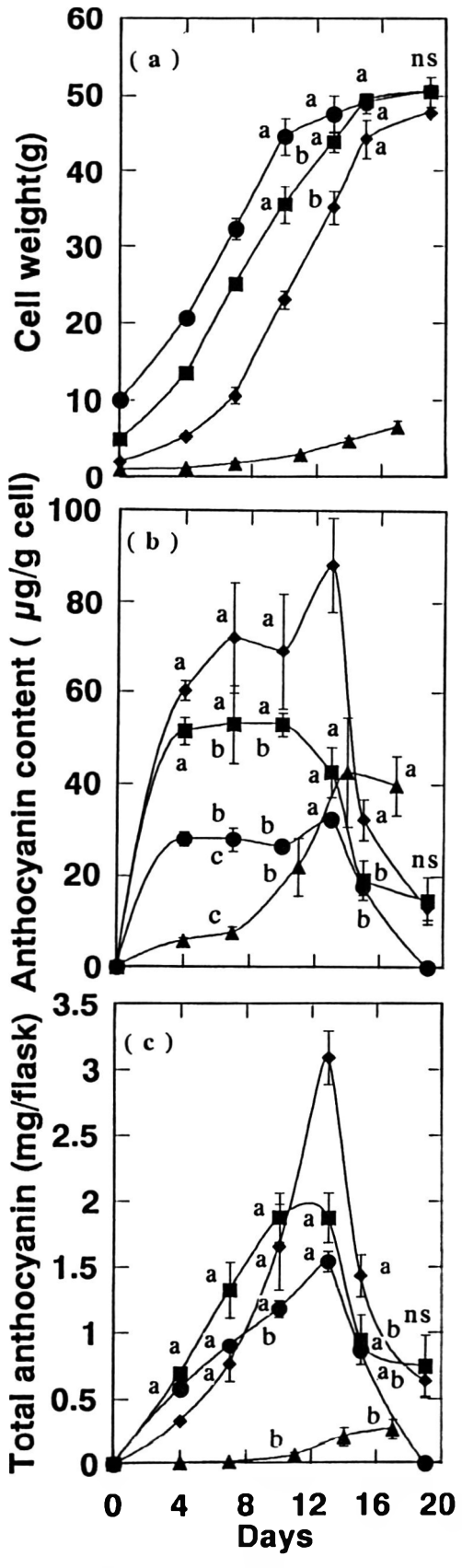

Fig. 1—Influence of inoculum sizes on cell growth and anthocyanin production in suspension cultures of strawberry. Cultures incubated on a rotary shaker (80 rpm) under continuous fluorescent light (8000 lux). Initial fresh cell weight, ●: 10g/100mL; ■: 5g/100mL; ◆: 2g/100 mL; ▲: 1g/100mL. Averages of three replicates; vertical lines represent standard error of replicates.[a] *[c] Means with same letter do not show significant difference at P = 0.01. ns: Not significant within the same day.*

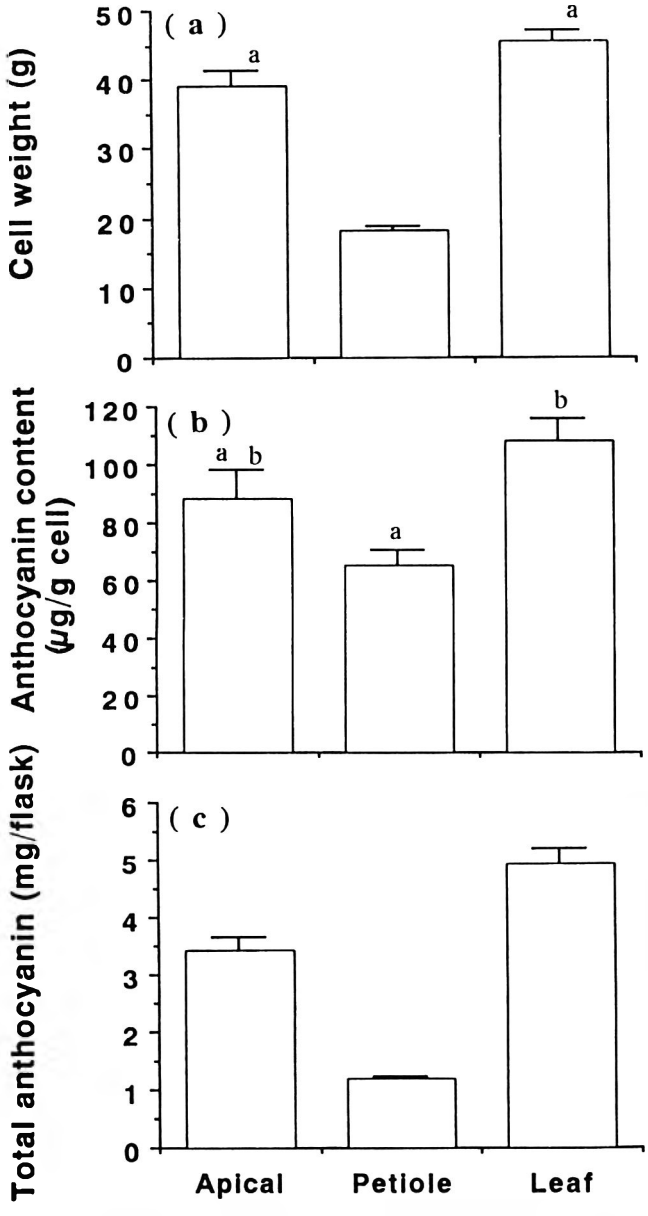

Fig. 2—Influence of various parts of strawberry on cell growth and anthocyanin production in suspension cultures. Cultures maintained in LS medium with (1mg/L) 2,4-D and (0.1mg/L) BA on a rotary shaker (80 rpm) under fluorescent light (8000 lux) at 25°C for 2 wk. Averages of 3 replicates, vertical lines represent standard error of replicates.[a-b] Means with same letter do not show significant difference at P = 0.01.

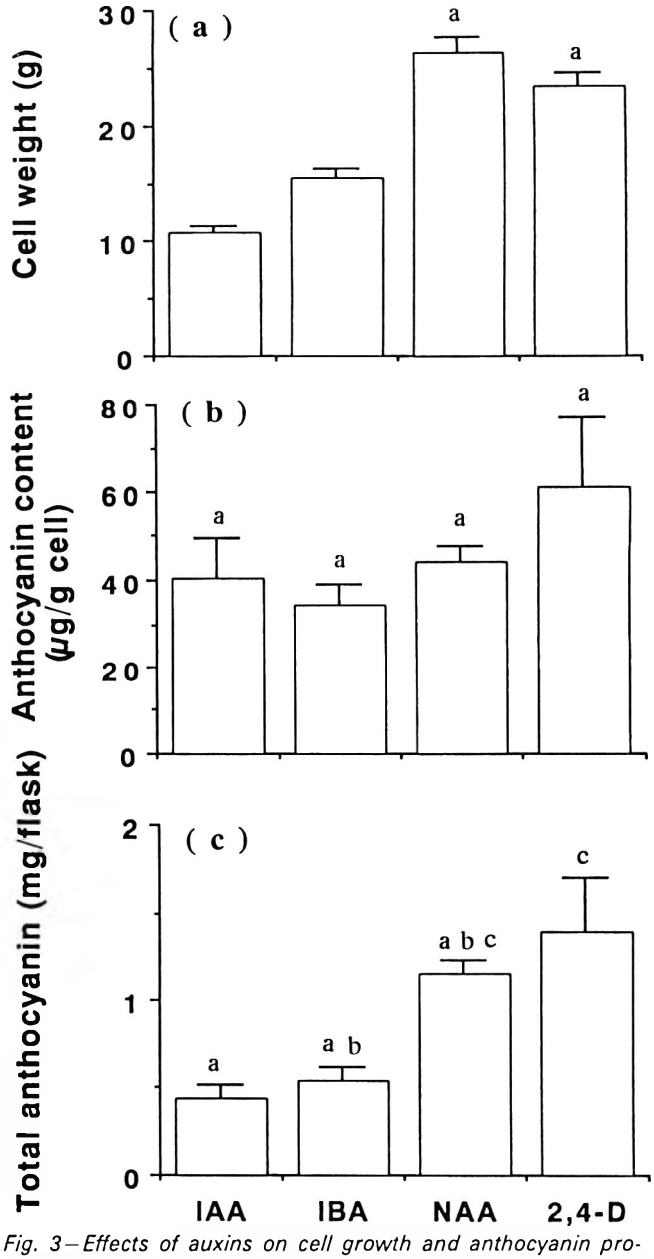

Fig. 3—Effects of auxins on cell growth and anthocyanin production in suspension cultures of strawberry cells. Cultures maintained in LS medium with auxin (1mg/L) on rotary shaker (80 rpm) under continuous fluorescent light (8000 lux) at 25°C for 2 wk. Vertical lines represent standard errors for three flasks.[a-c] Means with same letter do not show significant difference at P = 0.01.

Total anthocyanin yield was expressed as mg/100 mL of liquid LS medium in a 500 mL cell culture flask. Cells were separated from the culture medium by filtration through a nylon filter (30 µm) and then weighed. Results were expressed as fresh cell weight per flask.

Statistical analysis

Data were treated for multiple comparisons by analysis of variance with least significant difference (LSD) between means determined at the 1% level.

RESULTS & DISCUSSION

Callus induction

Different combinations of 2,4-D and BA (Table 1) after 2 months culture affected white friable calli on the agar medium. Their formation was observed only when the concentration of 2,4-D was 0.5 mg/L or higher and that of BA lower than 2 mg/L. These white friable calli were induceable when incubated at 800 lux. When placed at 3000 lux, the leaf tissue formed green or compact-surfaced calli. No differences in initiation of white friable calli were observed among leaf, apical meristem, and petiole tissues. Asahira and Kano (1977) and Hong et al. (1989) reported the induction of white friable callus from strawberry fruits. The former researchers obtained three types of calli (white friable, green firm, and pale green firm calli). The white calli were obtained in MS medium with either 0 or 0.1 mg/L of BA and either 1 or 5 mg/L of 2,4-D. The Hong group reported that the ability to initiate white friable callus was different among cultivars. They obtained the callus from "Aptos" and "Brighton" on MS medium containing various concentrations of 0.5, 1.0, 5.0, and 10 mg/L of 2,4-D.

In our present study, red pigments began to accumulate on the surface of each callus 2 wk after transfer of the white friable

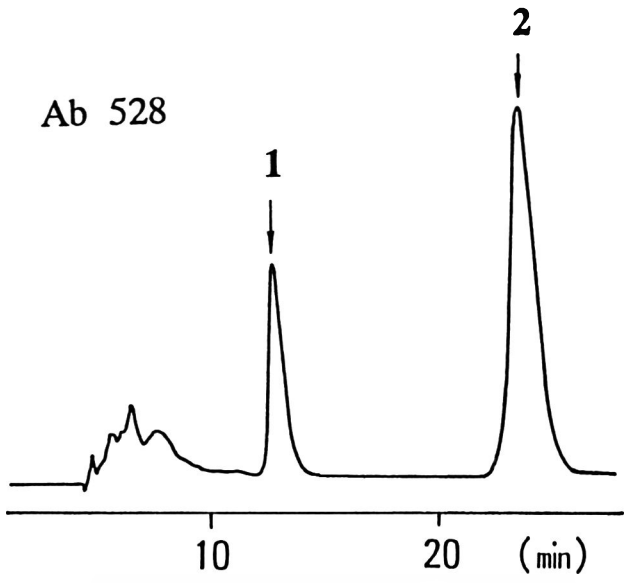

Fig. 4—HPLC separation of a crude 35% A solution extract from callus tissue derived from leaf.

Table 3—Assignment of the ^{1}H-NMR Spectra of 1 and 2. (10% TFAd-CD_3OD at 35°C)

Peak 1	9.04 (1H, S, H-4), 8.27 (1H, dd, J=9.5 & 2.5 Hz, H-6'), 8.07 (1H, d, J=2.5 Hz, H-2'), 7.04 (1H, d, J=9.5 Hz, H-5'), 6.91 (1H, dd, J=2.5 & 0.5 Hz, H-8), 6.68 (1H, d, J=2.5 Hz, H-6), 5.33 (1H, d, J=7.5 Hz, G-1), 3.70 (1H, dd, J=9.5 & 7.5 Hz, G-2), 3.57 (1H, t, J=9.5 Hz, G-3), 3.48 (1H, t, J=9.5 Hz, G-4), 3.58 (1H, ddd, J=9.5, 6.5 & 2.5 Hz, G-5), 3.73 (1H, dd, J=12.5 & 6.5 Hz, G-6a), 3.91 (1H, dd, J=12.5 & 2.5 Hz, G-6b), NOE G-1→H-4 (9% enhancement)
Peak 2	9.08 (1H, S, H-4), 8.28 (1H, dd, J=9 Hz & 2.5 Hz, H-6'), 8.26 (1H, d, J=2.5 Hz H-2'), 7.09 (1H, d, J=9.0 Hz, H-5'), 8.96 (1H, d', J=2.5 & 0.5 Hz, H-8), 6.00 (1H, b, d, J=2.5 Hz, H-6), 5.34 (1H, d, J=7.5 Hz, G-1), 4.04 (3H, S, OCH3), 3.94 (1H, dd, J=12.5 & 2.5 Hz, G-6a), 3.71 (1H, dd, J=12.5 & 6 Hz, G-6b), 3.98 (1H, dd, J=7.5 & 9.0 Hz, G-2), 3.59 (1H, ddd, J=12.5, 6, 2.5 Hz, G-5), 3.57 (1H, t, J=9.0 Hz, G-3), 3.45 (1H, t, J=9.0, G-4), NOE OCH3→H-2' (+13%), G-1-H-4 (+10%).

Table 4—Electronic Spectral Data of 1 and 2 in 0.01 % HCl–MeOH

Peak 1 (max.)	529nm,	282nm,	207nm,	E440/Emax=21%
Peak 2 (max.)	527nm,	281nm,	204nm,	E440/Emax=24%

callus from 800 to 8000 lux. In particular, a combination of 2,4-D of 1 mg/mL and BA of 0.1 mg/L induced best pigmentation (Table 2). Hong et al. (1989) obtained similar results, without testing the matrix of the phytohormones for pigment accumulation. Our study reconfirmed the formation of white friable callus and anthocyanin induction *in vitro*.

The time course and suitable inoculum sizes for anthocyanin production in suspended cultures from apical meristem (Fig. 1) indicated inoculum sizes influenced cell weight and anthocyanin production (Fig. 1a). Cell growth was remarkably stalled at inoculation levels <2 g/100 mL. The higher inoculum levels induced rapid cell growth during the first 2 wk and reached each stationary phase at about 15 days. These cells began to lyse by the end of the third week. It is important for anthocyanin production to preculture a callus for at least 3 wk under 800 lux. Bright red pigments were first observed in vacuoles of cells after 2 days in culture and they then accumulated in the cells. Anthocyanin content was remarkably influenced by inoculation size. The highest content (88 μg/g fresh cell) was obtained with 2g inoculum (Fig. 1b). In higher inoculum sizes of 5g and 10g cells, anthocyanin contents reached stationary low levels at 4 days. Anthocyanin content peaked at 2 wk in an inoculation size of 1g cells but its level was low. During the time course of period in which the anthocyanin content was monitored, a shoulder occasionally occurred (Fig. 1b). We cannot explain the reason for this. Considering total anthocyanin production per flask (Fig. 3c) inoculum size of about 2g cells was best for anthocyanin production in a 500 mL flask containing 100 mL medium. Total anthocyanin reached a maximum of 3 mg/flask at 2 wk. Inoculum sizes of 5g and 10g cells produced only about half the total anthocyanin compared with the 2g inoculum. Hong et al. (1989) reported the growth curve of cells in liquid suspension culture of strawberry fruits. That curve was almost the same as the cell growth-inoculum size relationship curve of our present study. However, the relationship between inoculum size and anthocyanin production was not established in their study.

Cell growth and anthocyanin production were compared among apical meristem, leaf and petiole tissues (Fig. 2). The petiole was considerably less effective than the other tissue parts in both cell growth and pigment formation (65.6 μg/g callus). Cell weight and anthocyanin content were almost the same between the apical meristem (88.2 μg/g callus) and the leaf callus (108.7 μg/g callus) (Fig. 2b). However, the total anthocyanin was significantly ($P<0.01$) higher in the leaf than the apical meristem (Fig. 2c). Hong et al. (1989) produced anthocyanin from immature strawberry fruits and reported the absorbance of anthocyanin content as 0.45/g callus at 520 nm. This led to an anthocyanin content of about 66 μg/g callus in their immature strawberry. These results indicated that, although anthocyanin was producible in culture from various parts of a strawberry, there were wide differences in ability for anthocyanin production between parts of strawberry. Production ability ranked as leaf, apical meristem, immature fruit, and petiole in descending order.

Of the four different auxins used, 2,4-D was most effective for anthocyanin production with the callus derived from apical meristem (Fig. 3c). It consistently stimulated cells to produce anthocyanin during 2 wk. IAA was also highly effective for anthocyanin production during only the first week (data not shown). Thereafter, anthocyanin production decreased, probably due to decomposition of IAA by light or other factors. Since 2,4-D was independently used, the total production of anthocyanin was low compared with the apical meristem in which 2,4-D was used together with BA.

Analysis of anthocyanin

Eight kinds of anthocyanins were detected with HPLC at 528 nm in all cultured callus tissues used. Two variations were isolated (Fig. 4) by using ODS column (Yoshida et al., 1990). Peak 1 anthocyanin (1) and peak 2 anthocyanin (2) comprised almost 15% and 70% of the total anthocyanin, respectively. No differences in anthocyanin composition and the number of major peaks were found among these calli, indicating that the control of the enzymatic function for anthocyanin production in cells was almost the same by dedifferentiation. Electronic absorption spectra (λ_{max}520 nm in 0.01N HCl-MeOH) of the pigments suggest that the nuclei of chromophores were cyanidin and/or peonidin. The structures of aglycone of 1 and 2 were determined by acid hydrolysis followed by HPLC analysis. By acid hydrolysis 1 gave cyanidin and 2 gave peonidin. FABMS of 1 gave a molecular ion peak (M^+) at m/z 449. The ^{1}H-NMR spectrum of 1 showed the presence of one cyanidin nucleus and one hexose. On the basis of H-4 (9.01, S), all of the signals at the aromatic moiety could be assigned by a homo spin decoupling experiment; the vicinal coupling of the sugar signals was $J_{1,2} \cong 7.5$ Hz, $J_{2,3} \cong J_{3,4} \cong J_{4,5} \cong 9.5$ Hz, indicating that the sugar was a β-D-glucopyranoside. By irradiation of the anomeric proton we observed +10% NOE at H-4, indicating that the linkage position of the sugar was OH at the 3-position of the nucleus. Consequently, 1 must be cyanidin-3-glucoside.

A molecular ion peak of 2 appeared at m/z 463. The NMR spectrum was very similar to that of 1 except for the presence of one CH_3O-. By irradiation of CH_3O and the anomeric proton we observed +15% NOE at H-2′ and +10% at H-4, respectively, indicating that the anthocyanidin was peonidin and the sugar was attached to OH at 4-position of the nucleus. These results confirmed that 2 is peonidin-3-glucoside. Each proton signal and the electronic spectral data are shown (Table 3 and 4, respectively).

There have been many studies on identification of anthocyanin pigments in intact strawberries. The main pigment has been identified as pelargonidin-3-glucoside (88%) and the second major pigment as cyanidin-3-glucoside (12%) (Wrolstad and Putnam, 1969). Hong and Wrolstad (1990) also reported relative percentages of cyanidin (24%) and pelargonidin (75%) based on total peak area from HPLC. However, the main anthocyanin produced in the present tissue cultures was a peonidin-derivative.

Although both pelargonidin- and cyanidin-type anthocyanins have been reported as major pigments in intact strawberry fruits, it is interesting that the callus tissues of strawberries were capable of producing peonidin- and cyanidin-type anthocyanins. Yamakawa et al. (1983) reported a difference in anthocyanin composition between cultured cells and intact organs in *Vitis* sp. They found that the intact pericarp contained malvidin- and peonidin-glucoside, whereas callus tissues from grape vines produced cyanidin- and peonidin-glucoside. Mizukami et al. (1988) also reported the difference in anthocyanin compositions between cultured cells and intact organs. Those reports suggested that the shikimic acid channel for producing anthocyanin was inhibited by an unknown factor during callus formation. As a result, peonidin- and cyanidin-type anthocyanins may be produced in the growth of callus tissues.

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Volatile Compounds from Raspberry and Strawberry Fruit Inhibit Postharvest Decay Fungi

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ABSTRACT

Fifteen volatiles released by red raspberries and strawberries during ripening were analyzed for the inhibition of postharvest decay fungi. Five compounds completely inhibited all fungi directly on fruit at 0.4 μL/mL.. Of these, benzaldehyde at 0.04 μL/mL completely inhibited isolated cultures of *Alternaria alternata*, *Botrytis cinerea*, and *Colletotrichum gloeosporioides*, while 1-hexanol, *E*-2-hexenal and 2-nonanone inhibited all three fungal species at 0.1 μL/mL. When added directly to media, 0.1% (v/v) benzaldehyde or *E*-2-hexenal inhibited all three species. Starch encapsulated 2-nonanone reduced fungal decay of raspberries and strawberries in enclosed containers after 7 days at 10°C.

Key Words: raspberries, strawberries, postharvest decay, flavor, modified atmosphere

INTRODUCTION

FRESH MARKET availability of small fruits such as raspberries (*Rubus idaeus* L.) and strawberries (*Fragaria ananassa*, Duchesne) is restricted due to rapid deterioration from fruit senescence and diseases after harvest. Several approaches for prolonged shelf-life have been used, including harvesting at earlier stages of maturity than are ideal for consumption (Sjulin and Robbins, 1987); controlled atmospheres and temperatures (Goulart et al., 1992; Sommer, 1985); gamma irradiation (Thomas, 1986); and biological control (Janisiewicz, 1988). The principal method of controlling postharvest diseases of such fruit is suppression of inoculum production and subsequent infection of the flowers and developing fruit (Eckert and Ogawa, 1988). Several fungal species (including *Alternaria alternata*, *Botrytis cinerea* and *Colletotrichum* spp.) constitute the majority of postharvest pathogens on small fruit and berries (Eckert and Ogawa, 1988). Preharvest applications of fungicides have been used to control postharvest fungal decay (Freeman and Pepin, 1977). However, the use of certain fungicides to control grey mold caused by *B. cinerea* has increased the frequency of diseases caused by *Mucor* spp. and *Rhizopus stolonifer* (Eckert and Ogawa, 1988). Additionally, strains of *B. cinerea* have developed resistance to several classes of fungicides (Eckert and Ogawa, 1988; Vali and Moorman, 1992). Postharvest application of fungicides is limited to adverse effects due to wetting the fruit and by stringent federal and stage regulations concerning use of available fungicides (Eckert and Ogawa, 1988).

Red raspberries and strawberries release many volatile compounds during ripening (Hirvi, 1983; Hirvi and Honkanen, 1982; Honkanen et al., 1980; Larsen and Poll, 1990; Pyysalo, 1976; Pyysalo et al., 1979). Some such compounds have antifungal activities (Fries, 1973; Pauli and Knoblauch, 1987; Farag et al., 1989; Hitokoto et al., 1980; Wilson et al., 1987). Volatile C_5–C_9 aldehydes in mature citrus fruit inhibited *Penicillium digitatum* (Davis and Smoot, 1972). Natural benzaldehyde was reported to protect peaches from *Rhizopus* rot (Wilson and Wisniewski, 1989). Acetaldehyde vapor decreased decay in raspberries and strawberries (Pesis and Avissar, 1990; Prasad and Stadelbacher, 1973, 1974). Although many such compounds are effective fungal inhibitors at relatively low concentrations, none is used commercially to prevent or delay fruit decay. Such natural volatile compounds may function as effective antifungal agents if sufficient concentrations could be maintained in the gas headspace surrounding the fruit. The objectives of our study were to evaluate 15 volatile odor compounds (which represented several chemical classes) released by raspberries and/or strawberries for inhibiting fruit decay fungi (*A. alternata*, *B. cinerea*, and *Colletotrichum gloeosporioides*) and to determine if they might be potential commercial antifungal compounds.

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MATERIALS & METHODS

Fungal cultures

Fungal cultures of *A. alternaria*, *B. cinerea* were obtained from the NCAUR culture collection. These cultures exhibited positive pathogenicity on healthy raspberries and strawberries. An isolate of *C. gloeosporioides* pathogenic on blueberries was obtained from the USDA/ARS Cranberry and Blueberry Research Center, Chatsworth, New Jersey. All cultures were maintained at 24°C and 95% relative humidity on V-8 juice® agar supplemented with 0.3% $CaCO_3$ and adjusted to pH 5.8. Spores of each fungus were removed from sporulating cultures by carefully brushing the surface of the media with a glass rod and suspending the spores in sterile water. The concentrations of spores in the suspensions were adjusted to concentrations of 10^6 to 10^7 spores/mL. The suspensions were mixed together to form a composite suspension which was used to inoculate all fruit in a given experiment.

Fruit

Untreated red raspberries (cv. 'Heritage') and strawberries (cv. 'Ozark Beauty') were harvested and used the same day. Berries were selected on the basis of uniform size, color, firmness, and were free of evident defects or diseases.

Bioassay system

A bioassay system used to test the effect of volatile compounds on fungal growth, both on intact fruit and on V-8 juice medium, was developed. The apparatus consisted of 75 mL glass jars into which either fruit or sterilized V-8 juice medium (10 mL) were added. These jars were placed in 275 mL airtight glass jars with aluminum foil cap liners which contained one 5.5 cm filter paper disk (Whatman No. 1) onto which appropriate amounts of the test compounds were added. The total gas headspace in the larger jars after addition of all components was 250 mL.

Treatment of inoculated fruit with volatiles

Fifteen compounds reported as major volatile components of red raspberries and/or strawberries and representing several chemical classes were evaluated for suppression of fungi directly on the fruit. These included aldehydes (benzaldehyde and *E*-2-hexenal), alcohols (benzyl alcohol, eugenol, geraniol, 1-Hexanol, *Z*-3-hexanol, linalool, and *Z*-3-nonen-1-ol), ketones [α- and β-ionone, 2-nonanone, and raspberry ketone (4-(4-hydroxyphenyl)-2-butanone)], an ester (methyl cinnamate) and a heterocycle containing both an alcohol and ketone moiety (furaneol; 2,5-dimethyl-4-hydroxy-3*(2H)*-furanone). These compounds were selected on the basis of preliminary screening of com-

pounds which had suitable volatility, low mammalian toxicity, and commercial availability. All chemicals were obtained from the supplier (Aldrich Chem. Co., Milwaukee, WI) and used without further modification. Red raspberries and strawberries were rinsed with sterile distilled water and allowed to dry before inoculation. Fruit was sprayed with the spore suspension to runoff. Single red raspberries or strawberries were placed in the 75 mL jars, and 100 μL each of a single test compound was added to a 275 mL jar and sealed. Fruit were kept at 10°C in the dark for 10 days then rated for development of fungi and for damage to fruit due to the treatments. Categories based on percentage of fruit surface area visibly covered with fungi were: 0, no detectable fungal growth; 1, 0–25% of fruit covered with mycelia; 2, 25–75% of fruit covered with mycelia; 3, > 75% of fruit covered with mycelia. Fruit damage rating categories were: 0, no detectable damage; 1, some surface damage; 2, moderate fruit damage with fluid loss; 3, extensive necrosis and heavy fluid loss. Each treatment was replicated five times and the experiment was repeated. As results from both experiments were similar, only results from one experiment are reported.

Treatment of isolated cultures with volatiles

Compounds which completely inhibited fungal growth on fruit at 0.4 μL/mL were benzaldehyde, 1-hexanol, 2-hexenal, 3-hexen-1-ol and 2-nonanone. These were tested to determine threshold levels to completely inhibit isolated fungal cultures growing on V-8 juice medium as volatiles, or when added directly to media. Compounds were bioassayed as volatiles by placing 7 mm diameter plugs from mycelia from actively-growing 1 wk-old cultures into the 75 mL jars on V-8 juice media, with the appropriate amount (0, 5, 10, 25 or 100 μL) of each compound added to the filter paper. The sealed jars were then placed in a growth chamber in the dark at 10°C for 7 days. Growth of fungi was on the plug only in many cases. Due to the difficulty of accurately measuring fungal growth in the jars, a rating system was developed as follows: 0, no growth of fungi, including plug; 1, growth on plug only; 2, small amount of fungal growth on medium; 3, most of medium covered with fungal mycelia; 4, all of medium covered. Compounds were assayed as media components by adding appropriate amounts of each compound to cooled V-8 juice media in 9.0 cm plastic petri dishes. Experiments were initiated by placing 7 mm plugs of mycelia in the center of the petri dishes. As the cultures were nearly circular, the radii (from the inoculum plug to one typical point on the growing margin) of the cultures were measured and expressed as a percentage of the control. In both cases, treatments were replicated five times and experiments were repeated before statistical analysis using analysis of variance. Data represent the results of single experiments as results were similar for both types of bioassays.

Starch encapsulation

Starch encapsulation of 2-nonanone to allow fast release of this compound was accomplished by thoroughly mixing 5g of 2-nonanone with 15g Miragel (pregelatinized cornstarch, A.E. Staley, Decatur, IL). Also a slow release formulation was made by mixing 15g Miragel with 2.5g glycerine followed by 5g of 2-nonanone. The resultant mixtures were packed into glass tubes (0.5 cm × 2.0 cm) and heat-sealed in polypropylene plastic bags (polypropylene allowed vapor phase 2-nonanone to pass which could be detected by gas chromatography; data not presented). Single tubes were placed in airtight 2.45L desiccator flasks which were fitted with a septa allowing headspace gas samples to be removed.

Headspace gas analysis

Gas headspace samples (1 mL) were collected with a 1 mL gastight syringe (Dynatech Precision Sampling Co., Baton Rouge, LA) and were analyzed by a Hewlett Packard Model 5890 gas chromatograph (Hewlett-Packard Co., Palo Alto, CA) using a capillary column (15 m × 0.25 mm) coated with 0.25 μm film (DB-1; J&W Scientific, Folsom, CA). Helium gas carrier flow through the column was 1 mL/min and the sample was injected into an 100/1 inlet splitter (total flow 100 mL/min). The injection temperature was set at 180°C, the flame ionization detector was set at 290°C and the oven was at 75°C. Peak areas were quantified by comparison to a standard curve prepared from the response of a range of dilutions of known concentrations of 2-nonanone in hexane.

Table 1—Fungal growth and fruit damage ratings of inoculated raspberry and strawberry fruit after 10 days exposure to 0.4 μL/mL volatile compounds

	Fungal rating[z]		Fruit damage rating[y]	
Treatment	Raspberry	Strawberry	Raspberry	Strawberry
Control	2.4 b[x]	3.0 a	2.0 b	2.7 ab
Benzaldehyde	0.0 f	0.0 e	0.7 c	1.3 cde
Benzyl alcohol	0.7 e	3.0 a	0.7 c	0.3 fg
Eugenol	2.7 a	3.0 a	3.0 a	2.7 ab
Furaneol	1.8 bc	2.3 c	0.7 c	0.3 fg
Geraniol	2.5 ab	2.3 c	2.3 b	2.0 bc
1-Hexanol	0.0 f	0.0 e	1.0 c	1.0 def
E-2-Hexenal	0.0 f	0.0 e	3.0 a	3.0 a
Z-3-Hexen-1-ol	0.0 f	0.0 e	3.0 a	3.0 a
α-Ionone	2.2 b	2.8 ab	1.0 c	0.7 efg
β-Ionone	2.0 b	3.0 a	1.0 c	0.3 fg
Linalool	1.8 bc	2.2 c	2.3 b	2.7 ab
Methyl cinnamate	1.3 d	1.2 d	0.7 c	0.7 efg
2-Nonanone	0.0 f	0.0 e	2.0 b	1.7 cd
Z-3-Nonen-1-ol	2.8 a	2.7 ab	1.0 c	1.0 def
Raspberry ketone	0.7 e	1.5 d	0.0 d	0.0 g

[z] Mean of fungal growth, 0 = no fungi present, 3 = fruit completely covered by fungal mycelia.

[y] Mean of fruit damage, 0 = intact, undamaged fruit, 3 = whole fruit necrotic and watersoaked.

[x] Mean separation among all values by Duncan's multiple range test, $p < 0.05$.

RESULTS & DISCUSSION

Bioassay system

The bioassay system was simple to use and provided a convenient means of sampling large numbers of volatiles simultaneously. Teflon cap liners were originally used instead of aluminum foil, but they were found to leak, and did not hold up well under autoclaving. We could also fit rubber septa onto the caps of the 275 mL jars so that headspace gases could be sampled for GC analysis.

Effect of volatiles on inoculated fruit

Results from exposure of inoculated raspberries and strawberries to the 15 compounds were compared (Table 1). Five compounds, benzaldehyde, 1-hexanol, *E*-2-hexenal, *Z*-3-hexen-1-ol and 2-nonanone, completely suppressed fungal growth on both raspberry and strawberry fruit. Benzaldehyde, 1-hexanol and 2-nonanone treatments slightly damaged fruit while E-2-hexenal and Z-3-hexen-1-ol treatments caused extensive tissue necrosis. E-2-hexenal, which is formed via the lipoxygenase pathway in plants, has been shown to inhibit germination of *A. alternata* and *B. cinerea* spores (Hamilton-Kemp et al., 1992). Benzyl alcohol suppressed fungal growth on raspberries but not on strawberries, while methyl cinnamate and raspberry ketone [4-(4-hydroxyphenyl)-butan-2-one] partially suppressed fungal growth on both types of fruit. Two of the treatments (eugenol and Z-3-nonen-1-ol) promoted greater fungal growth on raspberry fruit as compared to the control ($P < 0.05$). This may have been due to low antifungal activities of those compounds together with increased tissue damage which promoted fungal growth.

Inhibition of fungal cultures

The five compounds that completely inhibited fungi directly on fruit were tested to determine threshold levels for inhibition (Table 2). *B. cinerea* and *C. gloeosporioides* cultures were inhibited at lower concentrations than were *A. alternata* cultures. Among the five compounds, benzaldehyde and E-2-hexenal were the most toxic to all three fungal species, while Z-3-hexen-1-ol did not completely suppress growth when < 0.4 μl/ml.

Added directly to the media, the compounds were considerably less toxic than as volatiles (Table 3). While all the compounds inhibited fungal mycelia at 1.0% levels, only benzaldehyde and E-2-hexenal completely suppressed mycelial

Table 2—Response of three fungal species to vapor phase concentrations of volatile odor compounds

Treatment µL/mL	Fungal growth rating[z]		
	A. alternata	*B. cinerea*	*C. gloeosporioides*
Control	4.0 a[y]	4.0 a	4.0 a
Benzaldehyde			
0.02	2.3 de	0.0 e	0.0 c
0.04	0.0 g	0.0 e	0.0 c
0.10	0.0 g	0.0 e	0.0 c
0.40	0.0 g	0.0 e	0.0 c
1-Hexanol			
0.02	4.0 a	2.0 c	2.0 b
0.04	3.1 bc	0.0 e	0.0 c
0.10	0.0 g	0.0 e	0.0 c
0.40	0.0 g	0.0 e	0.0 c
E-2-Hexenal			
0.02	1.3 f	0.0 e	0.0 c
0.04	1.3 f	0.0 e	0.0 c
0.10	0.0 g	0.0 e	0.0 c
0.40	0.0 g	0.0 e	0.0 c
Z-3-Hexen-1-ol			
0.02	3.6 ab	4.0 a	4.0 a
0.04	3.5 ab	3.3 b	2.6 b
0.10	1.7 ef	0.7 d	0.0 c
0.40	0.0 g	0.0 e	0.0 c
2-Nonanone			
0.02	2.7 cd	1.0 d	2.0 b
0.04	1.3 f	0.0 e	0.6 c
0.10	0.0 g	0.0 e	0.0 c
0.40	0.0 g	0.0 e	0.0 c

[z] Mean of fungal growth, 0 = no growth, 4 = media completely covered.
[y] Mean separation among all values by Duncan's multiple range test, $p < 0.05$.

growth at 0.1%. The decrease in toxicity (about one order of magnitude) compared to the same compounds in the volatile state, may have been due to low solubilities. This would decrease the amount of the chemical that could come in contact with the fungi.

Examination of encapsulated 2-nonanone

2-Nonanone, while not as fungitoxic as several other compounds, was selected as a starch-encapsulated, slow release antifungal compound which might be used commercially to prevent premature decay of stored fruits. Reasons for selection of 2-nonanone over more potent compounds include: low mammalian toxicity (oral rat LD_{50}, 3200 mg/kg) (NIOSH, 1979); a pleasant, fruity/floral odor; resistance to rapid decomposition (benzaldehyde, E-2-hexenal and Z-3-hexen-1-ol are readily oxidized to less volatile and/or fungitoxic compounds); and adequate volatility. Benzaldehyde, while previously reported to be antifungal, lent unmistakable odor and taste sensations when used at antifungal levels. The results from storing red raspberries and strawberries in airtight containers for 1 wk at 10°C with both slow and fast release formulations were compared (Table 4). Although the fast release formulation had a higher level of 2-nonanone after 24 hr, both treatments contained the same headspace concentration after 7 days. The treated berries had no visible fungi, and no fruit damage, although fruit had a slight odor of 2-nonanone when first removed from the flasks. Sensory analyses by a taste-panel were not conducted but the berries had little off-flavor as judged by informal lab bench screening.

Commercial potential of slow-release, antifungal volatiles

The commercial potential for natural, antifungal compounds would depend upon several factors, including quality of treated fruit, consumer acceptance and added costs. Gamma radiation has been examined for use in the extension of shelf life of perishable fruits (Thomas, 1986). Two major obstacles in commercial use of gamma radiation have been consumer acceptance and damage to fruit at dosage levels which effectively control pathogens. Additionally, irradiated foods contained and/

Table 3—Radial growth of fungal cultures on media with added volatile compounds

Treatment (%)	Fungal growth (% of control)[z]		
	A. alternata	*B. cinerea*	*C. gloeosporioides*
Benzaldehyde			
0.01	91.7 ab	99.1 a	93.9 abc
0.10	0.0 e	0.0 e	0.0 f
1.00	0.0 e	0.0 e	0.0 f
1-Hexanol			
0.01	98.3 a	98.6 a	93.3 abc
0.10	70.0 c	47.9 d	69.5 d
1.00	0.0 e	0.0 e	0.0 f
E-2-Hexenal			
0.01	92.6 ab	88.1 bc	35.4 e
0.10	0.0 e	0.0 e	0.0 f
1.00	0.0 e	0.0 e	0.0 f
Z-3-Hexen-1-ol			
0.01	95.2 ab	93.0 b	81.7 cd
0.10	89.1 b	87.9 bc	84.2 bc
1.00	0.0 e	0.0 e	0.0 f
2-Nonanone			
0.01	85.2 b	100.0 a	100.4 a
0.10	48.7 d	83.0 c	95.7 ab
1.00	0.0 e	0.0 e	0.0 f

[z] Mean separation among all values by Duncan's multiple range test, $p < 0.05$.

Table 4—Response of raspberry and strawberry fruit after exposure to encapsulated 2-nonanone for 7 days at 10°C

Treatment	Headspace concentration (µg/mL) Of 2-nonanone ± S.E.	Fruit damage rating[z]	
		Raspberry	Strawberry
Control (7 days)	0.00 ± 0.00	2.3 a[y]	2.5 a
Slow release (24 hr)	0.04 ± 0.01	0.0 b	0.0 b
Fast release (24 hr)	0.11 ± 0.02	0.2 b	0.0 b
Slow release (7 days)	0.13 ± 0.01	0.4 b	0.0 b
Fast release (7 days)	0.13 ± 0.00	0.4 b	0.0 b

[z] Mean of fruit damage, 0 = intact, undamaged fruit, 3 = fruit completely necrotic and water-soaked.
[y] Mean separation among all values by Duncan's multiple range test, $p < 0.05$.

or released many radiolytic products, some of which had high mammalian toxicities or contributed to off-flavors (Nawar, 1986). Modified atmosphere packaging (MAP) using plastic films decreased strawberry fruit decay but contributed to off-odors and flavors (Shamaila et al., 1992). Those researchers also reported that unpackaged strawberries had the highest overall levels of desirable sensory attributes at all storage times tested, but the berries were infected with fungi (species unreported) after 6 days. Therefore it appeared that if normal O_2 and CO_2 levels could be maintained while fungal growth was being prevented, optimum sensory qualities would be maintained. Partially ventilated packaging to allow O_2 and CO_2 levels to remain near ambient levels while maintaining adequate levels of antifungal compounds might prevent decay without production of off-flavors and odors.

CONCLUSIONS

OF 15 VOLATILE compounds released by ripening red raspberries and strawberries, five completely inhibited growth of three common fungal species at 0.4 µl/ml. 2-Nonanone, selected for examination as an encapsulated slow-release compound, inhibited fungal growth on intact fruit without fruit damage. Because it has low mammalian toxicity, possesses a fruity/floral odor and resists rapid decomposition, it has potential for commercial development as a slow-release fungistatic compound.

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Control of Browning During Storage of Apple Slices Preserved by Combined Methods. 4-Hexylresorcinol as Anti-Browning Agent

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ABSTRACT

Effectiveness of 4-hexylresorcinol to inhibit enzymatic and nonenzymatic browning in apple slices preserved by combined methods was assessed during storage at four temperatures. Sodium sulfite and ascorbic acid-2-phosphate were used for comparison. Browning as measured by reflectance spectroscopy and based on visible light absorption was partially inhibited by 4-hexylresorcinol comparable to that of a fivefold sulfite concentration at 25°C. At temperature above 35°C, the inhibiting effect of 4-hexylresorcinol was minimal. Energy of activation of the browning reaction was 5–20 kcal/mol and was not affected by anti-browning treatment. Only induction time was delayed by the 4-hexylresorcinol and sulfite treatments.

Key Words: apples, browning, storage temperatures, 4-hexylresorcinol

INTRODUCTION

COMBINED METHODS of preservation restrain microbial growth through a combination of inhibition factors, such as low pH, water activity (a_w), and anti-microbial agents. A common characteristic of intermediate moisture foods (IMF) and combined methods is the incorporation of solutes, generally salt or sugar, to decrease the a_w of the food. The target is a range that, in conjunction with control of pH (with organic acids) and bacteriostatic and fungistatic agents (e.g. sorbic acid) would prevent growth of bacteria, yeast and mold (Karel, 1975; Levi et al., 1985; Leistner 1985; Bolin and Huxsoll, 1989; Alzamora et al., 1989; Cerruti et al., 1990; Rojas et al., 1991). Enzymatic and nonenzymatic browning reactions are the main factors that limit acceptance of product preserved by such combined method approach. An appropriate anti-browning agent is a key factor to enable production of non-refrigerated shelf-stable fruit products using combined method techniques.

Sulfiting agents have been the conventional chemicals to inhibit browning reactions in fruits and vegetables. However, there have been concerns over the possible harmful effects of sulfiting agents on sensitive consumers, particularly asthmatics, (Taylor et al., 1986). Consequently, the need for safer anti-browning agents has been sought to prevent or minimize the characteristic enzymatic and nonenzymatic browning of fruits and vegetables. The search for sulfite replacers has been promising. Several alternatives have been proposed to reduce browning in fresh and processed fruits and vegetables (Montgomery, 1983; Dziezak, 1988; Langdon, 1987; Pointing et al., 1972; Sapers et al., 1989).

Agents to control enzymatic browning include ascorbic acid derivatives and isomers (Sapers et al., 1991, 1990; Sapers and Ziolkowski, 1987; Bolin and Steele, 1987) and proteases, specifically ficin, to inactivate polyphenoloxidase (Labuza, 1989).

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Wide varieties of sulfite substitutes have lacked the versatility of sulfiting agents in controlling both enzymatic and nonenzymatic browning. A different approach to anti-browning compounds, resorcinol derivatives, have been described and patented (McEvily et al., 1991a, b; Frankos et al., 1991). Of the family of resorcinol derivatives, 4-hexylresorcinol has been effective in preventing shrimp blackspot due to polyphenoloxidase activity (McEvily et al., 1991b). Thus, 4-hexylresorcinol has commercial potential to control enzymatic browning mediated by polyphenoloxidase. 4-hexylresorcinol has a long history of use in pharmaceuticals and exhibits no systemic toxicity (Frankos et al., 1991). In addition, a low concentration of 4-hexylresorcinol, up to 25-fold lower than sulfites, was required to produce comparable shrimp blackspot inhibition (McEvily et al., 1991b).

The objective of our study was to examine the effectiveness of 4-hexylresorcinol as an anti-browning agent during storage of apples processed by combined methods as compared with other anti-browning agents that have been effective in preventing either enzymatic or non-enzymatic browning of fruits.

MATERIALS & METHODS

Fruit samples

'Red delicious' (*Malus domestica*, Borkh) apples were obtained from a commercial orchard in Yakima, Wash. Apples were placed in controlled atmosphere storage (1% O_2, 1% CO_2, 95% humidity, and 1°C) until used. For experimental purposes, fruit of similar firmness and solid content (12–14° Brix) were used. The storage time of the fresh apples in the controlled atmosphere chamber did not exceed 3 mo in any case.

Preparation of apple slices by combined method

Individual syrup solutions were prepared to contain the following anti-browning treatments:(a) 0.02% (w/v) hexylresorcinol (OPTA Food Ingredients, Inc., Cambridge, MA) and 0.25% (w/v) ascorbic acid, (b) 1% (w/v) ascorbic acid-2-phosphate (Nutri-Quest, Inc, Chesterfield, MO) and 0.25% (w/v) ascorbic acid, (c) 0.1% (w/v) sodium sulfite and 0.25% (w/v) ascorbic acid. A concentration of 0.02% (200 ppm) 4-hexylresorcinol was used because previous experience in our laboratory had shown best effectiveness at that concentration and also to minimize residual inhibitor in the fruit. In addition to the anti-browning agents, all sugar solutions included the general formulation of 0.2% (w/v) citric acid, 0.15% (w/v) sorbic acid and sucrose to make up a 52°Brix syrup. The general solution, was the blank, and that formula plus 0.25% (w/v) ascorbic acid was the control treatment. Control samples were included in all comparisons to estimate by difference the effectiveness of the resorcinol derivative.

Medium to large size apples were simultaneously peeled, cored and sliced by a mechanical apple peeler. The device produced uniform 1 cm thick apple slices. These were immediately placed in glass beakers with the sugar solution. Beakers of apple sections were transferred to a constant temperature orbital water bath shaker (Magni Whirl, GS Blue M Electric, Blue Island, IL.) and agitated at 150 rpm.

An inverted glass funnel was placed on top of the apple slices to keep the fruit continuously submerged in the sugar solution. The solutions were used with the fruit in a 4:1 ratio (w/w). The apple slices

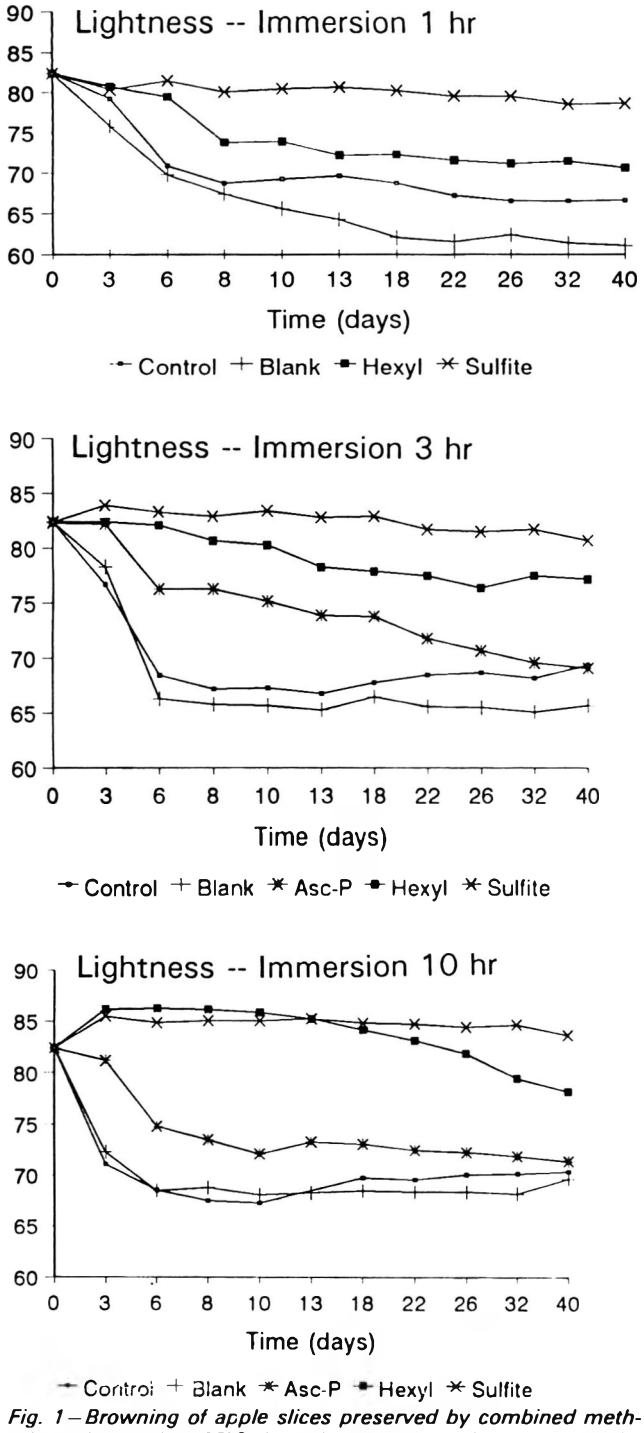


Fig. 1—Browning of apple slices preserved by combined methods and stored at 25°C. L values were used to measure the progress of browning.*

were osmotically treated by leaving them immersed for 1, 3 and 10 hr at 30°C. Upon removal, the treated slices were immediately blotted with absorbent tissue to remove excess solution. For each combination of anti-browning and immersion treatment (5x3), four sets of three apple slices were individually packaged in 2 mil thick plastic pouch. Each pouch was hermetically sealed under partial vacuum to maintain a minimal head space and to facilitate further color measurements. For statistical purposes, the three apple slices contained in each pouch were obtained from different apples. For storage studies, the pouches were stored up to 75 days in incubators (Precision Scientific Co.) at 25, 30, 35 or 45°C.

Browning measurements

Pouches containing apple slices were removed from the incubators and allowed to stand 30 min at room temperature (≈23°C) prior to color measurements. The surface color of each apple slice was measured using a Minolta CM-2002 Spectrophotometer (Minolta Camera Co., LTD, Chuo-Ku, Osaka 541, Japan) with 11 mm measurement aperture. The L*, a*, b* values were measured by the instrument (CIELAB for a 2° Standard observer) from which the metric saturation Suv (CIELUV) was calculated. Measurements were made through the plastic pouch at four fixed places on the apple slice. Each pouch contained three slices from three different apples. Thus, 12 readings were recorded from each of the 60 anti-browning-immersion, time-storage, temperature combinations (5x3x4).

Table 1—Lightness values for anti-browning treatments of apple slices preserved by combined methods and stored at different temperatures

Treatment comparison	Differences between means for L* value[a]			
	25°C	30°C	35°C	45°C
Sulfite-4 hexylresorcinol	3.2	2.9	5.9	2.6
Sulfite-asc. acid-2-P	7.8	6.7	8.5	3.8
Sulfite-control	11.9	9.1	10.5	6.4
Sulfite-blank	13.9	12.3	13.5	8.4
4 hexyl-asc. acid-2-P	4.6	3.9	2.6	1.1
4 hexyl-control	8.7	6.2	4.6	3.8
4 hexyl-blank	10.7	9.4	7.6	5.8

[a] All means are significant at the 0.01 level.

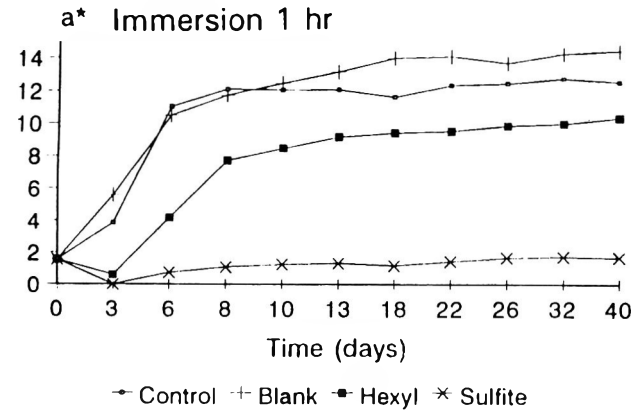
Statistical analysis

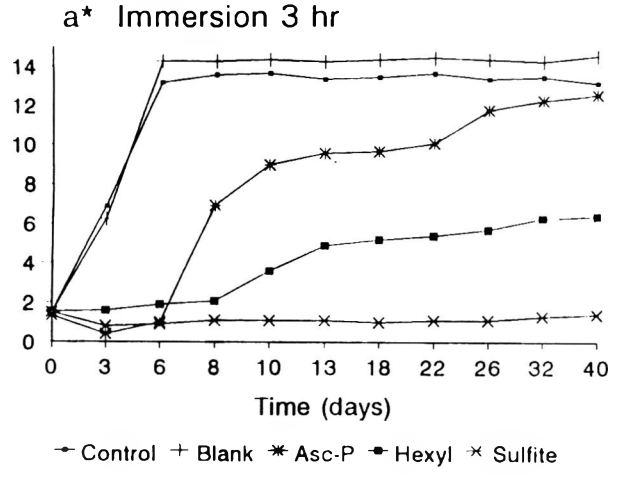
Analysis of variance was used to determine treatment response on browning using the GLM procedures of the Statistical Analysis System (SAS Institute, 1985).

RESULTS & DISCUSSION

THE SOLID CONTENT of treated fruit, for all treatments, increased from 12°Brix (fresh) to 29–30°Brix. The titratable acidity (as % malic acid) increased from 0.21–0.25% (fresh) to 0.55–0.58% while pH remained stable at 3.9–4.2. Slight changes in pH were observed (about 0.5 unit increase), in those treated samples that browned notably during storage.

Browning of the apple slices was measured by L* (lightness, a*(green-red), and Suv (metric saturation). A decrease in L* value and an increase in a* and Suv values are indicative of browning (Petriella et al., 1985; Bolin and Steele 1987; Sapers et al., 1989). No heat treatment was given to the apple slices; thus enzymatic activity of polyphenoloxidase was assumed. Four treatments, excluding the blank, were used for comparision to explore effectiveness of 4-hexylresorcinol as an anti-browning agent. For the L* changes of the apple slices during storage at 25°C, (Fig 1) only 40 days are reported since beyond that point the value remained generally unchanged except at higher storage temperatures (30, 35 and 45°C). Of the three treatments, sodium sulfite and 4-hexylresorcinol were most effective in preventing browning at 25°C. Ascorbic acid-2-phosphate (1%) was not very effective as browning occurred within a few days of processing (Fig 1). The difference between treatment means were significant ($p > 0.01$) and the pair comparison between treatment means indicated sodium sulfite was the best anti-browning agent with regard to L* and a* (not shown) changes during storage (Table 1). However, a more realistic estimation of anti-browning efficacy was the overall lag or induction period in which the apple slice did not brown enough to notably impair color quality. The induction period can be approximated in the curves as change in slope. On that basis, induction times in blank, controls and ascorbic acid-2-phosphate at 25°C were very short (<2 days) whereas induction time increased up to 12 days with 4-hexylresorcinol treatment and up to 14 days with the sulfite treatment for immersion time >3 hr (Fig 1). The value a* was also indicative of progress of browning during storage (Fig 2). The switch from green to red, also an indication of browning (Sapers and Ziolkowski, 1987; Bolin and Steele, 1987) was noticed as an increase in a* value (Fig 2), and followed an inverse trend to that observed for L*. The trend for Suv highly correlated with L* and a*





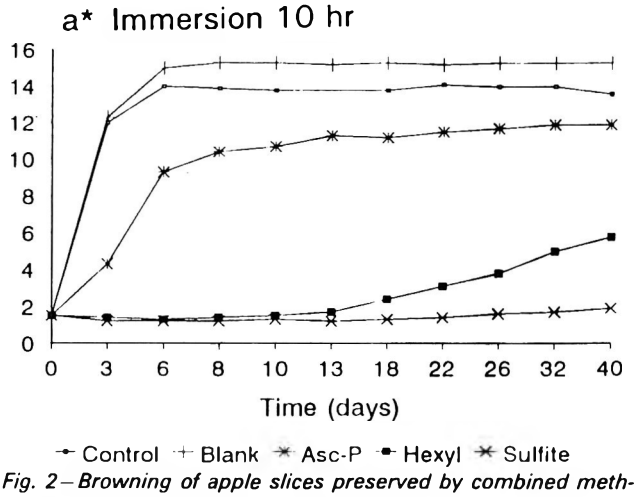


Fig. 2—Browning of apple slices preserved by combined methods and stored at 25°C. a values were used to measure the progress of browning.*

Table 2—Pearson correlation coefficient for L, a* and Suv parameters (N = 1750)*

	L*	a*	S_{uv}
L*	1.00	−0.938 (0.0001)	−0.937 (0.0001)
a*	****	1.00	.992 (0.0001)
S_{uv}	****	****	1.00

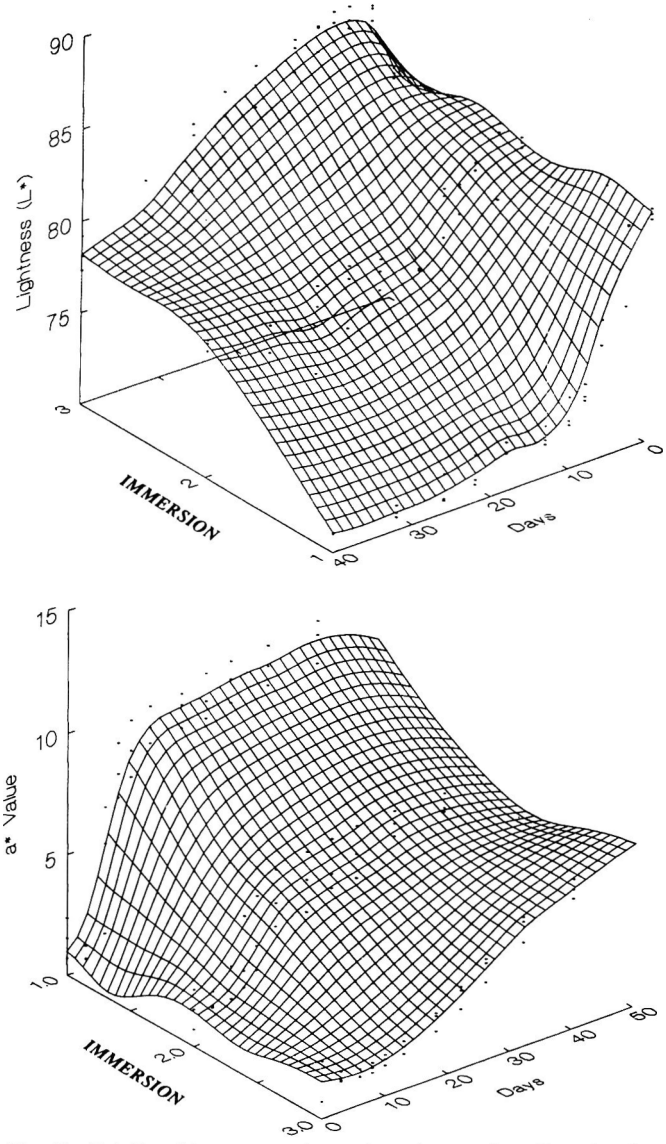


Fig. 3—Relationship among browning, immersion time treatment and storage time for apples slices treated with 4-hexylresorcinol and stored at 25°C.

values which indicated any of these parameters would be suitable for measuring browning. Pearson correlation values among those parameters were highly significant when all samples (1750 observations) were included (Table 2).

The length of immersion time for the apple slices in the treatment solutions influenced the rate of browning during storage (Fig 1). As immersion time increased, progress of browning (decrease of L*) was slowed for apple slices treated with sodium sulfite and 4-hexylresorcinol solutions. The decrease in L* value was more pronounced in the other treatment solutions. The dependance of immersion time on browning during storage is clearly illustrated (Fig 3) in a surface response plot. Two reasons could account for the relationship between browning and immersion time. One is the effect of a_w on browning. The other factor, more likely, is that an increase in immersion time led to an increase of inhibitor diffusion into apple tissue. That would be due to the differential chemical potential between the treatment solution and the apple tissue which has been reported to follow first order kinetics (Duckworth and Smith 1961). Moreover, the effect of a_w on browning within a narrow range (0.95-0.99) was insignificant (Petriella et al., 1985). The a_w of the apple slices at 1, 3, and 10 hr of immersion in the sucrose solution of 52°Brix fell within such range (0.975, 0.972 and 0.962, respectively).

The anti-browning treatments were mostly ineffective as storage temperature increased beyond the 35°C limit. The differences between treatment means decreased (Table 1) as stor-

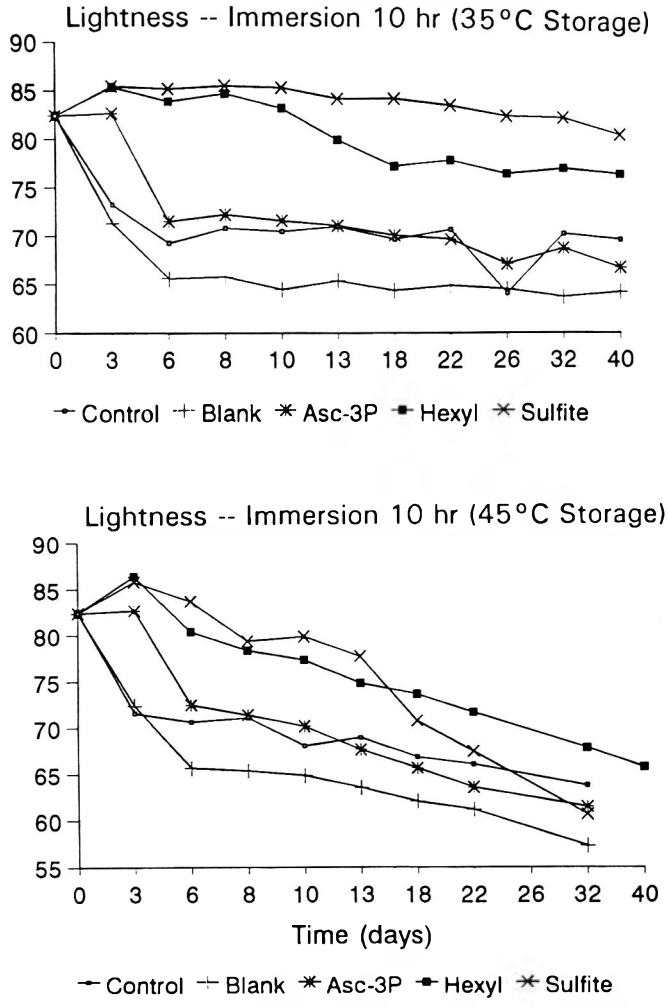


Fig. 4—Browning of apple slices preserved by combined methods. L values were used to measure the progress of browning.*

age temperatures increased. In addition, the parameters used to measure browning, L*, a* and S_{uv} showed progress of browning that coincided with visible darkening on the apple slice surfaces (only L* shown in Fig 4). Still, sodium sulfite and hexylresorcinol were more effective ($p > 0.01$) than ascorbic acid-2-phosphate and ascorbic acid in inhibiting browning at 35°C with induction times of 3, 8, 10 days (Fig 4, Table 1).

Despite ascorbic acid-2-phosphate and 4-hexylresorcinol being effective in preventing enzymatic browning, the inhibitory mechanisms are different. Ascorbic acid-2-phosphate reduces quinones generated by polyphenoloxidase and thus retards browning (Sapers et al., 1989), whereas 4-hexylresorcinol is a specific inhibitor of polyphenoloxidase (McEvily et al., 1991b). As mentioned, browning of apple slices preserved by these combined methods during storage was a combination of enzymatic and non-enzymatic browning. At higher temperatures, the nonenzymatic browning should predominate given the high Q_{10} (4–6) associated with browning reactions. High Q_{10} values indicate dependence of chemical reactions on high temperature (Labuza, 1982). At storage temperatures of 35°C, only sodium sulfite and 4-hexylresorcinol were more protective, while the other anti-browning treatments were mostly ineffective (see Fig 4). At 45°C, treated apples browned regardless of anti-browning treatments (Fig 4). Sodium sulfite, contrary to 4-hexylresorcinol is also effective against nonenzymatic browning. It forms hydroxysulphonate complexes that exhibit a much lower browning potential than the precursor intermediates (3-deoxyhexosulose and 3-4-dideoxyhexosulo-3-ene) that dehydrate to α, β unsaturated dicarbonyls and eventually form brown pigments or melanoidins (Wedzicha, 1987).

Table 3—Activation energies for browning in apples slices preserved by combined methods

Treatment	Activation energy (Kcal/mol)[a]		
	1 h°C	3 hr	10 hr
Sulfite-	18.9 ± 4.4	17.2 ± 3.8	20.1 ± 5.9
4-Hexylresorcinol	8.8 ± 2.6	9.1 ± 3.2	9.6 ± 3.7
Asc. acid-2-P	5.8 ± 4.9	5.2 ± 3.6	–
Control	7.5 ± 6.6	7.8 ± 1.2	6.8 ± 4.4
Blank	5.2 ± 3.9	6.1 ± 3.7	–

[a] Activation energy ± 95% confidence level. Rates of browning were determined by L* values.

The onset of browning during storage should correlate with depletion, oxidation, or chemical transformation of the anti-browning agent in the fruit tissue (Bolin and Boyle 1972). Depletion of ascorbic acid and derivatives such as ascorbic acid 2-phosphate occurs due to oxidation by quinones. Quinones are enzymatic products of polyphenoloxidase (Labuza and Saltmarch, 1981). Hydrogen sulfite, the predominant form of sodium sulfite at the pH of apple, is lost by irreversible binding, formation of sulfate or as gaseous SO_2 (Wedzicha, 1987). Once the residual concentration of reactive sulfite is low the onset of browning occurs with an activation energy characteristic of non-enzymatic browning reactions (15-25 kcal/mol Labuza, 1982; Wedzicha, 1987). The estimated activation energies of browning for apple slices treated with sulfite, 4-hexylresorcinol and ascorbic acid-2-phosphate were compared (Table 3). The data suggested that browning was probably enzymatic for all treatments excluding sulfite. Activation energy in the range 5–10 was consistent for the blank, control, ascorbic acid-2-phosphate and 4-hexylresorcinol. The sulfite treated apple slices probably browned by a nonenzymatic pathway.

The mechanism by which 4-hexylresorcinol interacts with polyphenoloxidase and possible effects on nonenzymatic browning as suggested by our results need to be determined. The synergistic effects of reducing substances such as ascorbic acid with 4-hexylresorcinol as well as the study of 4-hexylresorcinol residuals and stability during storage are also of interest.

CONCLUSIONS

4-HEXYLRESORCINOL in combination with ascorbic acid was an effective anti-browning agent that compared favorably to sodium sulfite at storage temperature 25°C, It could be used in lower concentrations to preserve color of apple slices processed by the combined method technique. A fivefold sodium sulfite concentration produced an equivalent browning inhibition to that of 4-hexylresorcinol at 25°C. Neither ascorbic acid alone nor ascorbic acid-2-phosphate were effective in inhibiting browning at any temperature. Anti-browning treatments were rendered ineffective when storage was at > 35°C.

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—Continued on page 826

Sorption of Ethyl Butyrate and Octanal Constituents of Orange Essence by Polymeric Adsorbents

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ABSTRACT

Sorption characteristics, rates and capacities of polymeric adsorbents (XAD-4, XAD-7, XAD-16, Duolite ES-865, Duolite S-761, Porapak-Q and XUS-43436) for ethyl butyrate and octanal were determined using model solutions. Sorption of these two principal components of aqueous orange essence, was evaluated utilizing the Freundlich isotherm model. Breakthrough curves were determined using XAD-16 as adsorbent. The capacity of XAD-16 for ethyl butyrate in the column system was 426 ± 12 mg/g. There was no column breakthrough for octanal even after 130 bed volumes. Most (91.4%) of the adsorbed ethyl butyrate was eluted from XAD-16 resin by 95% ethanol elution. Recovery of adsorbed octanal from the resin was 66.4%.

Key Words: adsorbent, Freundlich isotherm model, sorption, flavor, orange essence, ethyl butyrate

INTRODUCTION

POLYMERIC ADSORBENTS have been tested to remove organic substances from waste water (Patterson and Patzelt, 1981; van Vliet and Weber, 1981). They have also been applied in the food and flavor industries (Bryan et al., 1977; Di Cesare et al., 1987; Parliment, 1981). Recently, Matthews et al. (1990) and Manlan et al. (1990) utilized polymeric adsorbents for citrus juice debittering, and Ericson et al. (1992) evaluated the capacity of styrene-divinylbenzene adsorbents for recovering grapefruit oil from processing waste water.

Natural aqueous orange essences are flavor fractions obtained during orange juice concentration. Major flavor constituents of aqueous orange essence have been reported (Johnson and Vora, 1983; Kealey and Kinsella, 1979; Moshonas and Shaw, 1990). Aqueous orange essence is widely used by citrus processors to improve the flavor of concentrated orange juice. Orange concentrate containing added aqueous orange essence maintained acceptable essence flavor notes up to 30 months during frozen storage (Dougherty et al., 1974).

Polymeric adsorbents have potential applications for sorption of flavor constituents of aqueous orange essence. Our objective was to evaluate commercially available polymeric adsorbents for sorption rates and capacities for two major aqueous orange essence components (ethyl butyrate and octanal) from model solutions. One adsorbent was selected for column experiments, and the recovery efficiency of the compounds was calculated.

MATERIALS & METHODS

Physical properties of polymeric adsorbents

Polymeric adsorbents Amberlite XAD-4, XAD-7, XAD-16, Duolite ES-865 and Porapak-Q were obtained from Rohm and Haas Co. (Philadelphia, PA), XUS-43436 from Dow Chemical Co. (Midland, MI), and Duolite S-761 from Diamond Shamrock Co. (Cleveland, OH). XAD-4, XAD-16, Duolite ES-865 and XUS-43436 are nonionic hydrophobic divinylbenzene polymers. Porapak-Q is a weak polar adsorbent with ethylvinylbenzene-divinylbenzene as the major aromatic matrix. XAD-7 is a copolymer having an aliphatic matrix (polymethyl acrylate) and an intermediate polar surface. It is capable of binding both polar and hydrophobic compounds. Duolite S-761 is a weak-base phenol-formaldehyde hydrophilic absorbent with a phenolic hydroxyl group as the major hydrophilic functional group. The physical properties of these polymeric adsorbents are listed in Table 1.

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Polymeric adsorbents cleanup

Adsorbents were put in the cellulose thimbles and purified by extensive washing with 95% ethanol in a Soxhelt extractor for 2 hr intervals (8–10 hr total extraction time) until the ethanol rinse showed a nearly flat UV-scan from 200 to 250 nm using a Beckman DU-50 spectrometer. The cleaned adsorbents were dried overnight at room temperature (≈23°C) on filter papers and stored in glass bottles.

Preparation of model solutions

Model solutions for sorption rate determination were prepared to contain an aldehyde and an ester component of major aqueous orange essence as reported by Johnson and Vora (1983). A model solution (3L), 150 μg/mL ethyl butyrate and 100 μg/mL octanal in a 15% ethanol/water solution, was prepared. Model solutions used for sorption capacity experiments were prepared by adding 150–7500 μg/mL ethyl butyrate, and 50–175 μg/mL octanal to a 15% ethanol/water solution. The 7500 μg/mL and 175 μg/mL were the highest respective concentrations for ethyl butyrate and octanal soluble in the model solution. In column operation experiments, model solutions were prepared by adding ethyl butyrate (1695 μg/mL for the first two runs and 1411 μg/mL for the third run) and octanal (68 μg/mL for all three runs) to the 15% ethanol, 85% water solution.

Table 1—Physical properties of commercial polymeric adsorbents

Resin	Porosity (%)	Pore diameter (Angstroms)	Surface area (m^2/g)	Chemical group	Bulk density[d] (g/cc)	Surface group polarity
XAD-4[a]	51	60	725	divinyl-benzene	0.47	nonpolar
XAD-7[a]	55	80	450	acrylic-ester	0.42	polar
XAD-16[a]	58-63	144	860	divinyl-benzene	0.27	nonpolar
Duolite ES 865[a]	–	90	600	styrene-divinyl-benzene	0.36	nonpolar
Duolite S 761[b]	35	–	200	phenol-formaldehyde	0.42	polar
Porapak-Q[a]	–	75	582	ethyvinyl-benzene-divinyl-benzene	0.38	weak polar
XUS-43436[c]	–	–	500	styrene-divinyl-benzene	0.59	nonpolar

[a] Rohm and Haas
[b] Diamond Shamarock
[c] Dow Chemical
[d] Bulk densities were determined after resins were pretreated with 95% ethanol. All other values were obtained from manufacturers. All particle sizes were reported as 20–50 mesh.

Determination of sorption rate

The sorption rate of each adsorbent was determined by using a batch system containing 1 g of each adsorbent and 50 mL of model solution. The mixture was stirred with a magnetic stirring bar for 5, 10, 20, 40, 60, 80, and 100 min. After each time period, the solution was separated from the adsorbent by filtration through a Whatman filter paper no. 541 and analyzed for residual solute concentrations including total esters (ethyl butyrate) and total aldehydes (octanal). The fractional amounts of ethyl butyrate and octanal remaining in the model solution were plotted vs. time to form the sorption rate curve.

Sorption capacities

Sorption capacities were determined by mixing 50 mL of model solution at different ethyl butyrate and octanal concentrations with different weights of purified adsorbent (0.006–0.3g) at room temperature (≈23°C) for 24 hr in screw-top test tubes. After 24 hr reaction, the equilibrium ethyl butyrate and octanal concentrations in the solutions were determined and sorptions were calculated from differences of solute (ethyl butyrate or octanal) concentrations between the controls and each equilibrium solution. The sorption capacities calculated as mg solute/g adsorbent were plotted (log-log) Vs the equilibrium solute concentrations expressed as mg solute/mL solution to develop Freundlich isotherms. The Freundlich model is used in cases when heterogenous surface energies exist, where the energy of sorption varies as a function of surface coverage due to variation in heat of sorption (Patterson and Patzelt, 1981). The log form of the Freundlich isotherm can be expressed as:

$$\text{Log } Q_e = \text{Log } K + (1/n) \text{ Log } C_e$$

where Q_e = weight of solute adsorbed per unit weight of adsorbent at equilibrium concentration C_e, (mg/g), and C_e = residual solute concentration at equilibrium, (mg/mL).

The resulting line has a slope of 1/n and an intercept of Log K when equilibrium solute concentration (C_e) equals one. Patterson and Patzelt (1981) explained that the Freundlich equation intercept, Log K, could be used as a relative indicator of sorption capacity, and K is usually described as the distribution ratio (mg solute/g adsorbent) when C_e = 1. The slope (1/n) is an approximate inverse indicator of sorption intensity. The steeper the slope, the smaller the sorption intensity.

Column operation and breakthrough curves determination

A downflow fixed-bed system was used in column operation. Ten mL of the pretreated adsorbent, XAD-16 (dry base) was packed into a glass column (11 mm × 30 cm). Five to 10 bed volumes (BV) of water were added to rinse the sorption column before the model solution was pumped downflow through the column. The flow rate of the model solution was controlled at 25–30 BV/hr by a Cole-Parmer pump equipped with a model no. 7013 pump head and a Master flex no. 7013 Teflon B-44-4X tubing (Cole-Parmer Instrument Co., Chicago, IL). Fractions were collected by an ISCO Model Cygent fraction collector (Instrumentation Specialities Co., Lincoln, NE) at a constant volume of 10 mL. The influent and effluent concentrations were monitored. When the adsorbent was saturated (effluent concentration = influent concentration), the operation was discontinued. Breakthrough curves were developed by plotting effluent concentration vs elution volume. Three replications of the column experiment were conducted.

Column desorption

Following the sorption operation, the sorbed components were eluted from the column with 95% ethanol at a constant volume of 10 mL for each fraction. The desorption process was discontinued when eluate concentrations were <5% of the tube with the highest concentration. Elution curves of the test components, ethyl butyrate and octanal, were established for each run by plotting eluate concentrations of the compounds vs eluted volume. Mass balance of each curve was calculated and compared to the mass of sorbed compound to determine desorption efficiency.

Colorimetric analysis

Octanal content of fractions was measured as total aldehydes, determined using a spot test developed by Ismail and Wolford (1970). Aldehydes react with N-hydroxybenzene sulfonamide (HBS) in alkaline medium (KOH) to form a hydroxamic acid which produces a brown-purplish color complex with ferric (Fe^{+3}) ions of $FeCl_3$ in the presence of acid (HCl). The final colored complex has an absorption maximum at about 520 nm.

Total esters as an indicator of ethyl butyrate content, were determined using a colorimetric method reported by Attaway et al. (1967). Esters react with hydroxylamine hydrochloride in alkaline medium (NaOH). The product forms an orange-yellow color with $FeCl_3 \bullet 6H_2O$ in acid medium (HCl). The final rection color has an absorption maximum at 525 nm. Concentrations of total aldehydes and total esters were expressed as μg/mL. Three replicate analyses were conducted and average values were obtained.

Gas chromatographic analysis

After column extractions, concentrations of recovered ethyl butyrate and octanal were analyzed by capillary gas chromatography using a Perkin Elmer Sigma 3B chromatograph connected to a Spectra-Physics SP4400 ChromJet integrator. Chromatographic parameters were as follows: DB-5 capillary column (30 m × 0.32 mm i.d.), injection volume was 2 μL and split ratio 57:1, injection port at 200°C, flame ionization detector at 325°C, temperature program: 2 min, 40°C; 3.5°C/min to 250°C for 10 min, helium carrier gas at constant back pressure of 9.5 psi.

Standard curves of peak area vs. concentration (μg/mL) for octanal and ethyl butyrate were developed by duplicated injections at six concentrations (5–300 μg/mL). 1-Heptanol at 49.7 μg/mL was used as internal standard in all tests. Recovered products were diluted with 95% ethanol to provide peak areas that fell within the standard curve ranges. All GC analyses were conducted in duplicate and mean and standard deviation values were computed.

Mass balances and percent recoveries

In all desorption operations, percent recoveries of ethyl butyrate and octanal from the adsorbent were based on mass balances. This was accomplished by calculating quantities of the two constituents eluted in 95% ethanol and divided by the amounts of the two components sorbed on the column, then multiplied by 100.

Statistical analysis

Analysis of variance (ANOVA) on the slopes of isotherm regression lines was conducted using Duncan's Multiple Range Test (based on F test) (Ott, 1988). In each experiment, 3 replicates were conducted and the differences were considered significant at $p \leq 0.05$.

RESULTS & DISCUSSION

Sorption rate determination

The nonpolar adsorbents with higher surface area, such as XAD-4, XAD-16, XUS-43436 and Duolite ES-865, displayed faster sorption rates for ethyl butyrate and octanal (Fig. 1 and 2). By 40 minutes, all four nonpolar adsorbents had caused a reduction of ethyl butyrate to 10–15% and octanal 5–10% of original concentrations. The three polar adsorbents, XAD-7, Duolite S-761 and Porapak-Q exhibited different capacities for adsorbing ethyl butyrate and octanal depending on polarity of their surface groups. These adsorbents reduced ethyl butyrate to 30–50% and octanal to 20–50% of original concentrations. Generally, nonpolar adsorbents displayed higher ethyl butyrate and octanal adsorption from the model solution than did polar adsorbents. For all adsorbents tested, the fastest percent reductions occurred during the first 5 min, and equilibrium was approached at 20–40 min.

Sorption capacities of polymeric adsorbents

The sorption capacities of the adsorbents for ethyl butyrate and for octanal in model solutions were compared using the Freundlich model (Fig 3 and 4). Linear regressions for all tested adsorbents were derived and statistical analyses on the slopes (1/n) were performed. The overall data (Tables 2 and 3) indicate XAD-16, Duolite ES-865 and XUS-43436 displayed no significant differences in slope values (1/n). These

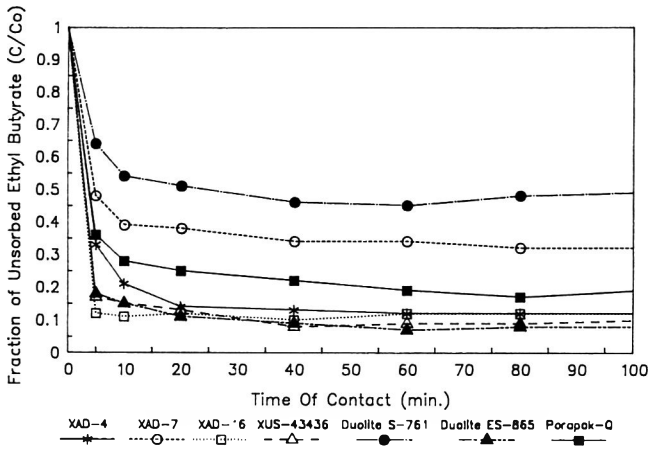

Fig. 1—Sorption rates of ethyl butyrate by various adsorbents using a model solution.

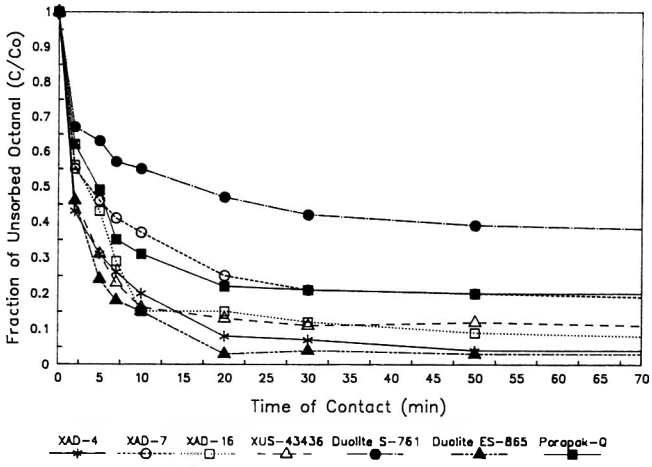

Fig. 2—Sorption rates of octanal by various adsorbents using a model solution.

Table 2—Freundlich isotherm parameters of resins for sorption of ethyl butyrate in model solution

Resin	Slope[b] (1/n)	Intercept log K	Distribution ratio (K)[a]	R^2
Duolite S-761	1.103A	1.980	95	0.996
XAD-7	0.964B	1.974	94	0.988
XUS-43436	0.974BC	2.617	415	0.998
Duolite ES-865	0.915BC	2.502	318	0.987
XAD-16	0.867C	2.603	401	0.991
Porapak-Q	0.754D	2.302	159	0.943
XAD-4	0.629E	2.434	271	0.977

[a] Units are mg solute/g resin when equilibrium solution concentration from Freundlich isotherm equation (C_e) is 1 mg solute/ml solution.

[b] Parameters followed by the same letter in the same column are not significantly different from each other ($P > 0.05$).

nonpolar adsorbents thus showed similar sorption intensities for ethyl butyrate (Table 2). Another nonpolar adsorbent, XAD-4, exhibited the highest sorption intensity for ethyl butyrate. Polar adsorbent XAD-7 displayed a similar intensity for ethyl butyrate sorption as nonpolar adsorbent Duolite ES-865 and XUS-43436. Based on the K values (Table 2) the three nonpolar adsorbents, XAD-16, Duolite ES-865 and XUS-43436, had higher capacities for ethyl butyrate than did the polar ones, XAD-7, Duolite S-761 and Porapak-Q. XAD-4 displayed a lower capacity (271 mg/g) for ethyl butyrate than did the other three nonpolar adsorbents.

The sorption capacities of each adsorbent for octanal in model solutions were derived from extrapolation of each isotherm line (Fig. 4). Extrapolation may not be valid over large concentration ranges, thus results obtained this way may not be reliable. However, the nonpolar adsorbents (XAD-16, Duolite ES-865 and XAD-4) generally displayed higher sorption capacities for octanal than did the polar adsorbents (Fig. 4). The low water solubility of octanal should enhance sorption by nonpolar adsorbents.

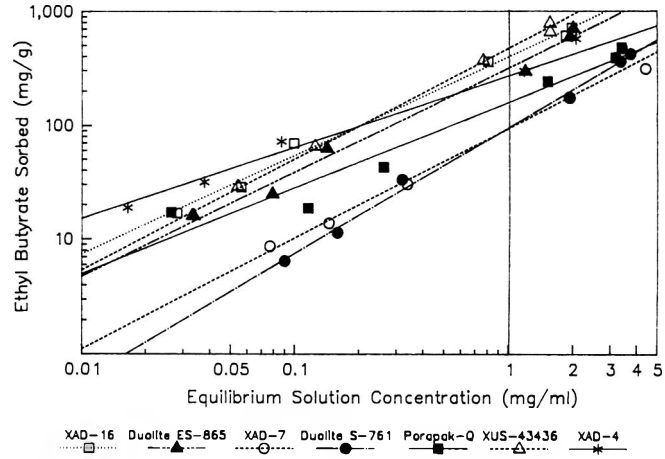

Fig. 3—Freundlich sorption isotherms of adsorbents for model solutions.

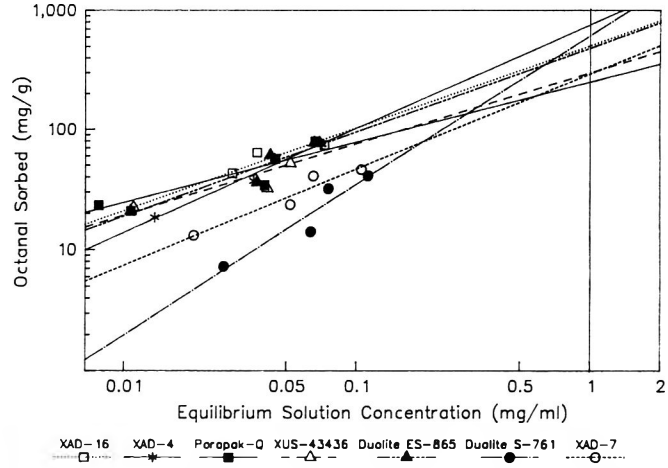

Fig. 4—Freundlich sorption isotherms of adsorbents for model solutions.

Table 3—Freundlich isotherm parameters of resins for sorption of octanal in model solution

Resin	Slope[a] (l/n)	Intercept log K	Distribution ratio (K)[b]	R^2
Duolite S-761	1.221A	2.751	563	0.898
XAD-4	0.896B	2.875	749	0.971
XAD-7	0.798C	2.467	293	0.930
XAD-16	0.692D	2.705	507	0.941
Duolite ES-865	0.705D	2.683	482	0.892
XUS-43436	0.597E	2.476	299	0.898
Porapak-Q	0.504F	2.398	250	0.777

[a] Parameter followed by the same letter in the same column are not significantly different from each other ($P > 0.05$).

[b] Units are mg solute/g resin when equilibrium solution concentration from Freundlich isotherm equation (C^e) is 1 mg solute/mL solution.

Breakthrough curves and column desorption

Breakthrough curves of model solutions are displayed in Fig. 5. Less than 3% leakage of ethyl butyrate was detected in each run of the downflow fixed-bed column experiments when the flow rates of model solutions were ≈30 BV/hr. The

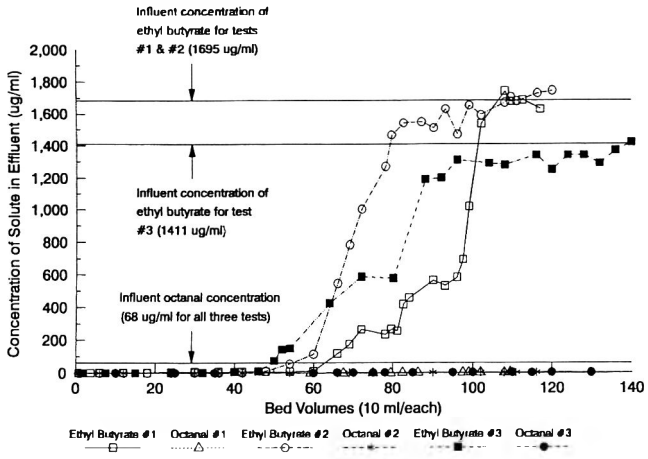

Fig. 5—Breakthrough curves of ethyl butyrate and octanal in model solution using XAD-16.

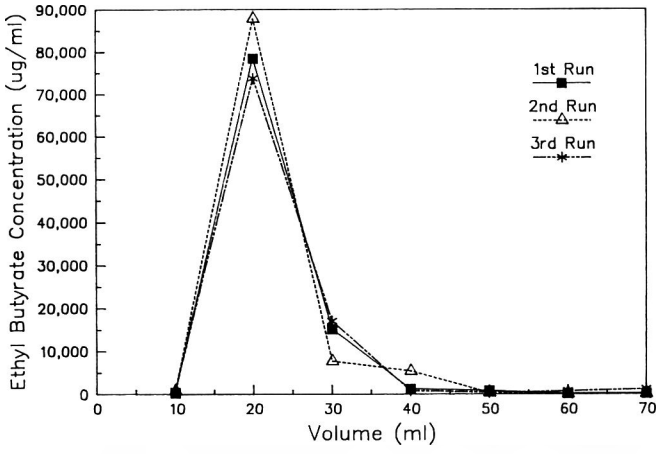

Fig. 6—Elution of ethyl butyrate in model solution using XAD-16.

capacity of XAD-16 for ethyl butyrate in the column system was 426 ± 12 mg/g. There was no column breakthrough for octanal even after 130 bed volumes passed through the column. This indicates the low concentration (68 μg/mL) of octanal in the model solutions and the strong affinity of the adsorbent for aldehydes.

Desorption profiles of model solutions from the column with 95% ethanol (Fig. 6 and 7) indicate that the mean percent of ethyl butyrate recovered from the XAD-16 resin was 91.4%. Only trace amounts of ethyl butyrate remained on the adsorbent after eluting with 70 mL 95% ethanol (Fig. 6). About 30% of the octanal remained on the adsorbent after 70 mL of 95% ethanol was used. The mean percent octanal recovered was 66.4% (Fig. 7).

CONCLUSIONS

NONPOLAR ADSORBENTS—XAD-16, XAD-4, Duolite ES-865 and XUS-43436—displayed faster sorption rates and higher sorption efficiencies (80–90%) for ethyl butyrate. Polar adsorbents Duolite S-761 and Porapak-Q exhibited more rapid sorption rates for octanal (20–30%). Sorption conformed to the

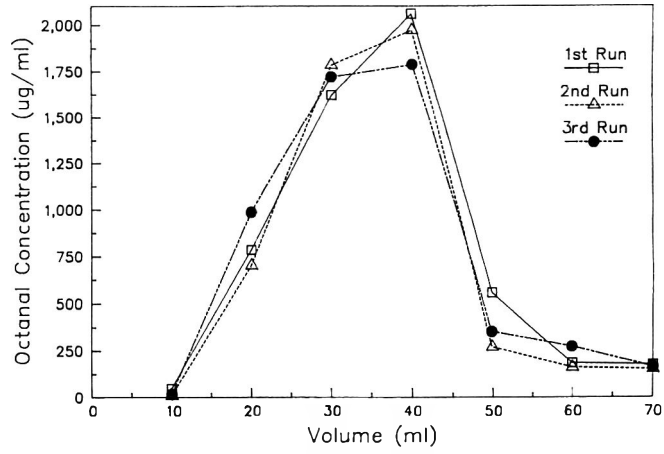

Fig. 7—Elution of octanal in model solution using XAD-16.

Freundlich isotherm model. The Freundlich isotherms distribution ratio for XAD-16 was 401 mg ethyl butyrate/gram resin and the capacity by downflow fixed bed was 426 mg ethyl butyrate/g resin. Recovery of ethyl butyrate from XAD-16 using 95% ethanol was higher (91.4%) than for octanal (66.4%). Polymeric adsorbents could be used in fixed-bed columns to recover flavor constituents from a model solution but application to commercial products requires further study.

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Storage Conditions Affect Quality of Raisins

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ABSTRACT

Raisins had a moisture content of ca 14% and were stored over an 11 mo period. The initial SO_2 content was ca 640 ppm. Moisture content, water activity, color, browning, texture, SO_2 content, soluble sugars, acidity and pH were periodically determined. A substantial decrease in SO_2 content was observed, especially in samples stored at room temperature (20–25°C) which had higher browning indexes. No substantial changes were detected in water activity, acidity and pH. No sugaring was observed. Refrigerated samples (stored at 4 and 11°C) were of higher quality in comparison with nonrefrigerated samples. Both were suitable for consumption after storage.

Key Words: grapes, raisins, dehydration, storage conditions

INTRODUCTION

AN IMPORTANT aspect concerned with dehydrated food products is their stabilization to chemical and physical changes as well as microbiological degradation which could occur during storage. Important nutritional and sensory problems occur during the preservation of raisins due to browning reactions. These are known to be caused by polyphenoloxidase (PPO), and also by nonenzymatic reactions. Kinetics of both types of browning are related to water activity, the maximum rate being around $a_w = 0.8$ (Aguilera et al., 1987). Moisture in commercial raisins varies between 12 and 18% (w.b.), low enough to protect against further browning and microbial growth (Bolin, 1980). Nevertheless, browning can appear during long storage, and is generally dependent upon product characteristics and storage conditions (Toribio and Lozano, 1984).

The quality of food depends not only on its original state but also on the extent of changes during processing and storage (Saguy and Karel, 1980). The influences of different variables such as temperature, time, light, water activity, etc., on the stability of a product have been reported from many studies. The aim of such studies has been to establish the best preservation conditions for extending shelf life of the food product, while preserving nutritional and sensory characteristics (Stein et al., 1986; Coffin et al, 1987; Lee and Nagy 1988; Onigbinde and Akinyele, 1988).

Both color and texture highly influence the market acceptability of food products and have been studied to a great extent in different products such as apples (Van Woensel and De Baerdemaeker, 1983); mashed potatoes (Faulks and Griffiths, 1983); okra (Stone et al., 1986); apple and pear (Sapers and Douglas, 1987), and raisins (Riva et al. 1986; Riva and Masi, 1989). Light-colored raisins are preferred by consumers. They are generally produced by treatment with SO_2. This prevents both types of browning through two types of reactions. These include reversible inactivation of carbonyl groups by the formation of hydroxysulphonates and irreversible sulphonation of the double bond a,b-unsaturated carbonylic intermediates in browning (Wedzicha, 1986).

The texture of fruits and vegetables is determined mainly by structural polymers of cellular walls, together with water activity and moisture content (Cheftel and Cheftel, 1989). Changes in these characteristics during processing or storage can also alter texture.

Our objective was to study the influence of storage conditions on the quality of dehydrated grapes (raisins) stored in airtight glass containers.

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MATERIALS & METHODS

Raw Material

Seedless grapes (Flame var.) cultivated on the island of Majorca were used. Fruit was washed and different pre-treatments were applied by subsequent dipping in a 0.15% NaOH (w/v) solution for 15 sec at 100°C, distilled water at 25°C for 5 min, a 0.3% citric acid solution for 3 min and finally, a 4% $K_2S_2O_5$ solution for 10 min (Cañellas et al. 1989).

Dehydration

Grapes were dehydrated in a pilot solar drier as described (Rosselló et al., 1990) to a final moisture content of ca 14% (g water/100g raisin).

Storage

Samples were placed in airtight glass containers (500 cm^3) filled to a ca 80% of their volume and stored under four different conditions. These were: at 4°C, at 11°C and at room temperature (20–25°C) in darkness as well as at room temperature in light. Several representative indicators of quality were measured at the beginning of storage (initial characterization) and at 3, 5, 8 and 11 mo. Longer storage was not considered, since after this period new raw material would be available.

Analytical methods

Measurements of the quality indicators were carried out on the contents of two containers stored at each storage condition which were homogenized. Each color measurement was performed five times on 20 different raisins and pH using 25 raisins. The rest of the measurements were performed in triplicate.

Texture was evaluated by a compression-extrusion assay in an Instron press, with Kramer cell, using a 5000 N head at a rate of 50 mm/min. Samples (18 grapes ≈ 20 g.) were used to calculate the value of the maximum force per mass unit with regard to compression and extrusion of the product. Color was measured in a HunterLab (Ultrascan model) spectrocolorimeter, with specular component included, C illuminant and observer with an angle of 10°. Results were expressed as L*, a* and b* values (CIELab coordinates). The SO_2 content was determined according to the method proposed by DeVries et al. (1986).

Browning measurements were carried out by the method described by Baloch et al. (1973) on 5g samples. This method is based on extraction of brown pigments in 50 mL acetic acid-formaldehyde aqueous solution (2%–1%, v/v) and measurement of absorbance at 420 and 600 nm. Browning is recorded as the difference between the two absorbances (measurement 1). In order to avoid possible interference of carotenoids and SO_2, an aliquot of the extract was treated with lead acetate and then absorbance measured by the same procedure (measurement 2).

Water activity was measured at 25 ± 1°C by refractometry, according to the method proposed by Steele (1987), using glycerol as hygroscopic liquid. Moisture content was performed by drying the sample in a vacuum drier at 70°C to constant weight, protein by

Table 1—Raisin initial measurements for control parameters

L*	32.3 ± 0.7
a*	6.5 ± 0.4
b*	3.0 ± 0.7
Texture (N/g)	68.1 ± 1.4
Sugar (g/100g)	64.8 ± 2.0
SO_2 (ppm)	640 ± 40
Browning (AcOH-HCOH)	0.043 ± 0.005
Browning (AcOH-HCOH-AcPb)	0.014 ± 0.001
Water activity	0.61 ± 0.01
Moisture (g/100g)	14.2 ± 0.7
Protein (g/100g)	2.8 ± 0.1
Fiber (Neutral detergent fiber) (g/100g)	4.2 ± 0.2

Kjeldhal (N x 6.25) and fiber content by the detergent method (Van Socst, 1979). Soluble sugars were determined by the dinitrosalycilic acid method (Miller, 1959) on the hydrolyzed samples, measuring absorbance at 540 nm.

Measurements of pH were performed directly on samples using a CRISON pH/mV-meter 501, equipped with a puncture electrode. Acidity was determined by titration with a 0.1M NaOH solution of an aqueous extract, expressed as grams malic acid/100g sample. The total amount of malic acid was enzymatically determined (Boehringer).

Statistical analysis

Analysis of variance (ANOVA) was performed on pooled data for the independent variables, storage time and temperature, as well as light on the physical and chemical parameters studied (Bisquerra, 1989; Best, 1990).

RESULTS & DISCUSSION

Sorption isotherm

The water sorption isotherm at 25°C was studied only in a moisture range within 10 to 45% (w.b.). Less than 10% w.b. raisins are not used commercially, thus data at lower values were not measured. The moisture range considered indicated a 0.5–0.9 water activity range.

The equilibrium data were fitted to the Henderson model (Iglesias and Chirife, 1982). This method was recommended by several researchers (Yanniotis et al., 1989; Schuchmann et al., 1990) for similar products. Using the method of Marquardt (Kuester and Mize, 1973) Eq. (1) was obtained, where the moisture content (W) was measured as a percentage of dry matter.

$$a_w = 1 - \exp(-0.184\ W^{0.575}) \qquad (1)$$

This isotherm at 25°C was in agreement with data reported by Saravacos et al. (1986) for sultana raisins, and by Bolin (1980) for Thompson variety. In that case the a_w values were slightly lower than for the Flame variety for the same moisture and temperature.

This type of isotherm is observed for high sugar foods (Ayranci et al., 1990). The shape of the isotherm is characteristic of grapes, figs, apricots and prunes (Tsami et al., 1990). In all those sugar is the main component, and for grapes it could be as high as 65–75% (d.m.). The remaining solids consist mainly of fibers and protein (Table 1).

At low moisture the shape of the isotherm is governed mainly by the water sorption of the biopolymers with strongly active sites. At higher water activities (greater than 0.6), levels of different sugars and their physical state (crystalline or amorphous) determine the water sorption in raisins (Saravacos et al., 1986; Tsami et al., 1990). Amorphous sugars sorb more water than crystalline sugars. For $a_w > 0.8$ an exudation of sugars (sugaring effect) is often found in raisins due to their slow dissolution. In our stored samples this was not observed.

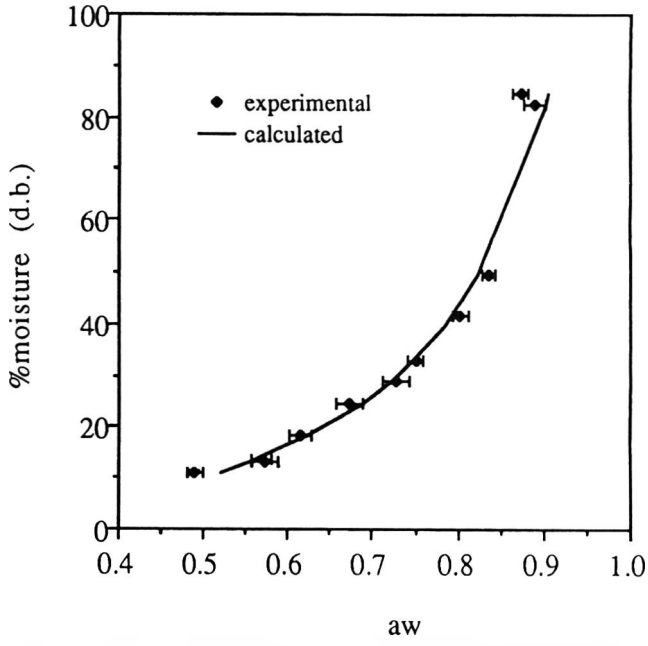

Fig. 1.—Water sorption isotherm for Flame raisins at 25°C (moisture content is expressed on dry basis). Each point is the mean of three replications.

Initial characterization

Measurements before storage for different control parameters on dehydrated samples were recorded (Table 1). Color characteristics of the dehydrated product were acceptable for marketing. The initial L* was high (32.3), compared with previous studies for samples dried under the same conditions without pretreatment ($L^* = 20$) and very similar to those obtained by treatment with K_2CO_3 + olive oil and $K_2S_2O_5/K_2CO_3$/ethyl oleate ($L^* = 30$) (Cañellas et al., 1989). Aguilera et al. (1987) reported L* values between 19.5 and 32.5 for sultana grapes by changing both temperature and blanching time. Such results are indicative of the great influence of pretreatment on the lightness of the product. Absorbance differences $\lambda_{420} - \lambda_{600}$ on the extracts in AcOH/HCOH were very low. This indicates that browning was negligible in the drying procedure we used.

Texture was highly influenced by moisture content as previously reported (Faulks and Griffith, 1983; Stanley and Aguilera, 1985; Bhattacharya et al, 1986). The mean value 68 N/g was used as a reference in order to evaluate changes in this property over storage. The moisture content of the sample (14.2%) was within the recommended commercial range. The corresponding water activity value, 0.61, seemed appropriate to maintain stability of the product against browning reactions and microbial growth (Bolin, 1980; Ziemke, 1980).

Storage effects

The variation of water activity during storage time (Fig. 2) showed a slight increase with time in samples stored at both 11°C and room temperature (≈ 23°C) from 0.61 ± 0.01 to almost 0.65 ± 0.01. The sample at 4°C had least variation in water activity. Application of factorial analysis to the data showed the influence of time was significant on water activity in all samples ($P < 0.001$), except that at 4°C. Within the working range of water activities (0.6–0.65) sugars are the main components governing water sorption. They are involved in Maillard reactions and consequently a decrease was observed during storage. This decrease in sugar content would influence water activity (Fig. 7). Moreover in Maillard browning reactions, water appears. This could also increase the a_w (Nursten, 1986). During storage sugars change from less stable amorphous structures to more stable crystalline structures, releasing

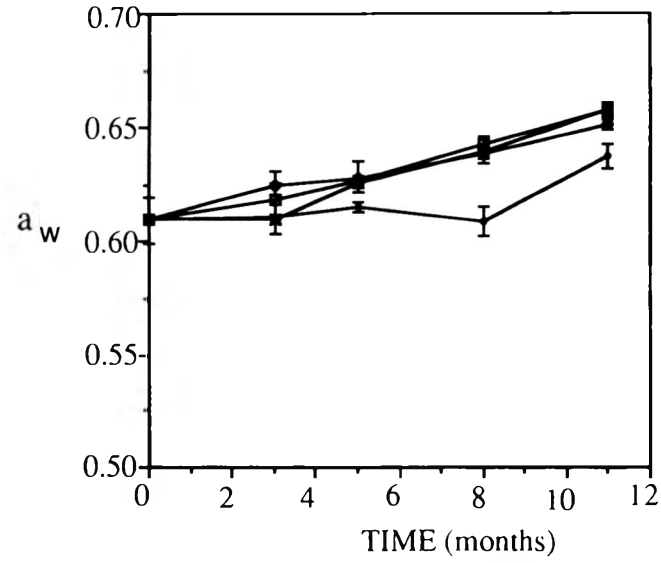

Fig. 2.—Influence of storage time on water activity of raisins. Storage conditions: ● 4°C in darkness; ✳ 11°C in darkness; ♦ room temperature with light; ⊡ room temperature in darkness. Variation coefficient: 1.6.

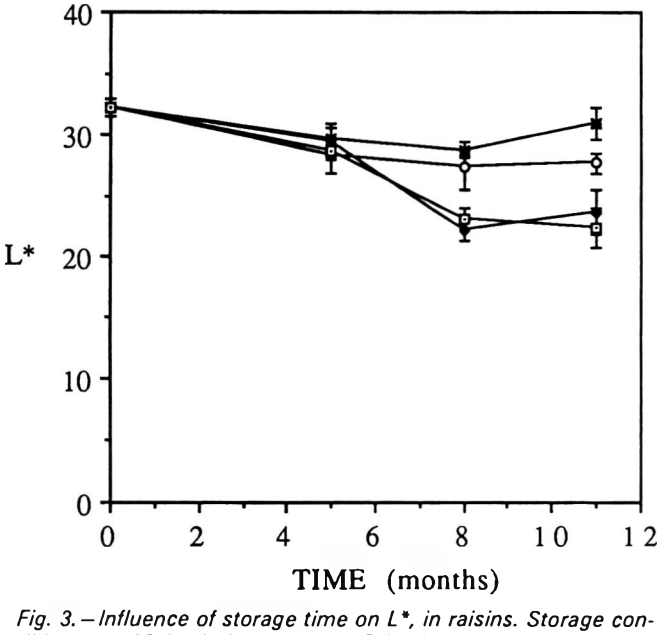

Fig. 3.—Influence of storage time on L, in raisins. Storage conditions: ● 4°C in darkness; ✳ 11°C in darkness; ♦ room temperature with light; ⊡ room temperature in darkness. Variation coeffiecient: 2.5.*

water. This factor also influences the increase in water activity observed, especially for nonrefrigerated samples (Cheftel and Cheftel, 1989).

There were no changes (Fig. 3) in the L* values over 5 mo. storage. McBride et al. (1984) conducted a sensory assessment on the variation of color in sultana grapes packed for commercial markets (in PVDC) stored at different temperatures. General acceptability of color was observed in samples processed at temperatures below 20°C. Rejection of samples increased when stored at higher temperatures, which our results confirmed. No influence of the presence or absence of light was detected on the samples at room temperature.

Values for a* and b* were considerably lower in all samples. For grapes of this cultivar subjected to the pretreatment, the variation of a* parameter was from 4 to 10, and b* from 3 to 9 which indicates a lcw proportion of red and yellow. These

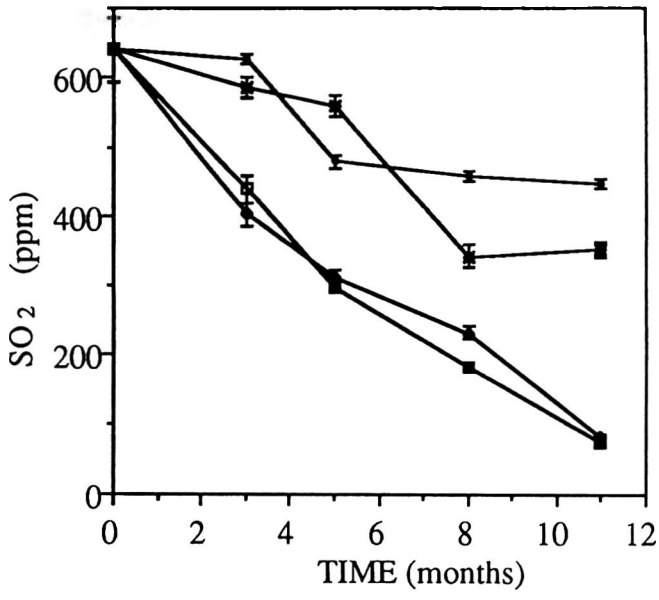

Fig. 4.—Influence of storage time on the SO_2 content (ppm) in raisins. Storage conditions: ● 4°C in darkness; ✳ 11°C in darkness; ♦ room temperature with light; ⊡ room temperature in darkness. Variation coefficient: 7.0.

parameters did not contribute notably to the description of the samples during the 11 mo period. Aguilera et al. (1987) obtained similar values for a* in dehydrated sultana grapes (3–7 units). However their b* values were higher (10–16 units) than those of the raisins of our study. Thus L* was a more important parameter for study of the evolution of the color in dehydrated grapes of Flame variety.

The initial sulphur content in the samples was 640 ± 40 ppm (Fig. 4). Refrigerated samples assayed at the end of the storage period contained a much higher total sulphur (IV) than those of non-refrigerated samples. Clustering of samples in 2 groups was observed. Refrigerated samples lost less than 50% of the initial sulphur content, with final values of 450 ± 30 and 350 ± 30 ppm at 4°C and 11°C, respectively. Samples maintained at room temperature (≈ 23°C) lost most of their sulphur. By the end of the storage period they contained only 82 ± 6 and 74 ± 5 ppm respectively. The influence of storage time and temperature on sulphur content was important in all samples, and it was considerably greater in samples at room temperature ($P < 0.0001$). A high temperature-time interaction was observed ($P < 0.0001$).

Considerable losses in SO_2 in stored dehydrated foods were reported to be influenced by storage temperature. This decrease was due mainly to the conversion of sulphur (IV) to sulphate through formation of organic (carbonylic and Schiff bases that appear at first stages of Maillard reactions) products. Also inorganic products are formed which do not decompose to sulphur (IV) oxospecies under the conditions of analysis or by reaction with oxygen in the package headspace (Davis et al., 1973; Wedzicha, 1984).

The influence of storage temperature on loss of SO_2 appeared to follow first order kinetics and the constant term varied according to the Arrhenius law ($r^2 = 0.995$). The activation energy was 12.1 kcal/mol. Such kinetics have been also shown by other dried fruits and vegetables such as carrots, potatoes, cabbage and apricots (Wedzicha, 1984).

Although absorbance values were lower in the extracts treated with lead acetate (measurement 2), both treatments showed a similar tendency in browning development. This was reflected in the correlation coefficient between the variables, 0.88 (Fig. 5). Samples refrigerated at 4° and 11°C in darkness were relatively stable, with few if any browning effects. However those stored at room temperature with and without light showed a

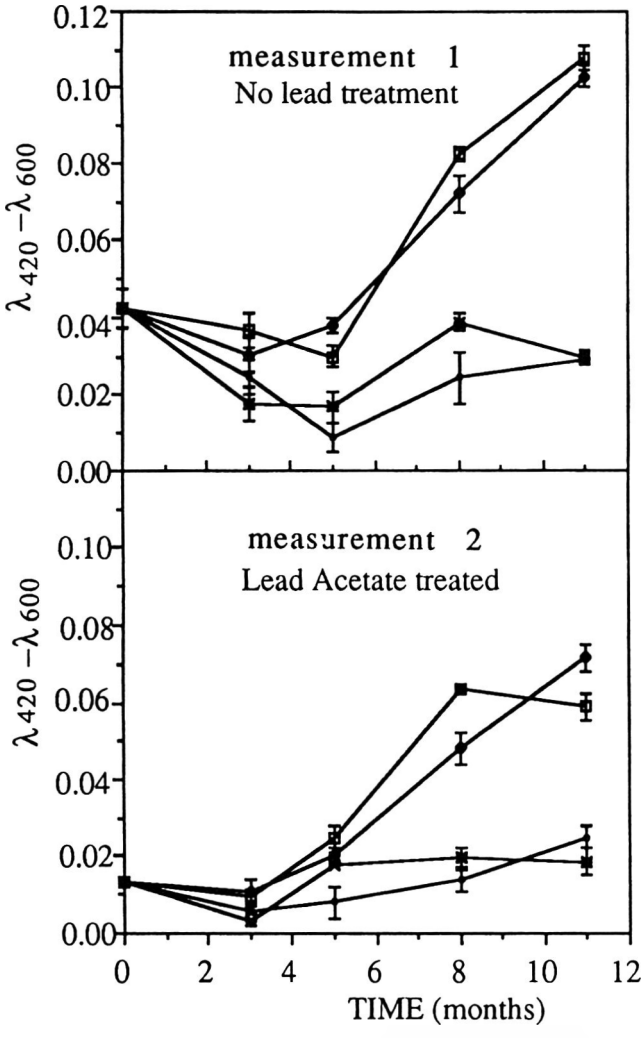

Fig. 5.—Influence of storage time on browning of raisins. Storage conditions: ● 4°C in darkness; ✳ 11°C in darkness; ♦ room temperature with light; ⊡ room temperature in darkness. Variation coefficient: 8.4.

considerable increase in browning, especially over a long time. During the first months of storage practically no browning was observed. This corresponded to the induction period reported previously with both dried fruits and vegetables (Davies et al., 1973). Once this period was over, the presence of browning could be related to the decrease in SO_2 content.

The influence of temperature was more significant with time, being negligible during the first 3 mo ($P<0.5$) and increasing afterwards ($P<0.1$ at 5 mo and $P<0.0001$ at 8 and 11 months). The steadiness in browning measurements on samples at 4° and 11°C could be explained. These samples experience much lower losses of SO_2 than those maintained at room temperature. Also browning kinetics depend on storage temperature (Toribio and Lozano, 1984).

No determination of the type of browning, whether enzymatic or nonenzymatic, was carried out. Nevertheless, we could assume that in a product with low SO_2 the browning effect observed was mainly nonenzymatic. This is because heat inactivates the PPO (Aguilera et al., 1987). Also for the water activity range during storage, the non-enzymatic browning rate would be much higher than the enzymatic one. For this type of non-enzymatic reaction, a zero-order reaction has been reported in many products such as fruits and vegetables (Saguy and Karel, 1980; Labuza and Kamman, 1983; Vega-Mercado and Barbosa-Cánovas, 1989).

An increase in average F_{max}/mass was observed with storage, from 68 ± 1 N/g to near 86 N/g for all samples except

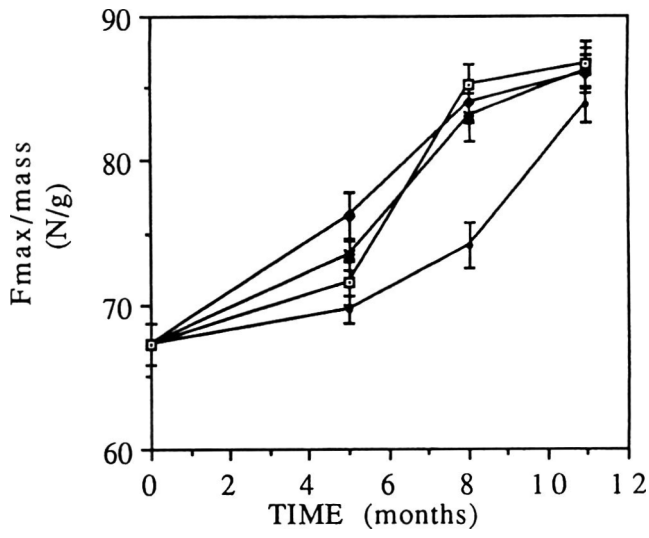

Fig. 6.—Influence of storage time on texture (N/g) in raisins. Storage conditions: ● 4°C in darkness; ✳ 11°C in darkness; ♦ room temperature with light; ⊡ room temperature in darkness. Variation coefficient: 2.3.

for that at 4°C. (Fig. 6). The rate at which the different samples attained this value differed depending on the temperature. Thus, the sample at 4°C appeared to maintain its initial texture for a longer time. In the first months of storage texture developed differently in all samples due to temperature and finally similar values were attained. Samples stored at 11°C and at room temperature were not significantly different. Since the water activity increase for the period was negligible, no influence of this parameter on texture was assumed (Bourne, 1987). No significant relationship was found between texture and other variables. The main reactions in sugar-rich products can produce important changes in color, sugar content, vitamin C, off flavor production, etc., but do not greatly influence texture (Nusrten, 1986). Nevertheless, changes in sugar state from amorphous to crystalline could explain the increase in hardness. The rate of change from one state to another increases with temperature. If held long enough, similar values for F_{max}/mass were attained since all samples had a very similar crystalline sugar content.

pH measurements in different raisins of the same sample varied widely. The variation coefficient was within a range near 10%, and pH differences were up to one unit. The average value for all samples was 4.2 ± 0.5. No significant pH changes were observed due to time or storage temperature. Total acidity was practically constant during storage, ca 16.5 meq acid/100g raisin, (1.1 g malic acid/100g raisin). The enzymatic determination of malic acid during storage was 0.38 ± 0.05g malic acid/100g raisin. No significant differences were observed in total acidity values or malic acid content between stored samples at different temperatures.

Sugar levels were stable in all samples during the first months of storage, then decreased at about 3 mo, especially in those samples stored at room temperature (Fig. 7). Losses with time were irrelevant, expressed as a percentage, since they were 10% at all storage conditions studied. In absolute values the decrease reached 5 units/100g. Changes in sugar content were mainly due to storage time ($P<0.001$) and to a lesser extent storage temperature ($P<0.5$). From a nutritional point of view, this decrease was not significant.

The maximim decrease appeared (Fig. 4 and 6) between the third and fifth month, although no increase in browning was found at that time ($\lambda_{420}-\lambda_{600}$). This increase appeared later. This was probably because in the first stages of non-enzymatic browning, unstable carbonyl compounds formed. These would

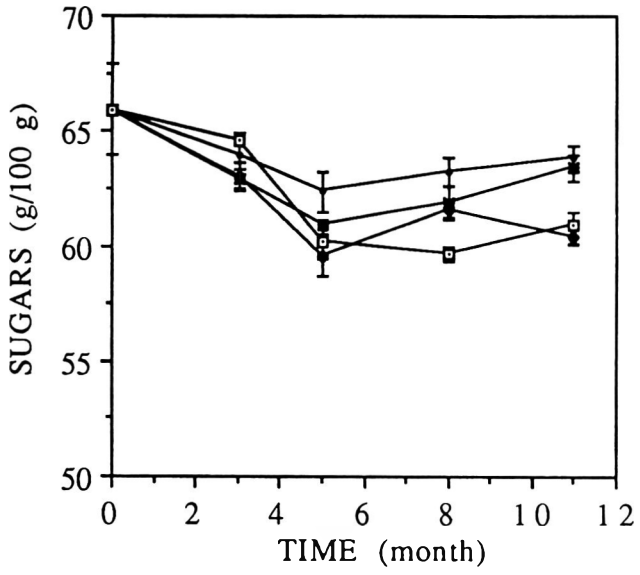

Fig. 7.—Influence of storage time on percentage of reducing sugars (b.h.) in raisins. Storage conditions: ● 4°C in darkness; ✳ 11°C in darkness; ♦ room temperature with light; ⊡ room temperature in darkness. Variation coefficient: 3.3.

react with dinitrosalicilic acid, used in sugar measurement. Such compounds produce browning at later stages.

An enzymatic analysis of glucose and fructose indicated an initial ratio of ≈1:1. After storage the amount of fructose was slightly higher than that of glucose. This confirmed the Maillard type reactions for reducing sugars and amine groups, where glucose reactivity is slightly higher than fructose (Hayashi and Namiki, 1985). During storage, no sugaring, i.e., crystallization on the surface of the raisin, was observed.

After storage both refrigerated and nonrefrigerated samples were satisfactory for consumption.

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Color Influences Flavor Identification in Fruit-flavored Beverages

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ABSTRACT

The influence of color on flavor was investigated using 310 untrained volunteers who each judged the flavor of 1 of 8 beverages. Artificially flavored raspberry and orange beverages were either left uncolored, or colored red, orange, or green. Color had a significant influence on the identification of both flavors, although every combination of color and flavor was identified correctly beyond the level expected by chance. Performance was degraded equally when beverages were uncolored, and facilitated equally when beverages were appropriately colored. Unusual color-flavor combinations reduced the identification of raspberry flavor more than that of orange flavor. The influence of color was particularly salient because tasters were aware that the color of the beverage might be inappropriate to its flavor.

Key Words: fruits, oranges, cordials, flavor, color

INTRODUCTION

FLAVOR is the sum of several sensations originating from the stimulation of taste receptors, olfactory receptors, and nerve fibres registering touch and chemical feelings. Food color is known to influence flavor. In short, "there is more to eating than taste" (Kroeze, 1990). The influence of color on flavor is interesting, because light reflected from a tastant does not directly influence any of the nerve fibres activated by the chemical and textural properties of food or drink. The integration of color information into the overall percept is a cognitive process that has adaptive significance since, as Pangborn (1967) pointed out, foods are largely discriminated and selected by eye in accordance with previous experiences. Nevertheless, relatively few studies have been undertaken to explore the influence of color on flavor.

Pangborn (1960) reported that although color did not affect sweetness judgements for colored aqueous solutions, among equi-sweet concentrations of pear nectar, green nectar was judged least sweet and uncolored nectar sweetest. Across a range of concentrations, green nectar was judged least sweet and red sweetest. Using untrained panelists, Johnson and Clydesdale (1982) demonstrated that some of the sweetness in a cherry-flavored beverage could be replaced by red color. Pangborn et al. (1963) found that food colorings added to a dry white table wine influenced the sweetness judgements of experienced wine tasters, but not of subjects who rarely used wine. Romeu and DeVicente, 1968 (cited in Hyman, 1983) reported that color differences in the yellow-orange range affected discrimination along a sweet-sour dimension for orange soda. Hedonic ratings for flavored yogurts reported by Norton and Johnson (1987) were higher for yogurts in which the intensity of color was enhanced than they were for naturally colored yogurts.

The influence of color on the identification, rather than the quality, of flavors has received little study. Maga (1974) found that the identification threshold for sweet, sour, salty and bitter solutions varied with color. Hall (1958) reported that miscolored sherberts were misidentified, and Hyman (1983) found that although identification of carbonated water was little affected by color, color-influenced responses were obtained on red, yellow and brown birch beer, with red color exerting greatest influence. Hyman concluded that carbonated water had too little taste to permit interaction between the taste stimulus and visual stimulus. In that study color was added to the carbonated water and birch beer by opening and resealing the bottles, and "flatness" of the beverage may have influenced identification of the product. DuBose et al. (1980) used trained panelists to examine the effect of color on identification of noncarbonated beverages. They reported that color influenced identification, although the data were not analysed statistically.

Our objective was to study the influence of color on identification of two different flavors using an uncarbonated product consisting of a packet-mix fruit-flavored beverage. Uncolored orange-flavored and raspberry-flavored versions of these beverages can be purchased "off the shelf," and have the advantage that the flavors are distinct and unconfounded with sensations associated with carbonation. Sachets of fruit-flavored beverage powder are common items in New Zealand households.

MATERIALS & METHODS

Subjects

The subjects were 310 untrained volunteers, 15 years of age or older, prospective students, parents of students, and members of the general public, (visitors to the University of Auckland Open Day). About two-thirds of the volunteers were women. The study was approved by the University Human Subjects Ethics Committee.

Materials

Uncolored, red, yellowish-orange and green versions of raspberry-flavored and orange-flavored beverages were prepared. Identical 1 L clear-plastic pitchers were used to contain the beverages, which were made up of filtered water mixed with powder (Vita-Fresh™ manufactured by Hansells (N.Z.) Ltd) in accordance with the manufacturer's instructions. In order to produce a batch of beverage colored appropriately for its flavor, the coloring agents listed on a colored version of the product were added to the uncolored beverage on a trial-and-error basis to make a match, by eye, to the colored version. These colorings were: for raspberry flavored beverage, powdered amaranth, (index number 16185); and for orange-flavored beverage, powdered tartrazine (index number 19140), and sunset yellow, (index number 15985). Once a satisfactory match was obtained to the colored version of the beverage, a similar amount of coloring was added to a pitcher of the other flavor (either raspberry or orange) to produce a beverage of the same color but alternative flavor. The green versions of each flavor were obtained by the addition of a few drops of liquid green coloring to two pitchers first colored the same yellowish-orange as the colored orange beverage. Use of the uncolored product as a base in every case ensured that the four batches of each flavor were identical, except for addition of the coloring agent. The three colored versions of each beverage were matched to hues from the Farnsworth-Munsell 100-Hue Test (Munsell Color, MacBeth Division, Maryland). The hues matched chips numbered 81, 15, and 23, (labelled red, orange and green throughout this report). In order to discourage participants from making assumptions based on the presence of exactly two duplicated color sets, a ninth pitcher, colored yellow, was included. The presence of the extra pitcher also served, by contrast, to identify the pair of yellowish-orange beverages as orange in color.

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Table 1—Flavor-labels applied to samples of raspberry-flavored and orange-flavored beverage, irrespective of color

Flavor label	Actual flavor	
	Raspberry	Orange
Apple	2	–
Blackberry	1	–
Blackcurrent	1	–
Boysenberry	1	–
Cherry	1	–
Citrus	–	2
Grape	1	–
Lemon	7	17
Lime	20	10
Mandarin	–	1
Mango	–	3
Orange	10	106
Passionfruit	1	1
Pineapple	11	9
Raspberry	94	6
Strawberry	1	–
Tropical	1	–
Nonfruit: sour	1	1
sugarwater	1	–
Totals:	154	156

This pair was a match to the manufacturers colored version of orange beverage. The pitchers of beverage were mixed the day before use and stored in a refrigerator overnight. They were brought to room temperature before use. During the course of the experiment samples were removed and discarded from the yellow pitcher from time to time to equate pitcher levels, but no samples from this pitcher were presented to subjects. The pitchers were numbered and set out in a row in plain view. About 10 mL of beverage was poured from a designated pitcher into an individual 40-mL disposable white plastic cup for each volunteer.

Procedures

Triangle tests. In order to verify the manufacturer's claim that the food colorings had no perceptible taste, pitchers of water were colored red, green and yellowish-orange as before, and then combined. This composite, containing all the colorings used in the study, was compared to plain water by means of a triangle test. Subjects were fitted with adjustable eye patches covered by a blindfold. Four untrained volunteers, staff and graduate students at the University of Auckland, each completed two blocks of 24 trials in which the six possible presentation arrangements were used equally often, and presented in random order. Out of a total of 48 trials the subjects correctly identified the "odd-man-out" 17, 15, 20 and 16 times. None of the scores indicated significant differentiation.

Flavor identification. No attempt was made to disguise the purpose for which data were collected. Subjects were instructed both verbally and in writing to identify the flavor of a single sample of beverage. The samples, labelled 1 to 9, were allocated sequentially as each subject volunteered. A poster stated "it is common knowledge that the experience of taste depends upon several factors in addition to chemical composition of the tastant. You are invited to participate in an investigation of the influence of color on flavor by judging the flavor of a sample of fruit beverage." The sample was poured from the designated pitcher in plain view of the subject. The range of judgements was unrestricted, although instructions identified the beverages as fruit-flavored. Subjects registered their decision on a response form that referred to a designated beverage by number and included the instructions "Your sample of fruit-flavored beverage will be either uncolored, colored appropriately for its flavor, or colored inappropriately for its flavor. Please write down below what you think its actual flavor is, and put this slip in the "ballot box."

Because volunteers were ignorant of the number of possible flavors among the nine pitchers on display, but knew their sample would be either uncolored, appropriately colored, or inappropriately colored, they had no reason to adopt any other goal than to strive to make a correct identification. Towards this end, because appropriately colored beverages were included, tasters had as much reason to accept the evidence provided by their gustatory sensations as they had to be cautious in making judgments based on expectations arising from sample color.

Statistical analysis of the subjects flavor judgements of the different samples were by means of two-tailed chi-square tests for independent samples.

Table 2—Proportion of tasters in the group who made correct identifications of flavor for each of eight combinations of color and flavor. Data are also given in fractional form

Color	Flavor			
	Raspberry		Orange	
Uncolored	0.553	(21/38)	0.512	(21/41)
Red	0.897	(35/39)	0.763	(29/38)
Orange	0.487	(19/39)	0.769	(30/39)
Green	0.500	(19/38)	0.684	(26/38)

Table 3—The proportion of correct identifications of flavor for four versions of raspberry and orange beverage with the proportion correct for every other version of the same flavor[a]

Raspberry				
	Uncolored	Red	Orange	Green
Uncolored	–	$\chi^2 = 9.86^{**}$	$\chi^2 = 0.12$	$\chi^2 = 0.05$
Red		–	$\chi^2 = 13.54^{***}$	$\chi^2 = 12.68^{**}$
Orange			–	$\chi^2 = 0.01$
Green				–
Orange				
	Uncolored	Red	Orange	Green
Uncolored	–	$\chi^2 = 4.32^{*}$	$\chi^2 = 4.66^{*}$	$\chi^2 = 1.76$
Red		–	$\chi^2 = 0.04$	$\chi^2 = 0.26$
Orange			–	$\chi^2 = 0.34$
Green				–

[a] d.f. = 1 in every case. Critical values of χ^2, two-tailed, with 1 d.f. are: at the $p = 0.05$ level of significance, 3.84; at the $p = 0.01$ level of significance, 6.64; and at the $p = 0.001$ level of significance, 10.83.
* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

RESULTS & DISCUSSION

IN ALL, 17 fruits and two non-fruits were named in the 310 judgements (Table 1). Five fruits accounted for 0.94 of the terms used to describe the flavors, whether correctly or incorrectly identified. These were raspberry, 0.323; orange, 0.374; lime, 0.097; lemon, 0.077; and pineapple, 0.065.

The dependent variable of most interest was the proportion of correct attributions of flavor for each combination of flavor and color. It is therefore pertinent to ask whether the artificial flavors were adequate examples of orange and raspberry flavors to serve as a basis for meaningful comparisons among combinations. The proportion of correct identifications expected by chance could not be determined precisely, because participants' flavor judgements were unrestricted. However, the number of different fruit flavors attributed to any one of the eight pitchers varied from 4 to 8. This could be used as a conservative estimate of the range of flavor labels expected if flavor identification was due to chance. By this reasoning the expected proportion correct should be in the range 0.250 to 0.125. The proportion correct of the total judgments for each sample was recorded in (Table 2) in fractional form. Flavor of each of the 8 pitchers was identified correctly well beyond the level expected by chance.

The influence of color on identification of flavor was examined for the raspberry and orange beverages separately by means of chi-square tests on the proportions (Table 2). Identification of each flavor was significantly affected by color. The effect of color can be accepted with greater confidence for raspberry flavor (χ^2 with 3 d.f.'s = 16.55 $p < 0.001$) than for orange flavor (χ^2 with 3 d.f.'s = 7.94 $p < 0.05$). The distinctive outcome for the raspberry-flavored beverage appeared to be a relatively high proportion of correct identifications for appropriately colored samples. The distinctive outcome for orange-flavored beverage appeared to be the relatively low identification of uncolored samples. These differences were explored formally by comparing the proportion correct for the four versions of each flavor with the proportion correct for every other version of that flavor. Chi-square tests for two independent samples, corrected for continuity, were employed (Table 3). Three comparisons involving raspberry-flavored beverage were significant beyond the $p = 0.01$ level,

Table 4—Proportion of correct identifications of flavor under comparable color manipulations for orange and raspberry flavors (d.f. = 1)

	Beverage (Color/Flavor)	χ^2
	Uncolored/Raspberry	
Versus	Uncolored/Orange	$\chi^2 = 0.02$
	Red/Raspberry	
Versus	Red/Orange	$\chi^2 = 1.48$
	Green/Raspberry	
Versus	Green/Orange	$\chi^2 = 1.96$
	Red/Orange	
Versus	Orange/Raspberry	$\chi^2 = 5.12^*$

* $p < 0.05$

while two comparisons involving the orange-flavored beverage were significant beyond the $p = 0.05$ level. For raspberry-flavored beverage, significantly more correct identifications of flavor were made to the red-colored version in the three comparisons in which it was a choice. In the case of the orange flavored beverage, significantly less correct identifications of flavor were made to the uncolored beverage in two of the three comparisons in which it was included.

It was prudent to ascertain whether the more pronounced effect of color on raspberry-flavored than on orange-flavored beverage was attributable to a difference in the ease with which the two flavors were identifiable. To this end, a further set of comparisons were made between outcomes for the two flavors under comparable color manipulations (Table 4). Proportion correct for uncolored samples could be regarded as baseline performance in the absence of color information, and was not significantly different for the two flavors. Appropriately colored samples of both flavors were also equally identifiable, as were green-colored samples of both flavors. Orange-flavored beverage colored red, however, was easier to identify as such than raspberry-flavored beverage colored orange. This difference accounted in large measure for the more pronounced effect of color on raspberry-flavored beverage (Tables 2 and 3). Because both flavors were equally well identified when uncolored, this could not be attributed to the artificial orange flavor being easier to identify per se and therefore less subject to color influences. Appropriately colored raspberry beverage was, equal or easier to identify than appropriately colored orange beverage (see Table 2). This trend appeared in accord with Hyman's (1983) finding of a greater ability of red than yellow to generate color-oriented responses in the identification of birch beer. However, with that explanation, one would expect evidence of a bias towards color appropriate responses in the attribution of flavor to red-colored orange beverage, compared to orange-colored raspberry beverage. Paradoxically only 5/38 responses to the red orange-flavored beverage related to red-colored fruit (raspberry in each case), while 16/39 responses to yellowish-orange raspberry-flavored beverage related to orange or yellow fruit (pineapple = 9, orange = 6 and lemon = 1).

The extent to which olfactory cues assisted identification of the flavor was unknown. In view of the fact that approximately equal numbers of correct identifications were made to uncolored raspberry- and orange-flavored samples, any such contribution unlikely affected the two flavors differentially. The volunteers were untrained consumers and were not observed to make deliberate use of olfactory cues. The testing bench was located in a well populated area alongside other displays, so the olfactory environment was complex.

CONCLUSION

CORRECT identification of artificial raspberry and orange flavors was degraded equally when beverages were uncolored, and facilitated equally when beverages were appropriately colored. However unusual color-flavor combinations (orange raspberry, or red orange) reduced the identification of raspberry flavor more than the identification of orange flavor. This was contrary to expectations based on reports that red coloring had a greater propensity to generate color-oriented responses to birch beer than did yellow coloring. With tasters who were alert to the fact that color might not be indicative of the actual flavor, the finding that color significantly influenced flavor judgements is particularly impressive, and emphasizes its important influence in the gustatory experience. This has obvious consequences in relation to acceptability and appeal of domestically and commercially prepared foods and beverages. In addition, individuals who have diminished taste sensitivity are likely to find attractively colored meals more palatable, because they evoke memories and expectations that may make a positive cognitive contribution to the perception of flavor.

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Base-Mediated Firmness Retention of Sweetpotato Products

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ABSTRACT

Sweetpotato strips vacuum-infiltrated with 0.01–0.15M solutions of Na_3PO_4, Na_2CO_3, NH_4OH, or NaOH prior to heat processing were firmer than untreated, heat-processed strips. Among the bases Na_3PO_4 and Na_2CO_3 were most effective. When base-treated, cooked tissue was adjusted to its normal pH range (5.9–6.2) and reheated, the retention of firmness did not decrease. Using calcium chloride solution in conjunction with base treatment further increased firmness retention. This process was applied on strips but could readily be adapted to other types of sweetpotato products ranging from dice to chunks.

Key Words: sweet potato, firmness, pectic, pectin methylesterase

INTRODUCTION

DIFFICULTY in controlling textural properties of processed sweetpotato products is a major reason for the small number of such products available in retail markets. Control of textural properties is essential. A successful commercial operation must produce consistent high-quality products from raw materials, ranging from freshly harvested roots to those which have been stored up to 10 mo.

Many published reports have described pureed sweetpotato products (Collins and Walter, 1992). However, there are few accounts of restructured products (Walter and Hoover, 1984; Hoover et al., 1983). For pureed/restructured products, texture control depends on change in the starch since cell to cell junctions and, consequently, product cohesiveness have been destroyed by maceration. Binders/thickeners can be added during processing to adjust texture for desired characteristics.

Control of firmness of intact sweetpotato tissue by vacuum-infiltration with acidulants prior to heat processing has been reported (Walter et al., 1992). However, this technique has limited usefulness because of changes in flavor due to acidulants. Also, when the tissue was readjusted to its normal pH (ca. 6) firmness retention was lost. Van Buren and Pitifer (1992) reported that snap beans, carrots, potatoes, cauliflower, and apples de-esterified with NaOH solution softened much more slowly when cooked at neutral or slightly basic pH than did untreated vegetables. The process they used included blanched tissue soaked for 3 days in dilute NaOH solution at pH 12.5. The process we independently developed explored the effects of several bases on raw sweetpotato tissue exposed for 20 min to an alkaline environment before exposure to heat and how each base affected firmness retention of the fully cooked material. Our objective was to develop a process to increase firmness retention of cooked sweetpotato tissue and avoid the disadvantages of the acid treatments.

MATERIALS & METHODS

JEWEL CULTIVAR sweetpotatoes harvested in 1989 and 1990 were utilized. The roots were cured and stored prior to use. The sweetpotatoes were hand-peeled, rinsed, and sliced into strips 0.9 cm thick × 0.9 cm wide, or 0.95 cm thick by 0.95 cm wide. Lengths were not controlled. For vacuum infiltration, strips were held beneath the liquid surface and the pressure reduced to 0.1 atm. After 10 min, the vacuum was released and the strips held for an additional 10 min. The solution was removed, and the strips were rinsed with water and blanched 2 min in water (100°C). For some experiments strips were frozen and, at a later date, fried in soybean oil for 3 min at 158–167°C (Fig. 1). They were then cooled and the shear force measured using a Kramer shear compression cell coupled to an Instron Universal Testing Machine (Walter et al., 1992). The crosshead speed was 200 mm/min. The shear force for each treatment was determined in triplicate. In other experiments, the shear force of blanched strips was measured in triplicate. Experimental details are outlined in Table 1.

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Effect of processing steps on firmness retention (Experiment 1)

Two groups of strips were prepared. One group was VI with water and served as the control for single, VI samples. The other group was VI with 0.05M Na_2CO_3. Both groups were blanched in water at 100°C for 3 min. A part of each group was then frozen. The remainder were fried in soybean oil for 3 min at 155–168°C and cooled. After 24 hr, the frozen samples were fried as described for nonfrozen samples. The force to shear samples was measured on raw, VI, blanched, fried, and frozen-fried strips for both groups.

Effect of bases on firmness retention (Experiment 2)

Depending upon the experiment, 0.01–0.15M solutions of Na_3PO_4, Na_2CO_3, NH_4OH, and/or NaOH were vacuum infiltrated (VI).

Effect of increasing pH of base-treated tissue on firmness (Experiment 3)

Five 300-g batches of sweetpotato strips were treated as follows: (1) VI with water, blanched 2 min at 100°C, VI with water, blanched 2 min at 100°C (control); (2) VI with 0.03M Na_2CO_3, blanched, VI with water, blanched (base control); (3) VI with 0.03M Na_2CO_3, blanched, VI with 0.1M acetate buffer (pH 6.1), blanched; (4) VI with 0.03M Na_2CO_3, blanched, VI with 0.1M acetate buffer (pH 5.1),

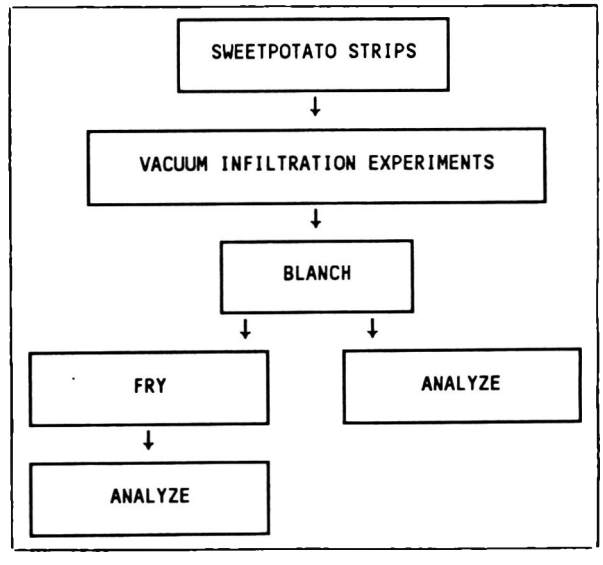

Fig. 1—Flow sheet for base treatment of sweetpotato strips.

Table 1—Details of experiments.

Experiment no.	Purpose	Concentration of base(s)	Analyses
1	Effect of processing steps on firmness	0.05M Na_2CO_3	Shear force; tissue pH
2	Effect of bases on firmness	0.01–0.015M Na_3PO_4, Na_2CO_3, NH_4OH, NaOH	Shear force; tissue pH; peptic substances; galacturonic acid; degree of esterification
3	Effect of firmness when tissue returned to original pH values	0.03M Na_2CO_3 and acetate buffers	Shear force; tissue pH
4	Effect of Ca^{+2} on firmness of base-treated tissue	0.3M Na_2CO_3 and $CaCl_2$ solutions	Shear force; tissue pH
5	Mechanism of base-mediated firmness retention	0.05M Na_2CO_3	Shear force; tissue pH; galacturonic acid; degree of esterification

blanched; (5) VI with 0.03M Na_2CO_3, blanched, VI with 0.1M acetate buffer (pH 4.1), blanched. The treated samples were held overnight in the refrigerator and the shear force and tissue pH measured.

Effect of calcium chloride on firmness of base-treated tissue (Experiment 4)

Seven 300-g batches of sweetpotato strips were treated as follows: (1) VI with water, blanched 2 min at 100°C, VI with water, blanched (control); (2) VI with water, blanched 2 min at 100°C, VI with 0.2% $CaCl_2 \cdot 2\ H_2O$, blanched; (3) VI with water, blanched 2 min at 100°C, VI with 0.6% $CaCl_2 \cdot 2\ H_2O$, blanched; (4) VI with 0.03M Na_2CO_3, blanched, VI with water, blanched (base control); (5) VI with 0.03M Na_2CO_3, blanched, VI with 0.1M acetate buffer (pH 5.3), blanched; (6) VI with 0.03M Na_2CO_3, blanched, VI with 0.2% $CaCl_2 \cdot 2\ H_2O$ in pH 5.3 buffer, blanched; (7) VI with 0.03M Na_2CO_3, blanched, VI with 0.6% $CaCl_2 \cdot 2\ H_2O$ in pH 5.3 buffer, blanched. The treated samples were held overnight in the refrigerator and the shear force and tissue pH measured.

De-esterification mechanism study (Experiment 5)

This study was performed to determine whether de-esterification caused by base treatment of sweetpotato strips prior to heating was enzymatic, nonenzymatic, or a combination. Four 300-g batches of sweetpotatoes were treated as follows: (1) VI with water, blanched and cooled, blanched (control); (2) VI with 0.05M Na_2CO_3, blanched and cooled, blanched (base control); (3) blanched and cooled, VI with water, blanched; (4) blanched and cooled, VI with 0.05M Na_2CO_3, blanched. Strip shear force and tissue pH were measured.

Tissue analysis

Dry matter was measured and alcohol-insoluble solids (AIS) were prepared as described by Walter et al. (1992). Using separate portions of AIS, water- and chelator-soluble pectins and total pectin were measured. Water- and chelator-soluble pectins were prepared and the anhydrogalacturonic acid content measured as described earlier (Walter et al., 1992). Total pectin content (as anhydrogalacturonic acid) was measured by a modification of the method described by Scott (1979). Duplicate 0.1-g samples of AIS were wet with 0.2 mL ethanol, placed in an ice bath for 15 min, 2 mL of cold, concentrated sulfuric acid were added and the mixture vortexed. An additional 2 mL of cold acid were added and the solution vortexed and then sonicated in an ice bath for 5 min. Samples were held at 4–5°C overnight. The viscous mixture was diluted to 50 mL with water and mixed. Aliquots were centrifuged 5 min in a tabletop centrifuge to remove particulate matter. For analysis, 0.125 mL of supernatant was mixed with 0.25 mL of 2% NaCl and 0.125 mL water in 16 × 120 mm test tubes. Then 2 mL of cold, concentrated sulfuric acid were added, and the tubes were heated at 70°C for 10 min and cooled in tap water. Next, 0.1 mL of a solution containing 0.1 g of 3,5-dimethyl phenol in 100 mL glacial acetic acid was added and the tubes held 15 min at room temperature (≈23°C). The absorbance at 400 mμ was subtracted from the absorbance at 450 mμ. This value was used to calculate the amount of galacturonic acid in the sample from a standard curve prepared with varying concentrations of galacturonic acid from 0.02 to 0.125 μM.

Pectin esterification was measured by a modification of the method of Wood and Siddiqui (1971). Alcohol-insoluble solids samples (<0.0800 g) were weighed into tubes, 2 mL of 4.0M NaOH were added, and 2 mL of water were added. The tubes were sonicated for 20 min in an ice water slurry, held at 5°C for 4.5 hr, 5.5 mL of cold, 6N H_2SO_4 added, and the mixture quantitatively transferred to a 25 mL volumetric flask and diluted to volume with water. Aliquots were centrifuged and 1.0 mL was put into 16 × 33 mm test tubes. The tubes were held in an ice water bath for ≥ 5 min, 0.2 mL of a 2% solution of $KMnO_4$ added, mixed, and, after 15 min, 0.2 mL of 0.5M sodium arsenite (in 0.12N H_2SO_4) and 0.6 mL water were added and mixed. After 1 hr, 2 mL of 0.02M pentane-2,4-dione (in a 50:50 mixture of 4.0M ammonium acetate and 0.1M acetic acid) were added, mixed, and held at room temperature for 1 hr. The absorbance at 420 nm was measured and the concentration of methanol calculated from a standard curve prepared with concentrations of methanol, ranging from 0.164 to 1.28 μM. To calculate the % methyl ester content, the molar concentration of galacturonic acid/g AIS was divided into the molar concentration of methanol/g AIS and the result multiplied by 100. Tissue pH was measured by blending duplicate 5-g samples of diced, blanched strips with 10 mL water and measuring the pH with an Orion model 701A meter.

Table 2—Effect of vacuum-infiltration of sweetpotato strips with basic substances on tissue shear force and pH prior to heat processing

Vacuum-infiltration solution	Shear force (kg)	Tissue pH
None	34.9 ± 6.1	6.07
Water	31.6 ± 2.7	6.05
0.01M Na_3PO_4	46.3 ± 2.0	6.25
0.05M Na_3PO_4	105.0 ± 9.9	6.94
0.1M Na_3PO_4	120.3 ± 11.3	7.39
0.15M Na_3PO_4	96.4 ± 7.2	7.95
0.01M Na_2CO_3	42.7 ± 5.5	6.13
0.05M Na_2CO_3	78.3 ± 7.6	6.52
0.1M Na_2CO_3	78.5 ± 1.1	6.93
0.15M Na_2CO_3	41.5 ± 3.5	8.00
0.01M NH_4OH	31.5 ± 0.7	6.26
0.05M NH_4OH	57.4 ± 7.5	6.69
0.1M NH_4OH	54.2 ± 5.5	7.7
0.15M NH_4OH	56.0 ± 4.4	8.07
0.01M NaOH	40.2 ± 3.7	6.2
0.05M NaOH	36.9 ± 1.3	6.94
0.1M NaOH	50.1 ± 2.2	8.11
0.15M NaOH	41.2	8.5

RESULTS & DISCUSSION

VACUUM INFILTRATION of sweetpotato strips with solutions of Na_3PO_4, Na_2CO_3, NH_4OH, or NaOH ranging from 0.01 to 0.15M prior to blanching and frying caused increased firmness retention when compared to untreated strips and strips VI with water (Table 2). Degree of firmness retention varied with both the base and its concentration. Firmness retention decreased in the order: Na_3PO_4 > Na_2CO_3 > NH_4OH > NaOH. Firmness retention increased as the concentration of base increased from 0.01 to 0.05M, or 0.10M. With the exception of NH_4OH, firmness retention decreased when base concentration increased from 0.10 to 0.15M, possibly reflecting basic hydrolysis of cell wall polymers. After blanching, surface erosion was observed when the base concentration was > 0.05M. However, sodium hydroxide solutions > 0.01M eroded the blanched tissue surface.

The force required to shear strips decreased as the degree of processing increased. Raw strips were slightly firmer than the VI strips (Fig. 2). After blanching, base-treated strips had lost about 49% of their firmness, while control strips had lost 83% of their firmness. Fried base-treated strips lost 52% of their firmness, while the fried control strips had lost 86% of

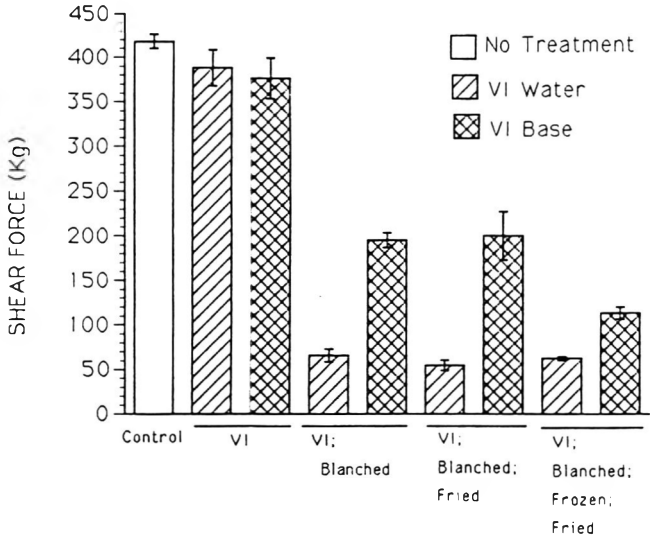

Fig. 2—Shear force of sweetpotato strips at various steps in the base treatment (0.05M Na_2CO_3) process. (VI = vacuum infiltrated).

Table 3—Galacturonic acid content, methanol content, and methanol/galacturonic ratio of AIS from sweetpotatoes vacuum-infiltrated with bases

Vacuum-infiltration solution	Galacturonic acid (μM/g AIS)	Methanol (μM/g AIS)	μM Methanol/μM galacturonic acid[a]
None	223.1	113.1	0.5609A
Water	238.7	114.5	0.4798B
0.01M Na_3PO_4	211.7	75.1	0.3548D
0.10M Na_3PO_4	186.7	43.2	0.2314E
0.01M Na_2CO_3	237.8	92.3	0.3884CD
0.10M Na_2CO_3	230.3	50.6	0.2816E
0.01M NH_4OH	237.4	109.4	0.4608B
0.10M NH_4OH	231.0	65.0	0.2816E
0.01M NaOH	236.1	104.2	0.4325BC
0.10M NaOH	182.3	44.1	0.2413E
MSD	16.5	15.2	0.0641

[a] Values within the same letter are not significantly different (P <0.05).

their original firmness. When the frozen strips were fried, the base-treated strips lost 71%, and the control lost 84% of original firmness. Freezing prior to frying caused a greater decline in firmness of acid-treated strips than that of control strips. These data showed that treating tissue with base prior to heating decreased heat-mediated softening.

Current theory is that softening occurs during cooking because pectic substances, responsible for holding plant cells together and for plasticizing cell walls, are cleaved causing cell separation and cell wall expansion (Doesburg, 1965; Van Buren, 1986). The mechanism postulated for pectin cleavage in the pH range (5.0-6.5) common to most processed vegetables is beta-elimination. This mechanism, in which an activated proton at C-5 is removed and the glycosidic linkage alpha to the carboxyl group is cleaved, was proven to be operative at relatively high pH values (Bemiller and Kumari, 1972), but direct experimental data for vegetable tissues is not available. At neutral pH, it is postulated that there are sufficient hydroxide ions present to promote beta-elimination at cooking temperatures. However, if the pH is lowered, the concentration of hydroxide ions decreases, the rate of reaction decreases, and subsequent softening typical of cooked vegetables decreases. A characteristic of decreased softening caused by tissue acidification is that it can be reversed if tissue pH is readjusted to 6 and it is reheated (Walter et al., 1992).

Other workers have reported that, for chelator-soluble carrot pectin, the higher the methyl ester content, the greater the rate of pectin chain degradation during heating at pH 6.1 (Sajjaanantakul et al., 1989). In an extreme case, boiling totally deesterified citrus pectin for long periods at pH > 6 pH did not

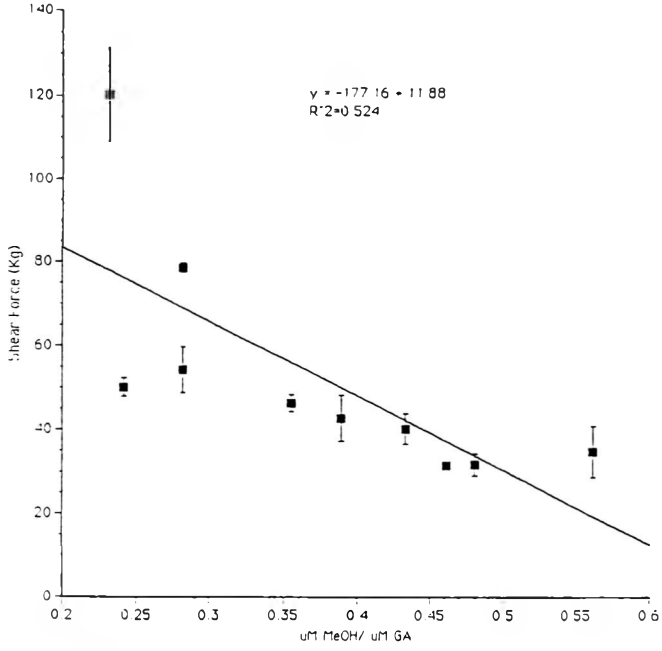

Fig. 3—Linear regression of shear force on μM methanol/μM galacturonic acid in base-treated sweetpotato strips.

cause pectin degradation (Van Buren, 1979). The rationale for this result was that the negative charge associated with the deesterified carboxylic acid strongly inhibited beta-elimination by destabilizing any developing negative charge at C-5 caused by hydroxide ion-mediated removal of protons.

Our results tend to support the theory that increased demethoxylation inhibits beta-elimination and, thus, decreases heat-mediated softening. Regardless of base, strips VI with 0.1M concentrations and blanched were firmer (Table 2) and more demethoxylated than untreated samples of those treated with 0.01M base solutions (Table 3) and blanched. However, strip firmness (measured by shear force) varied by base type. Maximum shear force declined in the order: Na_3PO_4 > > Na_2CO_3 > NH_4OH and NaOH. The fact that linear regression of shear force on μM methanol/μM galacturonic acid could explain only 52% (Fig. 3) of the variability in the model indicated that other factors were involved.

A characteristic of beta-elimination inhibition due to pectin demethoxylation would be that readjusting demethoxylated tissue to its normal pH (≈6) and reheating it should not result in increased softening. In contrast to the results of Van Buren and Pitifer (1992), we found this to be true. However, Van Buren and Pitifer (1992) lowered the tissue pH to ca. 3.5 before reheating, and this could have caused increased softening. In our study, tissue treated with 0.03M Na_2CO_3, blanched, then VI with water, and again blanched was firmer than the control and was slightly less firm than base-treated, blanched tissue VI with either pH 6.1 or pH 5.1 acetate buffers and then blanched (Table 4). When base-treated, blanched tissue was VI with pH 4.1 buffer and again blanched, it was significantly firmer than the other treatments. This firmness increase was greater than that resulting from treatments in which the final tissue pH was near that of the original tissue (i.e., either of the other buffers). This was probably due to tissue acidification (Walter et al., 1992) since its pH was 5.19, while the normal pH was 6.23.

Since base-treated tissue had an increased number of carboxylate groups, we wanted to ascertain whether incorporation of calcium ions into the second infiltration medium could increase firmness. Calcium has been shown to increase firmness in many processed vegetables (Van Buren, 1979; 1986). This effect, described by the "egg-box model," is ascribed to a mechanism in which divalent calcium crosslinks adjacent pec-

Table 4—Effect on shear force and tissue pH of readjusting base-treated sweetpotato tissue to original pH

Treatment[a]	Shear force (kg)	Tissue pH
Water (control) → water	50.0 + 10	6.23
0.03M Na_2CO_3 → water	85.3 + 12	6.56
0.03M Na_2CO_3 → pH 6.1 buffer	100.3 + 11.9	6.41
0.03M Na_2CO_3 → pH 5.1 buffer	104.0 + 9.5	5.87
0.03M Na_2CO_3 → pH 4.1 buffer	131.8 + 12.6	5.19

[a] Tissue was vacuum-infiltrated with the solution of the arrow and blanched. After cooling, the blanched strips were vacuum-infiltrated with the solutions to the right of the arrow, blanched, and the shear force and pH measured.

Table 5—Effect of $CaCl_2$ on shear force of Na_2CO_3-treated sweetpotato strips

Treatment[a]	Shear force (kg)	Tissue pH
Water (control) → water	69.3 ± 5.1	6.25
Water → 0.2% $CaCl_2$	65.3 ± 10.8	6.14
Water → 0.6% $CaCl_2$	83.0 ± 9.3	6.11
0.03M Na_2CO_3 → water	121.3 ± 7.5	6.77
0.03M Na_2CO_3 → pH 5.3 buffer	140.6 ± 6.1	6.11
0.03M Na_2CO_3 → 0.2% $CaCl_2$ in pH 5.3 buffer	162.5 ± 15.3	6.15
0.03M Na_2CO_3 → 0.6% $CaCl_2$ in pH 5.3 buffer	196.3 ± 14.9	6.09

[a] Tissue was vacuum infiltrated with the solution to the left of the arrow and blanched. After cooling, the blanched strips were vacuum infiltrated with the solutions to the right of the arrow, blanched, and the shear force and pH measured.

tin molecules via formation of ionic bonds with free carboxyl groups. The resultant three-dimensional network confers increased resistance to shearing forces. We found that treatment of sweetpotato strips with 0.03M Na_2CO_3, blanching the strips, and then VI with 0.6% calcium chloride solution in acetate (buffer (pH 5.3) resulted in almost a three-fold increase in shear force over control strips (Table 5). The buffer alone increased firmness by about 16% over Na_2CO_3 treated strips, and 0.2% calcium chloride increased firmness by another 16%. These results suggested that firming could be enhanced and tissue pH readjusted to the normal value by vacuum-infiltrating a second solution composed of acetate buffer (pH 5.3) and calcium chloride.

Vacuum infiltration with bases could have removed some of the pectic materials from the strips compared with VI with water. Thus, we measured the galacturonic acid content of water- and chelator-soluble pectin fractions and the galacturonic acid content of the total dry matter. We observed no consistent loss of pectic material (Table 6). For both water- and NaOH-treated samples, blanched pectic substances apparently increased over raw pectic substances, possibly due to extraction of endogenous sugars by blanch water. Some differences were observed between relative amounts of water-soluble pectins and chelator-soluble pectins. For raw tissue, vacuum infiltration with either base caused chelator-soluble pectins to be more abundant than water-soluble pectins. For blanched tissue, this was reversed, but in base treated, blanched tissue the amount of chelator-soluble pectin was still twice that in the blanched control strips. Thus, base treatment apparently converted some of the pectic materials from water-soluble to chelator-soluble.

The mechanism by which bases de-esterified methyl esters of polygalacturonic acid-containing polymers in sweetpotatoes could be either enzymatic or nonenzymatic. Research has demonstrated that sweetpotatoes have an active pectin methylesterase system (Baumgardner and Scott, 1965). Van buren and Pitifer (1992) held plant material in NaOH solution for 3 days to ensure that de-esterification was a non-biological process. To study this, we conducted an experiment in which we VI strips with 0.05M Na_2CO_3 either before or after blanching the tissue and measured the degree of esterification. Two sets of samples in which water was the infiltrating solution before and after blanching were also analyzed. We found that tissue treated with base prior to blanching was significantly less esterified than that which was blanched prior to treatment with 0.05M Na_2CO_3

Table 6—Galacturonic acid[a] content of raw and blanched sweetpotato strips vacuum-infiltrated with either water, 0.1M NaOH, or 0.1M Na_2CO_3

Galacturonic acid	Water	0.1M NaOH	0.1M Na_2CO_3
Raw tissue			
Water-soluble	115.7	65.0	72.6
Chelator-soluble	44.8	76.8	96.7
Total	229.5	214.6	238.4
Blanched tissue			
Water-soluble	155.6	99.8	88.9
Chelator-soluble	21.8	58.6	63.7
Total	258.2	228.5	219.7

[a] μMoles galacturonic acid/g dry matter.

Table 7—Effect of blanching on % methyl ester content of sweetpotato strips treated with 0.05M Na_2CO_3 solution

Treatment[a]	% Esterification[b]	Tissue pH
VI water → blanch → blanch	71.49A	5.83
VI Na_2CO_3 → blanch → blanch	52.08B	6.12
Blanch → VI water → blanch	74.75A	5.90
Blanch → VI Na_2CO_3 → blanch	64.21A	6.97

[a] Tissue was vacuum-infiltrated either before or after an initial blanch treatment.
[b] Values with the same letter are not significantly different ($P < 0.05$).

(Table 7). On that basis, we concluded that most of the de-esterification resulting from base treatment was enzymatic.

CONCLUSIONS

TREATMENT of sweetpotato tissue with either Na_2CO_3 or Na_3PO_4 prior to heat processing increased firmness retention. When base-treated tissue was VI with calcium chloride, tissue firmness was enhanced. Base-mediated firmness retention was effective on strips and could easily be adapted to many types of products ranging from dice to chunks.

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Supercritical CO_2 Extraction of β-Carotene from Sweet Potatoes

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ABSTRACT

Using supercritical CO_2 to extract β-carotene there was approximately a five-fold or a three-fold increase in amount of carotenoids extracted from freeze-dried tissue relative to the amount extracted from oven-dried or fresh tissue, respectively. The most efficient conditions were at 48°C and 41.4 MPa. Of the total carotenoid content ≈20% was inaccessible to supercritical CO_2. The HPLC carotenoid profile of sweet potatoes showed that the unextracted tissue contained 90% β-carotene, primarily as *all-trans* (ca. 99%). Supercritical CO_2 extracts contained up to 94% β-carotene. The isomer composition of β-carotene of supercritical extracts showed ≈ 14% 13-*cis* and 11% 9-*cis*.

Key Words: carotene, vitamin A, CO_2 extraction, sweet potatoes

INTRODUCTION

INTEREST is growing for obtaining β-carotene from natural sources because of consumer interest, its high provitamin A activity as well as its use as a colorant in foods. In food systems, β-carotene is also an effective antioxidant. According to reports and clinical studies, dietary β-carotene may be important in the prevention of some forms of cancer (Burton and Ingold, 1984; Menkes et al., 1986; Krinsky, 1988; 1989).

Sweet potatoes have a pleasant orange/yellow color because of their carotenoid content. β-Carotene is the predominant carotenoid of sweet potato tissue and is primarily found as *all-trans* β-carotene (Khachik and Beecher, 1987; Chandler and Schwartz, 1987; 1988). The *all-trans* form exhibits the maximum provitamin A activity (Sweeney and Marsh, 1973). Other carotenoid compounds such as α-carotene, γ-carotene, ζ-carotene, phytoene and phytofluene, have also been reported in sweet potatoes (Purcell and Walter, 1968), but at very low levels (usually less than 1%). Therefore, this readily available root crop could provide an excellent natural source of β-carotene.

Extraction of natural products with supercritical CO_2 (SC-CO_2) has received much attention. CO_2 leaves no chemical solvent residues in foods, allows for extractions at low to moderate temperatures, is nontoxic, low cost and readily available. Decaffeination of coffee (Zosel, 1974) and extraction of hops (Laws et al., 1980) are commercial food processing applications of SC-CO_2. Reduction of milk fat cholesterol (Lim et al., 1991), extraction of lipids and cholesterol from meat (Chao et al., 1991) and egg yolk (Froning et al., 1990) as well as recovery of crude oil from dry-milled corn germ, soybean and cottonseed flakes (List and Friedrich, 1989) have been reported. These reports reflect a strong interest in SC-CO_2 extraction of natural products which could lead to more commercial applications. Studies on the SC-CO_2 extraction of carotenoids include separation of carotene and lutein from alfalfa leaf protein concentrates (Favati et al., 1988), extraction of β-carotene from algae (Lorenzo et al., 1991), and solubility measurements of pure crystalline β-carotene over a range of pressures and temperatures (Cygnarowicz et al., 1990). The latter study also included the effect of various entrainers (i.e., ethanol) on the solubility of β-carotene in SC-CO_2.

Our objectives were to explore the potential application of SC-CO_2 for extraction of β-carotene from sweet potato tissue, and investigate the effects of pretreatment as well as extraction conditions. We also examined the effect of SC-CO_2 extraction conditions on composition of the carotenoid extract.

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MATERIALS & METHODS

Sample preparation

Sweet potato tissue, (Jewel) obtained from a local University farm,was sliced into strips, ≈ 2 mm × 10 mm × 15 mm. Strips were dried either in a freeze dryer or in a forced air oven at 70°C. The oven was also utilized to determine moisture content of raw and dried sweet potato. Moisture content was measured in duplicate samples kept in the oven until they reached constant weight.

Dried material was ground with a mortar and pestle and fractionated using a series of U.S. standard sieves No. 30, 40, and 60 (opening 0.630, 0.420 and 0.250 mm, respectively, Fisher Scientific Company). Particles passing through No. 30 but retained on No. 40 were defined as coarse, while those passing through No. 40 but retained on No. 60, were defined as fine.

Supercritical fluid extraction

Extraction of sweet potato carotenoids was performed in a continuous supercritical fluid extraction apparatus (Fig. 1 Superpressure Division of Newport Scientific Inc., Jessup, MD). Liquid CO_2 (commercial grade) was compressed with a double end, diaphragm type, variable speed compressor and pumped through the extraction vessel (6.5 cm i.d. × 25.4 cm long). The internal temperature of the extractor was monitored by an inserted thermocouple and regulated with a heater

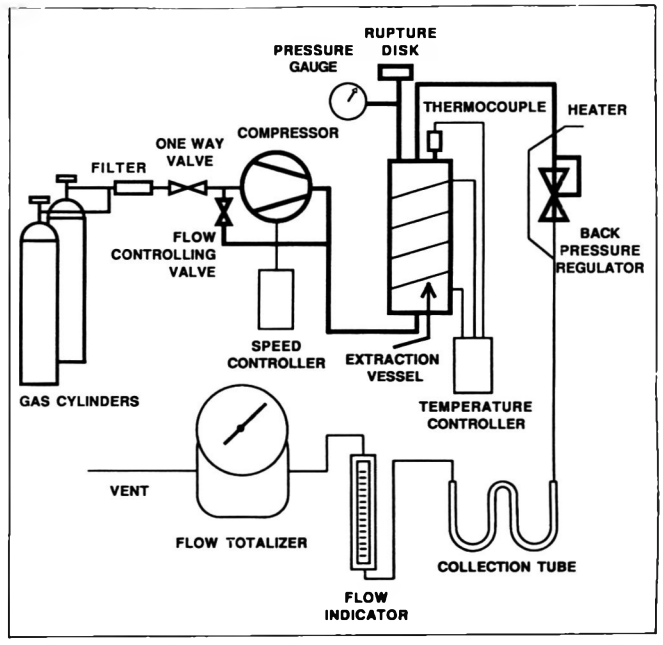

Fig. 1.—Schematic diagram of supercritical fluid extraction system.

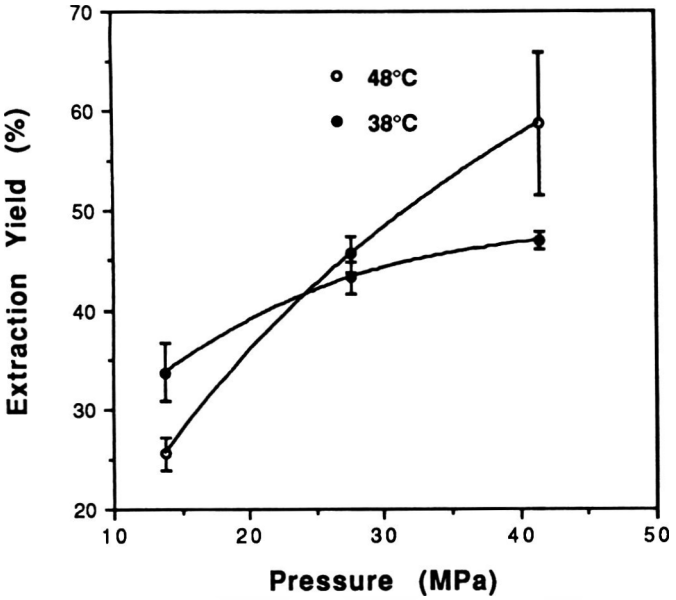

Fig. 2.—Effect of extraction conditions on the extractability of sweet potato carotenoids (fine powder of oven-dried tissue). Standard deviations are shown with error bars.

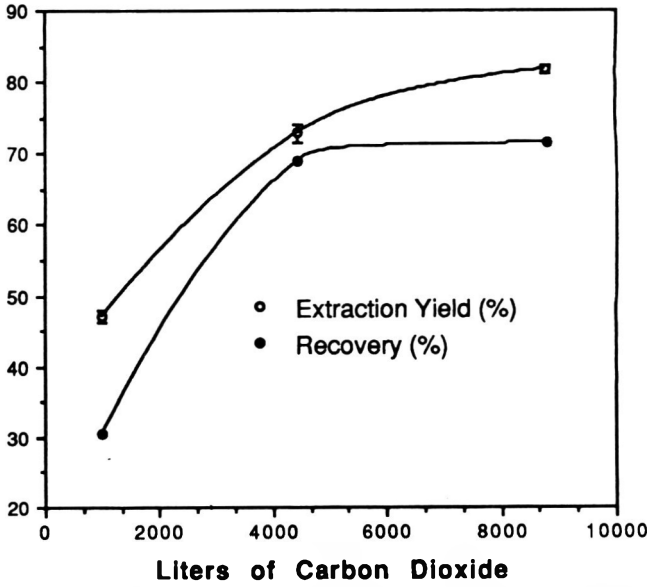

Fig. 3.—Volume of CO_2 consumed in the extraction vs. amount of carotenoids removed and amount of carotenoids recovered. Fine powder of oven-dried tissue was extracted at 41.4 MPa and 38°C. Standard deviations are shown with error bars.

Table 1—Effect of tissue pretreatment on efficiency of supercritical fluid extraction of carotenoids from sweet potatoes[a]

Pretreatment	Carotenoid content (μg/g d.b.)[d]	Carotenoids extracted (μg/g sample)	Carotenoids extracted (% d.b.)
Fresh	318.5 ± 3.5	43.6 ± 0.2	54.8 ± 0.2
Oven-Dried (forced air)			
fine[b]	164.5 ± 5.0	77.3 ± 2.1	47.0 ± 2.1
(coarse[c])	(164.5 ± 5.0)	(47.0 ± 5.7)	(28.6 ± 5.7)
Freeze-Dried			
fine	353.2 ± 2.1	227.6 ± 8.1	64.4 ± 2.3
(strips 2mm thick, 15 mm long and 10 mm wide)	(353.2 ± 2.1)	(105.0 ± 4.1)	(29.7 ± 1.2)

[a] Mean ± SD; extraction conditions were: 41.4 MPa, 38°C and 1000L of CO_2.
[b] Coarse particle passed through No. 30 sieve, but remained on No. 40 sieve.
[c] Fine particle passed through No. 40 sieve, but remained on No. 60 sieve.
[d] dry basis

controller (±1°C). The depressurized stream of SC-CO_2 discharged the extracted carotenoids in a double U-tube (collection tube) connected at the outlet of the extraction valve. The other end of the collection tube was packed with glass wool to retain the collected sample upon depressurization. The volume of CO_2 in the extraction was monitored by a flow totalizer.

Dried sample (strips or powder 1g) or raw sample (3g) was wrapped with a layer of filter paper (Whatman No. 1) and a layer of glass wool. Extraction experiments were conducted at 13.8, 27.6 and 41.4 MPa and 38°C as well as 41.4 MPa and 48°C. In all cases, 1000 L of CO_2 at 14–18 L/min (1 atm and 25°C), were passed through the extractor. The collection tube was kept in the dark and held at the temperature of dry ice resulting from expansion of the depressurizing CO_2. A few mL of hexanes were used to quantitatively transfer extracted carotenoids from the collection tube to a Teflon screw cap test tube that was flushed with nitrogen, wrapped with aluminum foil and stored @ −18°C.

Total carotenoid concentration

Total carotenoid content was determined by acetone:hexane extractions of sweet potato tissue (1g) according to the procedure described by Chandler and Schwartz (1988). Absorbance of the carotenoid extract at 453 nm was measured (Shimadzu UV-visible recording spectrophotometer, UV 240) and expressed as β-carotene based on the extinction coefficient ($E_{1\%}$) of 2592 for *all-trans*-β-carotene reported by De Ritter and Purcell (1981).

High performance liquid chromatography

Analysis of carotenoids was carried out on a high performance liquid chromatography (HPLC) system that consisted of: Waters Model 510 and 501 pumps, a U6K injector, and a 990 photodiode array detector (Waters Assoc., Milford, MA) interfaced with a NEC SX/20 workstation computer. Carotenoid extracts (200 μL) in hexanes (filtered through a 0.45 μm filter) were placed in a tube (6 × 50 mm) and the solvent was evaporated under a stream of ultra-high purity nitrogen. The dried carotenoid extract dissolved in 20 μL of ethyl ether and 80 μL of methanol was injected onto the HPLC system and separated on a Vydac 201 TP column (C_{18}, 5μ, 4.6 mm i.d. × 25 cm, Vydac, Hesperia, CA). The elution conditions (flow rate 0.7 mL/min; methanol for 5 min, followed by methanol-chloroform 94:6 in a step gradient) reported by O'Neil et al. (1991) were applied. The chromatogram was monitored at 410 nm along with absorption spectra from 300 to 500 nm. The percentage of carotenoid compounds was estimated from peak area measurements.

RESULTS & DISCUSSION

Pretreatment of sweet potato tissue

Tissue pretreatment affected (Table 1) the efficiency of carotenoid extraction at 41.4 MPa and 38°C. Moisture content, sample drying and particle size affected carotenoid yield. High moisture content (75%) of fresh samples lowered the efficiency of the extraction. Obviously, extraction from fresh samples (carotenoid content of 79.6 μg/g, fresh weight basis) resulted in inefficient use of the extraction vessel. Oven drying of the tissue prior to extraction increased the amount of carotenoids extracted/g sample. About 77 μg of carotenoids were removed from 1g of oven-dried sample, which was ≈33 μg more than that obtained from fresh samples. Oven drying, however, can cause detrimental effects on extraction. Substantial (about 50%) carotenoids were lost during oven drying due to oxidative degradation. Also a tough surface layer, formed by case hardening, was apparent in the dried samples. In preliminary work with drum dried sweet potato flakes, we observed severe case hardening. The carotenoid content of the drum-dried flakes was essentially inaccessible to SC-CO_2. The highest carotenoid yield, 228 μg of carotenoids/g tissue, was achieved in the extraction from freeze-dried sweet potato. This was about, a five-fold and a three-fold increase in the amount of carotenoids extracted from fresh and oven-dried sample, respectively. Results also show that grinding samples improved SC-CO_2 car-

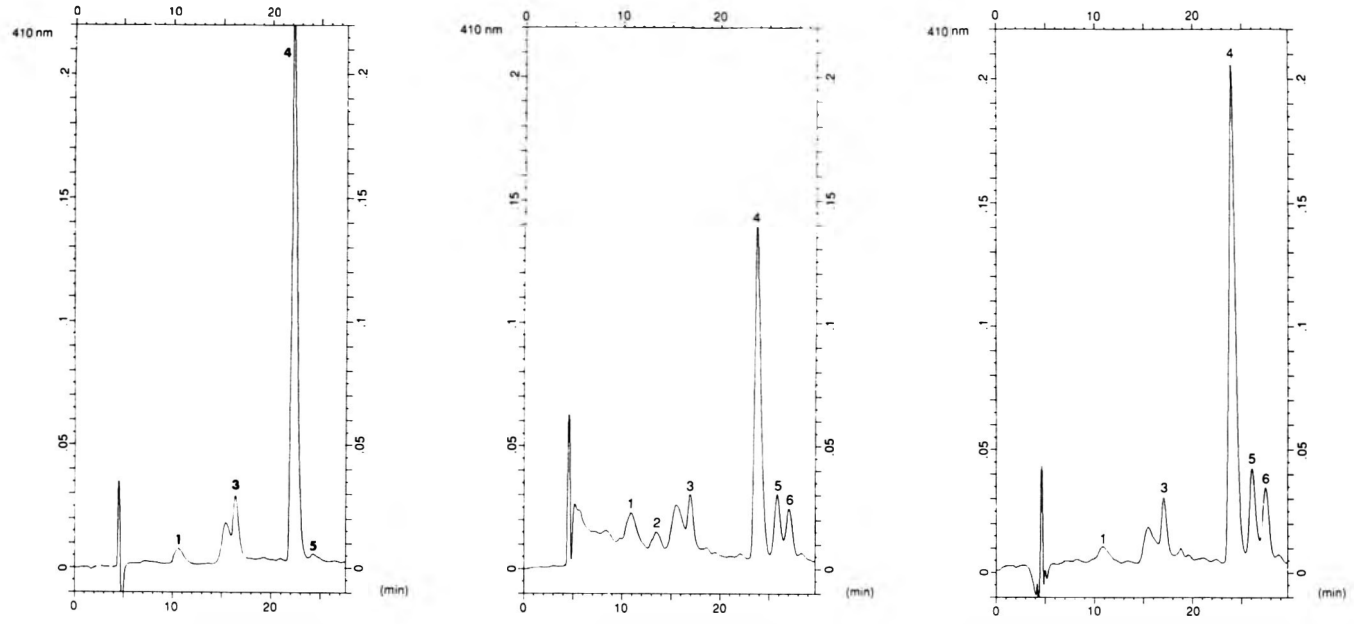

Fig. 4.—HPLC profiles of sweet potato carotenoids (freeze-dried tissue). (A) Hexene:acetone extracts (control); (B) SC-CO_2 extracts obtained at 13.8 MPa and 38°C; (C) SC-CO_2 extracts obtained at 41.4 MPa and 48°C. Peak identification: 1, 2, 3 polar carotenoids; 4, all trans β-carotene; 5, 13-cis *β-carotene; 6,* 9-cis *β-carotene.*

Table 2—Effect of SC-CO_2 extraction conditions on carotenoid composition (%)[a]

Treatment	β-Carotene	Other carotenoids[b]
Control[c]	89.3	10.7
Extracted with SC-CO_2		
13.8 MPa, 38°C	82.5	17.5
27.6 MPa, 38°C	88.5	11.5
41.4 MPa, 38°C	88.9	11.1
41.4 MPa, 48°C	93.6	6.5
Residual in tissue extracted at 41.4 MPa, 38°C	92.1	7.9

[a] Freeze-dried tissue
[b] Corresponds to peaks 1, 2 and 3 in figures 4A, B and C
[c] Extracted with hexane:acetone

otenoid extractability with that of fine powder of oven dried samples 65% higher than that of coarse powder. This was due to increase in surface area of sample and was in agreement with previous reports that increased area enhanced oil extractability and recovery from oil seeds (Snyder et al., 1984, Fattori et al., 1988).

Data indicated sample preparation was critical in extracting carotenoids from sweet potato tissue. Removal of moisture, minimizing case hardening during drying, and maximizing surface area assisted in achieving higher extraction yields.

Extraction conditions

At a constant temperature, the amount of carotenoids extracted increased as the extraction pressure increased (Fig. 2). This was apparently due to the increase in SC-CO_2 density. The effect of temperature on extraction was more difficult to assess than the effect of pressure. An increase in temperature of extraction results in increased solute vapor pressure. Density of SC-CO_2, at constant pressure, decreases as temperature increases but the magnitude of such density change becomes smaller at elevated pressures (Marentis, 1988). As shown (Fig. 2) at low pressure (13.8 MPa), lower temperature favored extraction. In contrast, at high pressure (41.4 MPa), elevation of temperature showed a positive effect on extraction, while at intermediate pressure (27.6 MPa) an increase of temperature showed no apparent effect. There is no clear explanation for these differences but solubility may be controlled by a balance between SC-CO_2 density and solute (carotene) vapor pressure changes as temperature is increased. Possibly the increase in vapor pressure of carotene from the 10°C increase in temperature could not compensate for the relatively high SC-CO_2 density drop occurring at 13.8 MPa. The increase in carotenoid vapor pressure, however, overcame the relatively small change of SC-CO_2 density caused by the 10°C increase in temperature at 41.4 MPa. At 27.6 MPa, the effect of a decrease in SC-CO_2 density seemed equally balanced by the increase in carotenoid vapor pressure.

The carotenoid extraction yield and recovery were related to the volume of CO_2 (Fig. 3) utilized in the extraction (41.4 MPa, 38°C) from fine powder of oven-dried tissue. The extraction yield was quantitated from differences in total carotenoid content of the tissue before and after extraction. Recovery was estimated from the amount of carotenoids collected. Increasing the volume of CO_2 from 1000 to 4500 L resulted in ≈ 30% increase in the amount of carotenoids extracted, and about a 35% increase in amount of carotenoids recovered. As the volume of CO_2 increased further, neither extraction yield nor recovery increased much beyond 80%. Apparently, ≈ 20% of the carotenoids present were not accessible to extraction by SC-CO_2.

Composition of SC-CO_2 extracted carotenoids

The HPLC profile of sweet potato carotenoids (freeze dried tissue) obtained with acetone:hexane extraction (control) and SC-CO_2 extraction at 13.8, 38°C and 41.4 MPa, 48°C were compared (Fig. 4A, B and C). *All-trans* β-carotene (peak 4) was identified by matching retention time and spectral characteristics with those of the *all-trans* β-carotene standard. Peaks 5 and 6 were assigned as the *13-cis* and *9-cis* isomers of β-carotene, respectively. These peaks exhibited the same retention characteristics (on identical column and elution conditions) and spectra with peaks identified as *13-cis* and *9-cis* isomers of β-carotene by O'Neil et al. (1991). The spectral characteristics of peaks 1, 2 and 3 clearly indicated that these peaks corresponded to carotenoid compounds. The retention times of peaks 1, 2 and 3 suggested polar carotenoids, most probably the oxygenated xanthophylls or products of carotenoid oxidation, although no further characterization was performed.

The sweet potato carotenoid composition was compared with

Table 3—Isomer composition (%) of β-carotene in SC-CO_2 extracts[a]

Treatment	*all-trans*	*13-cis*	*9-cis*
Control[b]	98.8	1.2	0
Extracted with SC-CO_2			
13.8 MPa, 38°C	76.5	12.9	10.6
27.6 MPa, 38°C	72.5	15.6	11.9
41.4 MPa, 38°C	75.5	13.9	10.6
41.4 MPa, 48°C	75.5	13.2	11.3
Residual in tissue extracted at 41.4 MPa, 38°C	97.2	2.8	0

[a] Freeze-dried tissue
[b] Extracted with hexane:acetone

the composition of SC-CO_2 extracts (Table 2). The composition of the carotenoids that remained in the tissue after extraction at 41.4 MPa and 38°C was also compared. Sweet potato tissue contained ≈ 90% β-carotene. SC-CO_2 carotenoid extracts, obtained at 27.6 and 41.4 MPa and 38°C, showed essentially identical percentages of β-carotene. The carotenoids (that remained in the tissue after extraction at 41.4 MPa and 38°C had a very similar β-carotene percentage with the control, verifying that extraction at 41.4 MPa and 38°C showed no selectivity for any carotenoid compounds. At 41.4 MPa and 48°C, however, some selective extraction of β-carotene was observed as a slightly higher percentage (94%) was measured. The only extracts containing considerably lower levels (82%) of β-carotene than the control were obtained at 13.8 MPa and 38°C. Solubility of β-carotene in SC-CO_2 at 13.8 MPa was low and as a result, slight oxidation occurring in the collection tube could have a major effect on relative proportions of the carotenoid peaks. Note that when isolated carotenoids were exposed to light during extraction or subsequent handling, extensive oxidation occurred that resulted in a broad HPLC peak eluting along with the injection front. Excluding light and keeping the collection tube at the temperature of dry ice (from depressurization of CO_2) eliminated the problem of oxidation.

The composition of geometrical isomers of β-carotene found in sweet potato and SC-CO_2 extracts was also compared (Table 3). SC-CO_2 caused considerable isomerization of β-carotene increasing the levels of *13-cis* and resulting in formation of *9-cis* isomers. In sweet potato tissue, ≈ 99% of β-carotene was in *all-trans* form. SC-CO_2 extracts (obtained under any conditions) were comprised of about 75% *all-trans*, 14% *13-cis* and 11% *9-cis* isomers. Heat could induce isomerization of β-carotene (Chandler and Schwartz, 1988). However, hexane extracts of sweet potato carotenoids placed in a water bath at 38°C for 70 min showed a minor increase in *13-cis* but no formation of *9-cis* isomers. Trace amounts (less than 2% of total β-carotene) of *9-cis* isomers were formed at 55°C for 70 min. There was no apparent explanation for the high increase in *13-cis* and the formation of *9-cis* isomers of β-carotene in the SC-CO_2 extracts. Analysis of carotenoids that remained in the tissue after SC-CO_2 extraction at 41.4 MPa and 38°C showed an isomer composition similar to the control. There was 2.8% *13-cis* and no *9-cis* β-carotene, indicating a protective effect of the tissue environment against β-carotene isomerization. Possibly in the supercritical phase, the conjugated double bond system of β-carotene facilitated rotation to the *cis*-isomers without resuming the *all-trans* form. Contact between β-carotene molecules (solubilized in supercritical phase) with the metal surface of the extractor might also promote isomerization. The surface of the vessel was at a higher temperature in order to maintain desired extraction conditions. To our knowledge, isomerization of such magnitude of β-carotene in SC-CO_2 has not been reported previously.

CONCLUSION

SC-CO_2 was used to obtain a natural carotenoid extract from sweet potato that contained β-carotene up to 94% (of total carotenoids) and was free of solvent chemical residues. A higher percentage of β-carotene might be possible by increasing the temperature of extraction beyond 48°C. Extraction of low moisture, ground tissue prepared under conditions that minimized case hardening was necessary to improve efficiency of extraction. Further work is needed to understand and minimize isomerization of β-carotene as levels of *-cis* isomers which form during isolation lower provitamin A activity of the extract.

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Optimizing Processing Conditions for Chemical Peeling of Potatoes using Response Surface Methodology

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ABSTRACT

Using Response Surface Methodology the effects of NaOH concentration (4-20%), process temperature (55–95°C) and time (1–7 min) was determined on the yield, peeling quality, unpeeled skin and total usage of NaOH. Also evaluated were titratable NaOH in the potato tissue, NaOH penetration and "heat ring" depths during one stage chemical peeling of potatoes (Huincul variety). The best peeling quality, maximum yield and minimum total usage of NaOH was obtained for the following ranges: concentration, 11–13%, time 5–5.70 min and temperature, 90–95°C. The maximum temperature for which the "heat ring" and NaOH penetration depths were equal was 72°C where, at 20% NaOH and 7 min, peeling quality was very good and "heat ring" was absent.

Key Words: potatoes, chemical peeling, lye peeling

INTRODUCTION

ECONOMIC and efficient peeling is very important in commercial processing of fruit and vegetable products (Reeve, 1976). Potatoes are commercially peeled by abrasion, high pressure steam, conventional caustic and dry caustic (Huxsoll et al., 1981). Among the different methods employed by industry, chemical peeling using NaOH is one of the most common and oldest. The selected method depends upon the type and variety of products to be processed and the capacity of the industrial plant. The chemical peeling of potatoes is a compromise between yield, peeling quality, NaOH consumption and the "heat ring" formed, with processing conditions empirically adjusted according to specific requirements.

Processors generally maintain the lye bath temperature as high as practicable while varying the immersion time and lye concentration to obtain desired peeling effects. While large processors use a high temperature lye process, the low temperature process has been used to some extent in the pre-peeled potato industry where the presence of a "heat ring" may be particularly objectionable (Huxsoll and Smith, 1975). In chemical peeling of potatoes three independent variables, temperature of peeling solution, concentration of NaOH and peeling time are important. Dependent variables are various, including product yield, peeling quality, NaOH consumption, "heat ring" thickness, etc. Thus, methodologies are needed for studying and optimizing such processes. Response Surface Methodology is a useful statistical technique for investigation of complex processes. Floros and Chinnan (1987,1988a,b) used it for optimization of a one and double-stage lye peeling of pimiento peppers.

Our objectives were: to evaluate the relationship between factors affecting chemical peeling of potatoes and responses that are relevant for understanding the process, to establish optimum processing conditions for yield, peeling quality and NaOH usage, and to determine conditions where NaOH penetration and "heat ring" depths were equal and peeling quality of potatoes was very good.

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THEORY

THE BASIC theoretical aspects of Response Surface Methodology (RSM) have been discussed by several authors (Cochran and Cox, 1978; Box et al., 1978; Thompson, 1982; John and Quenoville, 1977). It is assumed that n mathematical functions, f_k (k = 1,2,...,n), exist for each of the response variables, η_k, in terms of m independent processing factors, ξ_i (i = 1,2,...,m), such as (Floros and Chinnan, 1987):

$$\eta_k = f_k(\xi_1, \xi_2, \ldots, \xi_m) \quad (1)$$

In our case n = 8 and m = 3,

η_1 = peeling yield, PY
η_2 = peeling quality, PQ
η_3 = total usage of NaOH, total NaOH
η_4 = titratable NaOH in the potato tissue, titratable NaOH
η_5 = depth of NaOH penetration, NaOH Pt
η_6 = heat ring depth, HR
η_7 = unpeeled skin surface area, US
ξ_1 = NaOH concentration of peeling solution, C
ξ_2 = temperature of peeling solution, T
ξ_3 = peeling time, t

The exact functions (f_k) are either unknown or complex. However, second order polynomial equations of the following form can be assumed to approximate the true functions:

$$\eta_k = \beta_{k_0} + \sum_{i=1}^{3} \beta_{k_i}\chi_i + \sum_{i=1}^{3} \beta_{k_i}\chi_i^2 + \sum_{i=1}^{2} \sum_{j=i+1}^{3} \beta_{k_{ij}}\chi_i\chi_j \quad (2)$$

where β_{k_0}, β_{k_i}, $\beta_{k_{ij}}$ are constant coefficients and χ_i are the coded independent variables. In practice the levels of the independent variables change from one application to another. Therefore, the general designs are given in terms of standardized or coded variables (χ_i) which in any particular application are linearly related to ξ_i by the following equation (Floros and Chinnan, 1987):

$$\chi_i = 2(\xi_i - \bar{\xi}_i)/d_i \quad (3)$$

where

ξ_i = actual value in original units,
$\bar{\xi}_i$ = mean of high and low levels of ξ_i
d_i = spacing difference between the low and high levels of ξ_i

Table 1—Experimental design for three-variables-three leve's response surface analysis

x_1	x_2	x_3	Numbers of runs
±1	±1	0	
±1	0	±1	3 × 4 (combinations) = 12
0	±1	±1	
0	0	0	1 × 3 (replications) = 3
			Total runs = 15

Table 2—Independent variables and levels

Independent Variables	Symbol Coded	Symbol Uncoded	Levels Coded	Levels Uncoded
			1	20
Concentration, % NaOH	x_1	C	0	12
			−1	4
			1	95
Temperature, °C	x_2	T	0	75
			−1	55
			1	7
Time, min.	x_3	t	0	4
			−1	1

MATERIALS & METHODS

AN EXPERIMENTAL plan from the family of three levels designs suggested by Box and Behnken (1966) was used (Table 1). The independent variables (ξ_i), the coded variables (χ_i) and their levels are presented in Table 2. The levels of independent variables were set according to those normally used by industry.

Experimental procedure

Potatoes, Huincul variety, (weight 238.00 ± 5.37 g) (n = 130, $P < 0.05$), density 1.068 ± 0.006 g/cm^3 (n = 10, $P < 0.05$), and skin surface area 150.30 ± 10.21 cm^2 (n = 10, $P < 0.05$) were used. Potatoes were processed in pilot plant equipment with automatic control of temperature and agitation with compressed air. Potatoes (8) were placed in a specially designed basket and immersed in the NaOH bath; after the prescribed time, the basket was withdrawn, drained, and immediately two potatoes were used for determining titratable NaOH in the potato tissue, one for total usage of NaOH, one for NaOH and "heat ring" penetration depths, and the other four were placed under pressurized water for 30s, then we determined yield, peeling quality and unpeeled skin surface area as described below.

Peeling yield (%). By weighing potatoes before and after peeling

Peeling quality. Peeled potatoes were assessed on a scale of 1 to 7 developed by Miles Willard (Magnuson Engineers, Inc.), where optimum peeling was assigned grade 1 and with more than 90% of skin surface area unpeeled as grade 7:

Total usage of NaOH (g NaOH/100 g potato). One potato from a lot of eight chemically treated was used to analyze total Na^+. The treated potato was disintegrated and homogenized in a measured volume of water of known weight, in a Waring Blendor for 3 min. Weighed samples of the homogenate, were transferred to Kjeldahl balloons and digested in a solution (20 mL) of nitric acid, perchloric acid and sulphuric acid (3:2:1) in one part water until complete digestion. Digested samples were quantitatively transferred with distilled water to 100 mL flasks (They were previously filtered if necessary). The Na^+ content of samples was analyzed in an atomic absorption spectrophotometer IL-551, expressing results as g NaOH/100g potato. The Na^+ content in the fresh potato was subtracted from the result for treated potatoes.

Titratable NaOH in tissue (g NaOH/100 g potato). Two potatoes chemically treated were placed in a volume in excess of an agitated solution of NCl 0.1 N for 30 min at 20°C. The solution was titrated with HCl solution in order to calculate titratable NaOH and account for a blank sample of potato nonchemically treated.

Depth of NaOH penetration (mm). From the central part of one chemically treated potato a thin slice (1 mm) was cut and placed into a phenolphthalein solution. A piece of the colored potato was place over a mesh of known size and the depth of NaOH penetration was read using a magnifying glass.

Heat ring depth (mm). From the central part of one chemically treated potato, slice of 12 × 12 × 3.5 mm (including skin) were removed. They were fixed with Formaldehyde-acetic acid-ethyl alcohol, dehydrated with a series of ethanol solutions, embedded in paraffin, cut in 16 mμ sections with a Leitz Minot microtome, colored with PAS method (periodic acid-Schiff) and examined with an Olympus microscope, reading the depth of gelatinized starch with a micrometer.

Unpeeled skin surface area (%). The skin remaining on the potato after peeling was measured as area (cm^2)/potato by using specially prepared transparent papers and referring to the average potato surface area.

RESULTS & DISCUSSION

RESPONSES fluctuated between wide limits as may be seen in Table 3. Note that the mean for titratable NaOH in the potato tissue, for all runs, was 60% of the total usage of NaOH, and for higher temperatures the "heat ring" depth was greater than the depth of NaOH penetration.

Box and Behnken design was programmed and used for fitting the experimental data to second order polynomial Eq. (2), using a computer VAX 11/780. The regression coefficients (β_{ki}) obtained (Table 4) and analysis of variance (Table 5), indicate that the models developed were adequate for yield, peeling quality, total usage of NaOH, titratable NaOH in the potato tissue and heat ring depth, showing no significant lack of fit. For NaOH penetration depth the lack of fit was not significant at the 0.8% level. Further statistical analysis was performed to test the effect of one given factor on all parameters. Results revealed that all three process variables had a significant overall effect on all responses, except for "heat ring" thickness. There the temperature only had a significant effect. For other variables concentration (c) and time (t) had the most significant effects, while temperature (T) was less important.

The predictive models for product yield, peeling quality and total usage of NaOH were used for examination of the system behavior and determination of optimum conditions. The canonical analysis based on the stationary points resulted in the following equations for product yield, peeling quality and total consumption of NaOH (critical points for each surface are also

Table 3—Experimental data for chemical peeling of potatoes

Treatment[a]	Conc	Temp	Time	Peeling yield %	Peeling quality grade	Total NaOH g/100 g	Titratable NaOH g/100 g	Penetration of NaOH mm	Heat ring mm	Unpeeled skin %
	x_1	x_2	x_3	PY	PQ	Total NaOH	Titrat. NaOH	NaOH Pt	HR	US
1	1	1	0	78.61	1.25	1.547	0.896	2.16	3.89	0
2	1	−1	0	100.08	7.00	0.189	0.111	0.62	0	96.27
3	−1	1	0	96.15	4.75	0.076	0.026	0.62	3.70	38.18
4	−1	−1	0	100.19	7.00	0.036	0.025	0	0	100
5	1	0	1	79.30	1.50	1.475	0.882	2.67	3.30	0
6	1	0	−1	100.27	7.00	0.184	0.136	0.27	0	100
7	−1	0	1	99.69	6.00	0.081	0.033	0.10	3.46	84.15
8	−1	0	−1	100.46	7.00	0.043	0.018	0	0	100
9	0	1	1	74.83	1.50	1.23	0.805	2.84	6.29	0
10	0	1	−1	96.74	5.00	0.22	0.091	0.27	1.68	45.20
11	0	−1	1	98.87	5.00	0.153	0.081	0.96	0	47.14
12	0	−1	−1	100.33	7.00	0.100	0.043	0.05	0	99.47
13	0	0	0	86.66	3.00	0.285	0.265	1.24	2.84	1.96
14	0	0	0	90.12	3.50	0.368	0.308	1.22	1.18	8.49
15	0	0	0	88.94	3.25	0.376	0.320	1.24	2.89	4.86

[a] The experimental runs were performed in a random order.

Table 4—Regression coefficients

Coefficient	Peeling yield	Peeling quality	Total NaOH	Titratable NaOH	Penetration of NaOH	Heat ring	Unpeeled skin
β_{k0}	89.21**	3.25**	0.34**	0.30	5.10	1.23**	2.30
β_{k1}	−4.78**	−1.00**	0.39**	0.24**	−15.76**	0.62**	0.35×10^{-2}
β_{k2}	−6.64**	−1.69**	0.32**	0.19**	−32.44**	0.53**	1.95*
β_{k3}	−5.64**	−1.50**	0.30**	0.19**	−26.67**	0.75**	1.42
β_{k11}	3.38*	1.25*	0.69×10^{-1}	-0.10×10^{-1}	38.30**	−0.33**	−0.33
β_{k22}	1.14	0.50	0.49×10^{-1}	-0.23×10^{-1}	15.21*	-0.57×10^{-1}**	-0.48×10^{-1}
β_{k33}	2.31*	0.87*	0.33×10^{-1}	-0.19×10^{-1}	27.64**	−0.15	−0.26
β_{k12}	−4.36**	−0.87*	0.33**	0.20**	−8.61*	0.23**	-0.47×10^{-1}
β_{k13}	−5.05**	−1.12*	0.31**	0.18**	−21.04**	0.57**	0.40×10^{-1}
β_{k23}	−5.11**	−0.37	0.24*	0.17**	1.78	0.41**	1.15

* Significant at 5%
** Significant at 1%

Table 5—Analysis of variance for the eight response variables

Source of variation	Degrees of freedom	Sum of squares						
		Peeling yield	Peeling quality	Total NaOH	Titratable NaOH	Unpeeled Skin	Penetration NaOH	Heat ring
Linear Terms	3	790.04**	48.78**	2.80**	1.05**	16,095.3**	9.86**	46.49
Quadratic Terms	3	59.76*	8.47*	2.74×10^{-2}**	0.003	8,091.61**	0.45**	0.66**
Cross Product Terms	3	282.51**	8.69*	1.05**	0.40**	2,079.71*	2.22**	5.34
Lack of fit	3	1.98	1.03	0.015	0.00005	666.54*	0.18**	0.61
Error	2	1.20	1.25	0.005	0.0017	31.41	0.00026	1.89
Total	14	1,135.49	67.10	3.91	1.46	26,954.58	12.71	54.99
Coefficient of Determination. % (R^2)		99.72	98.27	99.49	99.88	97.44	98.59	95.45

* Significant at 5%
** Significant at 1%

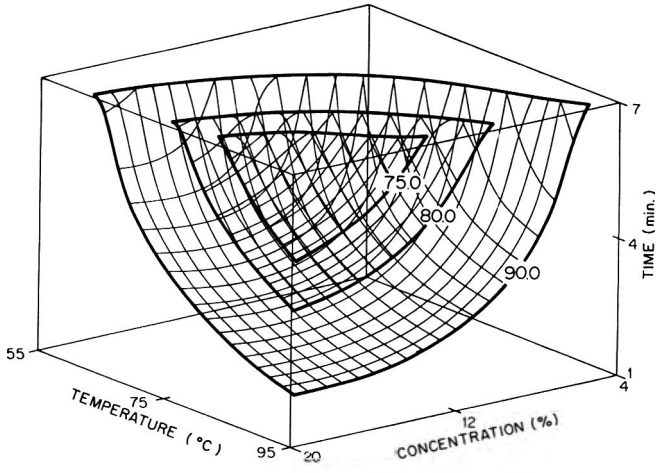

Fig. 1.—Response surfaces of product yield at constant values (75-80-90%) as a function of concentration, temperature and time.

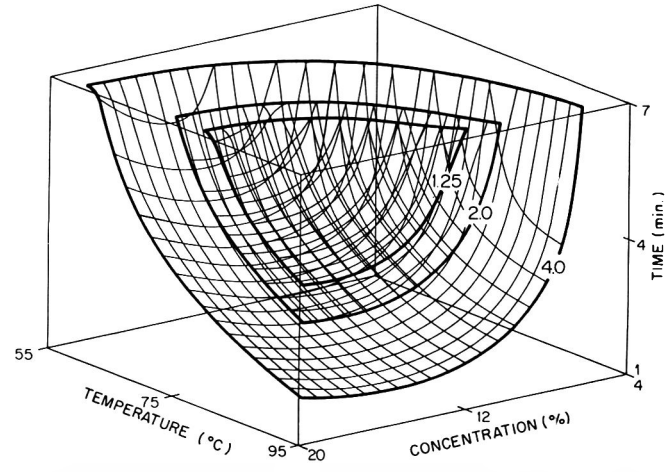

Fig. 2.—Response surfaces of peeling quality at constant values (1.25-2-4) as a function of concentration, temperature and time.

given):

$$PY = 98.434 - 2.684\omega_1^2 + 4.085\omega_2^2 + 5.428\omega_3^2 \quad (4)$$

$$\chi_{s_1} = -0.884, \quad \chi_{s_2} = -1.210, \quad \chi_{s_3} = -1.085$$

$$PQ = -22.531 + 0.058\omega_1^2 + 0.855\omega_2^2 + 1.711\omega_3^2 \quad (5)$$

$$\chi_{s_1} = 10.423, \quad \chi_{s_2} = 14.834, \quad \chi_{s_3} = 10.737$$

$$\text{Total NaOH} = 0.092 - 0.116\omega_1^2 - 0.078\omega_2^2 + 0.347\omega_3^2 \quad (6)$$

$$\chi_{s_1} = -0.452, \quad \chi_{s_2} = -0.512, \quad \chi_{s_3} = -0.520$$

Where, ω_1, ω_2, ω_3 (eigenvectors or cannonical variables) are linear combinations of the χ_i. The characteristic roots of Eq. (4) and (6) have mixed signs. This shows that stationary points exist in terms of saddle points, so movement away from these points would cause an increased or decreased response, depending upon direction of movement. Analysis of such ridge systems is not easy and is not always successful, especially with multi-response problems. Thus, a simpler approach was taken to explore and explain the system.

When the response surfaces for these models were plotted as a function of time (t) and concentration (c) keeping temperature (T) constant, almost plane surfaces were obtained. Thus, we decided to keep constant the responses at a fixed level, varying independent variables and plotting the system. The following alternatives, yield (75–80–90%), Fig. 1; peeling quality (1.25–2-4), Fig. 2 and total usage of NaOH (0.5-1-1.2%), Fig. 3 were developed. The figures show that the surfaces obtained were similar, and as independent variables increased, the yield decreased, the peeling quality was better and the total usage of NaOH increased. Since the Figures have the same coordinate systems (and equal scales), one can superimpose Figures of different responses, combining the most suitable surfaces according to experimental criteria. Two alternatives were chosen: first I) peeling quality 1.25 (the best obtained for the treatments performed), product yield, 80% and total usage of NaOH, 1% (Fig. 4); and second II) peeling

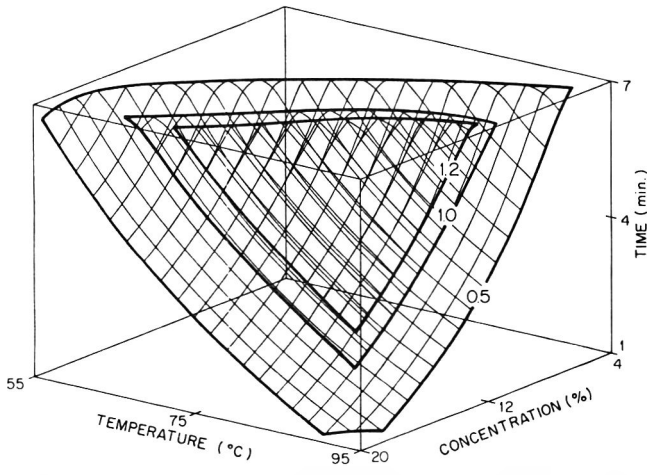

Fig. 3.—Response surfaces of total usage of NaOH at constant values (0.50-1.00-1.20%) as a function of concentration, temperature and time.

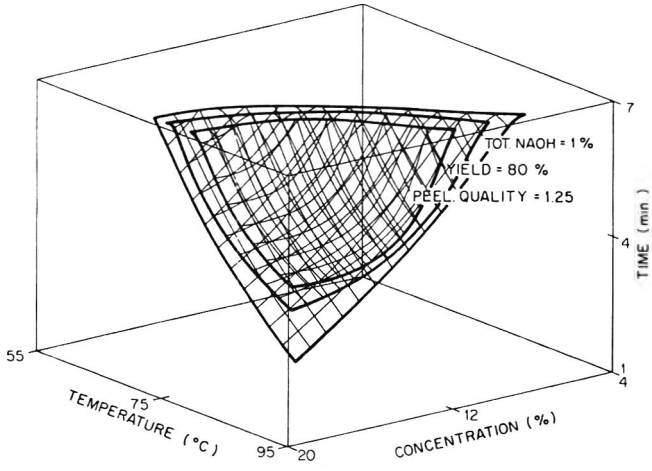

Fig. 4.—Simultaneous response surfaces of peeling quality (1.25), product yield (80%) and total usage of NaOH (1.00%) as a function of concentration, temperature and time.

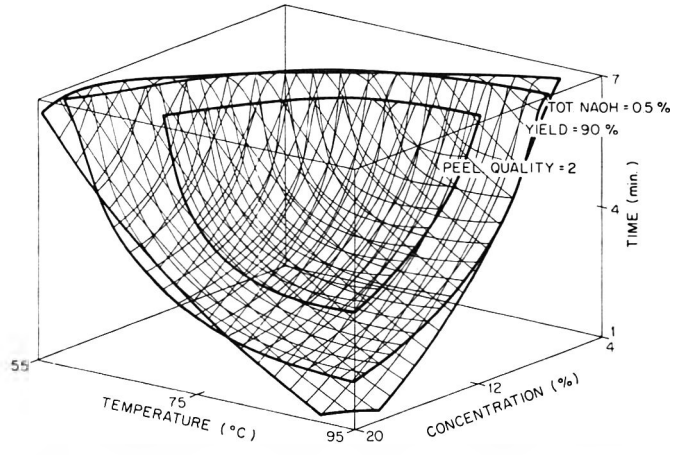

Fig. 5.—Simultaneous response surfaces of peeling quality (2), product yield (90%) and total usage of NaOH (0.50%) as a function of concentration, temperature and time.

quality 2 (very good quality), product yield, 90% and total usage of NaOH, 0.5% (Fig. 5).

Figures 4 and 5 graphically indicated the projected intersec-

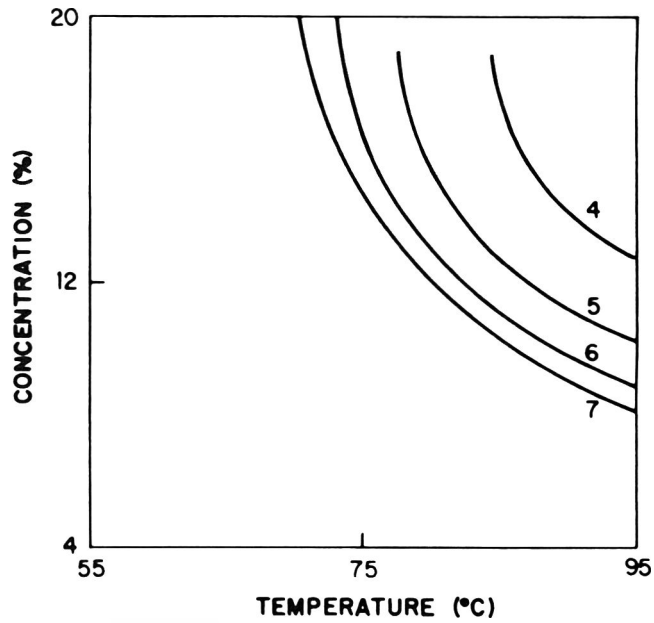

Fig. 6.—Contour plot of constant product yield, 83%, as a function of concentration and temperature, varying time from 4 to 7 min.

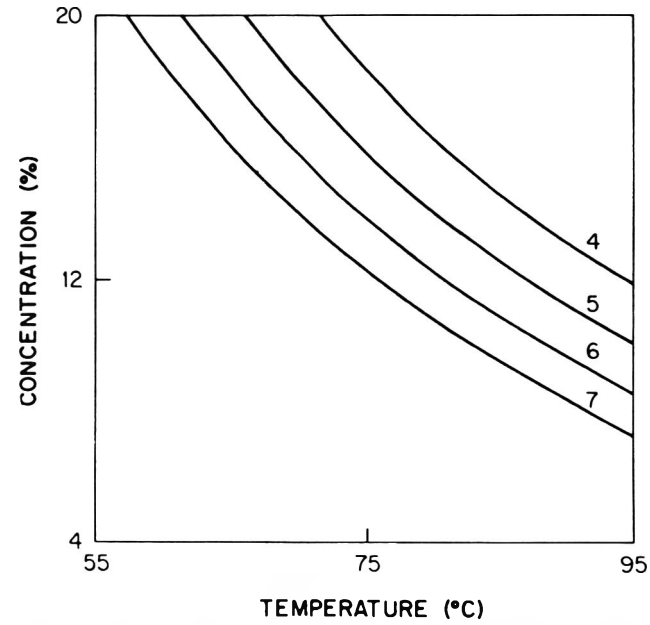

Fig. 7.—Contour plot of constant total usage of NaOH (0.70%), as a function of concentration and temperature, varying time from 4 to 7 min.

tions of surfaces in the respective axes, obtaining the following probable optimum intervals:

Alternative I)

x_1 = NaOH concentration : 11.20% – 19.20%
x_2 = Temperature : 77°C – 91°C
x_3 = Time : 4min – 7min

Alternative II)

x_1 = NaOH concentration : 12% – 19.20%
x_2 = Temperature : 69°C – 93°C
x_3 = Time : 3.46min – 7min

After this assessment, bidimensional plots were drawn, keeping constant the value of the response and representing con-

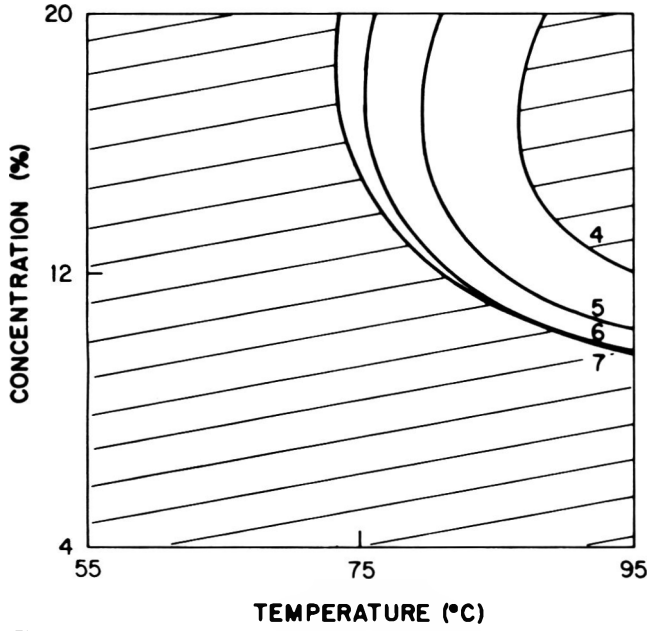

Fig. 8.—Contour plot of constant peeling quality (2), as a function of concentration and temperature, varying time from 4 to 7 min.

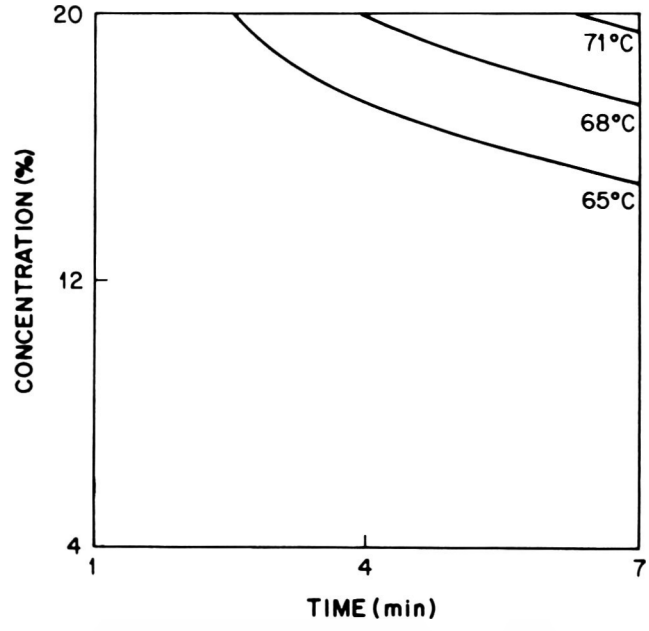

Fig. 10.—Concentration as a function of time and temperature as a parameter (65,68 and 71°C) for which the "heat ring" depth is equal to NaOH penetration depth.

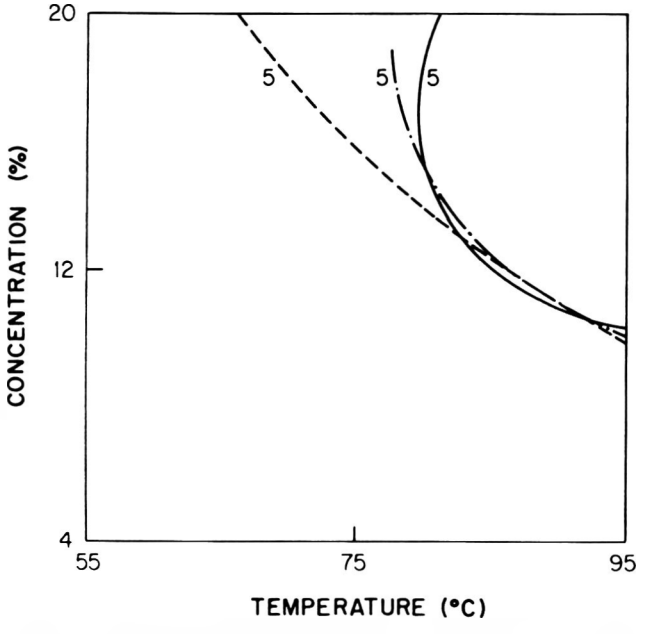

Fig. 9.—Superimposition of Figures 6, 7 and 8 to determine intersection point at time = 5 min.

centration as a function of temperature with time as the parameter. Fig. 6, 7 and 8 show examples for product yield, 83%, total usage of NaOH, 0.70% and peeling quality, 2; in Fig. 8 the shaded region is not appropriate for obtaining the established peeling quality. After this, superimpositions were made among plots for product yield (75 to 90%, increasing by 1%) total usage of NaOH (0.5 to 1.2%, increasing by 0.1%) and peeling quality (1.25 or 2) in order to obtain optimum conditions. For peeling quality 1.25 best responses were: product yield 80% and total usage of NaOH 1%, for processing variables: concentration 12.72%, time, 5.67 min and temperature, 95°C; while for peeling quality 2, they were: product yield, 83% and total usage of NaOH 0.70% and the independent variables, concentration, 11.04%, time, 5 min and temperature, 90.6°C (Fig. 9). Both points produced "heat ring", so it is important to choose conditions where the depth of heat ring is less. It was verified that 90.6°C and 5 min from the second point combined with a NaOH concentration 12.72% from the first point satisfied that condition. Results for the responses were following values: product yield, 80.74%, peeling quality 1.52 and total NaOH consumption, 0.87%.

These latter conditions should be adequate for processing potato products in which the formation of heat ring is not considered important. We also looked for conditions where the "heat ring" and NaOH penetration depths were equal. Figure 10 shows concentration as a function of time and temperature as a parameter (65,68 and 71°C), in which that condition was satisfied. The upper temperature limit was 71–72°C. It was verified that at 72°C, 20% concentration and 7 min, both depths were equal (2.60 mm) and no "heat ring" would appear after treatment with pressurized water. The peeling quality was 2.20 (very good), the product yield, 81.90% and the total usage of NaOH, 1.32%.

Following a common practice in industry, to use high temperature, high concentration and short time to avoid heat ring, we found that the minimum time necessary for obtaining a peeling quality of 2, was 3 min. The concentration was fixed at 20% and temperature at 95°C. In this last case the heat ring was very small. Table 6 shows various conditions that may be used in chemical peeling of potatoes. The third condition listed in Table 6 was experimentally tested in a pilot plant, obtaining peeling quality 1.50, product yield, 79.58% and total usage of NaOH, 0.97%. Predicted and experimental results were not statistically different at 1% significance levels. The other conditions were also experimentally verified for heat ring and NaOH penetration depths, with excellent agreement with predictive models.

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Table 6—Selected processing conditions for chemical peeling of potatoes

	Variables			Responses			
	Conc (%)	Temp (°C)	Time (min)	Peeling yields %	Peeling quality grade	Total NaOH g/100g	Observations
I)	12.72	95.0	5.67	80.00	1.25	1.00	Heat ring
II)	11.04	90.6	5.00	83.00	2.00	0.70	Heat ring (less than I)
III)	12.72	90.6	5.00	80.74 (79.58)[a]	1.52 (1.50)	0.87 (0.97)	Heat ring (similar to II)
IV)	20.00	72.0	7.00	81.90	2.20	1.32	No heat ring
V)	20.00	95.0	3.00	83.50	2.53	1.23	Very small heat ring

[a] Values in parentheses were obtained experimentally for model verification

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BROWNING OF APPLE SLICES DURING STORAGE. . .

From page 800

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Diffusion in Heated Potato Tissues

VASSILIS GEKAS, RICKARD ÖSTE, and INGRID LAMBERG

ABSTRACT

Data to optimize nutrient retention during processing of potatoes were obtained by measuring loss of solutes (glucose, fructose, citric acid, potassium, calcium and magnesium) by diffusion into blanch water. Apparent diffusivities were calculated based on Fickian diffusion. Magnesium and calcium did not follow the Fickian approach with the same accuracy as the others. A hindrance factor (K), the ratio of apparent diffusivity in potato to a published value was used. The hindrance factor was calculated at reference temperature 25°C to separate preheating effects from the direct effect of temperature on diffusion coefficients. A mathematical model for K(T,t) was fitted to experimental data for each solute. All solutes showed a sharp decrease of hindrance factor in the region 50–60°C. These models showed, however, that heating of potato affected diffusivities of the solutes to different degrees. Ions had a limit solution retention in the tissue higher than glucose, fructose or citric acid.

Key Words: potato, moisture, diffusion, blanching

INTRODUCTION

RAW POTATO TISSUE is rich in vitamins and minerals (particularly potassium). It has also adequate protein to energy ratio to meet nutritional needs of adult humans (Woolfe, 1987). Heat is needed to make starch digestible. However, heat-sensitive nutrients such as vitamin C are partly lost during processing. Low molecular weight nutrients such as potassium and iron, may also be lost to processing water through leaching (Mondy and Ponnapalam, 1983; Garrote et al., 1986). In industrial processing of potato products, a blanching step is often included to remove reducing carbohydrates and modify texture. Sometimes calcium salts are added to the blanch water to improve texture. Blanching conditions are usually established through empirical experiments to optimize product quality factors, usually color or storage stability against lipid oxidation. Little consideration may be given to nutritive value of the product. Blanching should be optimized for nutrient retention in addition to sensory properties and storage stability.

Several models are available for predicting loss of solutes from potatoes during blanching (Luna and Garrote, 1987; Garrote et al., 1986; Califano and Calvelo, 1983; Kozempel et al., 1983, 1982). Generally, these reports employ computer simulation of losses of reducing carbohydrates and nutrients through degradation and diffusion. A general model for potato processing which includes heat and mass transfer and nutrient degradation has been reported by Lamberg and Hallström (1986). They used experimental values of heat transfer coefficients in Bintje potatoes to numerically solve the non-steady state heat transfer equation by finite differences or finite elements. Tomasula and Kozempel (1989) have reported on the leaching of glucose, potassium and magnesium from French fry-cut Maine Russet Burbank and Maine Katahdin potatoes over a range of 45–90°C and modeled effective diffusivities as functions of temperature using an Arrhenius type and a polynomial expression.

Temperature distribution and the course of starch gelatinization in cylinders were reported by Lamberg et al., (1990). To extend usefulness of the model solute losses should also be accounted for. In preliminary experiments we found that the loss of low molecular weight substances from potato tissues heated in water was highly dependent on time-temperature history. Our objective was to measure apparent diffusivity of low molecular weight substances in preheated potato tissue at 25°C and to determine and describe the effect of temperature-induced changes in the tissue matrix on apparent diffusivity of low molecular weight solutes.

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MATERIALS & METHODS

Potatoes

Potatoes (Bintje; shape ellipsoidal; size: ≈ 50 × 80 mm, weight 1080–1090 kg/m^3) were obtained from a local storage plant (7°C, 95% RH) in southern Sweden (Kävlinge) during autumn 1987. The potatoes were stored at 8°C overnight in darkness.

Heat treatments

For each heat treatment, a cylinder (26 mm diameter) was cut from each of five potatoes, and machine sliced to 2 mm thickness. Slices were submerged in distilled water (7°C) for 15 min, dried with tissues and weighed. The thickness of each slice was measured with a micrometer. The 50 slices were placed, well separated from each other, in a plastic bag. The bags were sealed under vacuum and placed in a thermostated water-bath for a prescribed time and temperature of preheating then immediately transferred to an ice-bath. After cooling, the bags were equilibrated to 25°C for 15 min.

Diffusion experiments

The diffusion experiments were performed at 25 ± 0.2°C. The 50 slices were immersed in 200 mL deionized water (Milli-Q Reagent water systems, Millipore S.A., Molsheim, France) and were kept in motion through constant stirring. After 2, 5, 15, 30, 50, 70 and 90 min, a 100 mL sample of the extraction solution was withdrawn and immediately replaced with 100 mL of fresh, 25°C Millipore water. Each sample (10 mL) was lyophilized, weighed and kept for chemical analysis. The dilution was made in order to simulate the boundary condition of no solute at the slice surface.

After leaching, the slices were recovered, dried on the surface with tissue, weighed and homogenized in 20 mL ethanol. The homogenate was diluted to 50 mL in a volumetric flask and placed in a water-bath for 10 min. After cooling, a weight fraction (≈ 10 mL) was withdrawn for analysis of glucose, fructose and citric acid. The remainder was concentrated, lyophilized and subjected to assays of potassium, calcium and magnesium.

Chemical analysis

Glucose, fructose and citric acid were analyzed with enzyme-based commercial kits (Boehringer-Mannheim GmbH, Mannheim, Germany). Potassium, calcium and magnesium were analyzed using atomic absorption spectrometry (Varian AA 1275, Mulgrave, Vic., Australia). Assays were performed on samples taken during the diffusion experiment and on potato tissue after the diffusion experiment. The original amounts of solute in the tissue were also analyzed and amounts lost during diffusion were deduced from results.

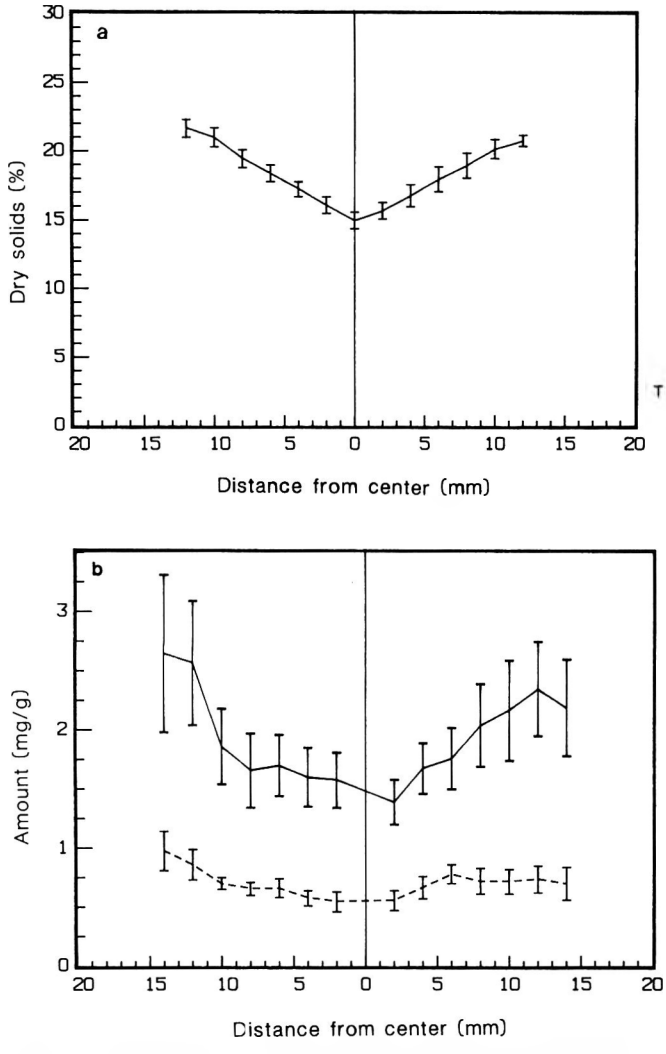

Fig. 1—Compositional variation within the potato tuber. Mean values with standard (n=10) in the slices (a) dry matter; (b) glucose (△) and fructose (–).

Calculations

The geometrical shape of the potato slices allowed the application of a one-dimensional solution of Fick's nonsteady state diffusion equation. Edge effects could be neglected since the ratio thickness to radius was less than 0.2 (Crank, 1975). Further, we assumed that the following conditions were met: initial uniform distribution of solutes in the slices, constant concentration of solute at the surface of the slice (equal to zero), constant and uniform thickness of slices, no chemical generation of solutes and diffusivity independent of solute concentration and time. Finally since all solutes were in very dilute concentrations the multicomponent effects could be disregarded (Gekas, 1992).

Then the following well known equation was used to calculate the apparent diffusion coefficient, D,

$$\ln\left(\frac{m}{m_0}\right) = \ln\frac{8}{\pi^2} - \frac{D\pi_i^2}{\lambda^2} \qquad (1)$$

where m is the amount of solute remaining in the tissue after time t and M_0 the original amount of solute in the tissue in the sample of initial thickness λ. Initial λ values were taken since preliminary experiments showed that the thickness of slices did not change substantially during heat treatment.

A diffusion hindrance factor was defined as $K = D_{H_2O}/D$, where D_{H_2O} is the published value of the diffusion coefficient of the solute in aqueous solution at 25°C. Empirical equations for the dependence of K on the parameters of the thermal pretreatment t (time in min) and T (temperature in °C) were obtained using a stepwise regression analysis procedure (Rand, 1983). The goodness of fit of the models was estimated by the square of the correlation coefficient, R.

Table 1—Initial proximate composition of heated potato slices

	Mean	Standard deviation
Dry solids (%)	17.6	1.51
Glucose (mg)	32.9	4.96
Fructose (mg)	23.9	5.63
Citric acid (mg)	39.6	4.58
Potassium (mg)	42.7	3.44
Calcium (mg)	0.49	0.35
Magnesium (mg)	2.70	0.32

RESULTS & DISCUSSION

Effect of dry matter variation

Establishing the best conditions for diffusion experiments required considering the possible effect of dry matter variation (Woolfe, 1987) in the samples on the original concentration of solutes in the potato slices. Raw slices from 10 different tubers were subjected to analysis. The content of glucose and fructose, as well as total dry matter (Fig. 1) increased gradually with distance from the center of the tuber. The concentration of glucose varied considerably in slices cut at about the same place in different tubers (C.V. > 20%, Fig. 1). This variation exceeded that of the total dry matter, and probably was a result of the dynamic equilibrium that regulates tissue content of glucose and fructose during storage (Burton, 1966). To account for both intra-and inter-tuber variation in solute concentration, and for possible variations in gross tuber composition, which might affect diffusion behavior, we used 10 slices from five different tubers for each experiment. To obtain homogeneity with respect to the internal structure of the tissue, central and outer slices were avoided. The original content of solutes (Table 1) in the heated potato slices and variations of concentration of all solutes were within expected ranges (Woolfe, 1987).

Diffusion coefficients. Results of the diffusion experiments (Table 2) showed a very good linear correlation between ln (m/m_0) of the various solutes and time of extraction for a large part of the experimental data (Fig. 2). When values for short leaching times were excluded (Sherwood, 1929), the square of the linear regression coefficients was close to 1 for glucose, fructose, citric acid and potassium. These results justified the approximation made to allow use of Eq (1) i.e. the diffusion to be Fickian. Apparent diffusion coefficients were then calculated for each solute at each of the different time-temperature treatments. Values for calcium, did not strongly support a linear approximation, as indicated by the comparatively low regression coefficients and higher standard errors. Thus, Eq. (1) was not perfect to model diffusion of this divalent cation. Nevertheless, for comparison, apparent diffusivities were calculated from the slope of the best linear fit to these values. Comparing the calculated values of apparent diffusivity at 25°C, with published values of diffusivity in pure water solution at this temperature (Table 3), suggested that the potato tissue constituted a considerable diffusion hindrance for low molecular weight solutes.

Hindrance factors. The hindrance factors (Table 4) summarize the effects of tortuosity, porosity, the (chemical) interaction factor and viscosity, which otherwise may be used to describe diffusion in heterogeneous media such as food (Schwartzberg and Chao, 1982). We further examined the hindrance factor by identifying the reasonably well fitting empirical mathematical model that would include the effects of both preheating temperature and -time.

Mathematical model. The primary goal of the empirical model was to fit the characteristic sharp decrease of K occurring with all solutes in the temperature region 55–60°C. A characteristic increase of D_{eff} or decrease of K has also been observed by Tomasula and Kozempel, 1991. They modeled D_{eff} as a function of temperature in which both the direct effect of temperature on diffusivity and the indirect effect though structural changes were taken into account. In our case we

Table 2—Measured apparent molecular solute diffusion coefficients, D ($m^2/sec*10^{11}$) and coefficient of correlation, R^2 and standard error of estimation for different heat treatments

Temp (°C)	Time (min)	Glucose D	Glucose R^2	Glucose Standard error (%)	Fructose D	Fructose R^2	Fructose Standard error (%)	Citric acid D	Citric acid R^2	Citric acid Standard error (%)
50	2	1.28	0.995	0.2	1.33	0.958	1.1	1.09	0.998	0.2
	5	1.49	0.998	0.2	1.52	0.998	0.2	1.43	0.998	0.3
	20	2.49	0.992	1.1	2.64	0.999	0.3	2.33	0.999	1.2
55	2	1.04	0.997	0.1	1.04	0.999	0.3	0.91	0.998	0.2
	5	1.76	0.994	0.4	1.49	0.996	1.4	2.26	0.999	0.1
	20	12.13	0.998	2.0	12.90	0.999	2.1	10.28	0.999	2.1
60	2	7.46	0.998	1.8	6.57	0.999	0.15	6.09	0.999	0.7
	5	18.60	0.998	3.2	17.62	0.996	2.1	20.41	0.997	2.8
	20	30.30	0.999	5.8	30.12	0.999	2.9	37.01	0.999	1.4
75	2	26.79	0.999	4.8	26.81	0.999	2.1	31.75	0.999	3.1
	5	27.10	0.999	4.2	26.53	0.999	3.2	26.72	0.998	4.2
	20	28.32	0.999	5.6	27.76	0.999	1.6	30.90	0.999	6.0
90	2	24.63	0.997	4.8	23.70	0.999	0.6	33.65	0.982	12.1
	5	28.27	0.998	6.0	31.32	0.999	3.0	27.07	0.999	3.1
	20	29.39	0.998	6.6	29.69	0.999	5.1	33.83	0.999	4.3

Temp (°C)	Time (min)	Potassium D	Potassium R^2	Potassium Standard error (%)	Calcium D	Calcium R^2	Calcium Standard error (%)	Magnesium D	Magnesium R^2	Magnesium Standard error (%)
50	2	1.68	0.999	0.3	0.27	0.999	0.2	0.08	0.980	0.4
	5	1.62	0.999	0.3	0.77	0.909	3.4	0.24	0.998	0.3
	20	2.88	0.999	0.1	1.56	0.668	25.6	0.47	0.986	4.0
55	2	1.36	0.999	0.3	0.56	0.999	2.8	0.14	0.964	0.1
	5	2.77	0.993	0.7	1.67	0.998	1.2	1.10	0.999	0.0
	20	10.29	0.994	0.9	15.23	0.998	4.3	10.16	0.997	0.8
60	2	7.07	0.999	1.2	2.75	0.951	4.7	4.50	0.999	0.3
	5	28.30	0.999	1.8	8.84	0.968	6.0	17.45	0.998	1.1
	20	46.55	0.994	3.1	10.27	0.962	3.3	23.10	0.991	3.2
75	2	40.22	0.999	1.8	13.43	0.956	6.4	22.30	0.996	1.7
	5	44.92	0.999	0.2	12.57	0.965	5.5	24.66	0.993	2.8
	20	38.76	0.983	2.8	11.66	0.955	2.8	23.96	0.992	3.1
90	2	35.13	0.999	1.4	12.48	0.984	7.6	16.79	0.996	2.7
	5	45.92	0.999	2.3	9.02	0.954	19.2	20.83	0.989	4.9
	20	40.99	0.991	3.1	16.40	0.978	6.2	24.77	0.981	9.3

wanted to include also the effect of time. This could be obtained by assuming the hindrance factor to decrease exponentially both with temperature and with time. The model suggested was

$$K - A = \exp(B - Ct - DT + EtT) \quad (2)$$

where K is the hindrance factor defined previously and A the asymptotic value at the levelling off occurring at high blanching temperatures and/or for prolonged time. The physical significance of the other constants is also interesting. C shows the effect of time and D the effect of temperature on the hindrance factor, E is to account for the tendency of the combined time-temperature effect to levelling off at high time and temperature values. If neither temperature nor time had an effect, K-A should reduce to exp(B); thus B is a measure of the hindrance effect in the untreated potato.

Furthermore the mathematical model indicates "first order reaction" kinetics for the reduction of the hindrance factor. Differentiation of the change in the hindrance factor (K-A) with time gives Eq. (3),

$$\frac{\partial(K - A)}{\partial t} = (ET - C)\exp(B - Ct - DT + EtT) \quad (3)$$

which may be identified as a first order reaction, for constant temperature, with the rate constant $\kappa = ET - C$:

$$\frac{\partial(K - A)}{\partial t} = \kappa.(K - A) \quad (4)$$

Thus the reduction of the hindrance factor has a linear temperature dependence. The magnitude of this temperature dependence is given by the constant E. The effect of temperature can be in a similar way (as with time) derived by differentiation of Eq. (2) with respect to temperature. Then the parameter giving the change due to the temperature would be Et-D.

Whereas the model gives good values in the region of the above mentioned sharp decrease it gives crude results for the two regions <55°C and >75°C. In these regions before and beyond the sharp changes in cellular structure, the experiment shows a tendency for a slight decrease of the hindrance factor. As another alternative, we have considered a sigmoidal model which could represent this behavior i.e. a gradual mild decrease in the beginning followed by a sudden sharp decrease, followed by a slight decrease in the end. This model could be described by the following equation:

$$K - A = \frac{\exp(B - Ct)}{1 + \exp(DT + Et - F)} \quad (5)$$

Such a model could be more safely used in predicting the blanching t-T conditions at which retention of a given solute could be favored compared to retention of another nondesired potato constituent. Unfortunately, application of the above model (using 6 constants instead of 5 for the previous one in Eq. (2) was not possible, due to the lack of experimental data, especially for T<55°C. We intend to follow these modelling ideas in the future.

Validity and discussion of the results of the model

Equation (2) gave coefficients of determination (R^2) in the range of ≈0.75 to 0.85 (Table 5). Most of the uncertainty, however, comes from the regions before and beyond the critical region 55–60°C where wide changes occur. However, this goodness of fit may be considered acceptable in a food science application, as suggested by Labuza (1983). The model showed that the hindrance factor was highly dependent on both time and temperature within the region of the validity of the model

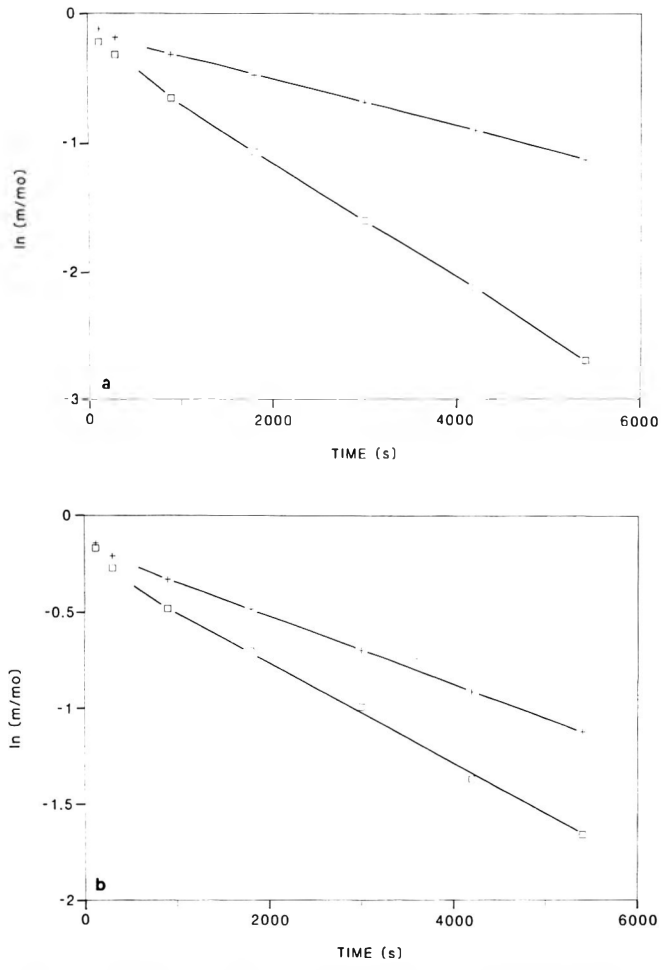

Fig. 2—Diffusion coefficient determination in heat-treated potato tissue. Figures show retained fraction (x/x_o) as a function of time at 25°C. (a) glucose, pretreatment conditions: + 60°C, 2 min; (—) 60°C, 5 min; (b) potassium, pretreatment conditions: + 55°C 20 min; (---) 60°C, 2 min.

(Table 5). This could be interpreted as the effect of changes of potato tissue structure induced by the temperature and time treatment. Such changes in tissue structure would reduce its ability to hinder diffusion.

The model was exponential with respect to both time and temperature. This might suggest that the basic phenomena affecting hindrance probably have been of a chemical nature. Major changes occurred around 50 to 60°C. At these temperatures, cell membrane proteins denature (Bartolome and Hoff, 1972). This would allow low molecular weight solutes to diffuse through cell walls. Further, after membrane denaturation, the intracellular water would flow into originally air-filled intercellular spaces, thus creating new channels of diffusion (Carlsson, 1989). Such changes should facilitate flow of solutes out of the tissue independent of chemical structure of the solute, i.e. it should affect different intracellular solutes to about the same extent. However, examination of the mathematical model showed that the hindrance factor was also solute dependent. It is interesting, for example, to compare the constant A of the model, (a measure of the residual hindrance after blanching) for the various solutes.

Glucose, fructose and citric acid molecules had lower A values than the ions, K^+, Mg^{++} and Ca^{++}. These three molecules showed similarities as far as other constants of the model were concerned. The similarity of the diffusive behavior of chemically neutral compounds glucose and fructose, as indicated by almost identical constants in the model (Table 3), seemed to agree with their structural similarities. Calcium constitutes a special case with the highest A value, i.e. highest tendency for residual retention, reflecting its possible reactions in the intermediate lamella during blanching.

Table 3—Solute diffusivity in water at 25 °C, D ($m^2/s*10^{11}$)

	D	Reference
Glucose	69	Schwartzberg and Chao (1983)
Fructose	69	Schwartzberg and Chao (1983)
Citric acid	50	Mills and Lobo (1989)
Potassium	199.4	Schwartzberg and Chao (1983)
Calcium	133.5	Schwartzberg and Chao (1983)
Magnesium	125	Schwartzberg and Chao (1983)

The separate effects of time and temperature on the hindrance factor also differed between the molecular species such as glucose and fructose and the ions such as K^+. Notably, the hindrance factors for glucose, fructose and citric acid had similar time and temperature dependence parameters (C and D). Potassium had a considerably lower time dependence (constant C) while the divalent calcium and magnesium had much higher C values. These considerable differences indicated that solute-specific interaction factors influenced the solute diffusion in the heated potato tissue. The ions such as Ca^{++} may interact with oppositely charged cell-wall components or participate in chemical reactions (Faulks, 1986).

Difference in behavior were found between potassium and fructose (Table 6). The time-and temperature-effect parameters (ET-C and Et-D respectively) are given for typical blanching conditions at low temperature-long time and high temperature-short time. This implied that a longer pretreatment could have a desired effect for improving K^+ retention in the tissue with simultaneous enhanced fructose loss. However due to reasons related to the applicability of the mathematical model more experimental work is needed to support this statement.

CONCLUSIONS

DIFFUSION of solutes in heated potato tissue appears to be hindered by tissue structure. This hindrance effect is dependent on time-temperature treatment of the tissue and was reduced upon heating. Further, the hindrance effect was dependent on the chemical nature of the solute. Molecular species showed different behavior from the ions. Calcium was more retained in the tissue after pretreatment than the other solutes. Also, in absolute terms, the reduction of potassium and the other ions' diffusion in the heated potato tissue, compared with diffusion in pure water was much greater than the reduction of glucose and fructose diffusion.

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Table 4—Solute hindrance factors, K for different heat treatments

Temp (°C)	Time (min)	Glucose	Fructose	Citric acid	Potassium	Calcium	Magnesium
50	2	53.82	52.00	45.87	118.97	490.80	1562.50
	5	46.18	45.45	34.87	123.46	172.48	514.40
	20	27.69	26.14	21.45	69.16	85.41	264.27
55	2	66.60	66.34	55.13	146.72	273.54	880.28
	5	39.20	46.25	22.08	71.98	79.89	113.74
	20	5.69	5.35	4.86	19.38	8.77	12.30
60	2	9.24	10.36	8.21	28.19	48.51	27.76
	5	3.71	3.92	2.45	7.05	15.11	7.16
	20	2.28	2.29	1.35	4.28	12.99	5.41
75	2	2.58	2.57	1.57	4.96	9.94	5.60
	5	2.54	2.60	1.87	4.44	10.62	5.07
	20	2.44	2.48	1.62	5.14	11.45	5.22
90	2	2.80	2.91	1.48	5.67	10.70	7.44
	5	2.44	2.20	1.85	4.34	14.79	6.00
	20	2.35	2.32	1.48	4.86	8.14	5.04

Table 5—Mathematical models for dependence of the diffusion hindrance factor (K) on temperature (T) and time (t) of pretreatment: ln (K−A) = B − Ct − Dt + EtT[a]

Solute	A	B	C	D	E	R^2[b]	Standard error (%)
Potassium	4.25	16.0	0.20	0.225	0.0022	0.810	11.2
Calcium	8.0	34.0	4.3	0.50	0.07	0.740	21.7
Magnesium	5.0	26.4	10.6	1.10	0.18	0.830	22.7
Citric acid	1.25	20.9	1.65	0.32	0.0275	0.850	15.6
Glucose	2.2	25.0	1.0	0.365	0.0080	0.765	27.4
Fructose	2.15	18.2	1.1	0.24	0.0085	0.810	19.4

[a] The models are strictly valid in the intervals T (50–90°C) and t (2–20 min).

[b] An overall coefficient of correlation (R^2) is given for the equation of each solute.

Table 6—Comparison of temperature and time effects on hindrance factors of fructose and potassium for two sets of blanching conditions.

Solute	Temperature effect (Et-D)[c]	Time effect (ET-C)[c]
LTLT conditions[a]		
Fructose	−0.07	−0.71
Potassium	−0.18	−0.09
HTST conditions[b]		
Fructose	−0.23	−0.37
Potassium	−0.21	−0.02

[a] Low temperature-long time (LTLT) conditions, T = 50°C, t = 20 min

[b] High temperature-short time (HTST) conditions: T = 90°C, T = 2 min.

[c] Temperature effect Et-D and time effect ET-C, according to empirical mathematical model used in this study. The minus sign indicates the decreasing character of both effects on the hindrance factor.

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Malic Acid Analysis in Cucumber Juice and Fermentation Brines in the Presence of Interfering Fructose

R.F. McFEETERS, R.L. THOMPSON, and H.P. FLEMING

ABSTRACT

A proccdure was developed for HPLC analysis of malic acid in the presence of an interfering fructose peak with an Aminex HPX-87H column. Fructose in cucumber juice or fermented cucumber brine was reduced to mannitol and sorbitol with sodium borohydride. The sugar alcohols eluted after malic acid and did not absorb light at 210 nm so that malic acid could be quantitatively determined either by refractive index or ultraviolet detectors. Lactic acid, acetic acid, and ethanol could also be determined in the sample after reduction of fructose.

Key Words: malic acid, cucumbers, fermentation, fructose, lactic acid

INTRODUCTION

MALIC ACID analysis in cucumber extracts and fermentation brines has been done by reversed phase HPLC (McFeeters et al., 1984). However, due to relatively rapid loss of resolution of malic acid from lactic acid on reversed phase columns, analysis on a polystyrene cation ion exchange column is preferred. Retention times of sugars and organic acids, including malic acid, are stable for many samples on this type column. Malic acid, the major organic acid in pickling cucumbers (McFeeters et al., 1982), is not completely resolved from fructose with the usual concentrations of sulfuric acid (≈ 0.01N) that resolve the other components of interest. Since the refractive index (RI) detector response for fructose is similar to that of malic acid, the malic acid peak may be obscured by a large fructose peak. Detection by ultraviolet (UV) absorption at 210 nm would be better, but fructose absorbs light to a small degree at that wavelength such that a large amount of fructose in a sample could prevent quantification of lower concentrations of malic acid. This is a common situation in analysis of fruit and vegetable products.

The same problem occurs in wines. Frayne (1986) obtained resolution of malic acid from fructose by using two Aminex HPX-87H columns in series but the method required longer analysis times and an additional column. Schneider et al. (1987) reported resolution with 0.0026N H_2SO_4 as the eluant, but 0.013N H_2SO_4 gave better resolution of other wine components. For cucumber fermentation brines, Lazaro et al. (1989) circumvented this resolution and detection problem by use of RI and UV detectors in series, and a set of simultaneous equations which used peak height values from both detectors to quantitate malic acid and fructose from incompletely resolved peaks. Our objective was to demonstrate the analysis of malic acid, lactic acid, acetic acid, and ethanol in cucumber juice and fermentation brines after reduction of interfering fructose to mannitol and sorbitol with sodium borohydride. Reduction would convert fructose to sugar alcohols which have a slightly longer retention time than fructose and no UV absorption. The result would be no interference in detection of malic acid by UV absorption. In addition, there was sufficient resolution of the malic acid from sugar alcohols such that it could also be quantitated with the RI detector. Sodium borohydride also reduces glucose to sorbitol.

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MATERIALS & METHODS

HPLC was performed with an Aminex HPX-87H column (7.8 × 300 mm) with a cation guard column (#125-0129, Bio-Rad Laboratories, Richmond, CA). The eluant solution was 0.01N H_2SO_4 at a flow rate of 0.8 mL/min. The chromatograph consisted of a Waters 6000 pump (Millipore Corp., Milford, MA); a 7125 injector with a 10 μL loop (Rheodyne Inc., Cotati, CA); a Waters column heater set at 65°C; a Varichrom variable-wavelength, visible/UV detector (Varian Instruments Inc., Palo Alto, CA) set at 210 nm; and a Waters, model 410, RI detector connected in series. Data were collected on two channels of a chrom-1AT A/D converter board and LabCalc chromatography software (Galactic Industries Corp., Salem, NH) installed on a Gateway 2000 (Sioux City, SD), model 486/25, personal computer. Peaks were analyzed using peak heights and external standards prepared in water.

Cucumber juice was prepared from commercial pickling cucumbers 38-44 mm in diameter (Daeschel et al., 1988) and frozen until use. Cucumber fermentation brine was prepared by fermentation of cucumbers with *Lactobacillus plantarum*. Cucumbers (38–44 mm in diameter) were covered with an equal volume of brine, which contained 106 mM acetic acid and 36 mM calcium hydroxide to give an initial pH of 4.6. No salt was added during fermentation. Jars were inoculated with 10^6 CFU/mL *L. plantarum* MOP3 from the laboratory culture collection. After fermentation, 4% NaCl was added to give a fermentation brine with NaCl concentration similar to that expected in pickel samples.

Solutions were prepared to contain known amounts of the compounds of interest added to cucumber juice or fermented cucumber brines with 4% NaCl and no detectable glucose or fructose. Malic acid, lactic acid, acetic acid, and ethanol were added to the cucumber juice in the range of 1 to 50 mM. Since there was a two-

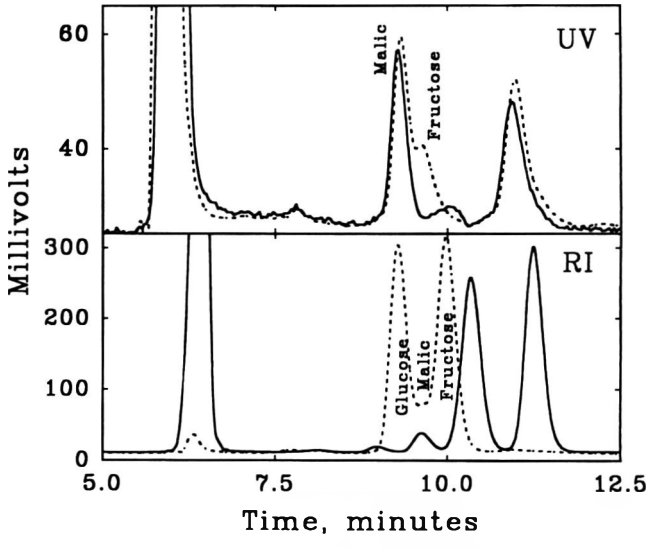

Fig. 1—Effect of sodium borohydride reduction of cucumber juice samples on the removal of interference from the malic acid peak by glucose and fructose. UV = ultraviolet detection at 210 nm; RI = refractive index detection. - - - - - before borohydride reduction; ——— after borohydride reduction.

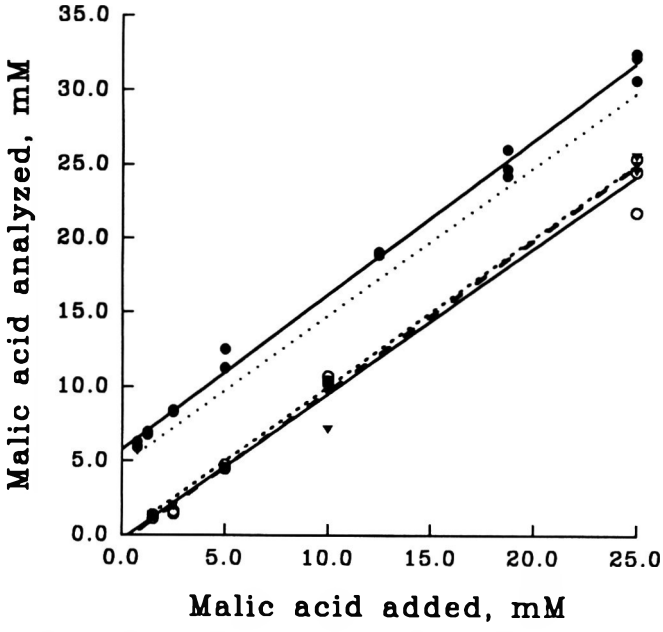

Fig. 2—Determination of malic acid by UV absorption at 210 nm in cucumber juice and fermented cucumber brine with 4% NaCl after sodium borohydride reduction of fructose and glucose. .... expected malic acid concentration; ▼ - - - ▼ malic acid in fermentation brine with 10 mM added fructose and glucose; ○ - ○ malic acid in fermentation brine with 50 mM added fructose and glucose; ● - ● malic acid in cucumber juice.

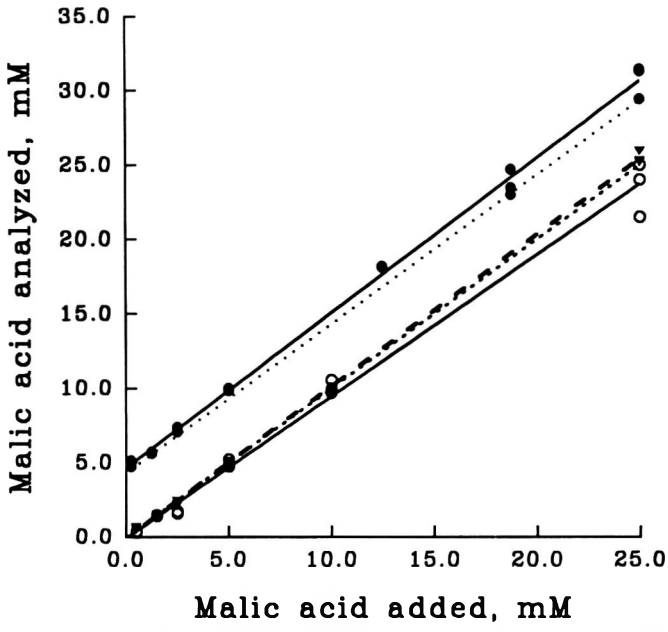

Fig. 3—Determination of malic acid by RI detection in cucumber juice and fermented cucumber brine with 4% NcCl after sodium borohydride reduction of fructose and glucose. .... expected malic acid concentration; ▼ - ▼ malic acid in fermentation brine with 10 mM added fructose and glucose; ○ - ○ malic acid in fermentation brine with 50 mM added fructose and glucose; ● - ● malic acid in cucumber juice.

fold dilution of samples during the borohydride reduction, the concentration range as analyzed was 0.5 to 25 mM. The juice contained ≈ 50 mM each of glucose and fructose after a 1:1 dilution resulting from addition of the compounds and water. The fermentation brine was also diluted 1:1 as a result of addition of glucose, fructose, malic acid, and ethanol. Two sets of solutions were prepared. Glucose and fructose (10 mM) were added to one set and 50 mM of sugars were added to the second set. Malic acid and ethanol were added in the range of 1 to 50 mM. Since the brine contained high levels of lactic and acetic acids, those compounds were not added.

Reduction of fructose and glucose was accomplished by preparing stock solutions of 285 mg $NaBH_4$ in 5.0 mL 0.25N NaOH, 1 mg/mL pronase (Sigma Chemical Co., St. Louis, MO) in water, and 7N H_2SO_4. A small hole was made with a hot, 27-gauge needle in the top of 1.5 mL, disposable plastic centrifuge tubes. Pronase (75 μL) was added to 200 μL of sample and then incubated for 2 hr at 40°C. Pronase treatment reduced foaming, particularly for cucumber juice samples. $NaBH_4$ solution (100 μL) was added to reduce sugars. Vigorous bubbling occurred due to release of hydrogen gas. The sample was again incubated for 2 hr at 40°C to assure complete reduction. To decompose remaining sodium borohydride, 25μL 7N H_2SO_4 was carefully added. Rapid release of

Table 1—Regression analysis of added versus analyzed concentrations of compounds in cucumber juice detected by UV absorption at 210 nm and by refractive index

Compound	Detector	Slope	Standard deviation of the slope	Intercept	Standard deviation of the intercept	r^2
Malic acid	UV	1.0449[a]	0.0121	4.5647	0.1458	0.9971
Malic acid	RI	1.0530	0.0154	5.5353	0.1865	0.9953
Ethanol	RI	0.7809	0.0643	1.9385	1.0937	0.9365
Lactic acid	UV	1.0623	0.0138	−0.1929	0.1924	0.9973
Lactic acid	RI	1.1234	0.0255	−0.4145	0.3886	0.9934
Acetic acid	UV	1.2032	0.0385	−1.1768	0.6551	0.9899
Acetic acid	RI	1.0383	0.0319	0.7954	0.4874	0.9878

[a] A slope for the regressions of 1.0 shows that the concentrations analyzed were equal to the added concentrations.

Table 2—Regression analysis of added versus analyzed concentrations of compounds in fermented cucumber brine detected by UV absorption at 210 nm and by refractive index

Sugars added to brine, mM	Compound	Detector	Slope	Standard deviation of the slope	Intercept	Standard deviation of the intercept	r^2
10	Malic acid	UV	1.0194[a]	0.0074	−0.0719	0.0828	0.9992
50	Malic acid	UV	0.9518	0.0218	−0.0768	0.2446	0.9917
10	Malic acid	RI	1.0092	0.0171	−0.3891	0.1779	0.9946
50	Malic acid	RI	0.9784	0.0219	−0.2831	0.2337	0.9911
10	Ethanol	RI	0.7993	0.0150	5.6002	0.1567	0.9933
50	Ethanol	RI	0.8153	0.0240	6.4600	0.2557	0.9847

[a] A slope for the regressions of 1.0 shows that the concentrations analyzed were equal to the added concentrations.

hydrogen again occurred. When bubbling stopped, tubes were centrifuged and the supernatant sample solution was injected. After addition of sulfuric acid, samples could be frozen until used. Reduction reactions were done in triplicate and analyzed for each level of malic acid added.

RESULTS & DISCUSSION

OVERLAID chromatograms with RI and UV detectors of cucumber juice before and after reduction with sodium borohydride (Fig. 1) showed that before reduction, the malic acid peak was completely obscured by glucose and fructose peaks in the RI chromatogram. After reduction, there was some tailing of the sugar alcohol peak into the malic acid peak, but the peak could be readily quantitated. In the UV/visible chromatogram, the malic acid peak was clearly visible, but there was substantial interference from the slight UV absorption by the large quantity of fructose present. Since reduction of fructose to sugar alcohols completely eliminated UV absorption in addition to shifting elution to a slightly longer time, no interference with malic acid remained. Complete conversion of both glucose and fructose to sugar alcohols by this reduction procedure was verified. We ran chromatograms of samples of sodium borohydride-treated cucumber juice and fermentation brine with up to 100 mM added sugars on a sugar analysis column (McFeeters et al., (1984). No trace of either glucose or fructose was observed, and the appropriate sugar alcohol peaks were present.

The relationship between added and analyzed malic acid with a UV/visible detector at 210 nm (Fig. 2) showed the analyzed malic acid was equal to the added malic acid, whether 10 mM or 50 mM glucose and fructose had been added to fermented cucumber brine with 4% NaCl. There was a linear relationship between the added and analyzed malic acid in cucumber juice samples as well. However, in the cucumber juice matrix the slope of the line was slightly greater than the expected value of 1.0 (Table 1). The intercept greater than zero is due to the malic acid naturally occurring in cucumber juice.

Analysis of malic acid in the same sets of samples with a RI detector gave essentially identical results (Fig. 3), provided the peak height was measured from the top of the peak to the chromatogram baseline. Calculation of the height of the malic acid peak using a shoulder-skimming integration algorithm resulted in under-estimation of the concentration. Due to interference from the leading edge of the large sugar alcohol peak which was present in the RI chromatograms (Fig. 1), 0.75 mM added malic acid could not be detected, but 1.5 mM malic acid was detected. With the UV detector where there was no response to sugar alcohols, the lowest level malic acid (0.5 mM) could be detected.

A linear response resulted from both detectors to the lactic and acetic acids added to cucumber juice (Tables 1 and 2). However, the slope of the lactic acid response was slightly greater than 1.0 with both detectors. There was a small amount of interference by the tailing edge of the sugar alcohol peak in the lactic acid peak with the RI detector. Acetic acid had a slope that was not different from 1.0 within experiemental error with the RI detector. However, the slope was 1.21 for the UV/visible detector. An unidentified peak (retention time 12.6 min) interfered with the acetic acid peak (retention time 13.2 min), but the reason for a slope so much greater than 1.0 is not known.

Ethanol could only be detected by the RI detector. In both cucumber juice and fermentation brines there was a linear response to the amount of ethanol added. However, the slope was only 0.8 for all three sets of samples analyzed. Since ethanol is volatile, possibly some loss was caused by evaporation during the sugar reduction reaction, particularly when vigorous release of hydrogen gas occurred. Intercepts greater than zero for the brine samples were due to the presence of a small amount of ethanol in the fermentation brine.

This method differs from previous approaches in that it does not rely upon manipulating the difficult fructose/malic acid resolution. Unlike Lazaro et al. (1989), two detectors were not required. Either the UV or RI detector was sufficient. This is the only method which allows analysis of malic acid on a RI detector alone when fructose is present. Sugar determinations must be done in a separate analysis. However, analysis of other compounds of interest in fermented samples may be done on the reduced samples.

CONCLUSIONS

SODIUM BOROHYDRIDE reduction of fructose and glucose allowed analysis of malic acid by either UV or RI detectors in cucumber juice and fermented brines containing NaCl. Other compounds of interest in the cucumber fermentation could also be analyzed. However, the sample matrix appeared to affect analysis in some cases such that preparation of standards in the matrix of interest may be required for best accuracy.

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Volatile Sulfur Compounds Formed in Disrupted Tissues of Different Cabbage Cultivars

H.-W. CHIN and R.C. LINDSAY

ABSTRACT

Thirty-eight cultivars of cabbage (*Brassica oleracea* var *capitata*) were analyzed for production of volatile sulfur compounds after samples were homogenized and held at 30 °C from 10 to 100 min. Allyl isothiocyanate was detected in most cultivars, and it was formed rapidly compared to methanethiol-related compounds. Patterns of methanethiol production varied among cultivars. Hydrogen sulfide formed rapidly compared to methanethiol-related compounds, but it was completely depleted after 40 min. Dimethyl disulfide and dimethyl trisulfide concentrations initally low (< 0.78 ppm), increased linearly through 100 min (to 3.3 ppm). Wide variations in abilities to produce volatile sulfur compounds were observed for the different cultivars.

Key words: cabbage, sulfur, volatiles, methyl sulfides

INTRODUCTION

CABBAGE (*Brassica oleracea* var *capitata*) and other cruciferous vegetables produce many volatile sulfur compounds upon tissue disruption. Allyl isothiocyanate is generally considered one of the desirable flavor components in cabbage where it provides characteristic fresh cabbage notes and hotness (Schwimmer, 1963). On the other hand, methanethiol, dimethyl disulfide, and dimethyl trisulfide are volatile compounds in cruciferous vegetables which are generally responsible for objectionable sulfurous aromas and overcooked off-flavors (Maruyama, 1970; Forney et al., 1991; Hansen et al., 1992).

Unpleasant aromas arising during cooking as well as those perceived during consumption have been considered important reasons for the relatively low per capita per annum consumption (0.8–1.2 kg) of sauerkraut in the United States (Anonymous, 1987). However, refrigerated fresh salads, including coleslaw, have become increasingly popular. Coleslaw and other salads containing cabbage sometimes have sulfurous aromas and excessive hotness which detract from consumer acceptance.

Because cruciferous vegetables have been identified among other foods as potential contributors of anticarcinogenic principles to the diet (Marks et al., 1991; Caragay, 1992; Zhang et al., 1992), considerable interest exists for approaches to minimize unpleasant flavors. Therefore, our objective was to determine the volatile sulfur compounds produced in several cabbage cultivars, and thereby determine the extent of genetic variation in the production of volatile sulfur compounds occurring in cabbage. Such information should be useful in selection of cultivars that do not produce excessive volatile sulfur compounds.

MATERIALS & METHODS

Plant materials

Cabbage heads were provided by Dr. Paul H. Williams of the Dept. of Plant Pathology, Univ. of Wisconsin-Madison. Cabbage varieties (38), including 24 established cutlivars and 14 new breeding lines, were planted in June, 1991, in Outagamie County, Wisconsin, and cabbage heads were harvested from mid-September to the end of October at an estimated optimal maturity for each. Cabbage heads were promptly cooled and then refrigerated at 4 °C until analysis (< 4 wk).

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Sample preparation

Samples (100 g each) were cut as wedges from cabbage heads. Samples were then coarsely chopped, and each was transferred to a Waring Blendor (Waring Products, New Hartford, CT) which contained 400 mL distilled water. Samples were blended for 30 sec at the highest speed. Each homogenate was transferred to a series of 140-mL flasks (100g cabbage homogenate/flask). The flasks were then sealed with silicone septa (Alltech Associates, Deerfield, IL) held by plastic screw-caps. The caps were drilled so that an opening was present to allow insertion of a gas-tight syringe for headspace sampling. The flasks were then held at 30 °C in a water bath (Cambridge Instruments, Buffalo, NY). Duplicate analyses were performed for each sample, and duplicate samples (2) were analyzed when available.

Headspace analysis

At various holding times at 30 °C, 4-mL headspace gas was withdrawn from flasks with a 5-mL gas-tight syringe (Hamilton Co., Reno, NV). Headspace gas samples were then injected into a Varian (Palo Alto, CA) model 3700 gas chromatograph (GC). The GC was equipped with a flame photometric detector (FPD) and a Varian 4270 integrator. Flow rates for air #1, air #2, and hydrogen were 80, 170, and 140 mL/min, respectively. A glass column (183 cm × 2 mm i.d.) packed with 40/60 Carbopack B HT 100 (Supelco, Inc., Bellefonte, PA), was used to separate the volatile sulfur compounds. Helium at 30 mL/min was the carrier gas. The column temperature was held initially at 40°C for 1 min, programmed to 180°C at 20°C/min, and finally held at 180°C for 10 min. Both the injector port and detector were maintained at 200°C.

Quantification of each volatile sulfur compound was carried out by preparing standard curves with authentic compounds. A series of flasks of the same type used for cabbage samples was also used for preparing standard curves. Each flask contained 100g distilled water, instead of cabbage homogenate, and a known amount of standard compound. The same amount of headspace gas (4 mL) was withdrawn and injected into the GC. A plot of the square root of peak area versus the total mass (μg) of the sulfur compound in the flask gave a linear curve. Minimum detectable concentrations for each volatile sulfur compound that was quantified were >15 ppb.

Differences in equilibrium distribution coefficients between the headspace and aqueous phases for water and cabbage homogenates were determined for each standard sulfur chemical following the general method of Nelson and Hoff (1968). Cabbage homogenates essentially free of volatile sulfur compounds were prepared by blending cabbage with acetone (1:4, w/v) to inhibit enzymic action. Acetone was removed under reduced pressure (< 10 mm Hg) at 35°C, and moist cabbage tissue was resuspended in distilled water at a concentration equivalent (solids basis) to that in samples employed for headspace analyses. Correction factors calculated after determinations were for allyl isothiocyanate 1.06, hydrogen sulfide 1.05, methanethiol 0.90, dimethyl disulfide 0.97, and dimethyl trisulfide 0.99.

Standard sulfur chemicals

The volatile sulfur compounds for preparing standard curves were obtained commercially, and each was 96+% pure. Methanethiol and dimethyl trisulfide were obtained from Eastman Fine Chemicals (Rochester, NY). Dimethyl disulfide and allyl isothiocyanate were

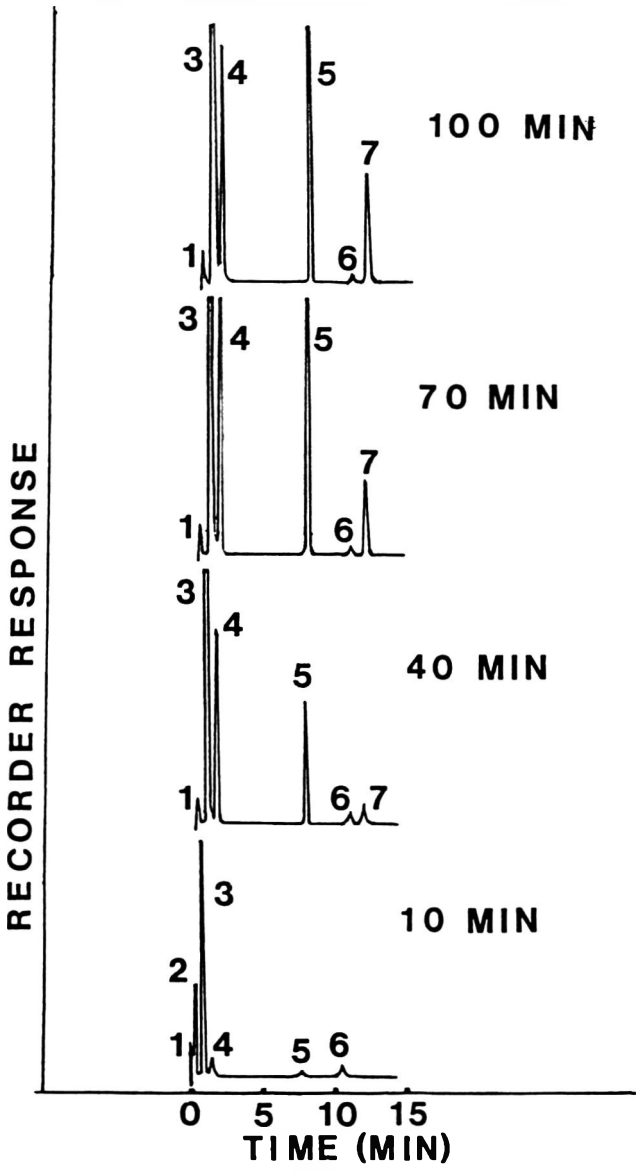

Fig. 1.—Flame photometric detector gas chromatograms of volatile sulfur compounds in the headspace of a selected fresh cabbage (Freja-21766) at various times (to 100 min) after blending with distilled water and holding at 30°C: (1) air peak; (2) hydrogen sulfide; (3) carbonyl sulfide; (4) methanethiol; (5) dimethyl disulfide; (6) allyl isothiocyanate; (7) dimethyl trisulfide.

obtained from Aldrich Chemical Co. (Milwaukee, WI). Hydrogen sulfide and sulfur dioxide were prepared by acidification of sodium sulfide (Aldrich) and sodium bisulfite (Fisher Scientific, Fair Lawn, NJ), respectively (Whitten et al., 1988). Carbonyl sulfide was prepared by hydrolysis of ammonium thiocyanate (Fisher) in dilute sulfuric acid (2*N*) at 60°C (Anonymous, 1966).

RESULTS & DISCUSSION

Hydrogen sulfide production

Hydrogen sulfide (H_2S), carbonyl sulfide (COS), methanethiol (CH_3SH), dimethyl disulfide (CH_3-S-S-CH_3), dimethyl trisulfide (CH_3-S-S-S-CH_3), and allyl isothiocyanate (CH_2=CH-CH_2-N=C=S) produced by cabbage were detected by the headspace GC technique (Fig. 1).

Hydrogen sulfide was detected in the cabbage headspace gas only at very early stages of analysis (Fig. 1; headspace sampled 10 min, 30°C, after tissue disruption). It was not found in other samples. Furthermore, not all cultivars produced detectable hydrogen sulfide. Among the 38 cultivars, 19 produced hydrogen sulfide in quantities that could be detected. Levels of hydrogen sulfide in those cultivars ranged from 0.02 to 0.39 ppm (Table 1).

Table 1—Volatile sulfur compounds produced by various cabbage cultivars 10 min after blending and holding at 30°C

Seed Co.	Cultivar	Concentration (ppm)[a] H_2S	CH_3SH	DMDS[b]	DMTS[b]	AITC[b]
AC[c]	Sanibel	0.02	0.02	0.02	ND[d]	2.5
AT[c]	Cheers	0.11	0.04	ND	ND	10.7
AT	58-543	0.17	ND	ND	ND	4.6
BS[c]	Carlton	ND	ND	ND	ND	5.9
BS	Cecile	ND	0.05	0.12	ND	9.4
BS	Hinova	ND	ND	ND	ND	8.6
BS	Krautpacker	ND	ND	ND	ND	49
BS	Rodolfo	0.02	0.02	0.05	ND	11.8
DA[c]	Bonnet-21396	0.02	0.05	0.38	ND	4.8
DA	Freja-21766	0.06	0.04	0.03	ND	0.7
DA	Parti-21656	0.08	0.05	0.17	ND	4.5
DA	Parti-23398	0.10	ND	0.12	ND	ND
DA	Parti-36462	0.04	0.05	0.05	0.04	0.4
DA	Parti-40155	ND	0.05	0.05	ND	0.7
DA	90-0321	0.11	0.05	0.05	ND	ND
FM[c]	King Cole	ND	0.06	0.08	0.25	1.0
FM	Titanic 90	0.14	0.05	0.19	0.30	0.9
FM	FMX 361	ND	0.05	ND	0.78	1.8
FM	FMX 378	ND	0.07	0.05	ND	1.1
FM	FMX 388	ND	0.05	0.05	0.25	1.2
HM[c]	Bravo	0.27	ND	ND	ND	5.2
HM	HMX 7271	0.14	0.03	ND	ND	3.5
HM	HMX 7273	ND	ND	ND	ND	4.7
NK[c]	CBO-16	ND	0.03	ND	ND	ND
NZ[c]	NIZ 90-956	0.08	ND	ND	ND	7.2
PS[c]	PSR 18589	0.27	0.04	0.08	ND	1.4
PS	PSX 63880	ND	0.04	0.08	ND	ND
RS[c]	Atria	ND	ND	0.08	ND	9.6
RS	RS 83106	0.39	0.03	0.05	ND	1.9
RS	RS 903005	ND	ND	0.10	ND	ND
RS	RS 903009	ND	ND	ND	ND	ND
RS	RS 903012	0.16	0.02	ND	ND	1.8
RS	RS 903015	0.06	ND	0.10	ND	1.7
RS	RS 903019	ND	0.05	0.22	0.50	ND
SA[c]	Tenacity	ND	ND	ND	ND	ND
SA	SCB 8401	ND	0.04	0.05	ND	0.7
SA	87G-100	ND	0.04	0.05	ND	1.6
SA	88G-72	0.06	ND	ND	ND	2.4

[a] Standard deviations based on pooled data were: 0.01 ppm for hydrogen sulfide; 0.02 ppm for methanethiol; 0.07 ppm for dimethyl disulfide; 0.02 ppm for dimethyl trisulfide; and 0.9 ppm for allyl isothiocyanate.
[b] DMDS: dimethyl disulfide; DMTS: dimethyl trisulfide; AITC: allyl isothiocyanate.
[c] AC: Alf Christiansen; AT: American Takii; BS: Bejo/Seedway; DA: Daehnfeldt; FM: Ferry Morse; HM: Harris-Moran; NK: Northrup King; NZ: Nickerson-Zwaan; PS: Peto Seed; RS: Royal Sluis; SA: Sakata.
[d] ND: not detected.

Presence of hydrogen sulfide in fresh and cooked cabbages has been generally accepted (Simpson and Halliday, 1928; Dateo et al., 1957; Bailey et al., 1961). The disappearance of hydrogen sulfide from samples of disrupted cabbage tissue is likely influential in development of unpleasant flavors and aromas. Hydrogen sulfide is very reactive (Badings et al., 1975), and it has been postulated to participate in formation of dimethyl trisulfide in cabbage (Maruyama, 1970). Additionally, Shaw et al. (1980) proposed that hydrogen sulfide was involved in the formation of carbonyl sulfide. Both dimethyl trisulfide and carbonyl sulfide were routinely encountered in our study.

Carbonyl sulfide production

Carbonyl sulfide was present in all 38 varieties, and it was the most abundant volatile sulfur compound in the headspace gases of the disrupted cabbage (Fig. 1). Bailey et al. (1961) and Shim and Lindsay (1990) reported that carbonyl sulfide was present in disrupted cabbage tissue, and its presence in our cabbage homogenates was verified by its inactivity with glyoxal (Josephson et al., 1983). The GC column packing material does not resolve sulfur dioxide (SO_2) and carbonyl sulfide (Anonymous, 1992). However, when glyoxal (500 ppm) was introduced into sampling flasks containing cabbage homogenates or standard samples of sulfur dioxide or carbonyl

Table 2—Volatile sulfur compounds produced by various cabbage cultivars 100 min after blending and holding at 30°C

Seed Co	Cultivar	Concentration (ppm)[a]				
		H_2S	CH_3SH	DMDS[b]	DMTS[b]	AITC[b]
AC[c]	Sanibel	ND[d]	0.06	0.19	0.85	2.5
AT[c]	Cheers	ND	0.03	0.24	0.45	10.3
AT	58-543	ND	0.03	0.39	0.90	5.1
BS[c]	Carlton	ND	ND	0.24	0.40	7.3
BS	Cecile	ND	ND	0.73	2.0	9.8
BS	Hinova	ND	0.02	0.24	0.43	9.5
BS	Krautpacker	ND	ND	0.29	0.85	45
BS	Rodolfo	ND	ND	0.17	0.30	8.9
DA[c]	Bonnet-21396	ND	0.18	0.68	0.43	3.2
DA	Freja-21766	ND	0.11	0.73	0.75	0.7
DA	Parti-21656	ND	0.02	2.1	2.4	4.8
DA	Parti-23398	ND	0.03	0.82	0.80	ND
DA	Parti-36462	ND	0.05	0.78	0.98	0.4
DA	Parti-40155	ND	0.11	1.1	1.1	0.6
DA	90-0321	ND	0.14	2.1	1.3	ND
FM[c]	King Cole	ND	0.05	0.87	1.5	0.9
FM	Titanic 90	ND	0.45	3.3	2.9	0.6
FM	FMX 361	ND	0.09	2.5	2.1	3.0
FM	FMX 378	ND	0.10	0.58	0.65	1.2
FM	FMX 388	ND	0.04	0.71	1.4	1.3
HM[c]	Bravo	ND	0.05	0.44	1.0	6.6
HM	HMX 7271	ND	0.04	0.39	0.60	3.9
HM	HMX 7273	ND	0.05	0.24	0.48	3.2
NK[c]	CBO-16	ND	0.04	0.68	1.0	2.0
NZ[c]	NIZ 90-956	ND	ND	0.34	1.2	9.6
PS[c]	PSR 18589	ND	0.36	1.8	1.4	1.2
PS	PSX 63880	ND	0.36	0.58	0.88	ND
RS[c]	Atria	ND	ND	0.53	1.1	9.0
RS	RS 83106	ND	0.07	1.1	1.3	1.8
RS	RS 903005	ND	ND	0.61	0.50	1.1
RS	RS 903009	ND	ND	0.24	0.30	ND
RS	RS 903012	ND	0.03	0.61	1.5	1.5
RS	RS 903015	ND	0.07	1.8	1.7	1.4
RS	RS 903019	ND	0.04	1.7	1.7	ND
SA[c]	Tenacity	ND	0.10	1.6	1.5	0.9
SA	SCB 8401	ND	0.11	0.45	0.58	0.6
SA	87G-100	ND	ND	0.41	1.4	1.7
SA	88G-72	ND	ND	0.63	0.95	2.9

[a] Standard deviations for the methods based on the pooled data were: 0.02 ppm for methanethiol; 0.10 ppm for dimethyl disulfide; 0.14 ppm for dimethyl trisulfide; and 0.9 ppm for allyl isothiocyanate.

[b] DMDS: dimethyl disulfide; DMTS: dimethyl trisulfide; AITC: allyl isothiocyanate.

[c] AC: Alf Christiansen; AT: American Takii; BS: Bejo/Seedway; DA: Daehnfeldt; FM: Ferry Morse; HM: Harris-Moran; NK: Northrup King; NZ: Nickerson-Zwaan; PS: Peto Seeds; RS: Royal Sluis; SA: Sakata.

[d] ND: not detected.

sulfide, the GC peak (retention time = 0.7 min) was altered only for the sulfur dioxide sample where it disappeared after 30 min. This verified the presence of carbonyl sulfide in cabbage homogenates, and no evidence was found for sulfur dioxide in the system.

The means of carbonyl sulfide formation is not clear, although two mechanisms have been proposed (Bailey et al., 1961; Shaw et al., 1980). Bailey et al. (1961) postulated that carbonyl sulfide may be formed from the hydrolysis of isothiocyanate, whereas Shaw et al. (1980) have speculated that H_2S could oxidize to SO_2 and may equlibriate with CO_2 to form carbonyl sulfide (COS) and water. Carbonyl sulfide is odorless (Anonymous, 1966; Jaddou et al., 1978), and because of its lack of effect on odors of cabbage, it was not quantified.

Methanethiol production

Among the 38 cabbage cultivars, 24 of them produced detectable amounts of methanethiol (Table 1) within 10 min after blending. After 100 min the number of cultivars that contained detectable methanethiol had increased to 28 (Table 2). However, not all 24 cultivars that initially produced methanethiol within 10 min still showed detectable amounts after 100 min. For example, Cecile and Rodolfo did not show methanethiol after 100 min (Table 2), though they initially produced 0.05 and 0.02 ppm of that compound, respectively (Table 1). Methanethiol has been shown to undergo oxidation to dimethyl disulfide in the presence of oxygen (Miller et al., 1973; Lindsay et al., 1986). That reaction probably accounts for the disappearance of methanethiol in the cabbage samples.

Methanethiol was generally present in disrupted cabbage in our current study, but earlier studies did not report methanethiol in cabbage. Dateo et al. (1957), using a mercuric cyanide trap, reported that no methanethiol was formed in raw or boiled cabbage. Similarly, Tressl et al. (1975) who gas chromatographically analyzed volatile compounds from cabbage by solvent extraction reported no methanethiol was formed. Since methanethiol is very volatile (b.p. about 6°C), the lack of detection in cabbage in the earlier reports probably was caused by losses occurring during analysis or insensitive analyses.

Dimethyl disulfide production

Dimethyl disulfide was first shown to be formed from a naturally occurring free amino acid, S-methylcysteine sulfoxide, through the action of acid hydrolysis at elevated temperatures (Dateo et al., 1957). Subsequently, Mazelis (1963) demonstrated the presence of an enzyme, cysteine sulfoxide lyase (C-S lyase), in cruciferous vegetables. This C-S lyase since has been generally recognized as responsible for the formation of dimethyl disulfide in raw cabbage. Except for differences in substrate specificity, this process is analogous to generation of flavors in garlic by a corresponding C-S lyase (Stoll and Seebeck, 1951).

All 38 cultivars produced dimethyl disulfide (Table 2), ranging from 0.17–3.3 ppm, during the holding period (100 min). However, only 23 varieties produced detectable amounts within 10 min after the cabbage was blended (Table 1). The slow appearance of dimethyl disulfide could be attributed to the fact that this compound is a secondary enzymic reaction product of the primary C-S lyase action on S-methylcysteine sulfoxide. The primary product, presumably methanesulfenic acid (Stoll and Seebeck, 1951), slowly converts to dimethyl disulfide and other sulfur-containing compounds. Methanesulfenic acid has not been isolated.

Dimethyl trisulfide production

Dimethyl trisulfide was also detected in the headspace gases of all 38 cultivars after 100 min (Table 2). The amount of dimethyl trisulfide ranged from 0.30 to 2.9 ppm. Only six produced detectable dimethyl trisulfide within 10 min (Table 1), and both dimethyl disulfide and dimethyl trisulfide had slow production. This suggested that dimethyl trisulfide also may be associated with a secondary reaction of the C-S lyase. No mechanism for trisulfide formation is generally accepted, but two mechanisms have been proposed. Maruyama (1970) proposed that the formation of trisulfides might result from the unstable sulfenic acids reacting with hydrogen sulfide. Boelens et al. (1971) speculated that disulfides might react with elemental sulfur to form trisulfides.

Allyl isothiocyanate production

Allyl isothiocyanate is formed by the thioglucosidase action on a glucosinolate precursor (Tookey et al., 1980). Other isothiocyanates (R-N=C=S) have been identified in cabbage (Bailey et al., 1967; Buttery et al., 1976; Van Etten et al., 1976), but the headspace-sampling technique and the GC conditions we used only allowed detection of allyl isothiocyanate (Figure 1). Most cultivars produced allyl isothiocyanate, but five varieties did not produce detectable amounts during the 100 min experimental period (Table 2). The levels of allyl isothiocyanate produced by disrupted cabbage after 100 min generally ranged from 0.4 to 10.3 ppm, with one exceptional cultivar (Krautpacker) yielding 45 ppm (Table 2). Note that all 5 cultivars from the Bejo/Seedway (BS) company had high levels of allyl isothiocyanate, while all the cultivars except Atria from the Royal Sluis (RS) company had low levels of

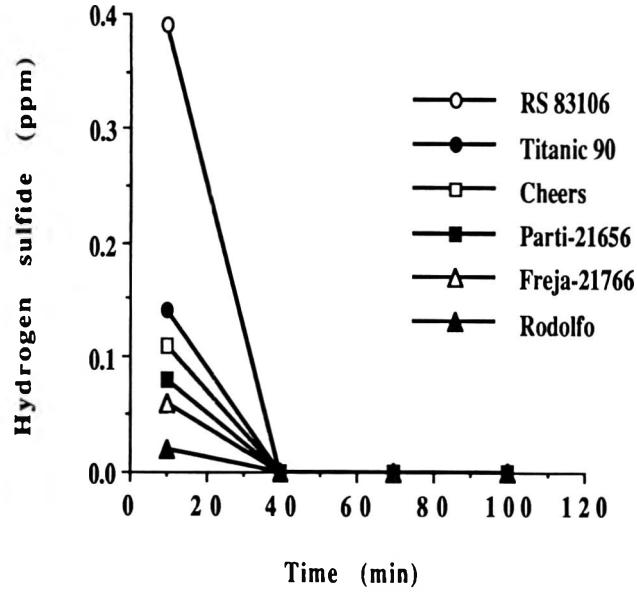

Fig. 2.—Rates of hydrogen sulfide production and disappearance in disrupted tissues of six selected cabbage cultivars.

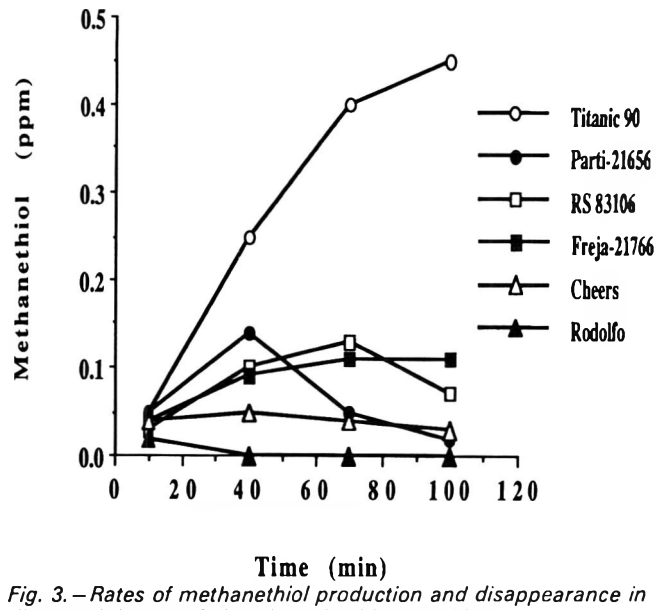

Fig. 3.—Rates of methanethiol production and disappearance in disrupted tissues of six selected cabbage cultivars.

this compound. Thus, breeding history may be reflected in the ability to produce allyl isothiocyanate.

Dimethyl sulfide

Bailey et al. (1961) reported the presence of dimethyl sulfide (CH_3-S-CH_3) in both freshly minced and dehydrated cabbages. This compound also has been reported to account for a very high proportion (about 34%) of total volatiles generated in cooked cabbage (MacLeod and MacLeod, 1968). However, under the conditions we used (30°C, pH 6.3, 100 min), none of the 38 cultivars produced detectable dimethyl sulfide.

McRorie et al. (1954) isolated a S-methyl methionine sulfonium salt from cabbage, and that compound was considered the precursor for dimethyl sulfide. Thermal decomposition of S-methyl methionine sulfonium salts has been shown to yield dimethyl sulfide (McRorie et al., 1954). However, Lewis et al. (1971) reported cleavage of S-methyl methionine sulfonium salts to dimethyl sulfide by a cabbage leaf enzyme fraction, and that enzyme had maximal activity at pH 7.8. The lower-than-optimum pH for the enzyme in the cabbage homogenates (pH 6.3) may have contributed to our lack of dimethyl sulfide detection. However, the sensitivity of the procedure for detecting dimethyl sulfide (to 15 ppb) indicates that this compound may not be a strong contributor to fresh cabbage flavors.

The impact of dimethyl sulfide on flavors of cooked or processed cabbage products is probably high, however. Analysis of freshly opened commercial canned sauerkraut for volatile sulfur compounds by GC-FPD showed that dimethyl sulfide accounted for about 75% of the total peak area of the chromatogram (data not shown).

Rates of production of volatile sulfur compounds

The rates of production of volatile sulfur compounds in six selected cultivars were compared (Fig. 2–6). Hydrogen sulfide (Fig. 2), as discussed previously, appeared in the headspace gases of some samples of disrupted cabbage only at very early stages of analysis, and completely disappeared after 40 min. Thus, the rates of hydrogen sulfide production and disappearance were very rapid. The rates of production of methanethiol (Fig. 3) were not consistent. For example, the level of methanethiol increased rapidly with time for Titanic 90, while it decreased with time in Rodolfo. For the other cultivars the concentrations of methanethiol increased at first and decreased later. This probably reflected conversion of methanethiol to dimethyl disulfide and dimethyl trisulfide (Miller et al., 1973; Lindsay et al., 1986).

The rates of production of dimethyl disulfide and dimethyl trisulfide were quite comparable (Fig. 4 and 5). These production patterns generally agreed with those observed for dipropyl disulfide in onions (Boelens et al., 1971) which is also an enzymic reaction product of a C-S lyase in *Allium* species. Boelens et al. (1971) showed that 15 min after chopping onions, the headspace gas consisted of very little dipropyl disulfide, but after 120 min dipropyl disulfide was the major volatile compound in headspace. Although a generally accepted mechanism for trisulfides formation has not been proposed, the data suggested that dimethyl trisulfide probably had a common precursor with dimethyl disulfide. Thus dimethyl trisulfide may also be a secondary reaction product of the C-S lyase present in cabbage.

Allyl isothiocyanate in cabbage (Fig. 6) reached near maximum concentrations soon after tissue disruption, although slight increases or decreases were often noted. The rapid appearance of allyl isothiocyanate when cabbage was blended illustrates a very rapid action of thioglucosidase on allyl glucosinolate precursor. Decompartmentalization of thioglucosidase and the glucosinolate substrates would provide a medium where fast conversion of precursors to flavor compounds could occur.

Importance of volatile sulfur compounds to cabbage flavor

Allyl isothiocyanate appeared to be important in very fresh cabbage flavor, since for most cultivars this compound was the major flavor-bearing sulfur compound detected soon after blending (Table 1, Fig. 6). However, some cultivars had limited production of this compound upon disruption. The average flavor threshold value for allyl isothiocyanate has been reported as 375 ppb in water (Buttery et al., 1976), and thus some cultivars would exhibit low cabbage flavor with no hotness.

It is also apparent that the impact of sulfide compounds (including hydrogen sulfide, dimethyl disulfide, and dimethyl trisulfide) on the flavor of very freshly chewed cabbage may be limited. Methanethiol could be important in very fresh cabbage flavor, since its threshold has been reported at 2 ppb in

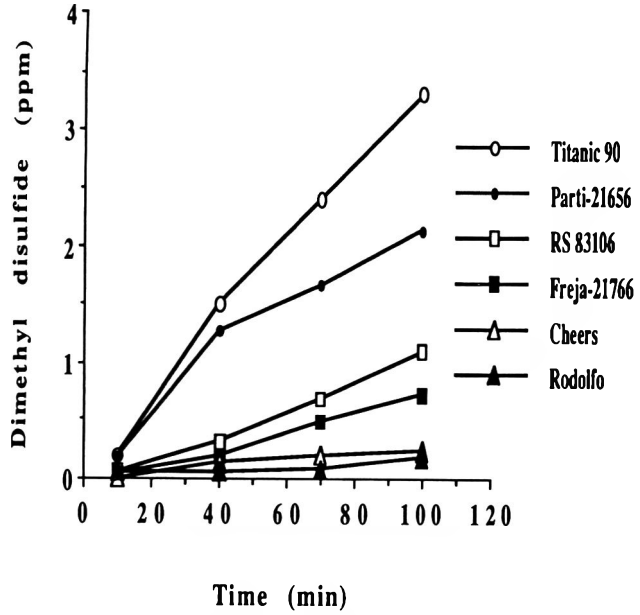

Fig. 4.—Rates of dimethyl disulfide production in disrupted tissues of six selected cabbage cultivars.

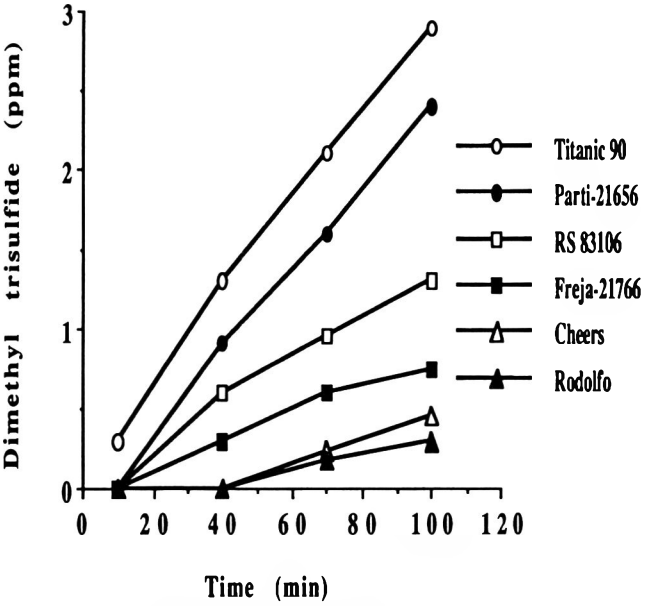

Fig. 5.—Rates of dimethyl trisulfide production in disrupted tissues of six selected cabbage cultivars.

water (Day et al., 1957). Several cabbage cultivars produced concentrations above the flavor threshold within 10 min after maceration of tissues. The odor of methanethiol has been described as "rotten cabbage" (Windholz et al., 1983).

The data clearly showed that some varieties of cabbage were capable of producing greater quantities of unpleasant volatile sulfur compounds than others. The observed increases in concentrations of dimethyl disulfide and dimethyl trisulfide were particularly important since those compounds contribute to putrid aromas (Lindsay et al., 1986). The flavor threshold for dimethyl disulfide in water has been reported at 12 ppb and that for dimethyl trisulfide at 0.01 ppb (Buttery et al., 1976). Thus, selection of cabbage cultivars which lack the ability to produce higher amounts of these compounds should result in fresh cabbage salads with more consumer appeal. Similarly, it might prove beneficial to prepare sauerkraut with cultivars that have low potential for production of volatile sulfur compounds.

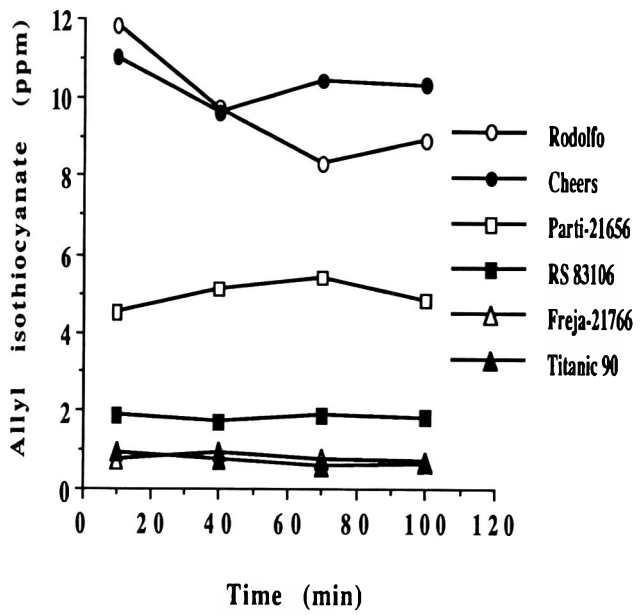

Fig. 6.—Rates of allyl isothiocyanate production and disappearance in disrupted tissues of six selected cabbage cultivars.

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—Continued on page 841

Accumulation of Chlorogenic Acid in Shredded Carrots During Storage in an Oriented Polypropylene Film

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ABSTRACT

Chlorogenic acid content of four samples of shredded carrots was measured and related to their flavor stability during storage wrapped in oriented polypropylene film (OPP). Chlorogenic acid was quantified by HPLC/UV. Shredded carrots accumulated chlorogenic acid faster in OPP film. This appeared related to higher sensory quality retention during storage.

Key Words: carrot, chlorogenic-acid, polypropylene, storage, modified atmosphere, packaging

INTRODUCTION

READY-TO-USE shredded carrots packaged in oriented polypropylene film (OPP) have not met consumer market requirements because of deterioration (off-odors, microbial spoilage) that occurs during storage. Shredded carrots may become slimy, lose firmness and produce off-flavors (Carlin et al., 1989). Such deterioration occurs at storage above 4°C, the temperature recommended by marketing agencies.

Previous studies have shown the importance of phenolic compounds in defense mechanisms of carrot tissues in response to infections or injuries (Rhodes and Wooltorton, 1978; Lewis and Garrod, 1983; Hoffmann and Heale, 1987). Babic et al. (1993) showed the accumulation of phenolic compounds in shredded carrots during storage. Among the hydroxycinnamic derivatives, chlorogenic acid amounted to 60% of total phenolics. Taylor and Zucker (1966) showed the relation between accumulation of chlorogenic acid and resistance of potato periderm tissues to infection by *Botrytis cinerea*. We hypothesized a relationship between chlorogenic acid and storage stability. Our objective was to measure changes in chlorogenic acid content of shredded carrots stored in OPP film during 7 days at 4°C. Resistance to spoilage was tested by storage at abuse temperature (10°C).

MATERIALS & METHODS

Plant material and processing

Four carrot samples from different cultivars were used, two samples from *Karotan* cultivar grown in different geographical areas, one sample from *Premia* cultivar and one sample from *Senior* cultivar. Carrots were washed with cool water (+8°C), trimmed, peeled with abrasives, disinfected in a 100 mg.L^{-1} chlorine solution, rinsed, shredded and spin drained. Shredded carrots (250g) were packaged in heat-sealed pouches (17 cm x 21 cm) made with an oriented polypropylene film (OPP), 35 μm thick, permeability to O_2 1200 mL.m^{-2}.day^{-1}.atm^{-1} at 25°C, vapor transmission rate 3.6 g.m^{-2}.day^{-1} at 38°C and 90% RH (Courtaulds Packaging, Avignon, France).

Storage conditions for phenolic compounds analysis.

Pouches of shredded carrots were stored at 4°C and analyzed after 0, 1, 3, and 7 days.

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Storage stability of shredded carrots.

Stability was measured by the amount of exudate released by shredded carrots in OPP film stored at 10°C. The rate of increase in exudate released was an indicator of rate of spoilage (Carlin et al., 1990). Exudate was measured in triplicate for each pouch of shredded carrots (4g) placed between two discs of filter paper and pressed for 10 sec by a 10 kg weight. Results were expresssed in g.100 g^{-1} of fresh matter (FM). Concentrations of O_2 and CO_2 inside the pouches were measured as in Babic (1992).

Quantification of chlorogenic acid

Shredded carrots (250g) were frozen in liquid nitrogen and crushed with a ball-grinder (type Dangoumau) for 1 min. The resulting powder (25g) was homogenized in 50 mL ethanol-water mixture (80/20, v/v) with sodium disulfite (0.5%). After stirring at 4°C for 15 min, the extract was filtered and the residue was treated two more times in the same way. The ethanolic filtrates were collected and ethanol was evaporated under vacuum. Pigments and lipids were removed by three successive petroleum ether extractions. Phenolic compounds were then extracted three times by ethyl acetate in the presence of ammonium sulfate (20%) and metaphosphoric acid (2%). The three ethyl acetate extractions were collected, dried on Whatman paper (phase separator silicone treated, cat. No. 2200185) and evaporated under vacuum. Residue was dissolved in methanol (1.5 mL). The methanolic extract was filtered through an Acrodisc filter (0.45 μm) before HPLC analyse.

Separation of phenolic compounds was by HPLC using a 3 μm C_{18} column (150 mm, 4.6 mm i.d.: Rosil from Alltech). The mobile phase (flow rate 0.8 mL.min^{-1}) consisted of acidified distilled water (solvent A) adjusted to pH 2.6 with orthophosphoric acid and of methanol/acetonitrile mixture (1.5/1, v/v) (solvent B). Best separation was obtained at 35°C with column pressure 120 bars using the gradient: 0 min, 12% B; 10 min, 12% B; 16 min, 15% B; 26 min, 15% B; 55 min, 60% B; 65 min, 70% B and 70 min, 70% B. Chlorogenic acid was quantified by external calibration (chlorogenic acid standard from Extrasynthese, Genay, France) by HPLC/UV at 325 nm. Values were determined on duplicate pouches and expressed in mg.100 g^{-1} of fresh matter.

Statistical analyses were done using STATITCF (ITCF, Paris, France) and STATGRAPHICS (STSC, Rockville, USA) softwares.

RESULTS & DISCUSSION

WIDE VARIATIONS in initial chlorogenic acid content were observed among the carrot samples (Fig. 1). Levels in *Karotan* 1 were different from those of other carrots (Tukey's test at level 5%). No difference was observed between *Premia*, *Karotan* 2 and *Senior*. In *Karotan* 2 chlorogenic acid was sevenfold higher (3.4 mg.100 g^{-1} FM) than in *Karotan 1* (0.5 mg.100 g^{-1} FM) and 1.7-fold higher than *Premia* and *Senior* (2 mg.100 g^{-1} FM). This variation might be due to different growing conditions in the fields (Chypman and Forsyth, 1971).

Chlorogenic acid content increased markedly in *Karotan 2* and *Senior* carrots after the first 24 hr storage, whereas it did not increase in *Karotan 1* and declined substantially between 1 and 3 days in *Premia* (Fig. 1). At 1 day, values were different from starting values for *Karotan 2* and *Senior* in contrast with *Premia* and *Karotan 1* (Student t test at level 5%). After 1 day storage, the chlorogenic acid contents of all carrot samples were significantly different and increases in chlorogenic acid,

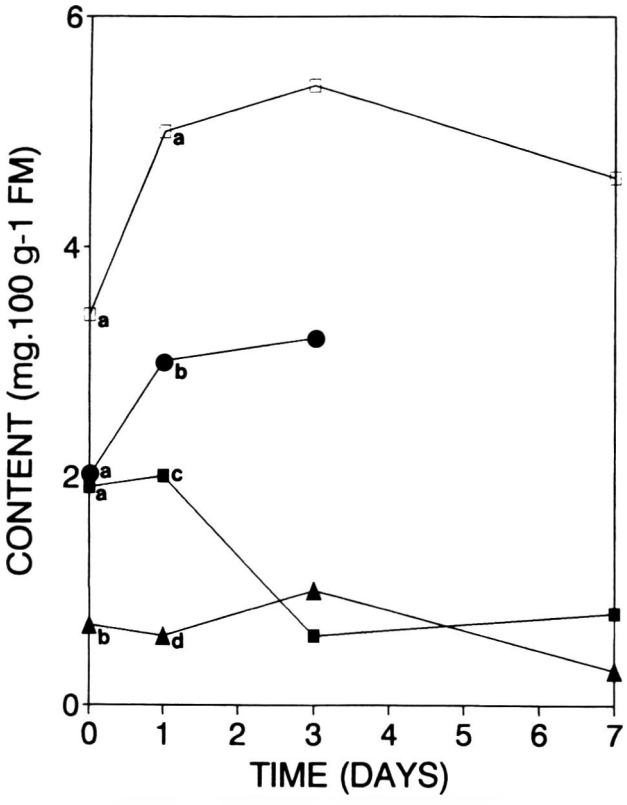

Fig. 1.—Changes in chlorogenic acid content in shredded carrots stored in OPP film at 4°C (n=2). Karotan 1 (▲), Karotan 2 (□), Premia (■), Senior (●).

as % of initial content were Premia, 6; Karotan 1, 0; Karotan 2, 47; and Senior, 50%.

During the first 3 days, exudate remained very low in all samples stored in OPP at 10°C (Table 1). At the end of storage, exudate increased sharply in *Premia* and *Karotan 1* carrots. The two samples had a "mashed" appearance, produced off-odors and clearly exhibited spoilage. In contrast, amounts of exudate in *Karotan 2* and *Senior* remained at the same level. After 7 days at 10°C, *Karotan 2* and *Senior* did not develop symptoms of spoilage (visual deterioration or off-flavors) in contrast with *Premia* and *Karotan 1*. Concentrations of O_2 and

Table 1—Changes in release of exudate (g.100 g^{-1} FM) of shredded carrots packaged in OPP film and stored at 10°C

Carrot sample	Time (days)		
	0	3	7
Premia	3.5	3.0	9.0
Karotan 1	3.0	3.0	13.0
Karotan 2	2.6	2.4	2.4
Senior	4.0	3.0	2.5

Standard deviation < 5% (n=3).

CO_2 evolved similarly in all pouches: respectively 1 and 25–30% at 3 days, 1 and 45–50% at 7 days.

Storage stability of carrot cultivars could not be characterized by its initial chlorogenic acid content, and shredded carrots with better storage stability were those which accumulated chlorogenic acid faster during the first 24 hr in OPP film. Therefore, the rate of accumulation of chlorogenic acid may be a useful index to select carrot cultivars for stability during storage under modified atmospheres.

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VOLATILE SULFUR COMPOUNDS IN CABBAGE. . .From page 839

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Controlled Fermentation of Spanish-type Green Olives

A. MONTAÑO, A.H. SÁNCHEZ, and A. DE CASTRO

ABSTRACT

Pure culture fermentation of Spanish-type green olives was developed. The method used no heat treatment, included chlorination of both fermentor and olives, used sterile lye, water and brine, and acidification with lactic acid before inoculation. *Lactobacillus plantarum* was used as test species. After 34 days fermentation, citric acid, mannitol and malic acid were completely degraded and ≈ 90% of available glucose and fructose, but <30% sucrose, were utilized. Fermentation products were D- and L-lactic acid, ethanol, succinic, and acetic acid with a calculated carbon recovery of 107.5%. D-lactic predominated over L-lactic acid. No differences were found between flavor of pure culture and naturally fermented olives, but there was a tendency towards preference of the latter.

Key Words: green olives, fermentation, *Lactobacillus plantarum*

INTRODUCTION

AMONG the main lactic acid fermented vegetable products, i.e. cucumbers, cabbages and olives, Spanish-type green-olives are the most economically important. Product uniformity and high quality are necessary if the market is to be increased and this may be achieved by using, as for other fermented foods, starter cultures of suitable species. Use of pure cultures of lactic acid bacteria (LAB) for green-olive fermentations was first suggested by Cruess (1937), and later by Vaughn et al. (1943), Borbolla y Alcalá et al. (1952), and others (Balatsouras et al., 1971; Pelagatti et al., 1975). Heat-shock treatment of olives before covering them aseptically with brine was developed by Etchells et al. (1966) to rid the fruits of naturally occurring and competitive microorganisms that interfere with fermentation. Subsequent inoculation with different species of LAB led to early and vigorous fermentations, especially when *Lb. plantarum* was used. Despite the experimental work, pure cultures are used on a limited commercial basis. Fleming et al. (1985) listed several factors which may account for this lack of commercial application. Harris et al. (1992) pointed out the necessity of developing a marked starter culture system to investigate the ecology of mixed culture fermentations.

Our objectives were: (1) to make available a system for pure culture fermentation of green olives without heat treatment, (2) to study substrate and product evolution in the brines in response to *Lb. plantarum* growth, and (3) to determine the effects on final product quality.

MATERIALS & METHODS

Preparation of Spanish-type green olives

Two replicate preparations were carried out on two consecutive days using locally grown olives of the Manzanillo variety. Leaves and damaged fruits were removed on receipt. The fermentation vessel (4,750g fruits plus 4,000 mL brine capacity) was designed and constructed (Fig. 1). It consisted of a PVC cylinder of 18.5 cm diameter and 30 cm height with a partially clear plastic cover provided with an inverted plastic U tube as gas outlet. The unit was kept sealed by a rubber ring between the cover and cylinder as well as suitable tightening screws. A rubber sample port for brine analysis, and a valve at the bottom for filling and discharging liquids by gravity completed the vessel. Before addition of olives, the fermentor and pipes were immersed in a solution of sodium hypochloride containing 300 ppm chlorine and then rinsed with tap water. Olives were washed thoroughly first under running tap water and then by immersion for 15 min in a solution containing 300 ppm chlorine. Finally they were rinsed again with tap water. The fermentor and olives were then dried on a laminar flow bench. The fermentor was aseptically filled with fruits and closed, in order to carry out the steps typical of Spanish-type green olive preparation, consisting of (a) lye treatment 4.0L. 2.16% NaOH for 6 hr at 24–25°C; (b) washing, 4.0L. tap water for 3 hr, and (c) brining, 4.0L. 11.4% NaCl. Lye, water, and brine had been previously autoclaved. Filling and discharging were performed using the chlorinated pipes connected to the bottom valves. The fermentor was kept in a lab environment (20–25°C). Before inoculation with *Lb. plantarum*, an acidification step (284 mL 1.2N lactic acid) was carried out to lower the initial alkaline pH of the brine to 6–7. Acidification was carried out 7 days after brining, by injecting the solution through the sample port.

Lactobacillus plantarum LP91 from the Instituto de la Grasa culture collection, originally isolated from green olives fermenting brine, was grown overnight at 32°C in MRS broth (Oxoid) containing 5% NaCl. Cells were harvested by centrifugation, washed, and finally resuspended in saline. They were injected into the fermentor one day after acidification. Initial populations were 0.8×10^6 and 1.3×10^6 CFU/mL brine in each replicate fermentor. Brine samples were taken with a sterile syringe and immediately used for microbiological analyses. Other samples taken at the same time were frozen (−30°C) for chemical analyses.

The traditional product was also prepared for sensory evaluation. Olives, lye treatment, washing and brining were identical as those used above, except that solutions were not autoclaved, aseptic precautions were not taken and neither acidification nor inoculation were performed. These fruits were subjected, therefore, to a natural fermentation without any control.

Microbiological analysis

Brine samples were always viewed by phase contrast microscopy, to look for microbial cells and, after inoculation, to confirm microbial cell morphology. In addition, samples were plated on standard method agar for aerobic plate counts, on Oxytetracycline-glucose-yeast extract agar to test for the presence of molds and yeasts, and on MRS (Oxoid) agar to count lactobacilli. All plates were duplicated and incubated at 32°C. If no growth had appeared after 5 days incubation microorganisms were assumed to be absent. Several samples were also inoculated into Differential Reinforced Clostridial Broth (Merck) for detection of viable spores.

Chemical analysis

The pH and sodium chloride content of the brines were determined by the routine methods (Fernández-Díez et al., 1985).

Glucose, fructose, sucrose and mannitol were analyzed by HPLC. The system consisted of a Hewlett-Packard Series 1050 liquid chromatograph equipped with a Rheodyne 7125 injector and a column heater, a Perkin Elmer Model LC-25 refractive index detector and a Hewlett-Packard Model 3396 Series II integrator. An Aminex HPX-87C carbohydrate analysis column (Bio Rad Labs.) held at 85°C was used for the separation. Deionised water was used as eluent at 0.9 mL/min. Concentrations were calculated by comparison of peak heights with external standards for each compound. Sample (2 mL brine) was passed through a 6 mL cationic-exchange column (Amberlite IR-120, H^+-form, 20-50 mesh, Merck) and then through a 6 mL anionic-exchange column (Amberlite IRA-93, free base, 20-50 mesh, Fluka AG). The columns were washed with deionised water and all eluates collected in a 125 mL volumetric flask. An aliquot (50 mL) was taken

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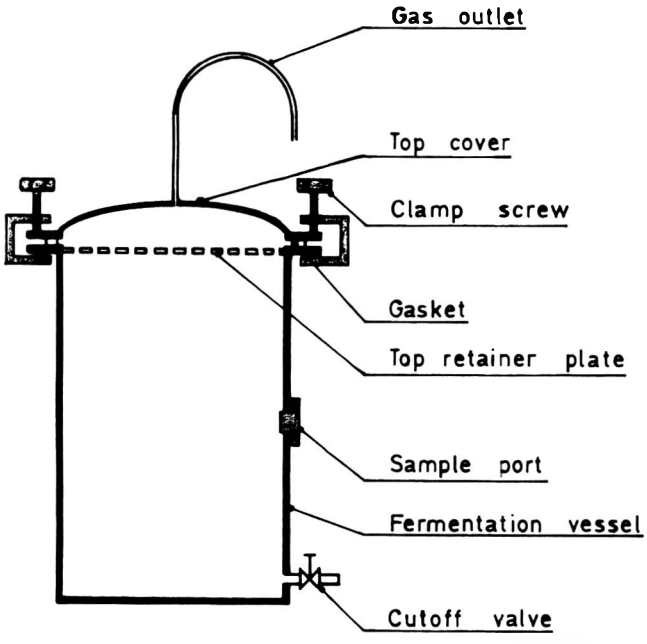

Fig. 1—Design of fermentor used for aseptic preparation of Spanish-type green olives.

Table 1—Chemical characteristics of the olive brine prior to inoculation (7 days after brining)[a]

pH	10.0
Sodium chloride (%)	5.6
Carbohydrates (g/L)	
Glucose	5.1
Fructose	3.1
Mannitol	1.4
Sucrose	0.5
Organic acids (g/L)	
Malic acid	0.8
Citric acid	0.4
Acetic acid	0.7
Volatile components (g/L)	
Methanol	0.4
Ethanol	0.08

[a] Values are means of two replicate fermentors.

to dryness on a rotary evaporator at 35°C and 2 mL deionised water was added to the residue. Finally, the solution was filtered through a 0.45 μm filter prior to injection (50 μL) into the chromatograph.

Acetic, malic, citric and succinic acids were also analyzed by HPLC, using a Spherisorb ODS-2 (5 μm, 25 cm * 4 mm i.d., Supelco) column protected by a C18 guard column (5 μm, 1.3 cm * 4 mm I.D., Teknokroma, Barcelona, Spain). Compounds were eluted with deionised water adjusted to pH 2.3 with phosphoric acid at 1.0 mL/min. Sample preparation included only a dilution stage (1:10 with deionised water) followed by passage through a minicolumn containing a cation-exchange resin (2 mL bed of Amberlite IR-120, H^+-form) to remove cations, mainly Na^+, and ensure that organic acids were in the protonated form. To avoid dilution on being passed through the resin, 6 mL of sample were added and discarded before collecting 2 mL for chromatographic analysis. An aliquot (50 μL) of this fraction was injected into the chromatograph after filtration through a 0.45 μm filter.

D- and L-lactic acid were determined using an enzymatic assay kit (Boehringer, Mannheim, Germany) according to manufacturer's recommended procedure. Ethanol and methanol were analyzed using the headspace method described by Montaño et al. (1990). Diacetyl and acetoin were also assayed by this method, except the GC column was programmed at 15°C/min. All analyses were performed in duplicate.

Sensory evaluation

Sensory evaluation was carried out by an 11-member trained panel. Pure culture and naturally fermented olives were previously packed in order to achieve the same salt (5% NaCl) and acid (0.5% as lactic acid) concentrations. Each panelist assigned number 1 to the preferred sample and 2 to the other, and the scores of the 11 panelists were summed. The series of numbers thus obtained were compared with tables (Kramer and Twigg, 1970) to determine which sample was significantly preferred.

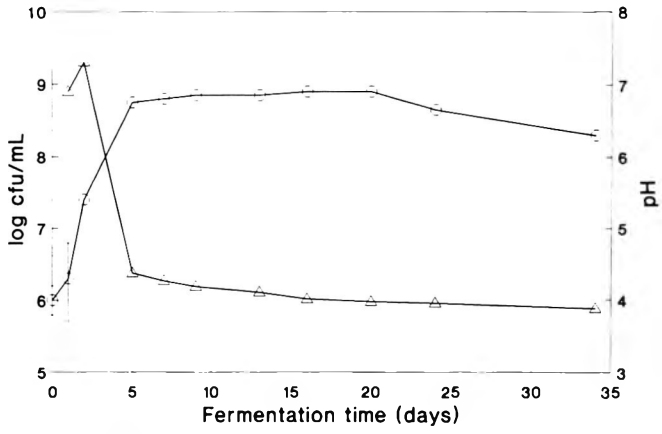

Fig. 2—Changes in pH (△) and microbial population (○) during Lb. plantarum *green olive fermentation. Points are means of duplicate fermentations. Where error bars are not visible, determinations were within the range of the symbols on the graph.*

RESULTS & DISCUSSION

Microbiological and chemical changes during fermentation

The aseptic brining procedure proved suitable for preventing microbial growth. Seven days after brining, none of the fermentors contained microorganisms, as was evident by visual inspection, microscopic examination, and plating. The composition of the olive brine prior to inoculation (Table 1) showed comparatively high amount of acetic acid. Its presence is attributable to the transformation of sugars following alkaline treatment (Borbolla y Alcalá et al., 1956). The composition shown scarcely differed from that obtained 5 days after brining which indicates that the compounds had already reached equilibration between olives and the brine.

Microbial growth and pH changes in brine after inoculation (Fig. 2) showed lactobacilli counts increased from 10^6 to almost 10^9 CFU/mL in 5 days. The population remained at that level for 15 days and then declined slightly. Around 5×10^7 CFU/mL were present 70 days after inoculation, and 10^4 CFU/mL after 250 days (data not shown). Microorganisms other than lactobacilli were not detected. We assumed, therefore, that the fermentation in both replicates was due to the *Lb. plantarum* starter culture metabolism. The pH increased slightly during the first 2 days due to diffusion of added lactic acid into fruits and residual NaOH still present the fruits diffused into the brines. However, as the starter culture grew, the pH values decreased rapidly, and was below 4.5 after 5 days post-inoculation. The final pH was 3.9.

Carbohydrate consumption started about 2 days after inoculation (Fig. 3). Glucose and fructose metabolism by *Lb. plantarum* occurred simultaneously but, at least initially, the consumption of glucose was faster than that of fructose. This agreed with results by Rodrigo et al. (1986) who studied controlled fermentations of cucumbers. Levels of glucose and fructose reached values below 0.1% in about 1 wk. Mannitol was also consumed, although more slowly, but sucrose degradation had scarcely occurred by 34 days after inoculation. The latter was consumed later (about 90% degradation after 70 days fermentation).

The main acid products of fermentation were D- and L-lactic acid, which increased during exponential growth and reached maximum values during the stationary phase (Fig. 4). The level of D-lactic was always higher than L-lactic acid, which

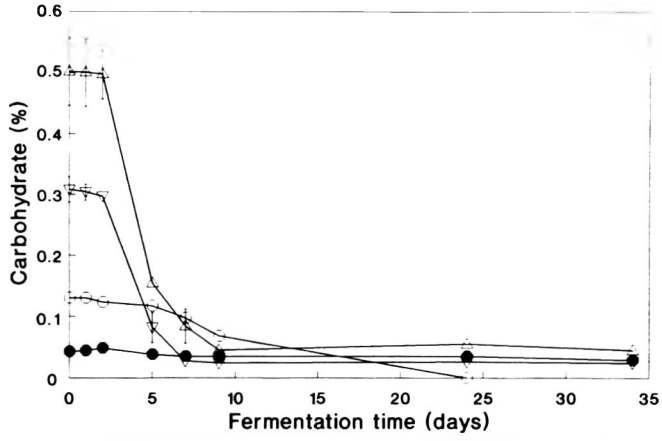

Fig. 3—Changes in the composition of carbohydrates during Lb. plantarum *green olive fermentation. (△) glucose; (▽) fructose; (○) mannitol; (●) sucrose. Points are means of duplicate fermentations. Where error bars are not visible, determinations were within the range of the symbols on the graph.*

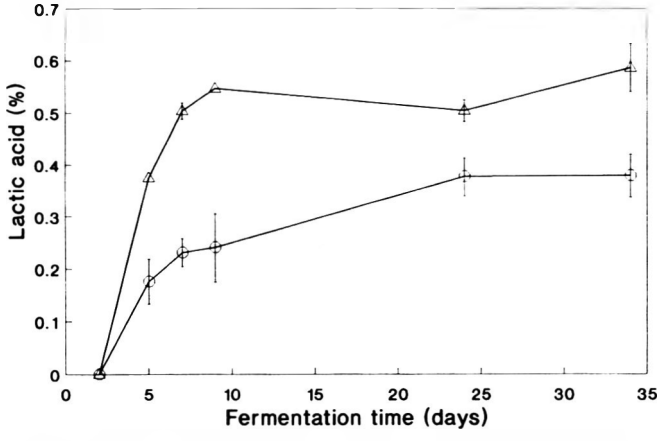

Fig. 4—Lactic acid production during Lb. plantarum *green olive fermentation. (○) L-lactic acid; (△) D-lactic acid. Points are means of duplicate fermentations. Where error bars are not visible, determinations were within the range of the symbols on the graph.*

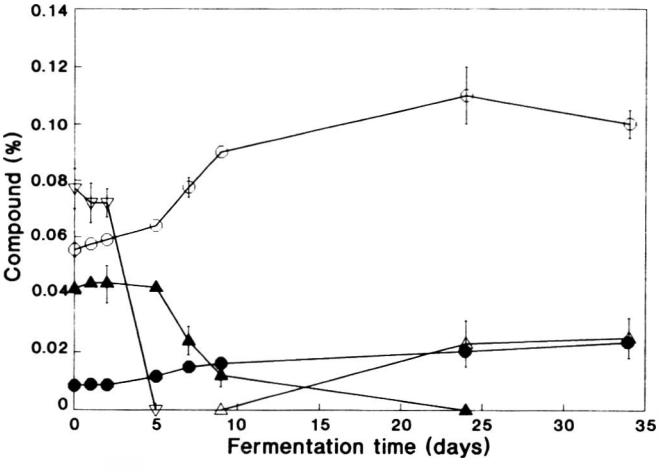

Fig. 5—Changes in the composition of organic acids and ethanol during Lb. plantarum *green olive fermentation. (▲) citric acid; (▽) malic acid; (△) succinic acid; (○) acetic acid; (●) ethanol. Points are means of duplicate fermentations. Where error bars are not visible, determinations were within the range of the symbols on the graph.*

Table 2—Fermentation balances from Spanish-style green olives, fermented by Lb. plantarum*, 34 days after inoculation*[a]

Substrate consumed (mM)[b]	
Glucose	25.4 (90)
Fructose	15.8 (93)
Mannitol	7.7 (100)
Sucrose	0.4 (27)
Malic acid	5.9 (100)
Citric acid	2.1 (100)
Product formed (mM)	
Lactic acid[c]	107.2
Acetic acid[d]	11.5
Succinic acid	2.1
Ethanol[d]	3.2
Carbon recovery (%)	107.5

[a] Values are means of duplicate fermentations.
[b] Percentage of substrate metabolized in parentheses.
[c] This represents the net increase in lactic acid over that added before inoculation.
[d] This represents the net increase in product over that present before inoculation.

agreed with results by Bobillo and Marshall (1991) for another strain of *Lb. plantarum* isolated from fermenting green olive brine in MRS broth. This indicated that for both strains D-lactate dehydrogenase was more active than L-lactate dehydrogenase (Fleming et al., 1985).

Changes in other organic acids and ethanol during fermentation (Fig. 5) showed malic and citric, the two major acids in raw olives (Vlahov, 1976), were degraded (100% degradation) during fermentation, but malic acid disappeared more rapidly. It was presumably degraded to lactic and CO_2, by a malolactic enzyme similar to that reported by McFeeters et al. (1982) in cucumber juice fermentations. Citric acid consumption did not begin until near the end of exponential phase of growth when the pH had fallen to about 4.5. This appears to be typical of homofermentative lactobacilli and, it has been suggested, may be due to passive transport of citrate (Drinan et al., 1976). Citric acid might have been degraded via the anaerobic pathway of citrate metabolism to form CO_2, acetate, acetoin, diacetyl and 2,3-butylene glycol (Hickey et al., 1983). However, neither acetoin nor diacetyl were detected. The production of succinic acid and ethanol (1 mmol of succinic acid and 1.5 mmol of ethanol were formed per mmol of citric acid utilized) suggested that citric acid may have been degraded via the pathway proposed by McFeeters and Chen (1986). That is, it served as electron acceptor for anaerobic mannitol metabolism.

Fermentation balance

Substrates consumed, products formed and carbon recovery 34 days after inoculation were compared (Table 2). As expected, carbohydrates were metabolized via the glycolytic pathway (1 mmol of hexose yielded 2.0 mmol lactic acid when corrected for malic acid conversion). The estimate for carbon recovery from fermentation was 107.5% which suggested that other substrates not measured may have been converted to the measured products. However, a greater number of replicates would be needed to determine whether this value is statistically different from 100%.

Product quality

When packed olives from both pure culture and natural fermentation were tasted, the Kramer test showed there were no significant differences ($p = 0.05$). Panelists who preferred the natural product mentioned its typical flavor, whereas olives from controlled fermentation were noted as "strange" in some way. On the other hand, this latter product was preferred by other panelists because they considered the naturally fermented olives to be too "strong" or "acid", despite the fact that both samples had the same acidity and salt content.

We concluded that the aseptic preparation method, along with the simple fermentor design used, was suitable for a pure culture fermentation in Spanish-type green olives. Heat treat-

—Continued on page 852

D-Erythroascorbic Acid in Bakers' Yeast and Effects on Wheat Dough

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ABSTRACT

Extraction of hydrated and freeze-dried bakers' yeast yielded ≈ 50 μg erythroascorbic acid (EAA) and 5 μg ascorbic acid (AA)/g dry yeast as determined by HPLC with electrochemical detection. D-EAA (82 ppm based on flour) slightly increased the flow of dough as rest time increased. Gluten isolated from a flour-water dough containing 82 ppm D-EAA or 100 ppm L-cysteine stretched at a faster rate than control gluten. EAA like AA did not change dough development time. Unlike AA, EAA showed neither oxidizing effect on dough and gluten nor improving effects on bread.

Key Words: Erythroascorbic acid, wheat, dough, yeast

INTRODUCTION

YEAST (*Saccharomyces cerevisiae*) fermentation of wheat dough decreases the flow of dough and increases its elastic character (Hoseney, 1986). L-Ascorbic acid (L-AA) elicits a similar response when added to dough at > 15 ppm (Elkassabany and Hoseney, 1980). Nick et al. (1986) reported that *S. cerevisiae* contained erythroascorbic acid (EAA). The naturally occurring material is thought to be the D-enantiomer because an isotopically labeled sample co-crystallized with chemically pure D-EAA (Liang et al., 1987).

The structure of D-EAA closely resembles that of L-AA (Fig. 1) suggesting this compound might cause the beneficial effects of yeast-fermentation on doughs. Our objectives were to determine the level of EAA in several samples of bakers' yeast and to determine the effect of EAA on mixing time, dough flow, and gluten stretching.

MATERIALS & METHODS

Materials

D-EAA (m.p. 132–135°C) was synthesized by the procedure of Liang et al. (1987). L-AA, HPLC-grade methanol, and sodium acetate were from Fisher Scientific, St. Louis, MO. All other chemicals were reagent grade. Compressed yeast was from Universal Foods Corporation, Milwaukee, WI. Three commercial, instant yeasts were obtained from SAF Products Corporation, Minneapolis, MN; Universal Foods Corporation, Milwaukee, WI; and Gist-Brocades, Charlotte, NC. Straight grade flour was milled from a composite of hard red winter wheat cultivars grown in the Central Plains (Ross Milling Co., Wichita, KS) and contained 11.9% protein on a 14% moisture basis (m.b.). For the gluten stretching test, unmalted flour containing 11.4% protein on a 14% m.b. was used.

High Performance Liquid Chromatography (HPLC)

The HPLC system consisted of a 6000A solvent delivery system (Waters Associates, Inc., Milford, MA) and an electrochemical (EC) detector (Model LC-4, Bioanalytical System, West Lafayette, IN) with a glassy carbon working electrode and a type TL-5A flow cell. EAA and AA in yeast were determined on a reverse-phase column

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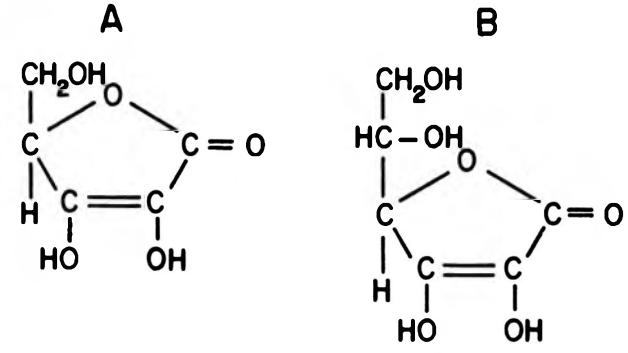

Fig. 1—(A) D-erythroascorbic acid (D-EAA) = (D-glycero-pent-2-enono-1,4-lactone). (B) L-ascorbic acid (L-AA) = (L-threo-hex-2-enono-1,4-lactone).

(Spherisorb, ODS-2, Rainin Instrument Co. Inc., Woburn, MA, 5 μm particle size, 250 × 4.6 mm). The analytical column was protected by a precolumn with a 40 × 4.6 mm ODS-10 cartridge (Bio-Rad Laboratories, Richmond, CA). The mobile phase contained 0.08M acetate buffer (pH 4.2), 5% methanol, 0.1 mM ethylenediaminetetraacetic acid, and 1.0 mM tetrabutylammonium phosphate. The column was maintained at 35°C, and the potential of the detector was set at + 0.72V vs a Ag/AgCl reference electrode.

Determination of EAA and AA in Bakers' Yeast

The recovery of D-EAA added to yeast was determined as follows. An aliquot of D-EAA stock solution in 3% metaphosphoric acid containing 0.08 mg D-EAA was added to compressed yeast (4 g, "as received") in aqueous metaphosphoric acid (5 mL). Then, 3% metaphosphoric acid was added to volume (10 mL), and the mixture was stirred on a magnetic stir plate at 25°C for 15 min to 2 h. After different extraction periods, the extract was centrifuged (27,000 × *g*, 5 min), and an aliquot (1 mL) of clear supernatant was pipetted into a volumetric flask (10 mL) and made to volume with cold, degassed 0.05 M perchloric acid. The resulting solution was filtered through a 0.5 μm micromembrane filter (Millipore Corporation, Bedford, MA) and 0.02 mL was injected into the chromatograph. The same methods were used for AA recovery.

Yeast was assayed for EAA and AA "as received" or after it was freeze-dried. Compressed yeast (4g "as received") or instant yeast (1.0g, "as received") was stirred with 10 mL of 3% metaphosphoric acid for 5 min at 25°C. After centrifugation, an aliquot (1 mL) of the clear supernatant was made to volume (10 mL) with cold 0.05M perchloric acid, and assayed as described. In the case of one instant yeast containing a very high level of AA, the sample weight was 0.1g and the aliquot (1 mL) of the metaphosphoric acid extract was diluted to 100 mL with 0.05M perchloric acid.

Freeze-dried compressed or rehydrated, freeze-dried instant yeast was ground with a mortar and pestle, and 1g of the yeast was treated as described, except the final volume of the diluted extract was 25 mL instead. Instant yeast (10 g) was hydrated with 25 mL of deoxygenized water for 5 min at 25°C and freeze-dried.

Spread test

Flour-water doughs were mixed with a pin mixer from 100 g flour to optimum consistency and development. Doughs were left standing at 30°C and 90% RH and, after 0, 1, 2, or 3 h, were mechanically rounded and permitted to rest 60 min at the same conditions. Spread

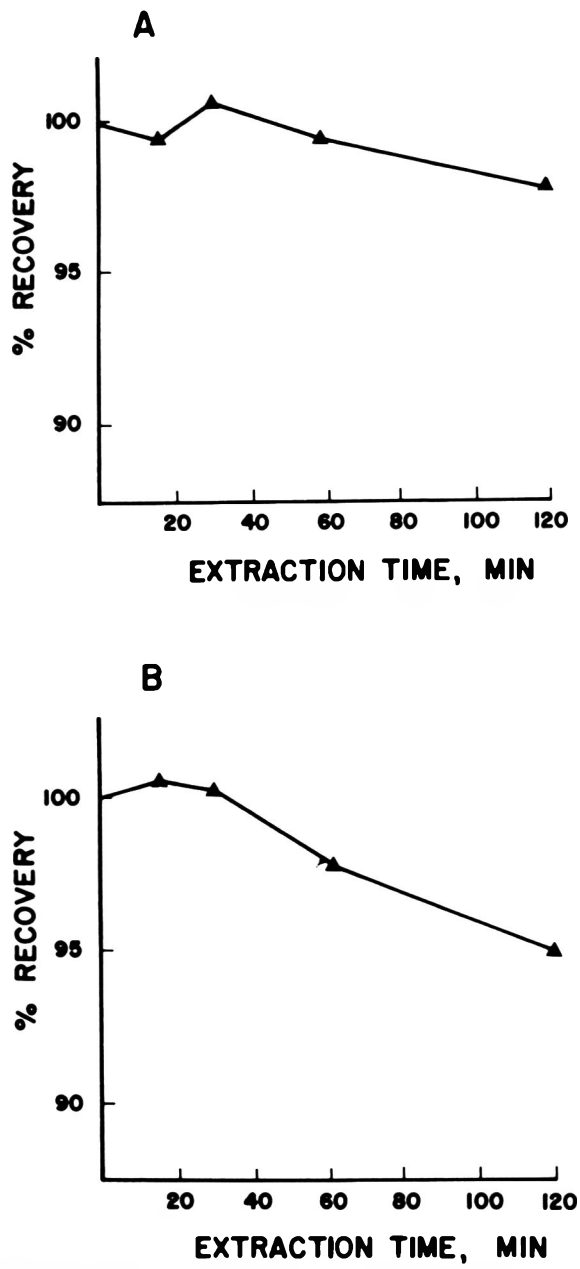

Fig. 2—Recoveries of (A) D-EAA and (B) L-AA added to extracts (3% metaphosphoric acid) of compressed yeast at 25°C.

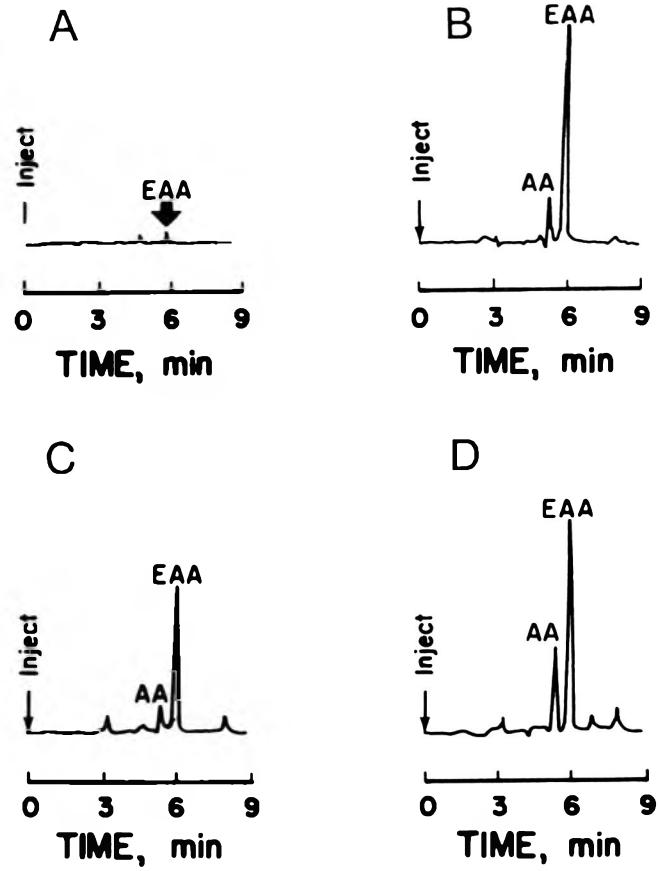

Fig. 3—HPLC-EC chromatograms of 3% metaphosphoric acid extracts of yeast: (A) compressed yeast, "as is;" (B) freeze-dried compressed yeast; (C) instant yeast, "as is;" and (D) freeze-dried instant yeast.

ratios then were determined by dividing the width of a molded dough piece by its height (Hoseney et al., 1979). Reported spread ratios are averages of 2 determinations.

Effects of D-EAA on mixing time

A 10-g mixograph, described by Finney and Shogren (1972), was used to study the effect of D-EAA on mixing time. Flour (10g on 14% m.b.) was placed in the mixograph bowl, and optimum water absorption for the flour was determined to be 6.2g from the shape of the mixogram. Then, various EAA levels were mixed with the dough while constant absorption was maintained. In the same manner, L-AA and L-cysteine hydrochloride were tested at levels equivalent to EAA on a molar basis.

Gluten strength by a stretching method

The gluten stretching apparatus consisted of four 1-L tubes (50 mm × 390 mm) filled with distilled water as described by Al-Obaidy (1986) and maintained at 32°C using a water bath. The top cover of each tube contained a small hook, from which gluten balls could be hung. Gluten samples were tested in duplicate. Gluten was washed from a dough mixed from 12.5g (on 14% m.b.) of flour, optimum water, and D-EAA (82 ppm), L-AA (100 ppm), or cysteine (100 ppm), as described by Chung et al. (1987), except for a change in water temperature from 30°C to 32°C. After being mixed to optimum development, the dough was kneaded under a stream of distilled water until the wash water appeared free of starch. The wet gluten was rolled

Table 1—Concentrations of EAA and AA in various yeasts

Yeast	Sample no.	EAA (ppm)[a]	AA (ppm)
Compressed:			
"As Received" (Fresh)	1	1.0	1.5
	2	4.0	4.5
	3	1.4	2.0
Freeze-dried	1A	50.9	2.4
	2A	52.0	4.9
	3A	35.2	5.4
Instant:			
"As Received" (Dry)	4[b]	18.1	2720.1[e]
	5[c]	26.9	8.2
	6[d]	11.7	4.3
Hydrated and Freeze-dried	4A	37.3	—
	5A	20.9	3.8
	6A	68.7	5.5

[a] Dry basis.
[b] Contains other ingredients: polyglycerol esters of vegetable fatty acids, potato starch, soybean oil. Manufacturer reported in a private communication that L-AA was added to yeast.
[c] Contains other ingredient: sorbitan monostearate.
[d] Instant Active Dry Yeast.
[e] L-AA was added by manufacturer.

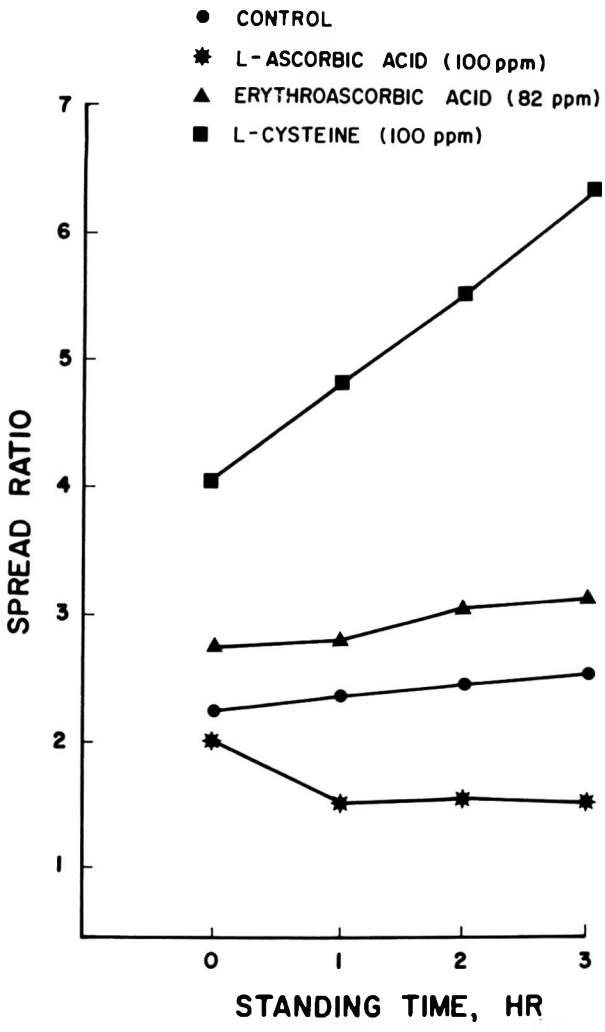

Fig. 4—Effects of additives on the spread of flour-water doughs; 100 ppm of L-AA, 82 ppm of D-EAA, or 100 ppm of L-cysteine hydrochloride.

between the hands to remove excess water and weighed. Two 2.5-g portions were cut from the same gluten sample, moulded into a ball with the fingers, and rested 10 min in distilled water at 32°C. The wet gluten ball was hooked through its center by a fishing hook, which was used to hang the gluten piece form the cover of the tube. A second fishing hook containing a 3-g weight was also hooked at the same point as the first hook. The distance of gluten stretching was recorded every 20 min, and the time for the gluten to reach the bottom of the tube (35 cm distance) was recorded.

RESULTS & DISCUSSION

Stability of EAA and AA in 3% metaphosphoric acid

Compressed yeast in 3% metaphosphoric acid had D-EAA or L-AA added to it to check stabilities in the extraction medium. D-EAA and L-AA were essentially stable in 3% metaphosphoric acid (pH 1.5) for up to 2 hr at 25°C. Recoveries of added D-EAA or L-AA were quantitative after 30 min stirring at 25°C (Fig. 2).

Assay of Yeast for AA and EAA

The recovery of D-EAA and L-AA added to compressed yeast in 3% metaphosphoric acid was linear over the range 0–60 μg/g yeast (dry basis); coefficients of variation were ± 2.3% for D-EAA and ± 1.2%, for L-AA.

Extraction of three samples of fresh compressed yeast with 3% metaphosphoric acid gave 1-4 ppm EAA (dry solids basis) and 1.5-4.5 ppm AA (Table 1). However, when the compressed yeast was freeze-dried prior to extraction, the samples gave 35–52 ppm EAA and 2.4–5.4 ppm AA. Probably the dry compressed yeast cells were lysed during freeze-drying or during the first stages of extraction. Typical chromatograms are shown in Fig. 3. The level of EAA extractable from the three samples of dry compressed yeast averaged at least one order of magnitude higher than that of AA (45.8 vs 4.2 ppm, Table 1). Nick et al. (1986) reported only EAA and no AA in two samples of *Saccharomyces cerevisiae*.

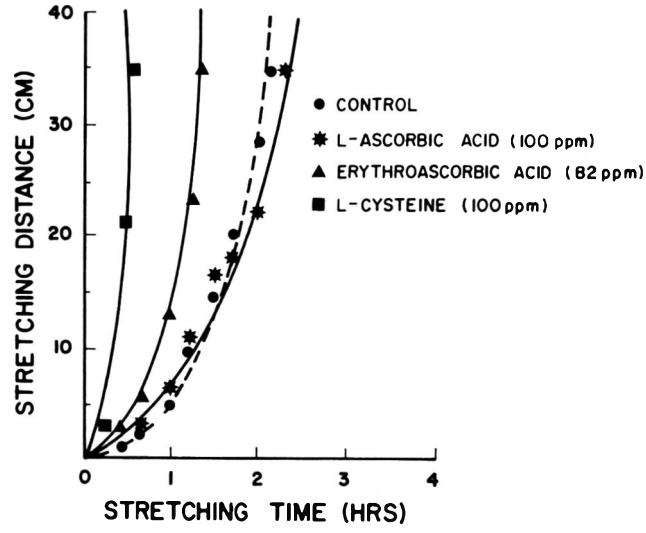

Fig. 5—Stretching time (hr) vs distance (cm) of gluten stretching. Glutens were obtained from optimally mixed doughs at optimum water absorption. The doughs contained no additives (control), 82 ppm D-EAA, 100 ppm L-AA, or 100 ppm L-cysteine hydrochloride.

Three samples of instant yeasts also gave a higher release of EAA (21–69 ppm) after hydration and freeze-drying than with no pretreatment (12–27 ppm). The instant, active dry yeast (Sample No. 6A) showed the highest discharge of EAA (69 ppm) and AA (6 ppm). The "as received" instant dry yeasts had much higher levels of EAA than "as received" compressed yeast. Either the instant yeasts were partially lysed when added to 3% metaphosphoric acid, or they contained lysed cells.

Effect of EAA on mixing and spreading of wheat dough

Mixograms showed that D-EAA, like L-AA, had little effect on mixing time at levels up to about 1000 ppm in typical dough (data not shown). The spread test (Fig. 4) showed that, compared to control dough, L-AA (100 ppm) strengthened dough and reduced its flow, whereas cysteine (100 ppm) greatly increased dough flow. On the other hand, D-EAA (82 ppm, equivalent to 100 ppm L-AA) gave a slight increase in dough flow with lay time. Pup-loaves baked with 82 ppm D-EAA had reduced volumes compared to control loaves whereas L-AA gave the expected increase in volume (data not given).

The spread test results indicated that D-EAA, like the stereoisomers of L-AA, did not oxidize dough. Reductic acid was the only analogue of L-AA that has been found to have an oxidative effect on wheat doughs (Lillard et al., 1982; Kieffer et al., 1990). The HPLC-EC assay of dough containing added D-EAA (20 ppm) showed a 96% recovery of D-EAA after 3 hr fermentation. Compressed yeast (2% based on flour) did not release detectable EAA in dough during 0-3 hr fermentation.

Effect of EAA on wheat gluten strength

The gluten washed out of an optimally mixed dough containing 82 ppm D-EAA had shorter stretching time (80 min)

—Continued on page 862

Water Absorption in Chickpea (*C. arietinum*) and Field Pea (*P. sativum*) Cultivars using the Peleg Model

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ABSTRACT

Moisture absorption in seven chickpea cultivars and three field pea cultivars was investigated at 5, 15, 25 and/or 42°C using the Peleg model ($M(t) = Mo + t/[K_1 + K_2t]$). The Peleg constant K_1 varied with temperature. At a given temperature, the lower the K_1, the more water was absorbed. The Peleg constant K_2 was almost unaffected by temperature and could be used to predict the equilibrium water absorption. A constant K_3 expressing the temperature effect on water absorption ($K_1 = K_3T + K_4$) was developed to distinguish two types of chickpea — Desi and Kabuli. All chickpeas had similar composition and initial moisture. The difference in water absorption rate was probably due to thickness and structure of the seed coat. The Peleg model could be used to predict water absorption in chickpea and field pea.

Key Words: chickpea, water absorption, Peleg equation.

INTRODUCTION

GRAIN LEGUMES are important sources of protein which are potential ingredients for many processed foods such as meat products, dairy blends and infant formulae. Regardless of end use, soaking grain legumes in water is common either to facilitate cooking or to enhance protein extraction. Since soaking is a long process and conditions affect cooking, nutritional qualities and physical properties of end products, the soaking process needs to be characterised for practical applications (Sefa-Dedeh et al., 1978; Kon, 1979). Water absorption in this soaking process needs to be predictable as a function of time and temperature.

Water absorption of soybean, pigeon pea, cowpea and rice has been investigated and several rather complex models, mainly based on Fick's law of diffusion, have been suggested to describe it (Hsu, 1983; Singh and Kulshrestha, 1987; Sefa-Dedeh and Stanley, 1979; Hendrickx et al., 1987). Peleg (1988) proposed a simple, empirical equation not derived from any set of physical laws or diffusion theories, to model water absorption of food materials. The model has been used to predict long range moisture absorption from experimental data obtained over a short time. The general application of the model has been confirmed by Sopade and Obekpa (1990) with soybean, cowpea and peanut. They reported that the model could adequately describe absorption behavior of these grain legumes at 2°C to 42°C.

Our objective was to evaluate the general applicability of the Peleg model and investigate the effect of grain composition and type on water absorption in several chickpea and field pea cultivars.

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MATERIALS & METHODS

SEVEN CHICKPEA (*Cicer arietinum*) and three field pea (*Pisum sativum*) cultivars were studied for water absorption characteristics. All chickpeas, consisting of three Desi (small grain) cultivars (Tyson, Dooen and Amethyst), three Kabuli (large grain) cultivars (Kaniva, Macarcena and Garnet) and a cultivar intermediate between Desi and Kabuli (Semsen) were grown in Kaniva, Victoria, Australia. Three cultivars of field pea (Dun, Dundale and Progretta) were grown in Dooen, Victoria.

Official AOAC methods (1985) were used for fat, moisture and ash determinations. Protein (Nitrogen x 6.25) was measured by a Leco Nitrogen Analyzer (Model FP228) and carbohydrates were calculated by difference. The size of each seed variety was determined by the number of seeds counted from 50g of dried seed sample.

For water absorption, seeds (10g) of each cultivar were randomly chosen and placed in an incubator for 30 min at 5, 15, 25 and 42°C before soaking in distilled water (75 mL) preadjusted to soaking temperature. Preliminary experiments showed that some cultivars of chickpea disintegrated after prolonged soaking at 42°C. Those cultivars were then soaked at 25°C only. Also, field peas did not absorb notable amounts of water at 5°C so were studied at 15, 25 and 42°C. Weight gains were measured after 2, 6, 12, 18 and 24 hr soaking by weighing soaked beans after centrifuging at 1,500 *g* for 10 min. Initial moisture was determined by heating 5g of each freshly ground flour in a forced circulation drying oven at 105°C to constant weight (2 hr). All measurements were performed in duplicate. The predicted water absorption was calculated using the following equation proposed by Peleg:

$$M(t) = Mo + t/[K_1 + K_2t] \quad [1]$$

where M(t) is the moisture content at time t, Mo is the initial moisture content, K_1, K_2 are constants, and

Table 1 – Chemical composition of chickpeas and field peas

Grain	Cultivar	Moisture %	Ash %	Fat %	Protein %	Carbohydrate[a] %	Relative size[b]
Chickpea Desi	Tyson	8.60	2.52	3.27	23.1	62.5	377
	Dooen	8.38	2.62	2.98	23.1	62.9	284
	Amethyst	8.34	2.59	3.29	23.3	62.5	391
Intermediate	Semsen	8.03	2.64	3.07	23.8	62.4	231
Kabuli	Kaniva	8.15	2.86	5.05	23.7	60.2	139
	Macareena	8.37	2.91	4.56	23.2	61.0	112
	Garnet	8.39	2.76	4.54	23.4	61.0	137
Field pea	Dun	8.85	2.71	0.98	24.1	63.4	215
	Dundale	8.73	2.63	0.88	23.9	64.0	233
	Progretta	8.52	2.55	0.97	24.0	63.0	186

[a] Carbohydrate calculated by difference.
[b] Number of seeds in 50g.

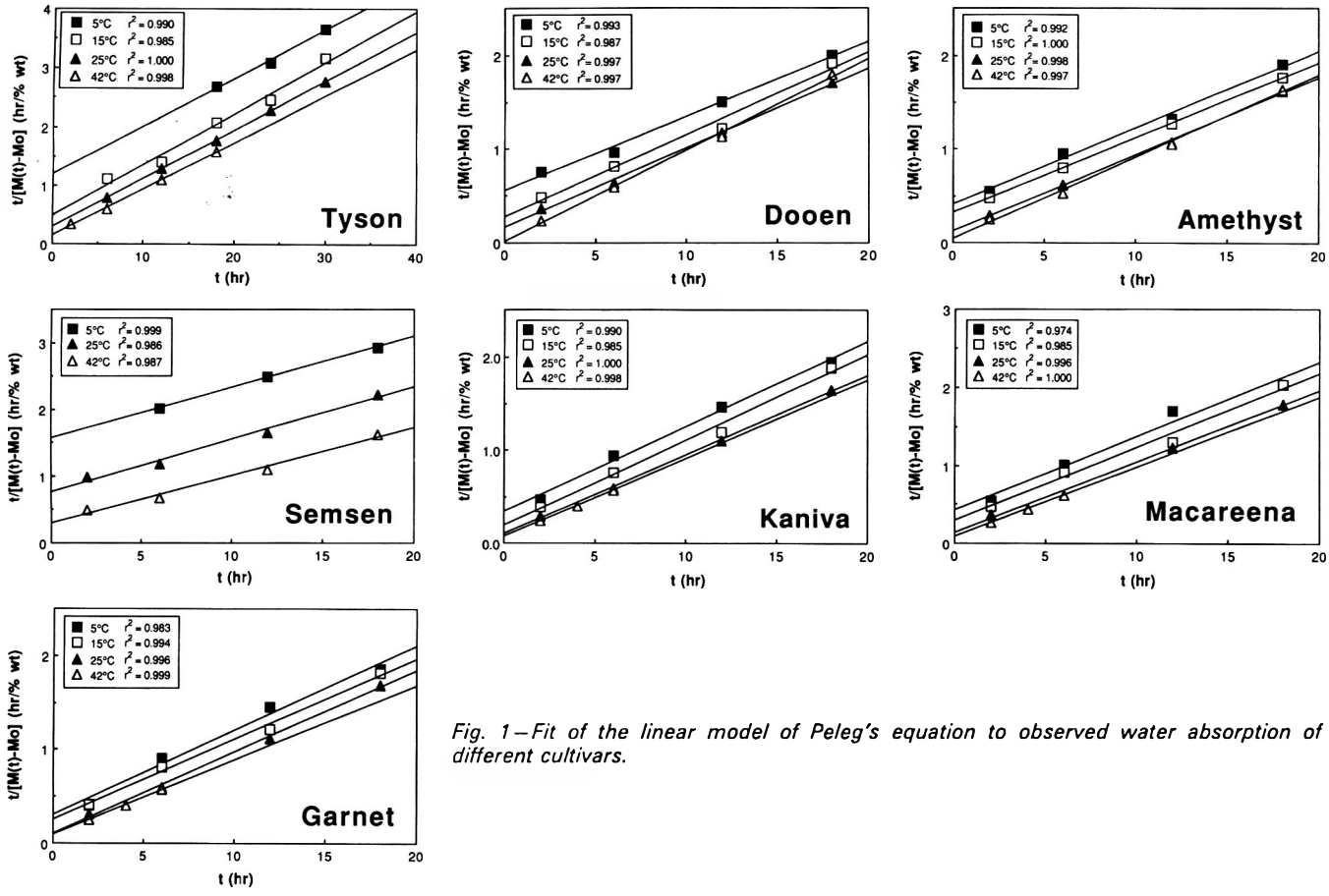

Fig. 1—Fit of the linear model of Peleg's equation to observed water absorption of different cultivars.

$$Meq = Mo + 1/K_2 \quad [2]$$

where Meq is the equilibrium moisture content as t ∞. Equation [1] can also be transformed to the linear relationship:

$$t/[M(t) - Mo] = K_1 + K_2 t \quad [3]$$

where $1/K_1$ is the initial rate of absorption. The unit of K_1 is hr/% weight and that of K_2 is the reciprocal of % weight.

RESULTS & DISCUSSION

GROSS chemical compositions of the seven chickpea and three field pea cultivars (Table 1) showed no differences ($P < 0.05$) in moisture, fat, protein, and carbohydrate among the cultivars. The fit of equation [3] to experimental data on water absorption of the seven chickpea cultivars was demonstrated (Fig. 1). Values of constants K_1 and K_2 derived from the linear fit (Table 2 and 3) showed regression coefficient ranges from 0.974 to 1.000 (Table 2) and degree of fit was generally good. The plots of t/ [M(t) - Mo] vs. t also showed no apparent curvature. Similar results were found for water absorption of field peas (Table 2 and 3). The capacity of the Peleg model [1] to predict the entire water absorption process, using limited data, is demonstrated in the characteristic moisture absorption curves for chickpea (Macareena and Dooen, Fig. 2) and field pea (Progretta and Dundale, Fig. 2) at three different temperatures. The moisture absorption curves show that water content increased with increased soaking time and temperature. Similar results have been reported for soybean, peanut, pigeon pea, rice and cowpea (Singh and Kulshrestha, 1987; Engels et al., 1987; Hendrickx et al., 1987; Sopade and Obekpa, 1990).

Data for the Tyson cultivar of chickpea at 5°C were not collected at soaking times < 12 hr since weight gains at shorter times were small and Peleg indicated that the model did not apply under such conditions (Fig. 1, Tyson). Generally, for the Desi type, the equation could give a fairly good fit after a

Table 2—Peleg constants (K_1, K_2) and initial absorption rate ($1/K_1$) of chickpeas and field peas

Grain	Cultivar	°C	K_1 (hr/% wt)	$1/K_1$ (% wt/hr)	K_2 (hr/% wt °C)
Chickpea Desi	Tyson	42	0.15	6.67	
		25	0.30	3.38	
		15	0.49	2.06	1.2×10^{-2}
		5	1.19	0.84	
	Dooen	42	0.01	72.3	
		25	0.16	6.12	
		15	0.28	3.62	1.3×10^{-2}
		5	0.55	1.82	
	Amethyst	42	0.04	27.6	
		25	0.12	8.26	
		15	0.32	3.13	1.1×10^{-2}
		5	0.41	2.45	
Intermediate	Semsen	42	0.20	3.39	
		25	0.76	1.31	3.4×10^{-2}
		5	1.56	0.64	
Kabuli	Kaniva	42	0.07	14.9	
		25	0.10	9.91	
		15	0.19	5.15	0.7×10^{-2}
		5	0.34	2.94	
	Garnet	42	0.09	11.0	
		25	0.10	10.2	
		15	0.25	4.02	0.6×10^{-2}
		5	0.30	3.36	
	Macareena	42	0.08	12.6	
		25	0.13	7.66	
		15	0.29	3.42	0.8×10^{-2}
		5	0.38	2.66	
Field pea	Dun	42	0.38	2.60	
		25	0.66	1.50	4.4×10^{-2}
		15	1.74	0.57	
	Dundale	42	0.37	2.67	
		25	0.68	1.48	3.3×10^{-2}
		15	1.33	0.75	
	Progretta	42	0.10	9.94	
		25	0.22	4.50	2.4×10^{-2}
		15	0.44	2.29	

Table 3—Peleg constant K_2 and calculated equilibrium moisture content (Meq) of chickpeas and field peas

Grain	Cultivar	°C	K_2 (% wt^{-1})	Mean K_2 (% wt^{-1})	Meq (% wt)	Mean Meq (% wt)
Chickpea Desi	Tyson	42	7.9×10^{-2}		13.7	
		25	8.2×10^{-2}		13.0	
		15	8.6×10^{-2}	8.2×10^{-2}	12.5	13.1
		5	8.1×10^{-2}		13.2	
	Dooen	42	9.7×10^{-2}		11.2	
		25	8.5×10^{-2}		12.7	
		15	8.8×10^{-2}	8.7×10^{-2}	12.3	12.9
		5	8.0×10^{-2}		13.4	
	Amethyst	42	8.8×10^{-2}		12.3	
		25	8.2×10^{-2}		12.3	
		15	8.0×10^{-2}	8.3×10^{-2}	13.3	12.9
		5	8.2×10^{-2}		13.0	
Intermediate	Semsen	42	7.1×10^{-2}		15.0	
		25	6.8×10^{-2}	7.5×10^{-2}	13.6	14.2
		5	7.6×10^{-2}		14.0	
Kabuli	Kaniva	42	8.4×10^{-2}		12.7	
		25	8.5×10^{-2}		12.6	
		15	9.1×10^{-2}	8.8×10^{-2}	11.8	12.2
		5	9.1×10^{-2}		11.8	
	Macareena	42	9.0×10^{-2}		12.0	
		25	9.1×10^{-2}		11.8	
		15	9.4×10^{-2}	9.2×10^{-2}	11.5	11.7
		5	9.5×10^{-2}		10.6	
	Garnet	42	7.9×10^{-2}		13.5	
		25	8.7×10^{-2}		12.3	
		15	8.6×10^{-2}	8.6×10^{-2}	12.5	12.5
		5	9.0×10^{-2}		11.9	
Field pea	Dun	42	6.9×10^{-2}		15.3	
		25	8.5×10^{-2}	7.1×10^{-2}	12.5	15.1
		15	8.8×10^{-2}		18.1	
	Dundale	42	7.3×10^{-2}		14.6	
		25	8.2×10^{-2}	7.6×10^{-2}	13.1	14.0
		15	7.4×10^{-2}		14.4	
	Progretta	42	7.7×10^{-2}		13.9	
		25	7.3×10^{-2}	7.1×10^{-2}	14.6	
		15	7.3×10^{-2}		14.6	14.4

rather long period of soaking (6–12 hr) and for the Kabuli type during the first 6–7 hr of soaking.

Differences in water absorption rate and water uptake among chickpea cultivars are exemplified by Macareena and Dooen (Fig. 2). In field peas, Progretta had a higher water absorption than the Dundale (Fig. 2). Sopade and Obekpa (1990) suggested that the difference in water absorption between soybean and peanut was due to differences in fat and protein content. However, differences in fat, protein and carbohydrate among our chickpeas and field peas were not significant (Table 1). The initial moisture content of the beans would affect the rate of absorption (Smith and Nash, 1961; Chittenden and Hustrulid, 1966). In our study, the difference in initial moisture was also insignificant. Consequently, there is probably a factor other than gross fat, protein and carbohydrate that contributes to differences in water absorption of different chickpea and field pea cultivars.

Seed size should be considered because water absorption rate is influenced by surface area. The smaller the seed, the larger the relative surface area for given seed weight (Ituen et al., 1985). The reciprocal of constant K_1 derived from equation [3] is used to present the initial absorption rate (Table 2). The results clearly indicated the effect of temperature on initial absorption rate which increased with increasing temperature. The large seed cultivars (Kabuli type) had higher absorption rates than the smaller seed cultivars (Desi type). Average $1/K_1$ values of three Kabuli cultivars at 15 and 25°C were 4.19 and 9.25% wt/hr respectively, while corresponding $1/K_1$ values of three Desi cultivars were 2.93 and 5.91% wt/hr respectively (Table 2). This finding could not be compared with other studies which focused on water absorption between different types of grain legumes rather than different cultivars. However, it suggested that seed size or water absorption surface of seed coat alone was not a major factor affecting water absorption, at least among chickpeas.

King and Ashton (1985) studied water absorption by whole soybeans and soybeans with seed coat partially abraded. They reported that the rate of absorption by the abraded seeds was more than that for unabraded seeds. Similar results have been reported by Singh and Kulshrestha (1987). Sopade and Obekpa (1990) reported no major difference in absorbed water between dehulled and whole peanuts. Chickpea seed coat is much thicker than that of peanut seed which contributes only 5% of seed weight while chickpea seed coat contributes up to 16.4% (Singh et al., 1981; Chavan et al., 1986). In an earlier study on water absorption of soybean, Smith and Nash (1961) reported that the principal factor controlling absorption in whole bean was the seed coat. The ratio of coat thickness to seed size and, to some extent, seed coat structure were the only obvious differences between the Desi and Kabuli chickpea types (Jambunathan and Singh, 1981). Polyacrylamide gel electrophoretic analysis did not distinguish two types of chickpea by protein patterns. Gross chemical composition also showed no significant differences. The seed coat in Desi seed contributed 16% of seed weight while in Kabuli seed the average was 7% (Singh and Jambunathan, 1981). The fact that all Kabuli chickpeas had unexpectedly higher water absorption than Desi chickpeas, suggested that thickness of seed coat and also the seed coat structure had critical effects on water uptake and absorption rate (Singh and Kulshrestha, 1987; Gandhi and Bourne, 1991).

A constant K_3 ($K_1 = K_3T + K_4$) was developed to describe the temperature effect on water absorption rate by plotting K_1 against temperature (Fig. 3). A straight line with K_4 as the ordinate intercept and K_3 as the gradient was obtained. This confirmed that the Peleg constant K_1 varied with temperature as reported by Sopade and Obekpa (1990). Note that at a given temperature, the lower K_1, the greater the amount of water absorbed.

The temperature effect on water absorption of Desi type chickpea was greater than that of the Kabuli type. All constants

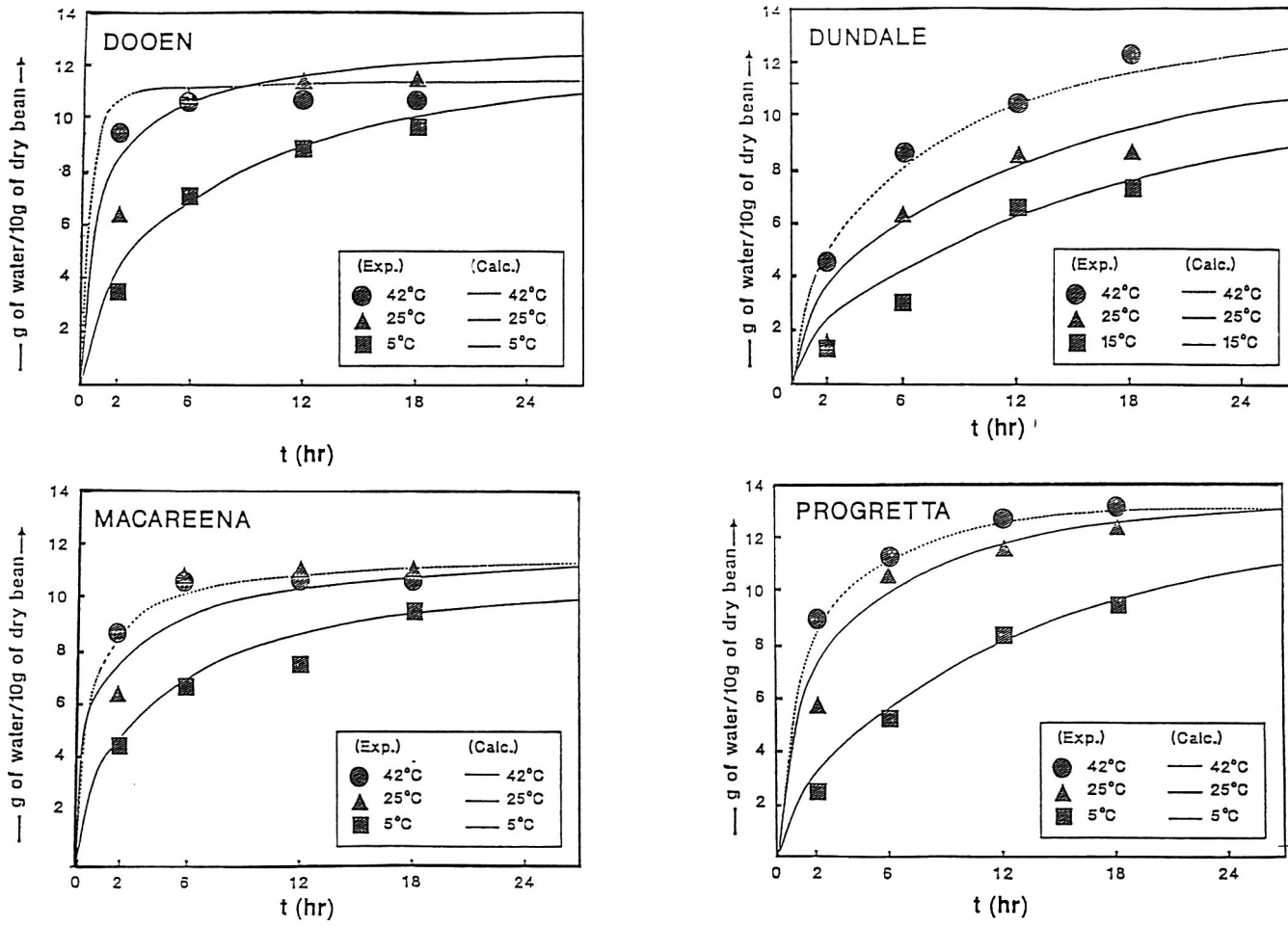

Fig. 2—Experimental and predicted water absorption characteristics of two chickpea cultivars (Dooen and Macareena) and two field pea cultivars (Dundale and Progretta).

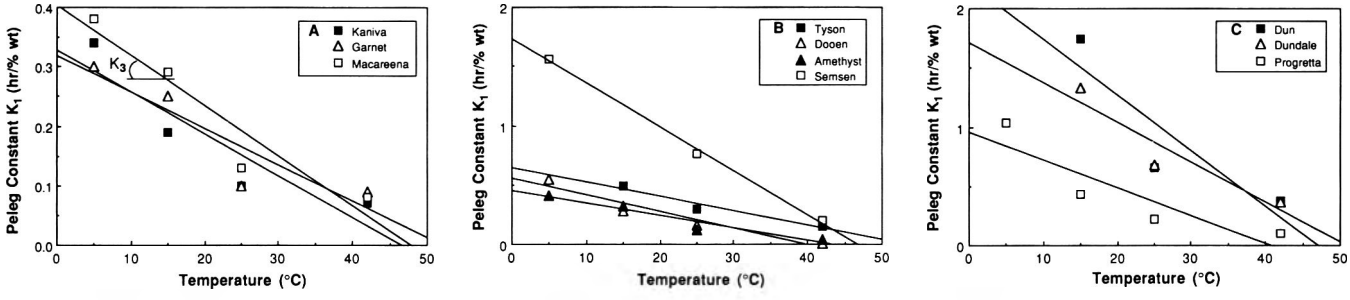

Fig. 3—Effect of temperature on the Peleg constant K_1 of chickpeas and field peas; (A) Kabuli type; (B) Desi type; (C) Fieldpea.

K_3 for the Kabuli type ($\leq 0.8 \times 10^{-2}$) were lower than those of the Desi type ($\geq 1.1 \times 10^{-2}$). Constant K_3 for the field pea ranged from $2.4 - 4.4 \times 10^{-2}$. There was clear correlation between constant K_3 and the seed number of different cultivars (Desi/Kabuli) as well as grain type (chickpea/field pea). Since there was no significant difference in protein pattern or gross chemical composition between Desi and Kabuli chickpea, this constant could serve as a characterizing parameter for grain type based upon water absorption behavior (Fig. 4).

In contrast to K_1 and K_3 constant K_2 was almost unaffected by temperature with mean values ranging from 8.2 to 9.2×10^{-2}% wt^{-1} for chickpea and 7.1 to 7.6×10^{-2}% wt^{-1} for field pea (Table 3). The independence of K_2 with respect to temperature indicated that the same equilibrium moisture content would be obtained regardless of soaking temperature. The equilibrium moisture content of each cultivar was obtained by inserting the K_2 value into equation [2]. The mean equilibrium moisture content of chickpea was 12.4% wt and that of field pea was 14.5% wt. Note that the Semsen cultivar with seed size nearer that of field pea than Desi or Kabuli chickpeas also had both K_2 value (7.5×10^{-2}% wt^{-1}), and equilibrium moisture content (14.2% wt) closer to those of field peas.

The Peleg model could be used to describe the water absorption of chickpea and field pea cultivars. The initial moisture absorption rate ($1/K_1$) and constant K_3 were influenced by soaking temperature. Constant K_2 was almost unaffected by temperature. Using only short term soaking data the Peleg model could provide a useful approach to calculate equilibrium moisture content of chickpea and field pea at any soaking temperature.

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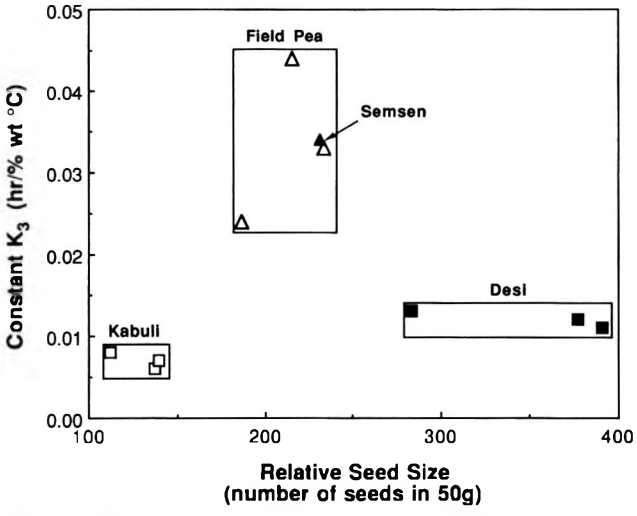

Fig. 4—Correlation between seed size of chickpea or field pea and constant K_3.

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ment to eliminate interfering microorganisms would not be necessary. Green olive fermentation by *Lb. plantarum* yielded lactic acid which almost exclusively provided the low pH needed for preservation. However, the formation of other products (e.g. flavor compounds) may be desirable for improving product quality. The use of a suitable mixed culture rather than a single lactic strain could be the most appropriate approach for accomplishing that.

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Oil Absorption and Sensory Properties of a Snack Food from Chickpea Genotypes

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ABSTRACT

Among the commonly consumed grain legumes the score on general acceptability of a snack food (*seviya*) was the highest for that from chickpea (*Cicer arietinum* L.) followed by lentil, pigeonpea and green gram, whereas the oil absorption by the product was highest for lentil followed by black gram, chickpea, and green gram. Different methods of dehulling did not cause any notable effects on oil absorption of chickpea *seviya*. The flour particle size, starch, and protein contents significantly influenced oil absorption of *seviya*. The oil absorption of the product differed significantly among genotypes, but clear cut differences were not found between desi and kabuli groups of chickpea genotypes.

Key Words: legumes, oil absorption, sensory properties, chickpea

INTRODUCTION

CHICKPEA (bengal gram or garbanzo bean), is an economical source of protein, calories, vitamins, and minerals for millions of people around the world. Chickpea cultivars are broadly divided into two groups, desi and kabuli. Desi seeds, generally yellow to black, are smaller and have a rougher surface than kabuli seeds which are usually large and light colored. Some chemical and nutritional differences between the two types have been documented (Singh et al., 1982, 1991; Singh, 1984). The nutritional quality, biochemistry, and technology of chickpea also have been thoroughly reviewed and summarized (Singh, 1985; Williams and Singh, 1987; Chavan et al., 1988).

Chickpea is the most important pulse crop in India considering production and utilization. It is processed and used in many forms and is popular with all sections of the population because of its taste and flavor. A large proportion of chickpea is processed into dhal, decorticated dry split cotyledons. Dhal is ground to a fine powder ('besan') used for a variety of snack foods and as an ingredient in preparation of special foods. Deep-fried products of chickpea besan include *seviya, muruku, bundia, pakoras, papad,* and *chips* and are quite common with chickpea consumers. These products can be quickly prepared and are generally used in fast food centers, restaurants, and homes. However, information on properties of chickpea consumed in such products is lacking. While frying, these products absorb considerable oil. Because of increasing cost of oil and from a health point of view, the preparation of deep-fried products using minimum oil would be highly desirable. Information is scarce on oil absorption and sensory properties of such deep-fried products of grain legumes and of chickpea in particular. Our objective was to examine the oil absorption and sensory properties of chickpea *seviya* by comparing them with *seviya* from other grain legumes, and to investigate factors that influence oil absorption. We also examined the relationship to desi and kabuli genotypes.

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MATERIALS & METHODS

SEED SAMPLES of newly developed genotypes and commonly grown cultivars of chickpea were supplied by the chickpea breeding unit at ICRISAT. The genotypes were grown at the ICRISAT Center during the postrainy season 1987/88 and 1988/89 in deep black soils without irrigation or fertilizer. After harvest, seed samples of the genotypes were stored in plastic bags at 5°C until used for analysis. Seed samples of other grain legumes were collected from local markets in Hyderabad. One soybean dhal sample (cv P-132) was obtained from G.B. Pant University of Agriculture and Technology, Pantnagar, U.P. All samples were stored under similar conditions. For frying, refined groundnut oil was purchased from the local market.

Dehulling methods

Whole seeds were processed into dhal using a laboratory-scale dehulling procedure employing a tangential abrasive dehulling device (TADD) as described by Singh et al. (1992) (model 4E-230 Venables Machine Works Ltd, Saskatchewan, Canada). In order to compare the effect of dehulling methods on oil absorption, dhal was prepared by removing the seed coat manually from whole seeds which had been soaked overnight at room temperature (25 ± 1°C). Whole seed samples were also decorticated to prepare dhal using a Prairie Regional Laboratory (PRL) mill, available at a community center near Patancheru, Andhra Pradesh, India. For this purpose, no water treatment was used, however, seeds were dried in an oven at 50°C before dehulling in the PRL mill. When soaking was a pretreatment, samples were dried in an oven at 50°C before dehulling. All dhal samples were ground to a very fine powder (80 mesh) using a Buhler mill (Buhler Miag, GmbH, Braunschweig, Germany).

Preparation of *pakora* and *seviya*

Two deep-fried products (*pakora* and '*seviya*') were prepared to compare the reproducibility of analysis of oil absorption of the products. For preparation of *pakora*, a 20-g flour sample was thoroughly mixed with 10 mL water to make dough. Small pieces of dough weighing 5–7g were dropped in preheated refined groundnut oil and fried at 230°C for 2 min using an electric heating coil. For preparation of *seviya*, similarly prepared dough was passed through a locally designed and fabricated extrusion plate of 3.5 cm diameter with 1.5 mm holes. The seviya was deep-fried at 230°C for 2 min in refined groundnut oil. Seviya (about 10 g) was dropped in preheated refined groundnut oil and fried at 230°C for 2 min. *Pakora* was initially prepared and compared with *seviya* for estimation of error of oil-absorption analysis. For subsequent studies *seviya* was used because the error of estimation of oil-absorption was very high for *pakora*.

Determination of oil absorption

Before determining oil absorption, excess oil was uniformly removed by retaining the product on the seive for several minutes, and this was followed by draining on absorbent paper. The sieve with 1 to 1.5 mm holes is traditionally used for removing oil. After removing excess oil, the product was broken into smaller pieces and used for oil extraction in a Soxhlet apparatus. The percentage oil absorption during frying was calculated after subtracting the original amount of oil in the flour. To study the effect of starch and protein on oil absorption, enrichment was made with starch and protein extracted from the same genotypes of chickpea. The starch fraction was isolated from dhal flour samples using the standard method (Schoch and Maywald, 1968). The globulin, the major protein fraction of chickpea, was isolated by extracting dhal flour with 0.5 M NaCl solution. The isolated globulin fraction was freeze-dried and used. The starch and protein

Table 1—Oil absorption of seviya of chickpea

Cultivar	Oil absorption (%) Range	Mean	SD[b]	CV[b] (%)
ICCV 1	29.2–32.6	30.9	1.34	4.34
ICCC 37	35.5–39.3	37.1	1.36	3.67
Annigeri	29.9–33.5	31.6	1.25	3.96
K 850	29.9–32.4	31.5	1.33	4.22
ICCV 2	31.5–35.6	33.5	1.36	4.06
ICCV 5	31.9–34.4	33.2	1.00	3.01
L 550	30.[illegible]–32.0	31.0	0.74	2.39
Mean	–	32.9	1.20	3.08

[a] Based on six determinations of each genotype analyzed on different days.
[b] SD = standard deviation; CV = coefficient of variation.

Table 2—General acceptability and oil absorption of seviya *of different legumes*

	Oil absorption[h] (%)	General acceptability score[i]
Chickpea	32.9[c]	3.2[e]
Lentil	38.5[e]	2.6[d]
Pigeonpea	25.1[a]	2.4[c]
Green gram	29.5[b]	2.3[c]
Cowpea	33.0[c]	2.3[c]
Horse gram	40.6[f]	2.1[bc]
Common beans	42.6[g]	1.9[b]
Soybean	42.7[g]	1.8[b]
Peas	37.2[d]	1.2[a]
Black gram	37.2[d]	1.6[b]

[a-g] Means in the same colomn not follwed by the same letter are significantly different from each other by Duncan's Multiple Range Test at the P<0.05 level.
[h] Average of three independent determinations.
[i] Average of 10 panel members using a rating scale of 4 = excellent, 3 = good, 2 = fair, 1 = poor.

fractions were added separately in different concentrations to dhal flour and *seviya* was prepared as described. To study the effect of flour particle size, dhal samples were ground using a Buhler mill and passed through sieves (60, 80, and 100 mesh, ASTM standard U.S.A. brass sieves) to separate flour fractions by particle size.

Sensory properties

Sensory properties of color, texture, flavor, taste, and general acceptability were evaluated by 10 trained panel members. They were selected on the basis of consistent performance in evaluation of the same product on three different days. Four samples and a control (local cultivar) were evaluated per session. The panelists cleansed their palates between samples with drinking water. Evaluation was carried out in individual booths under normal light at room temperature (25 ± 1°C). Freshly prepared samples were served at room temperature and 2 replications of the test products were evaluated by the panelists. The product was fried 2 min in refined groundnut oil at 230°C and freshly prepared samples were served for sensory evaluation. The following rating scale was used: 1 = poor, 2 = fair, 3 = good, and 4 = excellent.

Statistical analysis

For all determinations, 3 to 10 replicates were used. Data were analyzed using the Duncan's (1955) Multiple Range Test (SAS Institute, Inc., 1985).

RESULTS & DISCUSSION

DETERMINATION of oil absorption of any deep-fried product is generally cumbersome, time-consuming and might involve considerable error depending on conditions. Experiments on oil absorption of *pakora* showed that the standard error and coefficient of variation of analyses of oil absorption were beyond acceptable limits of analysis. For example, when analyzed on different days, oil absorption of *pakora* (genotype ICCV 2) ranged from 17.0 to 31.2%. This indicated that reproducibility would be very poor. On the other hand, oil absorption of *seviya* ranged between 31.5 and 35.6% on different dates (Table 1). Further, the coefficient of variation of analysis of different genotypes ranged between 2.4 and 4.3% for *seviya*

Table 3—Effect of methods of dehulling and pretreatments (soaking) on oil adsorption (%) of seviya *of chickpea*

Genotype	PRL-mill[c] (Without soaking)	TADD-mill[d] (4 hr soaking)	Manual method[d] (16 hr soaking
Desi			
ICCV 1	30.9[b]	29.4[a]	28.9[a]
ICCC 37	37.1[b]	34.7[a]	36.3[b]
Annigeri	31.6[b]	29.6[a]	32.1[b]
K 850	31.5[a]	30.7[a]	32.9[b]
Kabuli			
ICCV 2	33.5[b]	33.5[b]	32.8[a]
ICCCV 5	33.2[a]	33.2[a]	32.7[a]
L 550	31.0[a]	31.3[a]	33.8[b]

[a-b] Means in the same row not followed by the same letter are significantly different from each other by Duncan's Multiple Range Test at the P < 0.05 level.
[c] Mean of six independent determinations on dhal prepared by PRL mill.
[d] Mean of three independent determinations on dhal prepared by TADD mill (4 hr soaking) and manually prepared using forceps (16 hr soaking).

Table 4—Effect of flour particle size on oil absorption of seviya *of chickpea genotypes[d]*

Flour particle size (μm)	Oil absorption (%) Desi ICCV 1	Desi ICCC 37	Kabuli ICCV 2	Kabuli Market sample
250 (60 mesh)	47.1[c]	47.6[c]	46.5[c]	46.7[c]
180 (80 mesh)	44.6[b]	43.4[b]	44.8[b]	42.7[b]
150 (100 mesh)	32.6[a]	38.5[a]	34.6[a]	30.8[a]

[a-c] Means in the same column not followed by the same letter are significantly different from each other by Duncan's Multiple Range Test at the P < 0.05 level.
[d] Mean of three independent determinations, and results are expressed on a moisture-free basis.

oil absorption. Because of the highly acceptable standard deviation and coefficient of variation of analysis for oil absorption of *seviya* (Table 1), we further standardized procedures and continued studies on that product. Results of analyses on *seviya* should be applicable to other deep-fried products of chickpea such as muruku and bundia prepared in a similar way.

Results on *seviya* prepared using dhal flour of different legumes (Table 2) indicated large differences in oil absorption values of different legumes. Interestingly, the score on general acceptability of product was highest for chickpea followed by lentil, pigeonpea, green gram, and cowpea (Table 2). Among the commonly grown and consumed pulses oil, absorption was highest for lentil followed by black gram, chickpea, green gram, and pigeonpea (Table 2). From results on sensory analysis, it appeared that the deep-fried product of chickpea would be most acceptable among different legumes. Deep-fried products of chickpea have been reported to be very popular in India (Geervani, 1991) and Pakistan (Khan, 1991).

When different methods of dehulling and soaking whole seed in water, as a pretreatment for dehulling chickpea were compared (Table 3), mean oil absorption ranged from 31.8 to 32.7% indicating no large effects due to soaking treatments. The dry- and wet-methods of dehulling, the latter involving soaking as pretreatment, are commonly used for pulses including chickpea (Saxena et al., 1988). The results suggested that soaking as a pretreatment for dehulling would not adversely affect oil absorption of *seviya* products of chickpea. Also, results clearly suggested that oil absorption would not be influenced by different methods of dehulling chickpea such as PRL-mill (large-scale dehulling) and TADD-mill (laboratory-scale dehulling).

Chickpea dhal is converted into besan depending on grinding facility. Differences in degree of grinding and varietal differences would produce besan of different particle size. The particle size of several besan samples collected from local markets in Hyderabad ranged between 60 and 80 mesh. We observed that oil absorption decreased (P < 0.01) with a decrease in flour particle size (Table 4). Both desi and kabuli genotypes

Table 5—Effect of protein and starch on oil-absorption of seviya *of chickpea genotypes*[a]

Added conc of starch or protein (%)	Oil absorption (%)			
	K 850		Annigeri	
	Protein	Starch	Protein	Starch
0	31.5[d]	30.3[a]	39.0[c]	37.4[a]
10	29.4[c]	31.5[b]	34.7[b]	41.2[b]
20	27.4[b]	38.0[c]	34.3[b]	46.3[c]
30	26.2[b]	51.8[d]	30.9[a]	56.6[d]
40	24.0[a]	—	30.6[a]	—

[a-d] Means in the same column not followed by the same letter are significantly different from each other by Duncan's Multiple Range Test at the $P < 0.05$ level.
[e] Mean of two independent determinations.

Table 6—Oil absorption and sensory quality attributes of seviya *of desi and kabuli genotypes grown in 1987/88 postrainy season*

Genotype	Oil absorption[f] (%)	Sensory quality attributes[g]				
		Color	Texture	Flavor	Taste	General acceptability
Desi						
ICCV 1	30.9[c]	3.6[b]	2.9[b]	3.5[b]	3.5[b]	3.5[c]
ICCC 37	37.1[e]	3.4[a]	3.3[c]	3.6[b]	3.5[b]	3.5[c]
ICCC 42	36.9[e]	3.3[a]	3.2[c]	3.3[a]	3.3[a]	3.3[b]
K 850	31.5[a]	3.5[a]	3.0[b]	3.3[a]	3.4[b]	3.4[b]
Annigeri	31.6[c]	3.4[a]	2.8[a]	3.2[a]	3.4[b]	3.5[c]
Kabuli						
ICCV 2	33.5[d]	3.4[a]	2.8[a]	3.2[a]	3.4[b]	3.4[b]
ICCV 5	33.2[d]	3.4[a]	2.9[b]	3.4[b]	3.6[c]	3.6[c]
ICCV 6	30.3[b]	3.2[a]	2.7[a]	3.3[a]	3.2[a]	3.2[a]
L 550	31.0[c]	3.6[b]	3.0[b]	3.2[a]	3.4[b]	3.4[b]
Market sample	27.8[a]	3.7[b]	3.0[b]	3.4[b]	3.6[c]	3.5[c]

[a-e] Means in the same column not followed by the same letter are significantly different from each other by Duncan's Multiple Range Test at the $P < 0.05$ level.
[f] Based on six indepdendent determinations.
[g] Based on evaluation by nine panel members using a rating scale of 4 = excellent, 3 = good, 2 = fair, and 1 = poor.

exhibited the same trend. This could be related to the starch damage expected to occur as a result of grinding to finer particle sizes. Starch content appears important in oil absorption of the fried product.

Addition of isolated starch fraction to the flour sample of the same genotype (Table 5) increased ($P < 0.01$) the oil absorption of *seviya*. The oil absorption increased from 37.4% in control to 56.6% when 30% starch was added in case of Annigeri and a similar increase was noticed in K 850 (Table 5). Also, the addition of protein fraction to the flour sample of the same genotype decreased ($P < 0.05$) oil absorption of *seviya* in both K 850 and Annigeri. These results suggest that genotypes with low starch and high protein would have decreased oil absorption of deep-fried products. The development of high protein genotypes in grain legumes has often been suggested as a means to alleviate the protein calorie-malnutrition problem existing in some countries. Thus identification and development of high protein genotypes of chickpea would not only be beneficial in terms of nutritive value but would also help reduce oil consumption during preparation of their deep-fried food products.

To study genotypic differences with respect to oil absorption 5 genotypes each of desi and kabuli groups were analyzed. In the 1987/88 postrainy season, the mean oil absorption of desi genotypes was higher ($P < 0.01$) than those of kabuli genotypes, though the magnitude of difference was not very large. Also, there were no clear differences between the two groups of genotypes (Table 6). The desi and kabuli genotypes grown in the 1988/89 postrainy season did not reveal any differences in mean oil absorption of these 2 groups (Table 7). However, note that differences among genotypes were significant ($P < 0.01$) in both seasons (Tables 6 and 7). Results suggest that chickpea genotypes would differ for oil absorbing capacity of extruded product, whereas desi and kabuli genotypes could not be distinguished based on that characteristic. Generally, besan of desi genotypes is preferred for preparation of deep-fried products of chickpea. In comparison of sensory properties of *seviya* from desi and kabuli genotypes, no notable differences (Tables 6 and 7) were found. This suggested that depending on availability, either genotype could be used.

Table 7—Oil absorption and sensory quality attributes of seviya *of desi and kabuli genotypes grown in 1988/89 postrainy season*

Genotype	Oil absorption[f] (%)	Sensory quality attributes[g]				
		Color	Texture	Flavor	Taste	General acceptability
Desi						
Bheema	29.5[a]	3.6[d]	3.3[c]	3.4[d]	3.3[c]	3.3[c]
ICC 4958	31.7[b]	3.4[c]	3.2[b]	3.3[c]	3.3[c]	3.4[b]
ICC 4958	31.7[b]	3.4[c]	3.2[b]	3.3[c]	3.3[c]	3.4[b]
ICCV 88021	28.7[a]	3.2[b]	3.6[a]	3.1[b]	3.3[c]	3.3[c]
Phule G 5	33.8[d]	3.4[c]	2.9[a]	3.1[b]	3.3[c]	3.3[c]
Kabuli						
ICCV 895111	30.8[b]	3.7[d]	3.1[b]	3.2[c]	3.6[e]	3.4[b]
ICCV 89512	32.6[c]	3.3[b]	3.0[a]	3.0[b]	3.4[d]	3.1[a]
ICCV 89154	29.6[a]	3.0[a]	3.4[d]	3.1[b]	3.2[b]	3.2[a]
ICCC 32	29.6[a]	3.2[b]	3.3[c]	2.9[a]	3.0[a]	3.0[a]

[a-e] Means in the same column not followed by the same letter are significantly different from each other by Duncan's Multiple Range Test at the $P < 0.05$ level.
[f] Based on four indepdendent determinations.
[g] Based on evaluation by ten panel members using a rating scale of 4 = excellent, 3 = good, 2 = fair, and 1 = poor.

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Mineral Loss in Cowpeas [*Vigna unguiculata* (L.) Walp] by Pressure Heating in Water

F. RINCÓN, G. ROS, and J. L. COLLINS

ABSTRACT

White Acre cowpeas of four maturity levels were heated in water at 35 kPa for 0–40 min. As the cowpeas increased in maturity, Zn, Cu, Mn, Mg, and K increased, Fe decreased and Ca did not change. All minerals analyzed decreased with heating: Cu, Ca and Mg ($p<0.001$), Zn, Fe and K ($p<0.01$) and Mn ($p<0.05$). Minerals leached from the cowpeas at different magnitudes and rates. For example, K lowered 70–80% within 10 min; others lowered 20% or less. Loss of minerals across maturity stages was not uniform. This method reduced loss of certain minerals when compared to published values for the boiling water method.

Key Words: cowpea, southern pea, legume, minerals, cooking, heating, nutrients

INTRODUCTION

THE COWPEA [*Vigna unguiculata* (L.) Walp] is a legume consumed in many areas of the world (Akinyele et al., 1986; Fery, 1981). Cowpeas are a source of dietary protein, as well as other nutrients, including minerals (Lee, 1985; Rizley and Sistrunk, 1979; Scherz et al., 1986; USDA, 1986). Cooking in water is the common method for cowpeas, but modifications have been tried to reduce loss of soluble nutrients (Lee, 1985; Meiners et al., 1976; Rizley and Sistrunk, 1979; Uzogara et al., 1988). All studies reported significant mineral losses.

Many other legumes have been investigated for mineral loss during preparation. All reported losses of varying but important degrees (Borade et al., 1984; Latunde-Dada, 1991; Lopez and Williams, 1988; Pawar et al., 1986). Certain minerals are bound to non- or less-soluble substances, and their movement out of the bean is restricted (Haytowitz and Matthews, 1983; Lombardi-Boccia et al., 1991; Reddy et al., 1978; Sathe et al., 1984).

Cooking cowpeas in boiling water usually requires relatively long periods of time. Since pressure heating reduces cooking time, we undertook this investigation to determine the retention of selected minerals of four maturity levels of cowpea when pressure heated in a minimum amount of water.

MATERIALS & METHODS

Harvesting and preparation of cowpeas

White Acre cowpea was grown at the University of Tennessee Plant Science Farm, Knoxville, on clay loam soil, using standard cultural practices. Plots were irrigated as needed. Pods were harvested by hand over about 1 mo and separated into two maturity groups by color and dryness: succulent pods and mature dry pods. Succulent pods had differing degrees of green color, and the cowpeas did not rattle in the pods when shaken. Cowpeas were removed from the succulent pods with a roller-type sheller, model 'Little Sheller' (Taylor Mfg. Co., Moultrie, GA). The mature dry (MD) pods were yellow-brown, and the cowpeas rattled in the pods when shaken. The MD cowpeas were shelled by hand. After shelling, the cowpeas were frozen at −95°C in a Liquid Carbon Dioxide Freezing System, model BF300SD (Cryo-Chem Inc., Gardena, CA), packaged in polyethylene bags of 1 kg capacity and held at −17°C. When the harvesting season concluded, all cowpeas of a maturity group were combined, mixed for homogeneity, repacked and returned to −17°C.

The shelled succulent cowpeas were separated into 3 maturity groups by specific gravity. About 38L brine of 1.193 specific gravity (250g NaCl/L deionized water) were prepared in a plastic container 0.4 m high. The cowpeas were added to the brine where separation occurred within 30 min. The least mature cowpeas floated and were removed and classified as immature green (IG), while the most mature cowpeas settled on or near the bottom and were collected and classified as mature succulent (MS). All other cowpeas were suspended between the two previous groups, collected and classified as advanced green (AG). The cowpeas were rinsed in deionized water to remove surface NaCl. The MD cowpeas were rehydrated in deionized water 8 hr.

Pressure heating of cowpeas and preparation of analytical samples

Cowpeas of 200-g lots were heated in 1L deionized water (brought to boil before adding cowpeas) in a pressurized sauce pan (Mirro

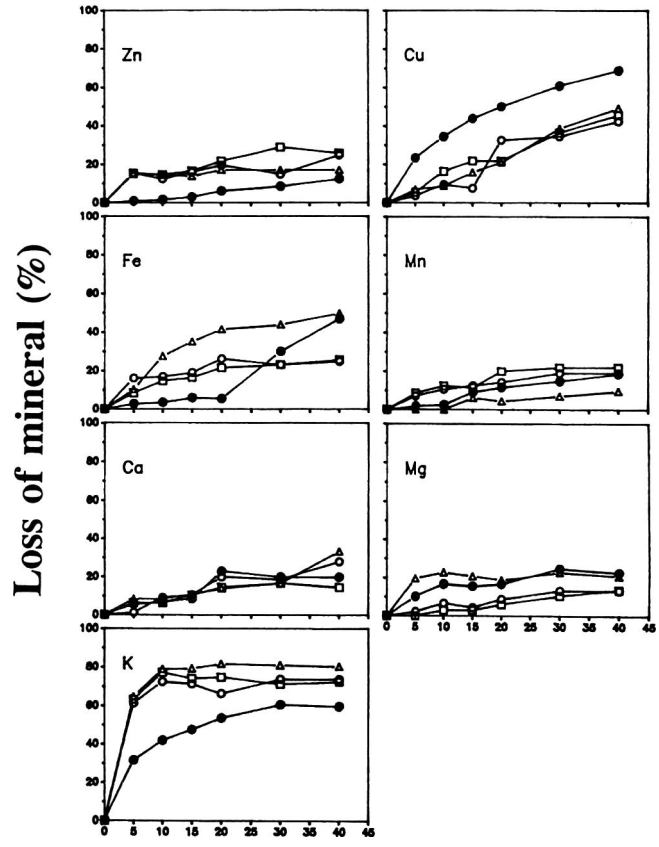

Fig. 1.—Loss of minerals from White Acre cowpeas during pressure heating in water as a function of maturity and heating time. Maturity: Immature green, ○; Advanced green, □; Mature succulent, △; Mature dry, .

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Table 1—Retention of minerals in cowpeas heated in water at 35 kPa up to 40 min

Mineral	Maturity of cowpeas				Significance[a]		
	IG	AG	MS	MD	Maturity	Heating time	Interaction[b]
	mg/100g, DWB[c]						
Zn	1.94 1.46	1.97 1.78	2.82 2.32	2.91 2.55	↑***	↓**	ns
	1.68	1.92	2.37	2.77			
Cu	0.54 0.30	0.55 0.30	0.57 0.29	0.64 0.20	↑***	↓***	ns
	0.43	0.43	0.47	0.39			
Fe	4.84 3.64	3.96 2.94	3.91 1.96	3.99 2.12	↓*	↓**	ns
	3.97	3.34	2.82	3.31			
Mn	0.84 0.68	1.05 0.82	1.14 1.03	1.54 1.51	↑***	↓*	ns
	0.72	0.92	1.08	1.46			
Ca	75.00 41.00	48.00 41.00	36.00 24.00	35.00 28.00	ns	↓***	*
	63.00	43.00	32.00	31.00			
Mg	45.00 39.00	66.00 57.00	101.00 80.00	137.00 106.00	↑***	↓***	ns
	42.00	63.00	84.00	116.00			
K	80.00 21.00	163.00 45.00	511.00 122.00	794.00 320.00	↑***	↓**	*
	36.00	62.00	180.00	460.00			

N = 8.

[a] Statistical differences by analysis of variance: ns = $P>0.05$; *, $P<0.05$; **, $P<0.01$; ***, $P<0.001$. ↑ = increase or ↓ decrease mineral content across maturity or heating time, respectively.

[b] Interaction: maturity × heating time.

[c] Upper data set; first value reports mineral content at 0 min heating and second value, at 40 min heating; lower value is mean of all heating treatments.

Aluminum Co., Manitowac, WI). Pressure was 35 kPa, and heating periods were extended up to 40 min, with 5- or 10-min intervals (see Fig. 1). Timing was begun when pressure was attained. After heating, the cowpeas were removed from the vessel, drained of liquid, spread thin and cooled by holding in room temperature (≈23°C) air.

To prepare analytical samples, cowpeas were dried in a Virtis Freeze Dryer, model FFP-15W5 (Virtis Co., Gardiner, NY) and pulverized in a Micro-Mill (Bell-Arts Products Co., Pequannock, NJ) to pass through a US Standard 30-mesh sieve. The powders were held in sealed, rigid plastic containers over desiccant at −17°C until analyzed.

Moisture and mineral determinations

Moisture was determined by the vacuum oven procedure at 70°C (AOAC, 1990). Selected minerals (see Table 1) were measured on 2-g samples which had been ashed in a muffle furnace (AOAC, 1990). The ash was dissolved in 25 mL of a mixture of water and acid (1:1, v/v), the acid consisting of HCl and nitric (1:1, v/v) (Rincón et al., 1990). The solution was passed through a Whatman GF/C glass microfiber filter into a 25-mL volumetric flask and brought to volume with the aqueous-acid mixture. The acids (suprapur grade) and mineral standards (Tritisol brand) were obtained from Merck Co., Darmstadt, Germany.

Mineral determinations were run on a Perkin-Elmer Atomic Absorption Spectrophotometer, model B-1100 (Perkin-Elmer Ltd., Überlingen, Germany). A 10-cm, single slot burner head and standard air-acetylene flame were used. Detection limit determinations of the American Chemical Society (1980) and Mottola (1984) were observed.

Experimental design and statistical analysis

Data of individual minerals were analyzed by analysis of variance as factorials of completely randomized blocks: 4 × 7 (maturities × heating periods) with two replications. An Olivetti personal computer, model M-240, equipped with SYSTAT (Wilkerson, 1986), was used to conduct the analyses.

RESULTS & DISCUSSION

THE COWPEAS of the IG, AG and MS maturity groups contained 71.9, 60.9 and 56.0% moisture, respectively, following brine separation. Since moisture in cowpeas decreased with advancing maturity, these values indicated three distinct groups. The MD group had 61.5% moisture after soaking 8 hr; however, pod conditions at the time of harvest confirmed that group was most advanced.

Stage of maturity and length of heating period affected the mineral content of the heated cowpeas, with exception of maturity effect on Ca. The maturity × heating interaction affected ($p<0.05$) Ca and K. Data were recorded within a maturity group for a given mineral (Table 1) indicating the amounts remaining in the cowpeas after 0- and 40-min heating and the average amounts which included all heat treatments. The average amounts of mineral increased ($p<0.01$) for Zn, Cu, Mn, Mg and K, decreased ($p<0.05$) for Fe and did not change for Ca as the cowpeas became more mature. Thus, the nutrient density for the specific minerals depended upon stage of maturity. Mechanical harvesting procedures collect cowpeas into 1 of 2 maturity groups: "succulents," consisting of differing amounts of both MS and MD cowpeas or "dries," consisting of low-moisture, mature cowpeas. Within each group, every mineral analyzed decreased during heating: Cu, Ca and Mg ($p<0.001$); Zn, Fe and K ($p<0.01$) and Mn ($p<0.05$).

When the content of individual minerals of raw White Acre cowpeas was compared to reported values (Akinyele, 1989; Lee, 1985; Longe, 1983; Rizley and Sistrunk, 1979; Scherz et al., 1986; USDA, 1986), we found comparable values except for Ca and K. The Ca content of White Acre was about 33% that reported by Lee (1985) and USDA (1986), and K was about 50 and 75%, respectively, of reported amounts. Published data showed wide differences in individual mineral contents of cowpeas. We hypothesized that such differences reflected the effects of several factors such as type of cowpea (White Acre is a creme-type), cultivar, amount and analysis of fertilizer, soil type and cultural practices. We were not aware of research covering these factors as they might influence mineral contents in cowpeas.

The minerals leached from the cowpeas into the liquid at different magnitudes and rates during heating (Fig. 1). For example, K of the succulent cowpeas lost 70–80% at 10 min heating, after which no further change occurred. The MD cowpeas lost less K, but the rate of loss increased to 30 min. Both Cu and Fe content were lowered less than K during heating but were lost more than the remaining minerals. Percentage of Cu and Fe losses amounted to 42–69 and 25–47, respectively, at 40 min with the loss depending upon stage of maturity. The remaining minerals of Zn, Mn, Ca and Mg lost about 25% or less at 40 min, while Ca lost up to 33%. Loss of individual minerals did not follow a uniform pattern across maturity stages. Cowpeas of MD, for example, lost smaller amounts of Zn and K and a greater amount of Cu than the less mature groups. Losses of remaining minerals followed no consistent pattern.

Haytowitz and Matthews (1983) reported that cooking in boiling water caused greater losses of K (24%) and Cu (15%) than those of protein-bound Zn (0%) and Fe (8%). Longe (1983) reported losses of 31% Ca and 22% Mg from mature cowpeas when cooked to desired tenderness. Lee (1985) reported greater

losses of Ca from MS and MD cowpeas heated in steam at 103 kPa for 19 min than from cowpeas of similar maturity groups heated in boiling tap water for 40 min. No explanation was presented, for the greater losses from steamed cowpeas. Lee also reported that the K content did not change in MS or MD cowpeas heated in steam, but decreased (22.5%) in MS cowpeas heated in boiling water.

Ideally, cowpeas should be cooked just long enough to tenderize them for eating. Longe (1983) reported the requirement of 74.5 ± 25.9 min to tenderize 13 cultivars of cowpeas in boiling water. Ros and Collins (1992) reported that 10-15 min heating in deionized water at 35 kPa was adequate for tenderizing White Acre cowpeas. We found (Fig. 1) that all minerals lost important amounts by 15 min. Of the average amounts lost at 40 min, percentages lost at 15 min were: Zn, 62; Cu, 43; Fe, 52; Mn, 57; Ca, 43; Mg, 63 and K, 95. Such losses provide evidence that important amounts of minerals of White Acre cowpea, and most likely other types and cultivars, may be lost when the cooking liquid is discarded. By consuming both cowpeas and the liquid, the total mineral component would be consumed and the effect of leaching of little importance.

We found that heating for 40 min did not cause the cowpeas to become a slurry. The seed coat remained intact, retaining essentially all the contents. After the longer heating periods, the liquid appeared slightly turbid but not cloudy or "soupy" as is often observed with boiled cowpeas after extended periods of time.

CONCLUSION

WHITE ACRE cowpeas of four maturity stages indicated with advancing maturity, Zn, Cu, Mn, Mg and K increased, Fe decreased and Ca did not change. All the minerals leached into the heating water but at different magnitudes and rates. An intact seed coat was retained and should reduce loss of minerals. Also, heating in a minimum of water for short periods should reduce such mineral loss.

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Heat Inactivation of Trypsin Inhibitors in Soymilk at Ultra-High Temperatures

K.C. KWOK, W.H. QIN, and J.C. TSANG

ABSTRACT

Soymilk samples at pH 7.5, 6.5 and 2 were subjected to heat treatment at 93°C and indirect ultra-high temperature. When heated at 93°C, 121°C and 132°C, trypsin inhibitor activity (TIA) in soymilk was more heat-labile at high pH than at lower pH. However, the effect of pH on rate of thermal inactivation was less pronounced when the holding temperature was increased to 143°C and 154°C. The point on a curve relating holding temperature and holding time, indicating inactivation of 90% of the TIA in soymilk at pH 6.5 in the range 93–154°C, coincided with the thermal-death-time curve of the organism putrefactive anaerobe 3679 at about 125°C.

Key Words: soymilk, trypsin, inhibition, enzymes, inactivation

INTRODUCTION

SOYMILK, the water extract of soybeans, was first used in ancient China around 2,000 years ago. Major improvements in soymilk processing technology, flavor improvement and aseptic packaging, have led to several of the largest food companies in the world broadening its markets and avenues of distribution. In soymilk processing, elimination of enzymic off-flavor development and destruction of growth inhibitors in raw soymilk are important concerns. Growth depression, pancreatic hypertrophy, hyperplasia and adenoma in experimental animals have been partly or fully attributed to soy trypsin inhibitors (TI) (Levison et al., 1979; Liener and Kakade, 1980; McGuinness et al., 1980; Rackis and Gumbmann, 1981). Proper heat treatment improves the nutritional value of soymilk by inactivation of TI (Liener, 1972) and by increasing the digestibility of soy proteins (Rackis, 1972). The extent of destruction of TI in soymilk for maximum nutritive value or protein efficiency ratio was reported to be 90% (Hackler et al. 1965). Overheating to completely remove proteolytic inhibitor activity reduces nutritive value of soybeans (Liener, 1972) and results in amino acid degradation and other deteriorative reactions. Therefore, precise control of thermal processes is critical to the preparation of soymilk with maximum nutritive value.

Previous reports on heat inactivation of TI in soymilk were mostly in the temperature range 93–121°C (Hackler et al., 1965; Wallace et al., 1971; Lei et al., 1981). Inactivation of 90% of the native TIA in soymilk could be achieved by heating for 60–70 min at 93°C or 5–10 min at 121°C. Johnson et al. (1980) studied the kinetics of heat inactivation of TI using a high temperature (121–154°C) short time (0–240 sec) process involving direct steam infusion of soymilk. Very limited other data are published on the heat inactivation of TI in soymilk in the ultra-high-temperature (UHT) range (132°C and above).

The pH of the heating medium is a factor in rate of destruction of soybean TI. Heating soymilk under alkaline conditions rendered TI more heat-labile (Badenhop and Hackler, 1970; Lei et al., 1981). Some TI fractions isolated from soybeans by DEAE cellulose chromatography were heat-stable in acid solution (Obara and Watanabe, 1971).

Our objective was to determine the effects of UHT heat treatments (132°C, 143°C and 154°C) at pH 2, 6.5 and 7.5 on the destruction of TIA in soymilk as compared to lower heating temperatures (93°C and 121°C).

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MATERIALS & METHODS

Soymilk preparation and pH

Soybeans of the Amsoy cultivar were soaked in water (bean-to-water 1:10) for 14 hr at 5°C. The soaked beans, along with the soak water were then blended by means of a Hobart vertical micro-cut emulsifier (model MCV-12) with circulation for 5 min. The slurry was discharged into a tank. The slurry was pumped through a Sperry filter press. The filtrate from the filter press was designated as water extract of soybeans, or soymilk. The soymilk prepared in this manner had a total solids content of 6.7%. Prior to heat treatment, the pH of the soymilk was adjusted to 6.5, 7.5, or 2 by adding 7% NaOH or 7% HCl solution.

Heat treatment at 93°C

About 50 mL of raw soymilk, adjusted to the desired pH, was placed in each of eight 25 × 150 mm screw-cap test tubes. One tube of extract served as the unheated or raw sample. The remaining seven tubes were heated in boiling water. At zero time (when temperature of soymilk reached 93°C), and at 10-min intervals up to 60 min, one tube was removed and immediately placed in a crushed ice bath. Raw and the heated soymilks were lyophilized in a laboratory freeze-drier for 48 hr and the dried product was stored in a screw-cap test tube at 5°C until analyzed for TIA.

UHT heat treatment of soymilk

A Mallory heat exchanger was used to heat soymilk. It consisted of a heating section, holding tubes, and a cooling section. In the heating section, the tubing was surrounded by a large pipe containing steam as heating medium. Ambient water was the coolant in the cooling section. Time required for the product to pass through each section was 6 sec. Each holding tube provided a measured holding time of 14 sec. Holding time could be varied by connecting 0, 1, 2, 3, or 4 holding tube(s). Batches of soymilk were processed at 121°C, 132°C, 143°C, and 154°C with holding times of 0, 14, 28, 42, and 56 sec. Heat-treated samples were lyophilized in a laboratory freeze-drier for 48 hr and the dried products were stored in screw-cap test tubes at 5°C until analyzed for TIA.

Assay for TIA

A 0.70-g sample of freeze-dried soymilk solids was dispersed in about 40 mL of distilled water at room temperature (≈ 23°C) and mixed in a small Waring Blendor for 1 min. The slurry was quantitatively transferred to a beaker, adjusted to pH 7.5 with 0.1M NaOH, transferred quantitatively to a 100-mL volumetric flask and made to volume with distilled water. The sample was stored in a refrigerator for ≥ 20 hr to allow complete hydration and extraction of the TI before assaying.

A modification of the Kunitz (1947) procedure developed by Hetrick (1970) using gelatin as a substrate was employed to measure the TIA of the freeze-dried soymilk solids. In that method, the activity of trypsin (extent of hydrolysis) was measured with and without soy extract in the reaction mixture by the change in formal titration of an

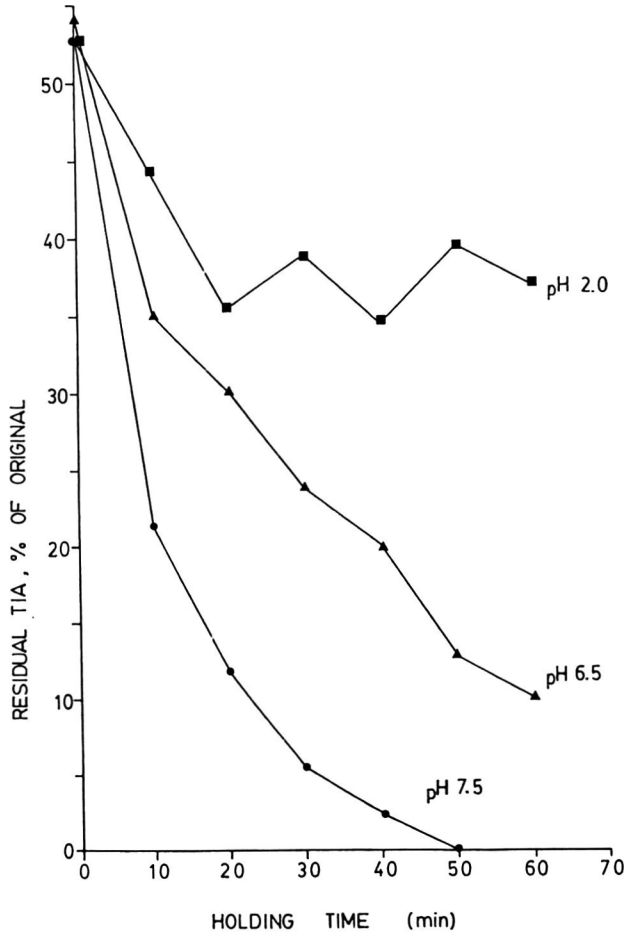

Fig. 1—Effect of pH and holding time at 93°C on TIA of soymilk.

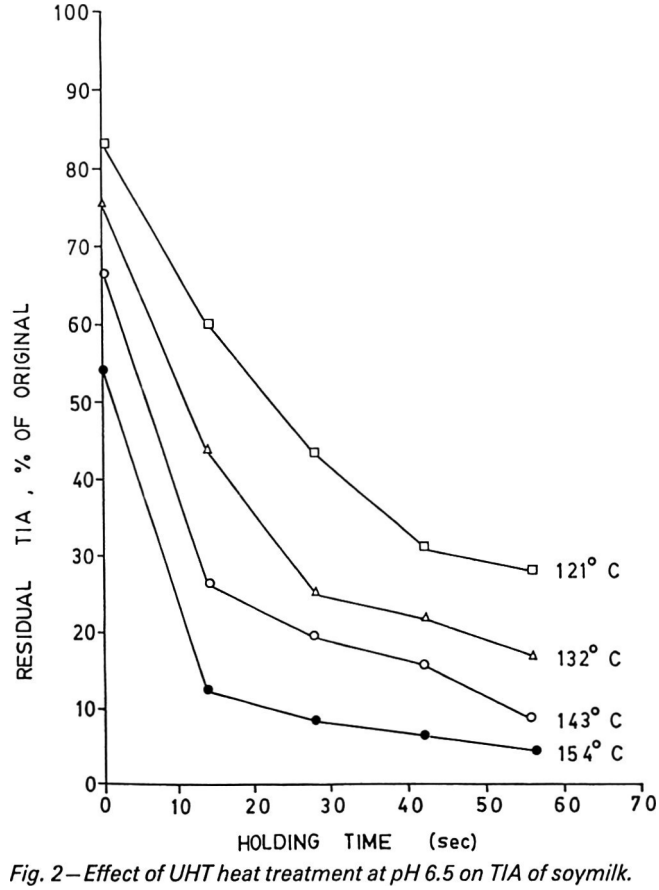

Fig. 2—Effect of UHT heat treatment at pH 6.5 on TIA of soymilk.

aliquot of the reaction mixture before and after 60-min incubation at 40°C. From the change in formal titration, the enzyme concentration for controls and reaction mixtures (with soy extract added) were determined from the standard curve for trypsin activity. The reduction in enzyme concentration represented the inhibition caused by that amount of soy extract. This was expressed as mg trypsin inhibited/mg soymilk solids. The percent residual TIA of the heated soymilk was calculated as TIA of the heated soymilk divided by the TIA of raw soymilk multiplied by 100.

RESULTS & DISCUSSION

Effect of pH and holding time at 93°C

On heating each of the pH-adjusted soymilks to 93°C (0 time samples), about 50% of the TIA (Fig. 1) was destroyed regardless of pH. The selection of pH 6.5 was made because this was the normal pH of water extracts of soybeans. The selection of pH 7.5 represented alkaline conditions that avoided cystine destruction on heating (Badenhop and Hackler, 1970). Use of pH 2 was selected because under these conditions soymilk could be heated without coagulation. On subsequent holding at 93°C, the rates of inactivation increased with increasing pH. The curves for heat inactivation at 93°C at pH 6.5 and 7.5 appeared to be first-order. The holding time at 93°C required to inactivate 90% of the TIA was 60 min for soymilk at pH 6.5 and 25 min for that at pH 7.5. The result at pH 6.5 was in agreement with Van Buren et al. (1964). They reported the required holding time at 93°C to inactivate 90% of the TIA in soymilk was 30–75 min for different lots of soymilk. With soymilk heated at pH 2, some additional TIA was inactivated during the first 20-min holding, but on extended holding the additional destruction of the TIA was very small, if any. About 35% of the original TIA remained after holding 60 min.

Our results could perhaps be explained on the basis of the known heterogeneity of soy TI. Individual soybean TI fractions vary in susceptibility to heat inactivation and rates of inactivation are a function of pH at which they are heated. Most of these fractions were more readily inactivated by heat in alkaline solution. The TI fractions isolated from soybeans by DEAE cellulose column chromatography were the most heat-stable of the soy TI fractions in acid solution (Obara et al., 1970; Obara and Wantanabe, 1971). Results (Fig. 1), which showed 35% of TIA remaining in the pH 2 soymilk after 60 min holding may be attributed to the more acid-stable and heat-stable fractions. Heating the soymilk at pH 7.5 showed the most rapid rate of destruction. The observation that soy TI was more heat-labile under alkaline conditions was consistent with reports of others (Wallace et al., 1971; Obara and Watanabe, 1971; Johnson et al., 1980; Lei et al., 1981). Heating in alkaline solution may cause more rapid destruction of disulfide bonds, which are important in the stability of soy TI (Dibella and Liener 1969). The Bowman-Birk acetone-insoluble inhibitor had a high cystine content (Birk, 1961).

Effect of pH and UHT heat treatment

Studying UHT heat treatment temperatures at pH 6.5 (0–56 sec), TIA in soymilk was mostly inactivated (Fig. 2) to 10% retained at 143°C for 56 sec and 154°C for 23 sec. The results showed that at pH 7.5, the initial rate of destruction of TIA in soymilk was very fast (Fig. 3). However at 132°C and 143°C, when the TIA had decreased to about 15% retained, there was little destruction upon further holding up to 56 sec. This was not expected considering that the TIA in soymilk was easily destroyed at 93°C at pH 7.5. At 154°C, however, TIA was satisfactorily inactivated in about 24 sec.

Previous results showed that some soy TI fractions were very heat-resistant at pH 2 at 93°C. Prolonged heating up to 60 min at that temperature did not destroy more than 65% of the TIA. From Fig. 2 and 4, it can be seen that the rate of

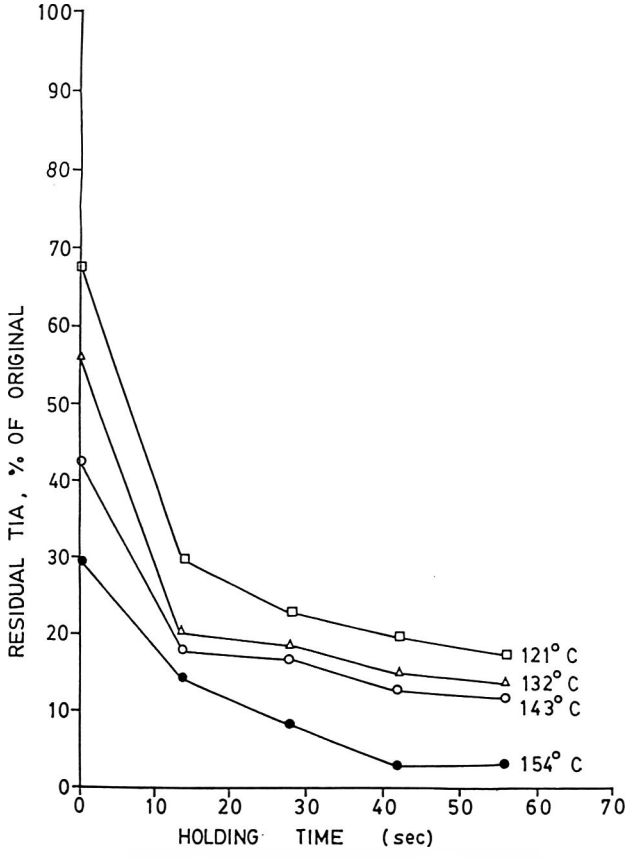

Fig. 3—Effect of UHT heat treatment at pH 7.5 on TIA of soymilk.

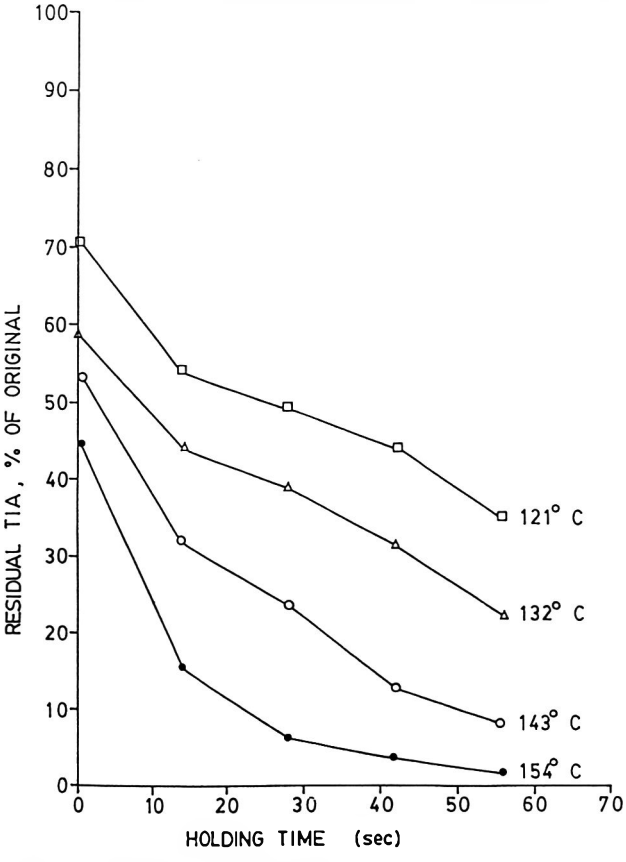

Fig. 4—Effect of UHT heat treatment at pH 2 on TIA of soymilk.

destruction was slower at pH 2 than at pH 6.5 when heated at 121°C and 132°C. When the holding temperature was increased to 143° and 154°C, however, pH had little effect on the rate of destruction. Therefore, we concluded that pH had more effect on rate of thermal inactivation of TIA in soymilk at low than at high temperatures. The 93°C heat treatment may have been too mild to cause destruction of the acid-stable TI fraction. At higher temperatures, more complete destruction of this TI fraction at pH 2 was accomplished.

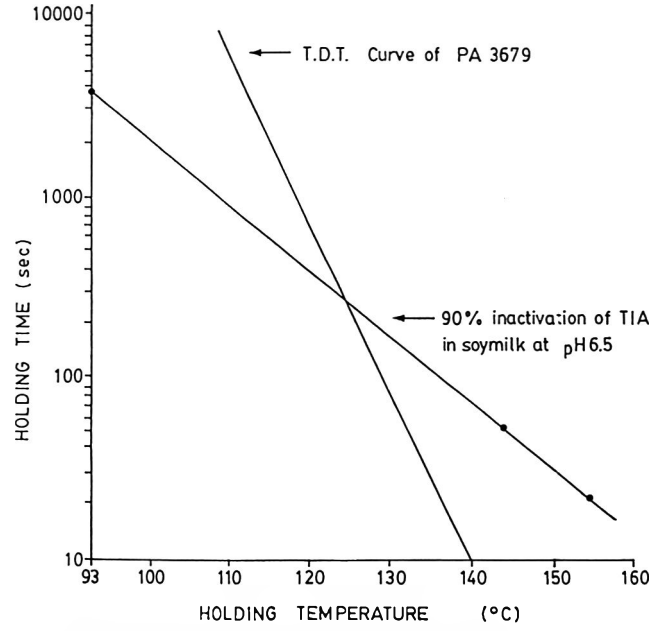

Fig. 5—Relationship between holding time and holding temperature for 90% inactivation of TIA in soymilk at pH 6.5. (Thermal-death-time curve of PA 3679 is included).

In order to determine the reaction order kinetics of UHT heat inactivation of TI in soymilk at different pH, log [TIA] was plotted vs the holding time (graphs not shown). At pH 6.5 and 7.5, the plots were curvilinear, indicating UHT inactivation of TIA in soymilk did not follow simple first-order kinetics. This was consistent with the report of Johnson et al. (1980) who modelled the kinetics of heat inactivation of TIA in soymilk with two separate linear segments separated by a curvilinear transitional period. The initial linear phase was attributed to the more heat-labile Kunitz inhibitor. The slower reaction represented by the final linear phase was attributed to the Bowman-Birk inhibitor.

Straight lines were obtained when Fig. 4 was converted to a semilog plot (graphs not shown). The linear relationship may suggest that all soymilk TI fractions respond in the same manner to UHT heat inactivation at pH 2. The overall kinetics of thermal inactivation at that pH appear to be first order.

Relationship between holding temperature and time necessary to inactivate 90% of TIA

The holding times required to inactivate 90% of the TIA in soymilk at pH 6.5 were 60 min, 56 sec, and 23 sec when heated at 93°C, 143°C, and 154°C, respectively. Combining these data, the relationship between holding temperature to inactivate TIA in soymilk to the 10% level appeared to be a log function of holding time in the range 93–154°C at pH 6.5 (Fig. 5). From the slope of the line, that by raising the temperature about 28°C (the Z value) the holding time required for satisfactory inactivation could be reduced tenfold. Figure 5 also indicates that at 121°C the holding time required was about 6 min. This was in confirmation of the work of Van Buren et al. (1964). The thermal-death-time curve of the organism putrefactive anaerobe (PA) 3679 used by Lo et al. (1968) in their study on heat sterilization of bottled soymilk was compared (Fig. 5). Note that, at temperatures below 125°C,

it required a longer time to kill PA 3679 than to inactivate TIA, but above 125°C, the situation was reversed. At about 125°C, inactivation of the TIA and sterilization of the soymilk should be accomplished with the same holding time. The results clearly show that a UHT process designed to produce a sterile product is inadequate to give complete destruction of TIA in soymilk. Unfortunately, manufacturers of UHT soymilk may have neglected this as some commercial UHT soymilk beverages have contained high residual TIA (Kwok and Qin, 1991). There is conflict between an ideal UHT process which would produce a commercially sterile soymilk with minimum nutrient degradation and a process which would satisfactorily inactivate TIA. Thus, optimization of the thermal process requires more kinetic data on nutrient degradation in UHT processes. Prolonged heating at high temperatures may destroy lysine, sulfur amino acids, and vitamins. Thus, it is advisable to heat-inactivate TIA in soymilk at a lower temperature (100°C) prior to the UHT process.

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 From page 847

than control gluten (132 min) (Fig. 5). This indicated that D-EAA increased gluten extensibility. Adding L-cysteine (100 ppm) was more effective in increasing gluten extensibility and shortened stretching time to 42 min. However, an equivalent amount of L-AA only slightly affected stretching behavior of gluten when compared to control gluten. As in the spread ratio test, these results showed that D-EAA had no discernible oxidizing effect on wheat gluten.

CONCLUSIONS

BAKERS' YEAST released at least 10 times more EAA than AA when the fully hydrated cells were freeze-dried and then extracted with 3% metaphosphoric acid at 25°C. Yeast did not exert its oxidative effect on wheat dough by releasing EAA during fermentation.

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Mention of a propriety product does not constitute an endorsement by the U.S. Department of Agriculture.

Lactobacillus salivarius for Conversion of Soy Molasses into Lactic Acid

JOSÉ-LUIS MONTELONGO, BRUCE M. CHASSY, and JEFFREY D. McCORD

ABSTRACT

The feasibility of conversion of soy molasses, a low value by-product of soy protein production, to lactic acid by fermentation with *Lactobacillus salivarius* was investigated. The basic environmental parameters affecting growth and lactic acid production were determined. Lactic acid production in soy molasses was optimal at pH 5.6 and 42°C. Addition of 0.5% yeast extract to soy molasses reduced fermentation time from 36 to 10 hr and increased lactic acid production by 30%.

Key Words: soy, molasses, lactic acid, lactobacillus, fermentation

INTRODUCTION

SOYBEANS contain about 30% carbohydrate, dry weight basis. Soy protein and oil are important commercial food ingredients but the carbohydrate portion is not well utilized and often presents a disposal problem. The principal use of the carbohydrate component has been in animal feeds where it contributes calories to the diet. Such feeds are used primarily for ruminants, since they can better utilize polysaccharides and α-galactosides than monogastric animals (Smith and Circle, 1978).

The immature bean contains readily digestible simple sugars such as glucose and galactose. However, the mature bean contains more complex soluble carbohydrates: 5% sucrose, 1% raffinose, and 4% stachyose (Snyder, 1987). The latter two are α-galactosides. Though sucrose can easily be digested through action of intestinal invertase, the α-galactosides are not hydrolyzed. Metabolism of such sugars requires the enzyme α-galactosidase. Due to absence of this enzyme in the human duodenal and small intestinal mucosa (Cristofaro et al., 1974), intact α-galactosides enter the lower intestinal tract. There, microflora ferment these sugars producing carbon dioxide, hydrogen and methane (Rackis, 1981) which results in flatulence and other undesirable side-effects in the host. Microflora capable of utilizing the α-galactosides were identified as clostridia (Steggerda 1968). Richards et al. (1968) demonstrated that the major bacterium responsible for fermentation of α-galactosides was *Clostridium perfringes*.

The potential undesirable effects of soybean-derived α-galactosides can be minimized through production of high quality soy protein concentrates, prepared by aqueous and ethanolic extraction which removes α-galactosides. The resulting by-product of the extraction and concentration process is a heavy brown syrup, soy molasses. This by-product contains soybean oligosaccharides; sucrose, raffinose and stachyose plus variable amounts of nitrogenous compounds and ash. This material has low economic value and is used as cattle feed additive (Wolf and Cowan, 1977).

An alternative use to increase the economic value of soy molasses may be to convert such carbohydrate portions of soy molasses to lactic acid. This organic acid, first reported by Schelle in 1790, derives its name from its presence in sour milk (Schopmeyer, 1954). Lactic acid is wide spread in nature (Vickroy, 1985), and has many applications in food and chemical industries. An interesting characteristic of lactic acid is the unusual property of spontaneous polymerization in aqueous solution (Lockwood et al., 1965). The polyester of lactic acid can be converted into biodegradable plastics (Lipinsky and Sinclair, 1986). This has encouraged much research for substrates and alternative strains to produce lactic acid at reduced costs and increased purity. About half the world production of lactic acid is produced from fermentation of carbohydrates using lactic acid bacteria (Vickroy, 1985). The carbohydrate content of the substrate determines the type organism used in the fermentation process. *Lactobacillus delbrueckii* ssp. *bulgaricus*, *L. casei*, or *Lactococcus lactis* (*Lc. lactis* was formerly called *Streptococcus lactis*) are used to ferment lactose, while *L. delbrueckii* ssp. *delbrueckii* and *L. leichmaneii* are used to ferment glucose (Gasser, 1970). *L. amylophilus* and *L. amylovorus* produce lactic acid directly from starch (Nakamura and Crowell, 1979; Nakamura, 1981). *L. delbrueckii* ssp. *bulgaricus* has also been used for production of lactic acid from whey permeate in high cell density bioreactors (Tejayadi, 1990).

L. salivarius has been reported to produce an α-galactosidase in the soluble fraction of the cell, which hydrolyzes melibiose, raffinose and stachyose (Mital et al., 1973). Nelson (1989), found the organism could rapidly ferment soy milk to produce a yogurt-like product. Our overall objective was to evaluate the suitability of *L. salivarius* to metabolize α-galactosides in soy molasses for production of lactic acid. Effects of pH and nutrient supplementation to the soy molasses on time of fermentation and yield of lactic acid, were determined.

MATERIALS & METHODS

SOY MOLASSES was donated by Central Soya Co. P.O. Box 112 Gibson City, IL 60936. *Lactobacillus salivarius* NRRL B-1950 was obtained from the culture collection (USDA Northern Regional Research Laboratory, Peoria, IL). Stock cultures were grown and maintained on Difco MRS Lactobacillus Media.

Controlled pH Fermentation

A Multigen 1.5 L (New Brunswick Co, New Brunswick, NJ) batch fermenter was used for all fermentations. A Jenco Model 6300 pH controller (Cole Palmer Co., Chicago, IL) was used to monitor pH and activate a peristaltic pump (ISCO Co., Lincoln, NE) to add 2.5N NaOH when pH of the fermentation broth dropped 0.1 pH unit below the set point. All fermentations consisted of a 2% solution of soy molasses (with and without addition of nutrients), were adjusted to the appropriate pH and autoclaved in the fermentation vessel for 15 min at 121°C. The fermentation was inoculated by using 1% by volume of 9 hr culture of *L. salivarius*. The temperature was controlled at 42°C and the culture stirred at 100 rpm to insure complete mixing of added NaOH. Samples were removed aseptically at intervals.

To evaluate the best approach for fermentation of the soy molasses, two groups of fermentations were performed. **Soy molasses medium.** This set of fermentations was done using a 2% soy molasses solution to study the effect of pH. Three pH values were tested: 5.6, 6.0 and 6.4. **Soy molasses plus yeast extract medium.** This set of fermentations was performed to study the effect of addition of nutrients to the fermentation broth at pH 5.6, 6.0 and 6.4. The media was prepared

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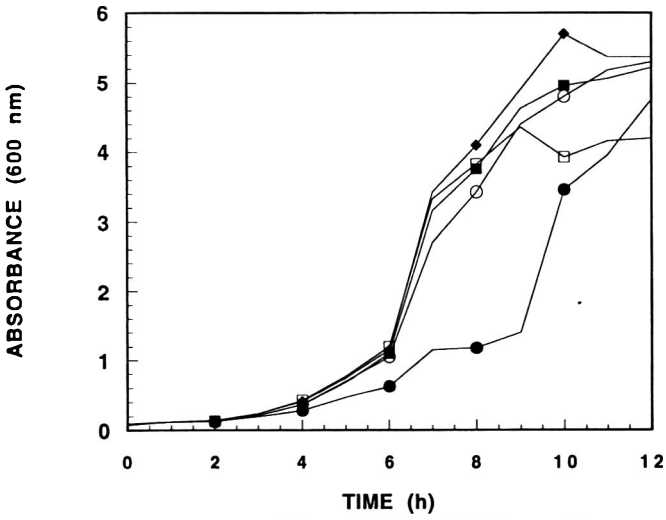

Fig. 1—Growth of L. salivarius *on different individual carbon sources: Galactose (●), Glucose (○), Fructose (□), Sucrose (■), Raffinose (♦).*

by addition of yeast extract to final concentration 0.5% in a 2% soy molasses solution.

Analytical methods

Analysis of carbohydrates. A Dionex HPLC (DIONEX Corporation, Sunnyvale, CA), system employing a PAD (Pulsed Amperometric Detector) equipped with a gold working electrode was used to analyze individual carbohydrates. These were separated on Dionex CarboPac PA-1 column (4 × 250 mm). The isocratic mobile phase was 120 mM NaOH. Flow rate was 1 mL/min. The sample was injected using a 20 μL sample loop Model 9125 Rheodyne Inc. (Cotati, CA). A Spectra-Physics SP 4290 integrator (Spectra-Physics Co., San Jose, CA) was used to record retention times and peak areas. External standards were prepared by dilution of specific amounts of sugars in deionized water. Least squares regression analysis was used to derive an equation from values reported for standard curves for each carbohydrate standard. Experimental results on carbohydrate consumption were calculated using equations derived from standard curves.

Lactic Acid. A YSI (Yellow Springs Instrument, Yellow Springs, OH) Model 27 analyzer equipped with a lactate oxidase membrane was employed for determination of lactate.

Number of Cells. Cells were counted using a Counter Chamber (Hauser Scientific Partnership Horsman, P.A. 19044), under a Nikon Optiphot microscope (Nippon Kogaku, K.K.).

Calculations. Equations were used to calculate consumption of carbohydrates and production of lactic acid with respect to time. These data and cell number were determined in duplicate and reported results are averages.

$$Su\ \% = \frac{[So] - [Sf]}{So} \times 100$$

$$Yield\ g/g = \frac{Po}{Su}$$

Where: [So] = Initial Substrate Concentration; [Sf] = Final Substrate Concentration; Su = % Disappearance of Substrate; Po = Product, measured as lactic acid.

RESULTS & DISCUSSION

A PRELIMINARY study was conducted to determine the ability of *L. salivarius* to metabolize α-galactosides and the more common carbon sources: sucrose, fructose, glucose and galactose. *L. salivarius* grew well in all carbon sources tested (Fig. 1). The conversion of each carbon source to lactic acid was >93%. A proximate analysis of soy molasses determined

Table 1—Fermentation (36 hr) of 2% soy molasses

pH	Carbohydrate Initial (g/L)	Carbohydrate Final (g/L)	Lactic acid produced (g/L)	Carbohydrate utilized (g/g × 100)	Yield Y/S (g/g × 100)
5.6	5.5	0.6	4.20	89%	85%
6.0	7.1	1.5	4.00	78%	72%
6.4	6.2	1.2	4.00	80%	80%

Table 2—Fermentation (10 hr) of 2% soy molasses with addition of yeast extract

pH	Carbohydrate Initial (g/L)	Carbohydrate Final (g/L)	Lactic acid produced (g/L)	Carbohydrate utilized (g/g × 100)	Yield Y/S (g/g × 100)
5.6	6.5	0.09	5.5	98%	86%
6.0	6.8	0.14	5.4	98%	81%
6.4	6.0	0.12	4.95	98%	84%

the general composition. The carbohydrate composition was ca. 32% dry weight bases (d.b.). Soy molasses contained 19% sucrose, 11% stachyose and 2% raffinose (d.b.) by chromatographic analysis. Composition of amino protein was 5% (d.b.) and ash content was 2.5% (d.b.). These results indicated that soy molasses might have sufficient nutrient content for use as a complete fermentation media. In a preliminary fermentation experiment, excellent growth was observed in nondeproteinated soy molasses without the need for additional growth supplements such as yeast extract. However, monitoring growth proved extremely difficult due to excessive turbidity of insoluble material. Optical density could not be used for quantitation of cell growth. As an alternative, cell growth was determined by direct cell counting.

In the absence of pH-control, growth on various concentrations of soy molasses resulted in low conversion to lactic acid at concentrations >2% solids (data not shown). In order to determine if growth of the bacteria was inhibited by a drop in pH caused by build up of lactic acid in the medium, a set of pH controlled experiments was performed with and without additional nutrients. Rogers and Whittier (1928) reported that bacterial growth and lactic acid production was higher when pH was constant throughout the fermentation.

Fermentations using Soy molasses medium

Experiments performed without addition of nutrients (Table 1) showed pH of 5.6 appeared to be optimal for production of lactic acid. Results agreed with those of Tejayadi (1990) for *L. delbrueckii* subsp. *bulgaricus*. The highest concentrations of lactic acid and highest yields/g substrate utilized were observed at pH 5.6. The yield of lactic acid remained below expectations, and fermentation times were rather long (36 hr). Using single carbohydrates as carbon sources, efficiency was >90%. However, with soy molasses, the highest efficiency was 85% conversion of carbohydrate content to lactic acid. These results indicated that additional factors affected conversion of carbohydrates to lactate.

The rate of pH change (ΔpH)/hr and lactate production was monitored throughout this experiment (data not shown). Lactate concentration clearly was important in the overall efficiency of conversion of carbon source to lactic acid. This was indicated by the pH independent sharp drop in acid production that occurred at a specific lactate concentration, 3.0 g/L lactic acid. Growth inhibition was also observed at this lactate concentration. There were two possible explanations for this. One was that under these conditions the concentration of lactic acid was inhibitory. Rogers and Whittier (1928) reported that lactate inhibition could be related to the concentration of undissociated form of the end product (lactic acid). Friedman and Gaden (1970) studied the growth of *L. delbrueckii* in a dialysis batch culture system. They demonstrated that lactate was in-

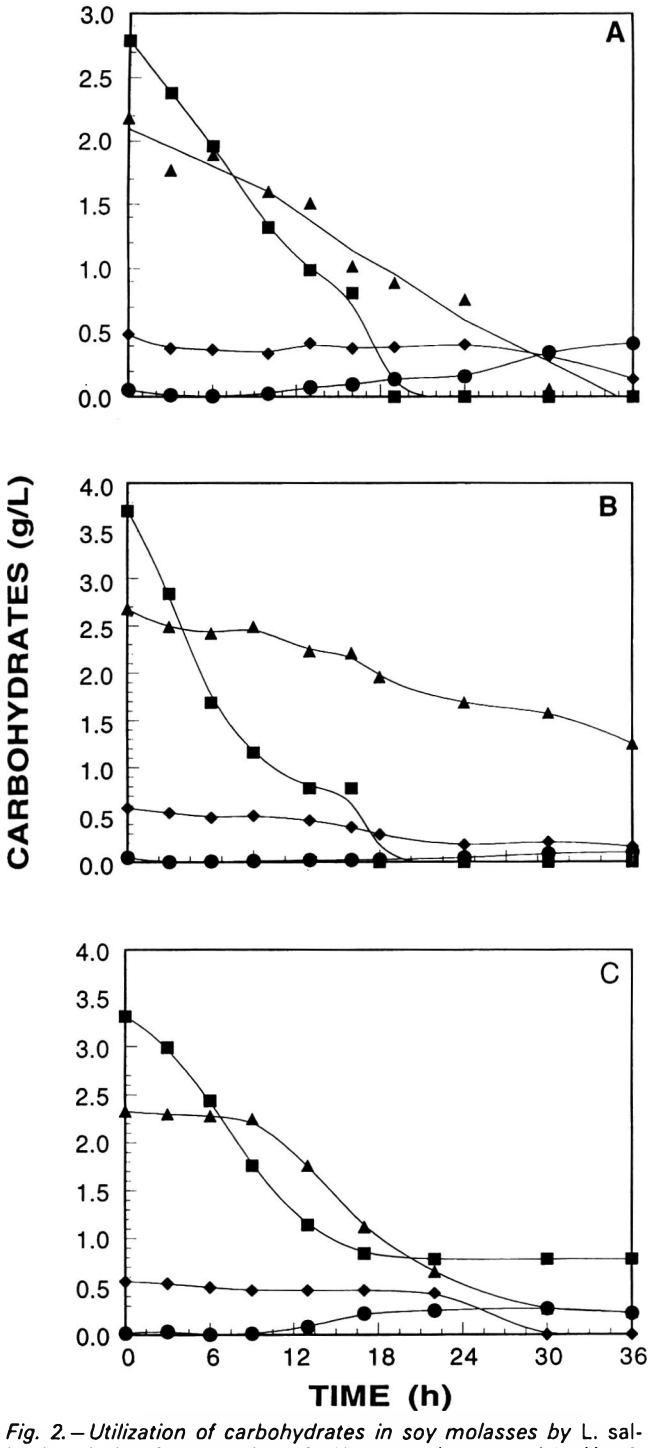

Fig. 2.—Utilization of carbohydrates in soy molasses by L. salivarius *during fermentation of 2% soy molasses at (a) pH 5.6, (b) pH 6.0, and (c) pH 6.4. Galactose/Glucose (●), Sucrose (■), Raffinose (♦), Stachyose (▲).*

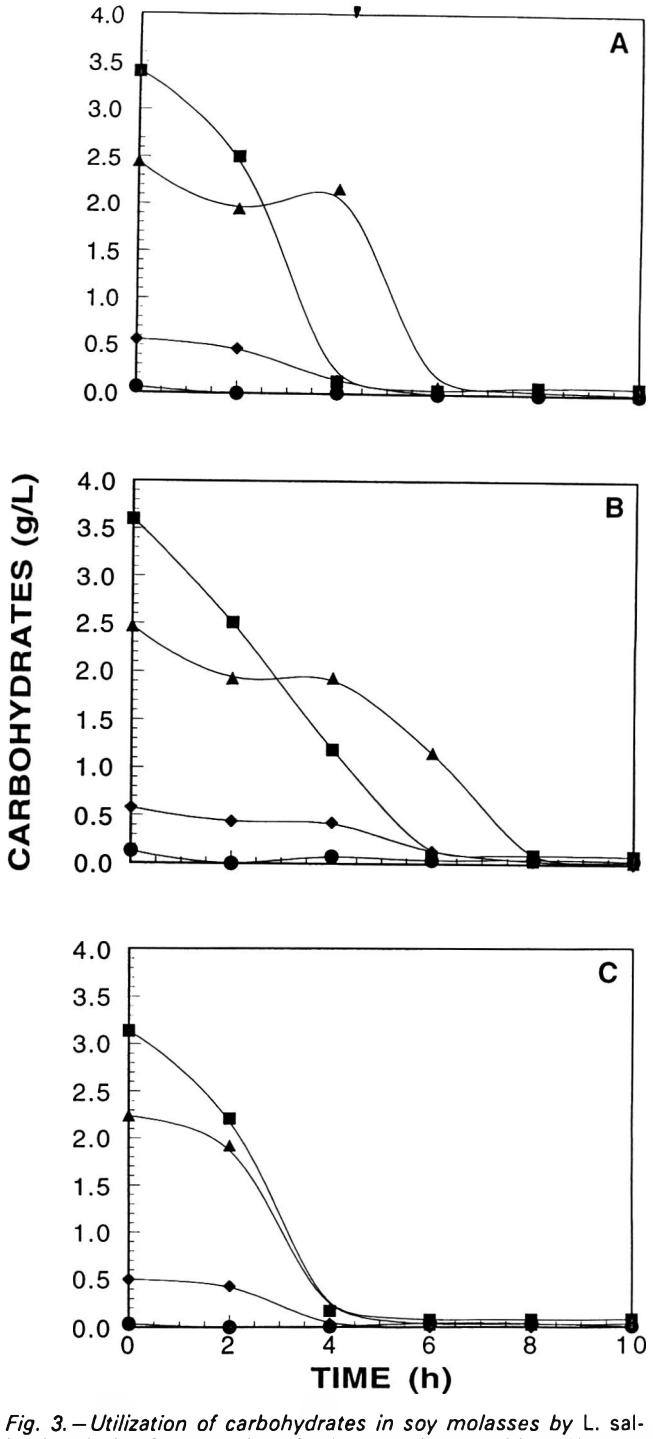

Fig. 3.—Utilization of carbohydrates in soy molasses by L. salivarius *during fermentation of 2% soy molasses with 0.5% yeast extract added at (a) pH 5.6, (b) pH 6.0, and (c) pH 6.4. Galactose/Glucose (●), Sucrose (■), Raffinose (♦), Stachyose (▲).*

hibitory. Using dialysis they maintained the concentration of the lactate in the fermentation media at very low levels. Under those conditions they observed that the specific growth rate, cell concentration and production of lactic acid were significantly higher. The other explanation for the rapid decrease in ΔpH could have been nutrient limitation. Since the only source of nutrients was the diluted soy molasses, possibly at a specific cell density, a required nutrient was completely utilized independent of pH. Bibal et al. (1988), demonstrated that the end product lactate was not the only factor affecting growth of *L. lactis* ssp. *cremoris* (*Lc. lactis* ssp. *cremoris* was formerly called *S. cremoris*). Nutritional limitations of the media may influence the response of the strain to lactic acid.

The optimum growth pH for *L. salivarius* NRRL B-1950 was pH 6.0 under these conditions. The shortest lag period, as well as the highest production of acid/hr, were observed at pH 6.0. However, the overall efficiency of lactate production was lower than that at pH 5.6 (Table 1). The utilization of each specific carbohydrate present in soy molasses was determined by liquid chromatography of samples taken throughout the course of fermentation (Fig. 2a–c). Growth and lactic acid production were slower at pH 5.6 and 6.4 than at pH 6.0;

however, stachyose, the major α-galactoside, was completely utilized. At pH 6.0, the carbohydrates were rapidly and completely utilized, with exception of stachyose which was still detected after 36 hr.

Fermentations with soy molasses plus yeast extract

In an attempt to improve conversion of carbohydrates to lactic acid, a set of pH-controlled experiments were performed to evaluate effects of added nutrients. In these experiments, 0.5% yeast extract (Difco Laboratories, Detroit, MI) was added to the 2% soy molasses media. The fermentation results were compared with those observed without addition of yeast extract.

Upon addition of 0.5% yeast extract to 2% soy molasses, the highest lactic acid yield was again observed at pH 5.6 (Table 2). At pH 6.4, the fermentation rate was higher than that at 6.0 and 5.6. This may have been due to higher production of cell mass at pH 6.4, as noted from higher cell counts. The time of fermentation for all three pH values was reduced from 36 hr to 10 hr by addition of yeast extract. Lactic acid production was increased by 30% with addition of 0.5% yeast extract. These results were consistent with those of Bibal *et al.* (1989) who reported that inhibition of lactic acid production by lactate was dependent on medium composition. *Lc. lactis* ssp. *cremoris* was more tolerant to higher concentrations of lactate when the medium was supplemented with yeast extract. At pH 6.4, all sugars were completely utilized in 4 hr (Fig. 3 a–c). Nevertheless, the highest yield of lactic acid was not observed at that pH. The α-galactosides were completely utilized in 6 hr at pH 5.6, while at pH 6.0, 8 hr were required for complete utilization of carbohydrates.

Note that the maximum cell production was achieved at pH 6.4. The most rapid utilization of carbohydrates was also observed at pH 6.4. More carbohydrate was apparently used for production of biomass at pH 6.4, since the final lactic acid concentration was lower at pH 6.4 than at 5.6 and 6.0. The highest production of lactic acid and best sugar conversion efficiency was at pH 5.6, possibly due to lower diversion of carbohydrate to production of cell mass. At pH 6.0 the production of cells was similar to that at pH 5.6, but the total concentration of lactic acid was lower at completion of fermentation.

CONCLUSIONS

Using a low value processing by-product, soy molasses was feasible for the production of a higher value end product, lactic acid. pH of fermentation, product inhibition and nutrient addition were important in controlling yield and efficiency of the fermentation. pH 5.6 was preferable for production of lactic acid, while the production of cell mass was favored at pH 6.0 without addition of yeast extract and at pH 6.4 with the addition of yeast extract. Apparent inhibition of growth occurred in the absence of nutrient supplementation. Nutrient limitation probably contributed to decreased lactate production. Addition of 0.5% yeast extract to fermentation broth reduced fermentation time from 36 to 10 hr and increased final production of lactic acid by 30%.

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Ca^{2+}-Induced Gelation of Pre-heated Whey Protein Isolate

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ABSTRACT

Addition of $CaCl_2$ to pre-heated whey protein isolate (WPI) suspensions caused an increase in turbidity when pre-heating temperatures were ≥ 64°C. Pre-heating to ≥ 70°C was required for gelation. WPI suspensions which contained $CaCl_2$ became turbid at 45°C and formed thermally induced gels at 66°C. Thermally and Ca^{2+}-induced gels showed significant time/temperature effects but the penetration force values in the Ca^{2+}-induced gels were always lower. However, Ca^{2+}-induced gels were higher in shear stress at fracture. The Ca^{2+}-induced gels had a fine-stranded protein matrix that was more transparent than the thermally induced gels, which showed a particulate microstructure.

Key Words: milk, whey, gelation, calcium, texture

INTRODUCTION

THE ABILITY to produce gel matrices with specified water-holding ability and textural properties is very important to the food industry. The industry uses different biopolymers (e.g., proteins and carbohydrates) to produce gels which vary in textural characteristics, appearance and gel point. Those factors are determined by type of biopolymer, formation conditions (e.g., heating), chemical conditions (e.g., pH), and interactions with other food ingredients (Clark and Ross-Murphy, 1987; Ziegler and Foegeding, 1990). Whey proteins (WP) are used to make certain types of cheese, and as a functional ingredient added to nondairy foods such as sausages and bakery goods (deWit, 1984, 1989). An important functional property of WP is the ability to form thermally induced gels, capable of holding large amounts of water (Schmidt et al., 1978). Characteristics of WP gels may vary from firm elastic to curd-like, depending on preparation techniques (Schmidt et al., 1978).

Physical properties of thermally induced WP gels depend on salts in the protein suspensions used for gelation. Maximum gel hardness values for WP concentrate was observed with 11 mM $CaCl_2$ or 200 mM NaCl; higher salt concentrations reduced hardness (Schmidt et al., 1978). Similar results were reported by Mulvihill and Kinsella (1988) for β-lactoglobulin, which represent 68–74% of the protein in WP concentrates and isolates (Morr and Foegeding, 1990). Maximum shear stress was found with 20 mM $CaCl_2$ or 50 mM NaCl in gels made from WP isolate (Kuhn and Foegeding, 1991).

Whey proteins require heating or addition of a destabilizing agent (e.g., urea) to cause gelation (Xiong and Kinsella, 1990). However, other biopolymers (mainly carbohydrates) can form gels at room temperature (≈23°C) when specific ions are present. One biopolymer with this property is alginate, which can form a gel when $CaCl_2$ is added (Clark and Ross-Murphy, 1987). This gelation mechanism is used to form products, such as restructured onion rings, where an alginate-mixture is injected into a water bath containing calcium (Whistler and Daniel, 1985).

Under certain conditions, WPI (10% w/v protein, pH 7.0, 80°C for 30 min) and β-lactoglobulin (10% w/v, pH 8.0, 90°C for 30 min) did not form thermally induced gels unless the protein suspension had added salts (Mulvihill and Kinsella, 1988; Kuhn and Foegeding, 1991). However, it was not determined whether such solutions would gel when salts were added after heating. Our objective was to determine whether "pre-heating" treatments could cause whey protein isolate to form a gel at room temperature after addition of $CaCl_2$. Effects of heating conditions, $CaCl_2$ concentrations and application method were investigated. The rheological and microstructural properties of gels were compared to thermally induced whey protein gels.

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MATERIALS & METHODS

Transition temperatures

The effects of heating on turbidity and gelation of aqueous whey protein isolate (WPI) (Le Sueur Isolate, Le Sueur, MN) suspensions which contained $CaCl_2$ during heating, or had $CaCl_2$ added after heating, were studied. Two lots of commercial WPI were used (A and B). Lot A had protein concentration 88.8% and lot B 89.0% (by Macro-Kjeldahl AOAC, 1984, using N factor 6.38). Protein suspensions were prepared (8% w/v) in deionized water, adjusted to pH 7.0 with 0.1M NaOH or HCl and diluted (1:1) before or after heating with a 20 mM $CaCl_2$ solution, resulting in suspensions containing 4% (w/v) protein and 10 mM $CaCl_2$. For treatments where $CaCl_2$ was added after heating, the 8% protein suspensions were heated in borosilicate glass tubes (13.5 mm i.d.), cooled 1 hr at 25°C, and 20 mM $CaCl_2$ solution was added for a final concentration of 4% (w/v) protein and 10 mM $CaCl_2$. This method for forming Ca^{2+}-induced gels allowed for complete mixing prior to gelation.

The tubes containing WPI suspensions (with or without $CaCl_2$) were heated at 0.5°C/min from 25° to 90°C in a water bath with a temperature programmer (Neslab ETP-3, Neslab, Portsmouth, NH). Samples were removed at predetermined temperatures (measured with thermocouples inserted in the geometrical center of two samples). The suspensions/gels were allowed to cool to 25°C and stored at 4°C overnight. Gel penetration force or suspension turbidity was measured the next day. Gels were equilibrated to room temperature and penetration force was determined by the back-extrusion method of Hickson et al. (1982). Briefly, a 9-mm-diameter steel rod mounted on an Instron universal testing instrument (Model 1122, Instron Corp., Canton, MA) was used to rupture the gels. The probe penetrated 20 mm into the gel at a constant speed of 100 mm/min. The force required to rupture the gel (first peak) was determined and expressed as penetration force. The turbidity (optical density at 400 nm) of suspensions that did not gel was measured with a spectrophotometer (Shimadzu UV-240, Kyoto, Japan). The experiment was replicated twice, once with each of the different lots of WPI.

Heating temperature and time

The effect of heating time (5, 10, 20, 40, 60 and 80 min) at four different temperatures (60°, 70°, 80°, and 90°C) was studied. The same two lots of WPI were used, in two replications, at the same protein and $CaCl_2$ concentrations described above. WPI samples were heated in borosilicate glass tubes, either in the presence of $CaCl_2$ or without (i.e., $CaCl_2$ added after cooling), and gel penetration force was determined.

Fracture properties

The fracture properties of WPI gels with different $CaCl_2$ concentrations, added before or after heating, were determined. Thermally induced WPI gels were prepared by heating 10% (w/v) protein sus-

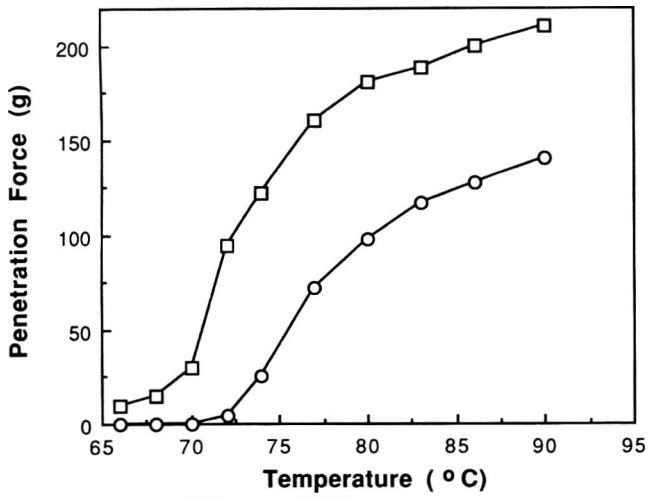

Fig. 1—Effect of heating temperature on gel (4% WPI and 10 mM $CaCl_2$) penetration force. Calcium was either present during heating (□) or absent during heating but added after heating (○). Mean values for two replications.

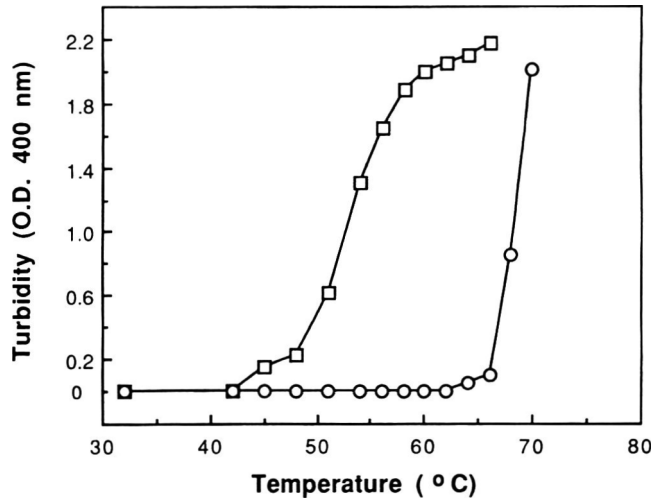

Fig. 2—Effect of heating temperature on turbidity ($OD_{400\ nm}$) of 4% WPI suspensions in the presence of 10 mM $CaCl_2$. Calcium was either present during heating (□) or absent during heating but added after heating (○). Mean values for two replications.

pensions containing 10 mM $CaCl_2$ in polycarbonate tubes (19 mm i.d.) pre-coated with sanitary lubricant spray (Haynes Co. Cleveland, OH), for 10 or 30 min at 80°C. Gels were cooled for 30 min at room temperature (≈23°C) removed from tubes and stored in air-tight containers overnight at 4°C. The Ca^{2+}-induced gels were prepared by heating 10% (w/v) protein suspensions in polycarbonate tubes for 10 or 30 min at 80°C, cooling to room temperature, transferring the suspensions into dialysis tubes (Spectra/Por #1 membrane, 6000–8000 molecular weight cut-off, Spectropor, Los Angeles), and dialyzing against different $CaCl_2$ concentrations (10, 40, 100 and 150 mM $CaCl_2$) for 14 hr. The following day, true shear stress and strain were determined by torsional fracture testing (Kim et al., 1986). Gels were cut into 28-mm-long cylinders and mounted on plastic disks with cyanoacrylate glue (Krazy Glue, B. Jadow and Sons, Inc., New York, NY). The gel cylinders were ground into capstan shapes with a minimum diameter of 10 mm, and twisted to fracture at 2.5 rpm with a Torsion Gelometer (Gel Consultants, Raleigh, NC). True shear stress and shear strain at fracture were calculated from the torque and angular displacement (Diehl et al., 1979). The experiment was replicated twice, once with each lot of WPI.

Scanning electron microscopy (SEM)

Samples from the torsional fracture test were prepared for SEM evaluation by cutting small blocks (1 × 2 × 2 mm), fixing them for 2 hr in 2% glutaraldehyde in 0.1 M HEPES buffer (pH 7.0), rinsing with buffer, post fixing for 4 hr in 1% OsO_4, rinsing and dehydrating in a graded series of ethanol (50, 70, 80, 90 and 100%). Dried samples were critical point dried using CO_2, fractured and mounted on stubs, which were later coated with palladium/gold (Hummer V, Anatech Ltd., Alexandria, VA). The samples were examined by SEM (Philips 505T) at 10 kv.

Rheological transitions

A Bohlin VOR rheometer (Bohlin Reologi AB, Lund, Sweden) with a concentric-cylinder-fixed bob and rotating-cup measuring cell (C 14) attached to a 11.2 gcm torsion bar was used for rheological measurements. All experiments were conducted in oscillation at a frequency of 0.05 Hz and a strain of 0.1. In thermal scanning experiments, suspensions containing 4% protein and 10 mM $CaCl_2$ were heated from 25–80°C at 1°C/min, held at 80 for 15 min, cooled to 25°C at 1°C/min, and held at 25°C for 20 min. The Ca^{2+}-induced gels were prepared by mixing a preheated (30 min at 80°C) 8% (w/v) protein suspension 1:1 with a solution of 20 mM $CaCl_2$ and monitoring gelation at 25°C for 12 hr. Two trials were run for each lot of protein. Values are means of four runs/treatment.

Statistical analyses

Data were analyzed using the Statistical Analysis System (SAS Institute, Inc. 1982). The General Linear Model procedure for regression analyses, ANOVA procedure for analysis of variance and Duncan's multiple range test for ranking the means were used.

RESULTS & DISCUSSION

Transition temperatures

Thermally induced whey protein gelation in the presence of $CaCl_2$ has been well documented (Hagget, 1976; Schmidt et al., 1978; Kuhn and Foegeding, 1991; Foegeding et al., 1992) and serves as reference for comparison with the little reported phenomenon of Ca^{2+}-induced gelation of pre-heated WPI suspensions. The major differences between the two systems can be seen in Fig. 1. The first is the gelation point and the second is the difference in gel penetration force. WPI suspensions containing $CaCl_2$ started to form a gel at 66°C, whereas WPI heated without $CaCl_2$ (i.e., $CaCl_2$ added after heating) did not form a gel until 72°C. Thus a higher temperature (or more time) was required to achieve $CaCl_2$ sensitivity in the preheated proteins. The requirement for additional heating could be due to irreversible or slowly reversible changes in protein structure and/or aggregation. This hypothesis is supported by changes in turbidity observed during the initial stages of heating (Fig. 2). The suspensions containing 10 mM $CaCl_2$ had detectable turbidity at 45°C. This indicated that the initial formation of protein aggregates, which must be the result of $CaCl_2$-mediated protein-protein interactions, started at 45°C or lower. WPI suspensions without $CaCl_2$ added did not show any change in turbidity when heated up to 95°C (data not shown). However, when $CaCl_2$ was added after heating, turbidity was observed in suspensions heated to ≥ 64°C (Fig. 2). Thus, changes in protein structure occurring between 45–62°C that caused Ca^{2+}-induced aggregation were reversible to the extent that Ca^{2+}-induced aggregation at 25°C was slowed and turbidity was not detected.

The second major difference between the two gels was the penetration force required to rupture the gel (Fig. 1). Those formed in $CaCl_2$-containing suspensions had higher penetration force values; about 1.5 to 2.0 times greater than the Ca^{2+}-induced gels. However, the similar shapes of the two curves suggest common factors for the two gelation processes.

To better understand differences between the gels, the effects of duration of heating at three different temperatures (70°, 80° and 90°C) were studied. In the thermally induced gels (Fig. 3), temperature affected ($P < 0.05$) the penetration force. As temperature increased penetration force increased, and longer duration of heating at 70° or 80°C resulted in higher penetration

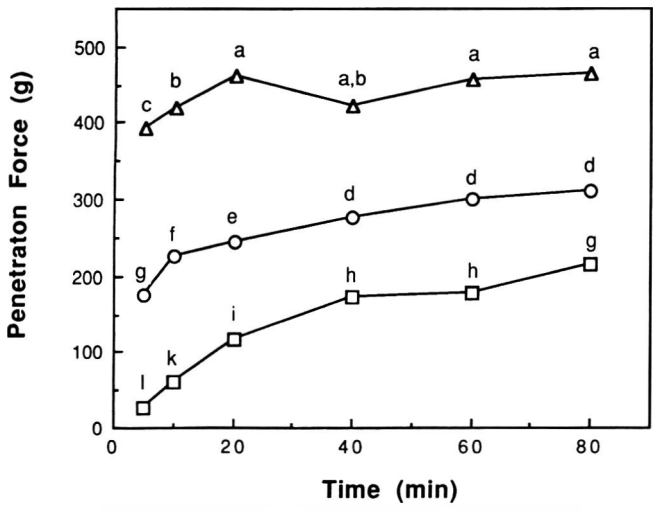

Fig. 3—Effect of heating temperature and holding time on gel penetration force of WPI gels prepared by heating 4% protein suspensions with 10 mM $CaCl_2$. Gels were formed at 70°C (□), 80°C (○) or 90°C (△). Different letters (a-l) adjacent to a value indicate a significant difference ($P<0.05$) among values. Mean values for two replications.

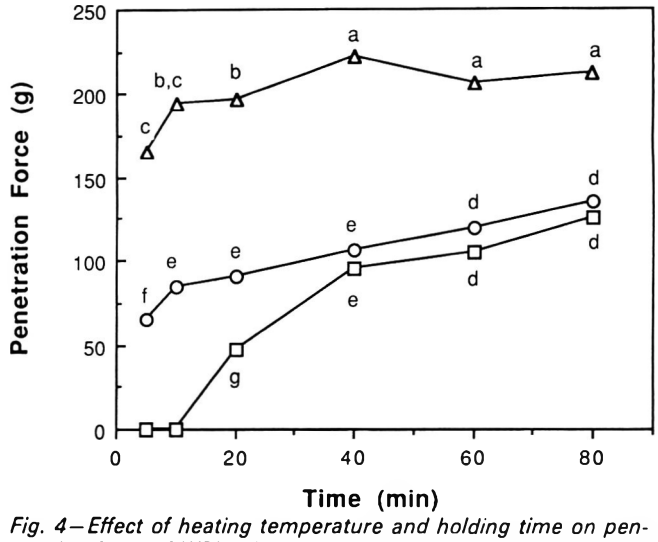

Fig. 4—Effect of heating temperature and holding time on penetration force of WPI gels prepared by heating 8% protein suspensions, cooling them to room temperature and adding an equal volume of 20 mM $CaCl_2$. Pre-heating temperatures were 70°C (□), 80°C (○) and 90°C (△). Different letters (a-g) adjacent to a value indicate a significant difference ($P<0.05$) among values. Mean values for two replications.

force values. However, at 90°C a plateau was reached after 20 min, indicating no further improvement. The same trend was observed in the 80°C treatment but at a later stage (after 40 min). This suggested that both kinetic and thermodynamic factors contributed to gel penetration force. When $CaCl_2$ was added after heating (Fig. 4), a temperature effect was observed. The 80° and 90°C treatments showed a pattern similar to that in thermally induced gels (Fig. 3). However, penetration force values for the Ca^{2+}-induced gels were lower ($P<0.05$) than thermally induced gels. In addition, the force/time slopes at different temperatures showed Ca^{2+}-induced gels were less sensitive to duration of heating.

At 70°C, a heating period of >10 min was required for gelation (Fig. 4). After 20 min at 70°C, gel penetration force was lower than that from the 80°C treatment but after 40 min the two temperatures produced gels of similar penetration force. This is important in understanding transitions taking place in WPI during heating which make the protein susceptible to Ca^{2+}-induced gelation. Gels produced from pre-heated WPI required higher heating temperature and/or time than thermally induced gels formed in the presence of $CaCl_2$. This was also shown (Fig. 1) where, at a heating rate of 0.5°C/min, a gel was formed at 66°C in the presence of $CaCl_2$, and at 72°C when $CaCl_2$ was added after heating. However, holding the WPI suspension at 70°C more than 20 min could also result in sufficient protein unfolding and/or aggregation to allow Ca^{2+}-induced gelation (Fig. 4). Longer holding at 70°C and 80°C resulted in further increases in gel penetration force.

Fracture properties

Penetration force values were used to detect general changes in gel structure. These values are empirical since factors affecting force and deformation associated with this method are not fully understood. To obtain fundamental information on fracture mechanics, true shear stress and strain at fracture were determined by torsional fracture testing (Diehl et al., 1979). At the point of fracture, true shear stress is the force (shear or tensile) per unit area and true shear strain is the deformation (shear or tensile) per length unit. These mechanical properties (true shear stress and true shear strain at fracture) correlated with sensory texture notes (Montejano et al., 1985), and therefore could be indicators of textural changes. Protein concentration was increased to 10% (w/v) since WPI gels formed with 4% (w/v) protein were too fragile to use in torsional fracture testing. Using 10% (w/v) protein WPI suspensions required another means of incorporating $CaCl_2$ into the pre-heated samples. At protein concentrations above 4% and/or $CaCl_2$ concentrations above 10 mM, gelation could occur on contact of the two solutions and therefore adequate mixing was not possible. Therefore, dialysis, which allows for slow diffusion-controlled changes in the ionic environment, was used. Note that 10% (w/v) WPI suspensions which were not heated did not gel at any $CaCl_2$ concentrations used for dialysis.

Results from torsional fracture testing (Table 1) indicated that duration of heating (10 or 30 min) affected shear stress of the Ca^{2+}-induced gels. This was determined by linear regression. Slopes of the lines representing shear stress and $CaCl_2$ concentration (graph not shown) for each heating period were significantly different ($P<0.001$) for the Ca^{2+}-induced gels. The shear stress values for thermally induced gels were similar to results previously reported (Kuhn and Foegeding, 1991). The shear stress values of thermally induced gels were lower than the $Ca_2{}^{+}$-induced gels, confirming previous results indicating that these were two different types of gels. However, those results differed from our penetration force values (Fig. 1, 3 and 4), which showed thermally induced gels to be "stronger" than Ca^{2+}-induced gels. The differences are probably related to protein concentration (4 vs 10%), Ca^{2+}-induced gelation method (mixing vs. dialysis) and physical properties measured (penetration vs torsional fracture). Note that the penetration test was used to detect "general" changes in gel struc-

Table 1—Effect of duration of heating at 80°C and calcium level on shear stress and strain at fracture.

$CaCl_2$ (mM)	Shear stress (kPa) *Heating time (min)* 10	30	Shear strain *Heating time (min)* 10	30
I. $CaCl_2$ added after heating[h]				
10	25.4[a]	29.9[d]	2.03[b]	1.99[b]
40	29.5[d]	42.2[b]	1.73[c]	1.57[d]
100	30.1[d]	50.2[a]	1.48[e]	1.48[e]
150	32.9[c]	51.5[a]	1.50[e]	1.40[f]
II. $CaCl_2$ present during heating				
10	16.3[f]	18.7[f]	2.33[a]	1.88[c]

[a-g] Means (n = 16) within each measurement (stress or strain) followed by the same superscript are not significantly different at the 95% level.

[h] $CaCl_2$ was added by dialysis after cooling to room temperature.

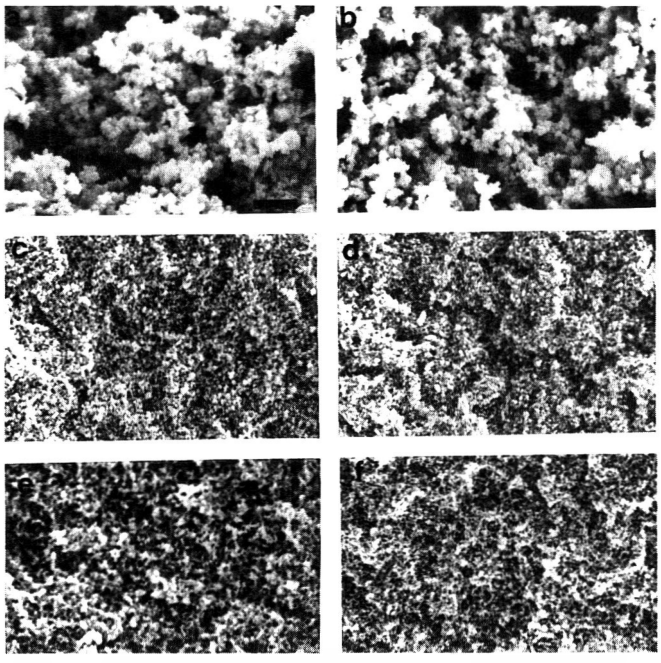

Fig. 5—Scanning electron micrographs of 10% WPI gels. Thermally induced gels were formed from suspensions containing 10 mM $CaCl_2$ by heating at 80°C for (a) 10 min or (b) 30 min. Ca^{2+}-induced gels were made by heating 10% WPI suspensions (10 or 30 min), cooling and dialyzing against 10 or 150 mM $CaCl_2$. Treatments were: (c) 10 min heating and 10 mM $CaCl_2$, (d) 30 min heating and 10 mM $CaCl_2$, (e) 10 min heating and 150 mM $CaCl_2$, and (f) 30 min heating and 150 mM $CaCl_2$. Bar = 2 μm.

ture, and fracture properties were determined because they are associated with consumer perceptions of texture.

Strain values decreased as $CaCl_2$ level increased, and regression analysis comparing the two heating periods indicated they were not different ($P > 0.05$). Generally, stress increased and strain decreased as $CaCl_2$ concentration increased (Table 1). A decrease in strain and increase in stress was found in thermally induced WPI gels as duration of heating or protein concentration increased (Foegeding, 1992). These changes appeared to be associated with transformation from a highly deformable incipient gel, (where strands had less restricted movement), to one with a more extensive network and less deformable strands.

Gel microstructure

Micrographs of selected treatments (Fig. 5) show the most apparent differences were among thermally induced gels (Fig. 5a and b) and the Ca^{2+}-induced gels (Fig. 5 c-f). Thermally induced gels showed a particulate microstructure composed of bead-like particles attached to each other. Such structure was shown in thermally induced WP concentrate gels (Beveridge et al., 1983) and in β-lactoglobulin gels (Stading and Hermansson, 1991; Langton and Hermansson, 1992). In contrast, the Ca^{2+}-induced gels had a completely different microstructure which consisted of a fine network of protein strands. This was consistent with our observation that Ca^{2+}-induced gels were less opaque than thermally induced gels. Differences in light transmission properties of gels have been associated with gel microstructures (Hermansson, 1988). Fine-stranded gels are generally less opaque than particulate gels. The fine-stranded gels are usually formed by an ordered association of molecules and, if dimensions are very small, a transparent gel can be formed. Such was the case in the 12% β-lactoglobulin gel made in distilled water at pH 4 or 6 (Stading and Hermansson, 1991). Such gels had a fine-stranded microstructure and were

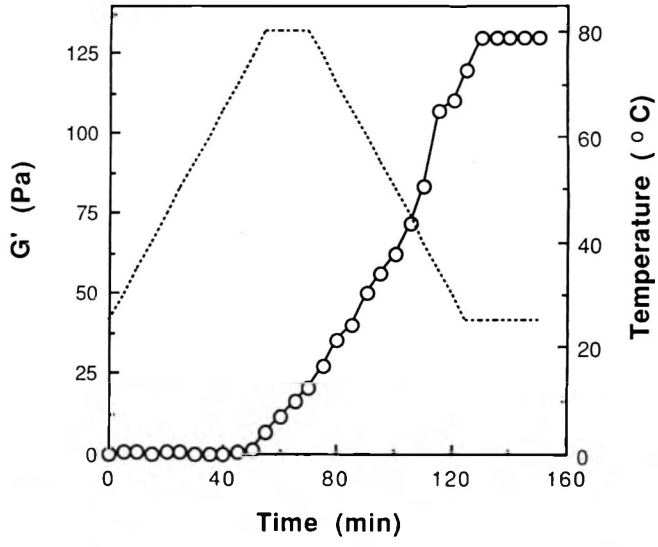

Fig. 6—Changes in storage modulus (G', ○) during heating and cooling of a 4% WPI, 10 mM $CaCl_2$ suspension. Dotted line indicates temperature.

less opaque than particulate gels formed at pH 4–6. Gels with particulate microstructures are usually opaque and size of particles can vary, depending on environmental conditions and type of protein. However, the particles are generally uniform in size within each gel structure (Hermansson, 1988). Gels with particulate microstructures were produced in our study by thermally induced gelation of WPI suspensions containing $CaCl_2$. The general microstructures of gels formed by heating for 10 or 30 min (Fig. 5a and b) were similar, indicating that the basic structure was formed within the first 10 min of heating.

The Ca^{2+}-induced gels showed a fine-stranded structure affected by the amount of $CaCl_2$ and duration at 80°C. The main difference was larger openings in the structure of the 150 mM treatment (Fig. 5e and f) compared to the 10 mM treatments (Fig. 5c and d). These gels differed in shear strain and shear stress values (Table 1). Short duration of heating (10 vs 30 min) appeared to result in slightly larger pore openings in the gel matrix. This was more obvious in the 150 mM $CaCl_2$ treatments (Fig. 5e vs f) and was also seen in the 10 mM $CaCl_2$ treatments.

Rheological transitions

Thermally induced and Ca^{2+}-induced rheological transitions in WPI suspensions were followed dynamically using a constant strain rheometer. Rheological properties were determined during heating (25–80°C, at 1°C/min), holding (80°C, for 15 min), cooling (80 to 25°C, at 1°C/min), and holding (25°C for 20 min) (Fig. 6), and at 25°C (for 12 hr) in Ca^{2+}-induced gels (Fig. 7). Thermally induced transitions in storage modulus (G') were similar to those observed when heating a 7% (w/v) β-lactoglobulin suspension containing 20 mM $CaCl_2$ (Foegeding et al., 1992). G' started to increase at 70–75°C, and further heating to 80°C resulted in a sharp increase (Fig. 6). Holding the gels at 80°C for 15 min and subsequent cooling to 25°C caused a further increase. An increase in G' upon cooling was reported in ovalbumin and soy protein isolate gels (Van Kleef, 1986).

Ca^{2+}-induced gelation was detected after 30 min at 25°C, as shown by an increase in G' and a decrease in phase angle (Fig. 7). The phase angle indicates the relative amounts of viscous and elastic elements in the gel (i.e., an elastic solid has 0° and a viscous fluid 90°). The change from a viscoelastic fluid to a viscoelastic solid can be used to indicate gelation. Accordingly, the fluid to gel transition was complete 36 min

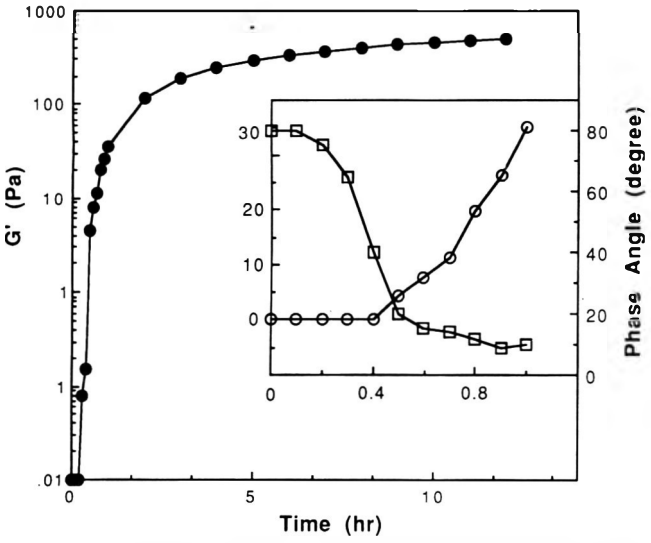

Fig. 7—Rheological changes in storage modulus (G', ○) and phase angle (□) during Ca^{2+}-induced gelation of a 4% WPI suspension by addition of 10 mM $CaCl_2$. Temperature was held constant at 25°C for 12 hr. Insert shows phase angle and G' transitions on an expanded time scale. Mean values for two replications.

after mixing the preheated (30 min at 80°C) WPI suspension with $CaCl_2$. The gelation pattern for that treatment was very different from that in thermally induced gel. When $CaCl_2$ was present during thermally induced gelation a sharp increase in G' was observed above 65°C and G' values kept on increasing slowly during cooling but did not increase when held at 25°C for 20 min (Fig. 6). In Ca^{2+}-induced gelation, G' values continued to increase at 25°C for 12 hr after mixing (Fig. 7).

CONCLUSIONS

PREHEATED WPI suspensions can form gels at 25°C by addition of $CaCl_2$. The Ca^{2+}-induced gel was very different from thermally induced WPI gel formed by heating a $CaCl_2$-containing WPI suspension. Ca^{2+}-induced gels had a fine-stranded microstructure, whereas thermally induced gels had a particulate microstructure consisting of bead-like particles. Ca^{2+}-induced gels formed by mixing protein and $CaCl_2$ solutions had lower gel penetration force values than similar thermally induced gels. However, shear stress at fracture was higher for Ca^{2+}-induced gels formed by dialyzing against a $CaCl_2$ solution. Both Ca^{2+}-and thermally induced gelation required proteins to reach a certain degree of unfolding. However, a higher temperature was required to cause Ca^{2+}-induced gelation.

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Model for Gelatinization of Wheat Starch in a Twin-Screw Extruder

W. CAI and L.L. DIOSADY

ABSTRACT

Wheat starch was processed in a co-rotating twin-screw extruder, at moisture contents 25% and 30%, screw speeds 200 and 300 rpm, feed rate 30 kg/hr, and barrel temperature settings 100, 120, 140, and 160°C. Degree of starch gelatinization at each point along the extruder channel was determined by sampling and analyzing material inside the extruder. Kinetics of the gelatinization during extrusion was investigated. A first-order rate equation was developed to predict degree of starch gelatinization during extrusion. The rate constant was a function of both temperature and shear stress.

Key Words: wheat, starch, gelatinization, modeling, extrusion

INTRODUCTION

EXTRUSION COOKING is popular for processing cereals, snacks, pet foods, and many products that utilize starchy and/or proteinaceous raw materials. Although it is important in extrusion cooking of starch-based products, starch gelatinization during extrusion cooking is not fully understood. Profiles of gelatinization of starch along an extruder channel have not been reported, although this is critical to understanding starch modification and development of comprehensive models of extrusion cooking. Most reported research concerns effects of major extrusion conditions on the extent of gelatinization rather than understanding and quantitative description of kinetics of the process. Lawton et al. (1972) investigated the effects of 15 variables including screw geometry and operating parameters on gelatinization of corn starch. The important variables were moisture content, temperature, screw speed and screw geometry. Other researchers have reported similar findings (Anderson et al., 1969a, b; Chiang and Johnson, 1977; Gomez and Aguilera, 1983, 1984; Owusu-Ansah et al., 1983; Bhattacharya and Hanna, 1987).

Very few studies have reported kinetics of starch gelatinization at low moisture contents where extrusion cooking is usually performed. Based on measurements using differential scanning calorimetry, the kinetics of starch gelatinization at high temperature and low moisture content were reported to be pseudo-first-order for corn meal (Burros et al., 1987), and pseudo-zero-order for waxy corn starch (Wang et al., 1989). However, the cooking environment for starch in an extruder channel is not the same as in a differential scanning calorimeter, since shear forces in the extruder channel can not be reproduced in the calorimeter cell. Bhattacharya and Hanna (1987) published the results of an investigation of the kinetics of starch gelatinization during extrusion with a single-screw extruder. They found that the process followed pseudo-zero-order reaction kinetics and the rate constants decreased with decreasing temperature. Lai and Kokini (1991) indicated that starch gelatinization during extrusion followed a first order reaction. However, no model has been reported to predict the degree of starch gelatinization during extrusion. Fundamental data are needed to develop a complete understanding. Our objective was to investigate the profiles of starch gelatinization along a twin-screw extruder channel and to develop a first order model of starch gelatinization in terms of temperature, shear stress and mean residence time in the cooking zone.

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MATERIALS & METHODS

Materials and extruder conditions

Whetstar-4, a commercial wheat starch from Ogilvie Mills Ltd, was used. A Baker-Perkins MPF-50D, pilot-scale, co-rotating intermeshing twin-screw extruder with a smooth barrel was used (Fig. 1). The L:D (barrel length:barrel inside diameter) ratio was 15:1. The barrel had five temperature-controlled sections heated by electricity and cooled by a refrigeration system. The barrel was horizontally split. The screws were built up of screw elements and lobe-shaped kneading discs, which could be assembled on the shafts for different screw configurations. Material was fed by a K-Tron loss-in-weight feeder. The screw configuration used is shown in Fig. 1 and described in Table 1A. Two circular dies, each 4 mm diameter were fitted in the die plate for every experiment. An alternative screw configuration without kneading discs, (Table 1B), and 6 mm dies were used only for visual investigation of effects of geometry parameters on location of starch transformations. The extruder was operated with a constant feed rate of 30 kg/hr. Screw speeds were 200 or 300 rpm. Moisture contents were 25 or 30%. Four sets of barrel temperature profiles were selected (Table 2).

"Dead-stop" operation

The horizontal split design of the extruder allowed the collection of samples inside the extruder channel with a "dead-stop" run. When the extruder was running at steady state as indicated by steady values of screw torque and die pressure, the extruder was suddenly stopped by stopping feed supply, screw rotation, and electrical heating. The barrel was cooled by the refrigeration system to ambient temperature and dismantled very quickly. Samples were then taken along the extruder channel.

Determination of mean residence time

Using the method described by Kirby et al. (1989), the mean time for each small segment inside the extruder was determined by determining a mass balance on the segment:

$$t = \frac{M}{Q_m} \quad (1)$$

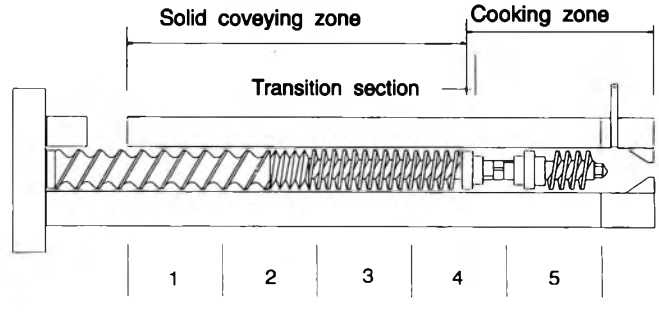

Fig. 1—Functional zones along the extruder channel, screw configuration (A).

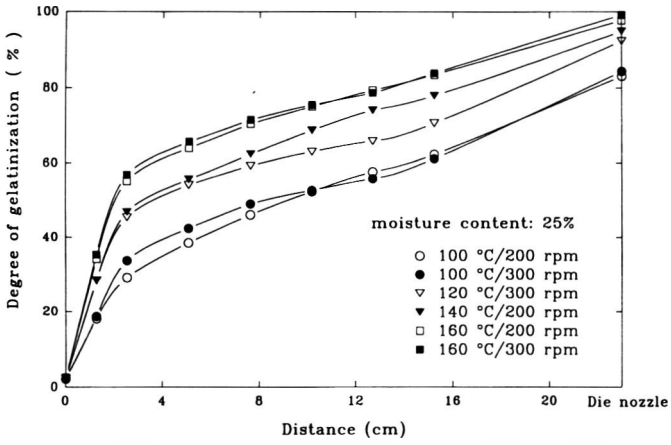

Fig. 2—Profiles of starch gelatinization as a function of distance along extruder channel from beginning of the cooking zone. Moisture content: 25%

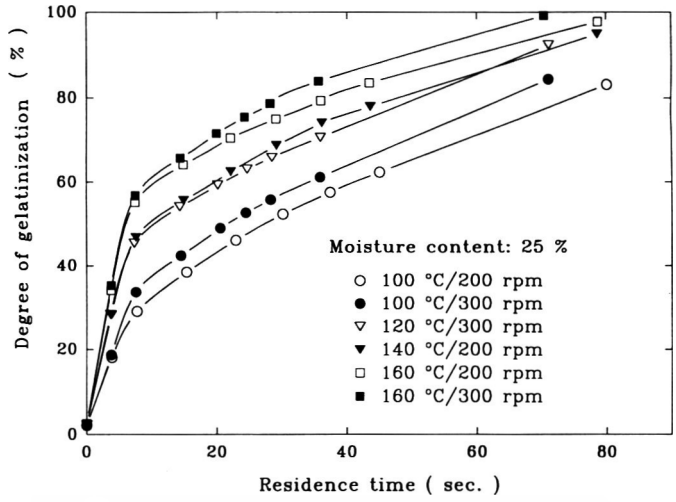

Fig. 3—Profiles of starch gelatinization along the extruder channel as a function of residence time from beginning of the cooking zone. Moisture content: 25%.

Table 1—Screw configurations

Screw configuration (A):
12" twin lead, 1" pitch, self-wiping screws;
2" twin lead, 1/2" pitch, self-wiping screws;
10" single lead, 1/2" pitch, intermeshing screws;
4" 8 × 90° kneading discs;
2" single lead, 1/2" pitch, intermeshing screws.
Screw configuration (B):
12" twin lead, 1" pitch, self-wiping screws;
2" twin lead, 1/2" pitch, self-wiping screws;
16" single lead, 1/2" pitch, intermeshing screws.

Table 2—Temperature profiles of the five barrel heating sections (Fig. 1)

Final section temp (°C)	Temperature profile (°C)
100	27 / 51 / 75 / 100 / 100
120	27 / 58 / 89 / 120 / 120
140	27 / 64 / 101 / 140 / 140
160	27 / 71 / 115 / 160 / 160

where: t = mean residence time in a small segment, M = mass hold-up in the segment, Q_m = mass flow rate. To avoid problems due to variability of moisture content during sample collection, moisture content of all samples was determined and all weights were converted to dry basis.

Degree of gelatinization

The degree of gelatinization was defined as the weight ratio of gelatinized starch to total weight of sample. The determination of degree of gelatinization was based on the method described by Birch and Priestley (1973), based on formation of a blue iodine complex by amylose released during gelatinization. A sample (0.04g) was dispersed in 50 mL 0.060M KOH solution and then gently agitated for 15 min. The slurry was centrifuged and 1 mL aliquots of supernatant were removed, mixed with 9 mL 0.00667M HCl. Then 0.1 mL iodine reagent (1g iodine and 4g potassium iodide/100 mL water) was added, and after mixing the absorbance a_1 was read at 600 nm in the spectrophotometer against a reagent blank. The estimation was repeated using 50 mL 0.4M KOH solution, and 9 mL 0.0445M HCl to obtain absorbance a_2. The degree of gelatinization was calculated by the ratio of the two absorbances a_1 and a_2. Reported results are averages of three replicated analyses.

RESULTS & DISCUSSION

Location of starch gelatinization in extruder channel

By the "dead-stop" shutdown and quick opening of the barrel, three different sections were observed: a solid powder section and a melt section, between which there was a very short transition section (Fig. 1). From this observation and the analytical results of samples obtained along the extruder channel we could describe the extruder channel, from the product transformation viewpoint, in terms of two zones: a solid conveying zone and a "reaction" or cooking zone. From the inlet to the start of the kneading disc section, starch only partially filled the screw channel and remained in powder form. Results showed that starch gelatinization did not occur in this zone. At about the last flight of the conveying screw before the kneading disc section starch completely filled the screw and was transformed from powder to melt within a very short section, defined as the transition section. The length of the transition section was about 2–2.5 cm. No significant difference in length was observed for the range of operating conditions used. The cooking zone was defined as the region between the beginning of the transition section and the die nozzle. Results on the samples obtained along the extruder channel revealed that starch gelatinization in the extruder channel started at the beginning of the cooking zone and continued throughout the entire cooking zone, (Figs. 2 and 3).

The length of the cooking zone was determined by the position of the transition section. The transition section was always at the beginning of the first fully filled segment. This was due to the increased energy input into this segment. The critical variable for transition of starch in the extruder was the temperature of the starch which did not increase rapidly up to the first fully filled segment. In that segment the heat transfer area between starch and barrel was increased to the maximum due to filling of the channel, and the increased mixing action improved heat transfer. Meanwhile, the increased shearing action developed heat through dissipation of mechanical energy.

Locations of the fully filled segments were affected only by screw configurations and die size in the range of extrusion conditions used. For the screw configuration with a kneading disc section (Fig. 1), two local filled segments were observed. One was at the beginning of the kneading disc section due to the sudden decrease of conveying capacity of the kneading discs. The other was close to the end of the screw because of the back pressure of the die. Between these two filled segments the screw was not always fully filled. The degree of fill was mainly dependent on screw speed. Lower screw speeds resulted in more complete filling of this section. When no kneading discs were used (configuration (B), Table 1), the transition section was at the beginning of the only fully filled segment close to the end of the screw and the position was affected only be die diameter. When 4 mm dies were used, the transition section started at the fourth flight from the screw end. When the die diameter was increased to 6 mm, the position of

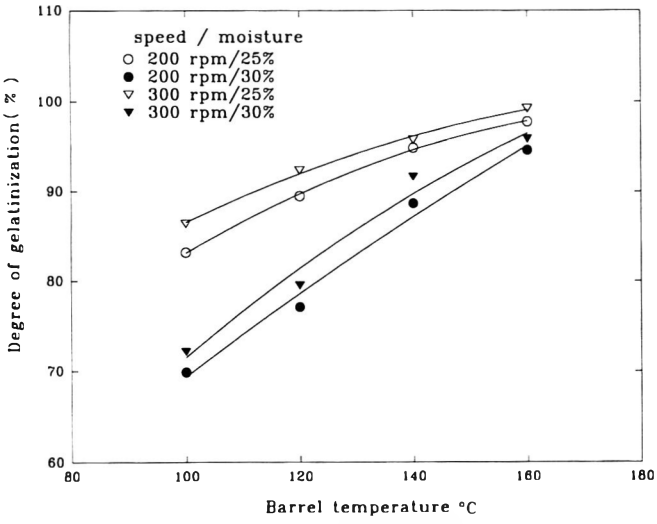

Fig. 4—Effects of operating conditions on gelatinization of the extrudate.

Table 3—Results of regression analysis with d (1-f)/dt = − k(1 − f)^m

Experimental conditions	Inc. transition section		Exc. transition section	
	Order of reaction	Cor. coef.	Order of reaction	Cor. coef.
100°C / 200rpm / 25%	2.067	0.9048	1.192	0.9853
100°C / 300rpm / 25%	2.383	0.9064	1.173	0.9267
120°C / 300rpm / 25%	2.253	0.9248	1.140	0.9487
140°C / 200rpm / 25%	1.868	0.9225	1.063	0.9572
160°C / 200rpm / 25%	1.816	0.9224	1.022	0.9628
160°C / 300rpm / 25%	1.713	0.9374	1.028	0.9482
100°C / 200rpm / 30%	2.188	0.8671	1.160	0.9817
100°C / 300rpm / 30%	2.257	0.8758	1.164	0.9630
120°C / 200rpm / 30%	2.375	0.8604	1.105	0.9279
140°C / 300rpm / 30%	2.015	0.9252	1.183	0.9753
160°C / 200rpm / 30%	1.821	0.9143	1.002	0.9384
160°C / 300rpm / 30%	1.921	0.9097	1.035	0.9570

Table 4—Correlation coefficients of regression on analysis with integral form of the kinetics

Experimental conditions	The first-order reaction ln(1 − f) = −kt + b		The second-order reaction 1/(1 − f) = −kt + b	
	Excluding transition section	Whole cooking zone	Whole cooking zone	Excluding the last datum
100°C / 200rpm / 25%	0.9924	0.9893	0.9177	0.9958
100°C / 300rpm / 25%	0.9878	0.9842	0.9184	0.9869
120°C / 300rpm / 25%	0.9776	0.9756	0.8637	0.9834
140°C / 200rpm / 25%	0.9784	0.9797	0.8139	0.9913
160°C / 200rpm / 25%	0.9584	0.9679	0.7468	0.9865
160°C / 300rpm / 25%	0.9575	0.9655	0.7368	0.9965
100°C / 200rpm / 30%	0.9964	0.9942	0.9588	0.9965
100°C / 300rpm / 30%	0.9959	0.9937	0.9568	0.9955
120°C / 200rpm / 30%	0.9933	0.9860	0.9466	0.9901
140°C / 300rpm / 30%	0.9960	0.9717	0.9061	0.9895
160°C / 200rpm / 30%	0.9806	0.9794	0.8160	0.9851
160°C / 300rpm / 30%	0.9840	0.9724	0.8419	0.9864

the transition section moved toward the screw end by about two flights. Because of the flexibility of screw configurations for twin-screw extruders, one could predetermine the required length of cooking zone by selecting the location of restrictive screw elements (kneading discs or reverse screw elements).

The "dead stop" operation required a notable amount of time, 4–5 minutes. However, it required a relatively short period of time for the barrel to cool down to below 80°C where the reaction essentially stops. We never found any samples inside the extruder with a higher degree of gelatinization than that of the extrudate for the same run. This indicated that the effect of "time delay" on the analytical results was not important. Furthermore, the developed kinetics were applicable to extrudates that were not affected by "time delay".

Starch gelatinization in the cooking zone

The profiles of starch gelatinization in the cooking zone for some extrusion runs were plotted as a function of distance (Fig. 2) and residence time (Fig. 3). The degree of gelatinization at first increased sharply, corresponding to the very short transition section. After that (or in the rest of the cooking zone) starch gelatinization occurred more gradually.

The rate of disappearance of ungelatinized starch can be described by the following equation:

$$\frac{d(1 - f)}{dt} = -k_g(1 - f)^m \qquad (2)$$

where f = degree of gelatinization, t = residence time counted from beginning of the cooking zone, k_g = gelatinization rate constant and m = reaction order. The order of reaction was determined by regression analysis of experimental results. Since all starch gelatinization took place in the cooking zone, only data from the cooking zone were used. At first, all nine experimental points obtained in the cooking zone were used to obtain the reaction order for each run. The regression analyses gave fractional orders of reaction around 2 (Table 3). The reaction order was totally different from those published. We assumed the difference was due to the complex starch transformation in the transition section, because starch gelatinization took place rapidly at first and then more gradually (Fig. 2 and 3). Regression analysis was applied again without the data in the transition section, and the reaction orders were around 1 with better correlation coefficients (Table 3). This indicated that the transformation of starch in the transition section may occur though a different mechanism than in the rest of the cooking zone where starch gelatinization might follow pseudo-first-order kinetics. The integral forms of both first order and second order rate laws were applied to fit the data to further investigate kinetics (Table 4). The first-order form gave better correlations for the data excluding the transition section than for the whole cooking zone, while the second-order form showed better correlations for data excluding the last datum (extrudate). Regression results seemed to suggest that starch gelatinization in extruders followed more than one reaction mechanism, described initially by pseudo-second-order kinetics and then transiting to a pseudo-first-order reaction.

Effects of temperature and shear

During extrusion cooking both temperature and shear are responsible for starch gelatinization (Lai and Kokini, 1991). This was confirmed by our results. The influence of operating conditions (Fig. 4) showed starch gelatinization increased sharply with increasing barrel temperature when moisture was higher (30%), and increased gradually when moisture was lower (25%). Chiang and Johnson (1977) reported similar results for extrusion of wheat flour with a single-screw extruder. Although higher screw speeds reduce residence time, an increase in screw speed from 200 to 300 rpm increased degree of gelatinization for all give combinations of moisture content and barrel temperature used. This confirmed that shear is a significant contributor to starch gelatinization. The effect of shear was also supported by the observed effect of moisture content on gelatinization at lower barrel temperatures when the mechanical effect dominates, and shear stress is responsible for most changes (Davidson et al., 1984). The viscosity of the melt is higher at lower moisture contents resulting in increased mechanical shear stress and thus increasing degree of gelatinization. Results agreed with those of Gomez and Aguilera (1983, 1984) and Bhattacharya and Hanna (1987).

Kinetics model for starch gelatinization

Starch gelatinization in an extruder does not follow a simple reaction mechanism. The reaction follows a pseudo-second order rate law in the transition section, followed by a pseudo-first order rate law. The transition section was too short to obtain a range of well defined samples, define second order rate constants and define the shape of the transition from second to first order. To provide reasonably accurate data on degree of gelatinization for the extrudates that may be substituted into starch degradation models, a pseudo-first-order reaction was used to describe starch gelatinization. The first-order form gave a better fit of experimental data for the complete cooking zone, than did the second-order form (Table 4). The treatment seemed justified since a pseudo-first-order mechanism was also previously suggested (Burros et al., 1987; Lai and Kokini, 1991). Accordingly, the gelatinization model was represented by the equation:

$$\ln(1 - f) = -k_g t \quad (3)$$

In a shearless environment the temperature dependence of the rate constant was assumed to conform to the Arrhenius equation (Burros et al., 1987):

$$k_g = k_0 \cdot e^{-(\Delta E/RT)} \quad (4)$$

where ΔE is the thermal activation energy, T the temperature, R the ideal gas constant, and k_0 is the pre-exponential factor. However, starch gelatinization in an extruder channel takes place in a shearing environment. Both published and our results suggested that shearing has a significant effect on gelatinization. Since starch gelatinization is due to the rupture of hydrogen bonds (Birch and Priestley, 1973), the theories for the effect of shear stress on bond rupture could be adapted to describe the effect of shear stress on gelatinization. Butyagin (1971) suggested that the dissociation of the strained bonds could be described by a first-order reaction where the rate constant followed the Arrhenius law. For thermal decomposition ΔE is the same as the bond energy ΔE_0. When the bonds are mechanically strained, ΔE is replaced by the expression for strength of the elastically deformed bond. Bueche (1960) indicated that shearing action caused tensile stress on the bonds and ΔE could be expressed as:

$$\Delta E = \Delta E_0 - F\delta \quad (5)$$

where F is the tension on the bond and δ is a distance approximately equal to the distance the bond would stretch before breaking. Therefore, the rate constant for starch gelatinization during extrusion may be represented as:

$$k_g = k_0 \cdot \exp[-(\Delta E_0 - \beta\tau)/(RT)] \quad (6)$$

$$\tau = \eta\dot{\gamma} \quad (7)$$

where τ is shear stress, β activation volume, $\dot{\gamma}$ shear rate, and η apparent viscosity. From Eq. (6) we can see that when $\tau = 0$, Eq. (6) simplifies to the equation for a shearless environment, i.e. Eq. (4). This suggests that starch gelatinization in the extruder is basically a thermal process and the effect of shear stress in the extruder mainly contributes to the rate constant by "activating" chemical bonds so that starch is more susceptible to thermal influence. Therefore, the overall gelatinization process can be described by:

$$f = 1 - \exp\{-k_0 \exp[-(\Delta E_0 - \beta\tau)/(RT)]t\} \quad (8)$$

The starch melt is a non-Newtonian fluid and η can be described by the power law model of Harper et al. (1971):

$$\eta = 78.5 \exp(2500/T) \exp(-7.9M)^{-0.49} \quad (9)$$

where M is the moisture content.

This equation gave a good approximation of the viscosity of wheat starch during extrusion cooking with a single-screw extruder (Davidson et al., 1984; Diosady et al., 1985). No simple equation to estimate average shear rate is available for twin-screw extruders. In a single-screw extruder, the screw geometry is relatively simple and the average shear rate was often approximated by $\dot{\gamma} = (\pi D\omega)/H$, where H is the average screw channel depth, D is the diameter of the screw, and w is the screw speed. We can see that D/H is geometry dependent and this equation could be generally expressed as

$$\dot{\gamma} = G\omega \quad (10)$$

where G is the geometrical coefficient. Shear rate is proportional to the screw speed. For a single-screw extruder, $G = (\pi D)/H$. Although the geometry of the screw channel in a twin-screw extruder is more complex than that in a single-screw extruder, for a given screw configuration G is constant, and thus

$$\tau = \eta \cdot \dot{\gamma} = 78.5 \exp(2500/T) \exp(-7.9M)(G\omega)^{0.51} \quad (11)$$

The experimental results of the degree of gelatinization of extrudates were used to estimate the parameters of Eq. (8), giving $k_0 = 5716.3\ S^{-1}$, $\Delta E_0 = 45636.9$ J/kg-mol, and $\beta \cdot G^{0.51} = 1.784 \times 10^{-3} m^3/kg\text{-mol}$, with correlation coefficient $R^2 = 0.944$ and mean square of errors $s^2 = 0.0699$. (Since G is constant for the given screw configuration and its value is unknown for this work, $G^{0.51}$ was combined with β for the regression analysis). Figure 5 shows the comparison of the experimental values for the degree of gelatinization of extrudates with predicted values calculated by Eq. (8). Starch gelatinization during extrusion cooking could be predicted with reasonable accuracy by the first-order model where the rate constant was a function of both temperature and shear stress. Unfortunately, while this first-order model empirically predicted the extent of gelatinization of extrudates, it did not adequately represent the true reaction mechanism in the extruder channel.

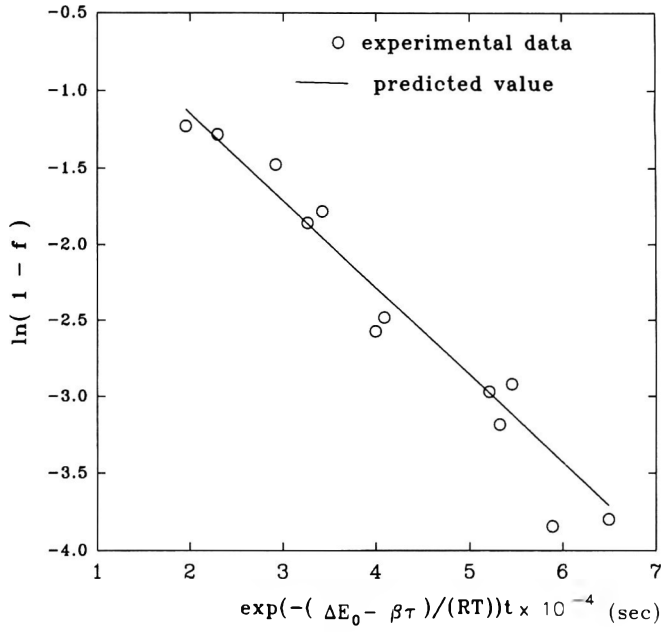

Fig. 5—Comparison of experimental values for degree of gelatinization of extrudates.

CONCLUSION

THE EXTRUDER CHANNEL could be divided into two major functional zones: a solid conveying zone and a cooking zone. The physical transition from solid to melt occurred in a relatively short section at the beginning of the cooking zone.

—Continued on page 887

Dynamic Rheological Measurement of Structure Development in High-methoxyl Pectin/Fructose Gels

M.A. RAO and H.J. COOLEY

ABSTRACT

Incipient structure development (SD) in 65% fructose—0.5, 0.75, and 1% high—methoxyl (HM) pectin gels (pH 2.7) being cooled (50–10°C) were measured in terms of dynamic viscosity (η^*) at 1Hz. SD rates (poise/min) were higher at lower temperatures, higher pectin concentrations, and when pectin was hydrated for 16 hr. Below the gel temperature, SD rates in 1% gel followed the Flory–Weaver model. SD in stored 0.5% gels during 22 days was strongly affected by storage temperature (2–38°C) and pH. SD rates in pH 2.7 gels were positive above, but negative below 18°C.

Key Words: rheology, pectin, gel structure, viscosity

INTRODUCTION

HM PECTIN/SUGAR gels are formed by a combination of hydrogen bonding and hydrophobic effects (Oakenfull, 1991). The latter are affected by the solutes (e.g., sugars) used and the temperature (Oakenfull and Scott, 1984). At the molecular level, pectin gels may be considered to be homogeneous and may be described as "association networks" (Clark et al., 1990) as opposed to the particulate nature of many denatured protein gels (Clark and Ross-Murphy, 1987).

Small amplitude oscillatory (dynamic) rheological tests have been used for studying the structure development (SD) of many food gels: gelatin, *i*-carrageenan, proteins, and milk (Doublier et al., 1992; Ross-Murphy, 1991). In a dynamic rheological (DR) test, the energy stored (G′, Pa) and the energy dissipated (G″, Pa) by a test sample are determined during a sinusoidal strain cycle (Ross-Murphy, 1984; Doublier et al., 1992; Rao, 1992) so that both the elastic and the viscous properties are measured. Another rheological property of interest is the complex viscosity (η^*); where, $\eta^* = G^*/\omega$, where $G^* = \sqrt{(G')^2 + (G'')^2}$ and ω is angular frequency. It is important that the test be conducted under small strains, typically 1–10%, so that the data are obtained in the linear viscoelastic range (Ross-Murphy, 1984).

Because high fructose corn syrup is being used to make pectin gels, fructose was chosen to prepare gels for our study. Earlier, we (Rao et al., 1993) pointed out that gel formation in HM pectin/fructose systems is a relatively slow process, that the gels can be classified as weak gels, and that the temperature at which the gel state is reached can be determined according to the Winter and Chambon (1986) criterion using DR tests. The objectives of this study were to determine incipient SD rates in 65% fructose—0.5%, 0.75%, and 1% gels being cooled from 50°C to 10°C and SD rate in 0.5% gels stored for 22 days at different temperatures. The effect of hydration of pectin on SD rates and magnitudes of DR properties was also examined.

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MATERIALS & METHODS

HM PECTIN-65% FRUCTOSE, 0.1 moles NaCl dispersions were made in a round bottom flask equipped with a reflux condenser and a magnetic stirrer. In gels made with unhydrated pectin, first crystalline NaCl (AR grade, Mallinckrodt, Paris, KY) (1.13g) was dissolved in sodium citrate buffer (0.05M, 3.0 pH) (97.5 grams) under agitation followed by fructose (Crystar, A.E. Staley) (162.5g) at room temperature (≈23°C). NaCl was added to control the ionic strength of each dispersion (da Silva et al., 1992). The mixture was then heated to 100±1°C and the pectin (Genu Pectin, type BB, rapid set 150°, Hercules Inc., Newark, DE) was (2.5g) added slowly. The mixture was held under agitation at 100±1°C for 8 min. The galacturonic acid content and the degree of methyl esterification of the pectin were 55% and 49%, respectively.

For hydrated pectin dispersions, first NaCl was dissolved in sodium citrate buffer, and pectin was dispersed and slow agitation with a magnetic stirrer was continued for 16h at room temperature (≈23°C). Fructose was added under agitation as the dispersion was heated to 100±1°C. The mixture was held under agitation at 100±1°C for 8 min. To obtain 0.5% and 0.75% pectin dispersions, the amounts of buffer, fructose, pectin and NaCl were adjusted keeping the total weight constant. The pH of the dispersions was 2.68.

A cone (4 cm diam, 2°) and plate system of a Carri-Med rheometer (Valley View, OH) connected to an IBM PS2/30 computer, and Carrimed 50 software were used to obtain rheological data. A solvent trap was placed on top of the cone to minimize moisture loss.

Incipient SD rate

Hot fructose-pectin dispersion was placed on the plate of the rheometer set at 50°C and the plate was raised to form the set gap with the cone. Temperature sweeps over 50–10°C were conducted in 2h at 1Hz and 3% strain. Magnitudes of time, temperature, G′, G″, η^*, and tan (δ) were recorded. From the time–η^* data, SD rates (poise/min) were calculated after cubic spline interpolation with a FORTRAN program on a computer (Macintosh IIsi, Apple Computer, Cupertino, CA) as a function of temperature. On separate samples, frequency sweeps 0.01–10Hz (0.06–63 rad/s, 3% strain) were conducted at 50, 40, 30, 20, 15, and 10°C. Existence of linear viscoelastic range at each temperature was checked from torque sweeps at 5Hz. Each torque sweep was conducted in 5 min, and a frequency sweep in 30 min.

Long-term SD

Hot fructose-hydrated pectin dispersions (pH 2.7) were poured into glass vials (16 mm i.d., 50 mm height). Additionally, a dispersion with pH 3 was prepared. The stoppered vials were stored for 22 days in chambers at 2, 11, 18, 25, and 38°C. The vials were removed periodically for DR tests. The top crust in a vial was carefully removed and discarded, and a representative sample was placed on the rheometer plate. Frequency sweeps were conducted at 10°C. Time (day)–η^* data at 25°C, 5 Hz, were used in estimation of SD rates (poise/day).

RESULTS & DISCUSSION

Frequency sweep data on unaged gels

Frequency sweep data on unaged gels served two purposes. They showed that magnitudes of G′ and G″ of hydrated pectin gels were higher than those of unhydrated pectin at 10°C and 20°C (Figs. 1 and 2) for 0.5% and 1.0% pectin gels, respectively. Data on 0.75% gel are not shown. The considerable scatter in the G′ values of unhydrated 0.5% pectin was due to

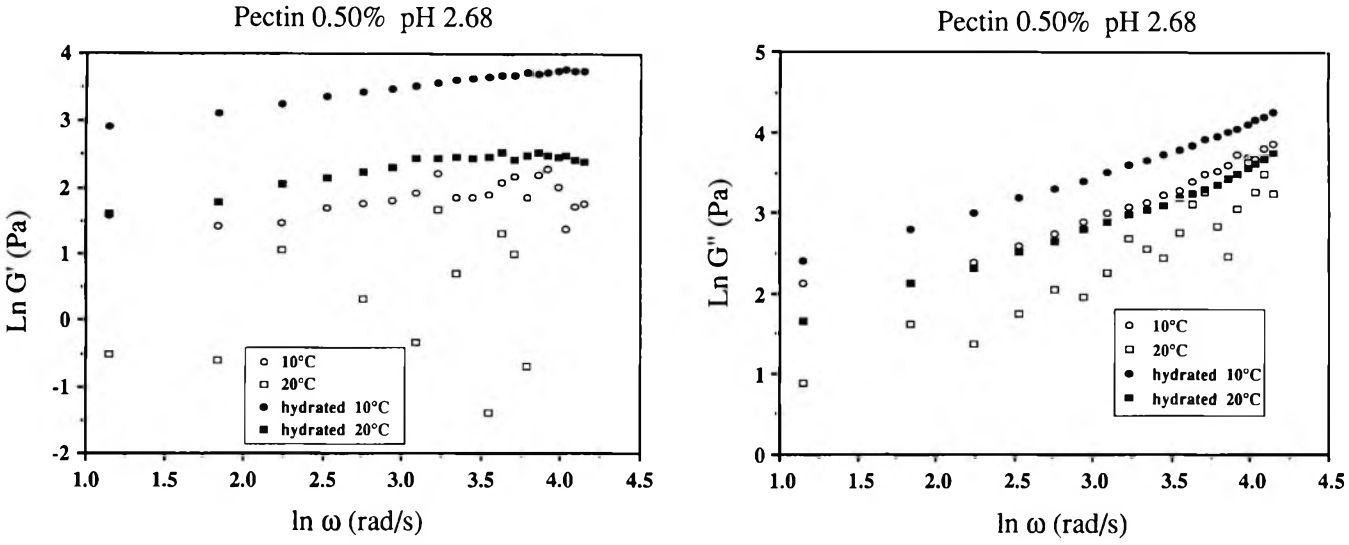

Fig. 1—Relationships of ln G′ and ln G″ to ln ω data on hydrated and unhydrated 0.5% pectin, 65% fructose. Data taken at 10°C and 20°C. G′ and G″ values of hydrated pectin sample were higher than those of the unhydrated sample.

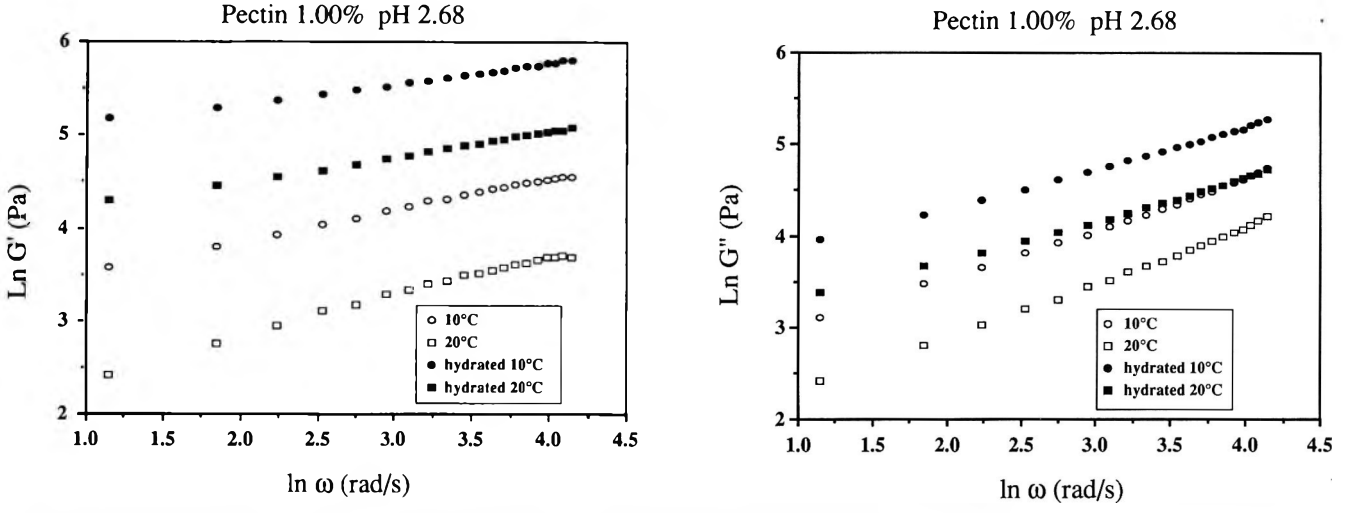

Fig. 2—Relationship of ln G′ and ln G″ to ln ω data on hydrated and unhydrated 1.0% pectin, 65% fructose sample. G′ data does not show the elastic plateaus at high ω values seen in Fig. 1.

its very weak gel nature. As expected, magnitudes of G′ and G″ increased with pectin concentration. Further, G′ data of the 1% gel did not show elastic plateaus at high ω values seen for the 0.5% gel. Sweeps of unaged gels were also used in evaluation of the temperature (T_m) at which gel state was reached using the criterion of Winter and Chambon (1986) (Rao et al., 1993). At the gel state, G′ and G″ are proportional to ω^x, where ω is the oscillation frequency and the common exponent x is <1, over a wide range of frequencies, i.e., plots of ln G′ and ln G″ against ln ω would be parallel lines (not shown). The magnitude of T_m was employed in testing the model of Flory and Weaver (1960) as discussed later.

Incipient SD rates

In temperature sweeps, with almost all gels, both G′ and G″ were positive over the temperature range, so magnitudes of η* were considered reliable measures of gel strength. Tests on incipient and long-term SD were replicated, and trends in results were reproducible and the numerical values of rheological parameters were in reasonable agreement.

Although there was an overall increase in η* as the temperature was reduced from 50°C to 10°C, incipient SD rates were low especially in the 0.5% and 0.75% gels, and there was considerable scatter in magnitudes (Fig. 3). SD rates calculated from time-η* data without cubic spline interpolation and using simple forward difference numerical method showed much more scatter (not shown). Such results could be attributed to the slow SD in pectin gels (Oakenfull and Scott, 1985; Rao et al., 1993) so that incipient SD rates along the temperature path were very low. Further, the negative and positive rates reflect breakage and reformation of bonds during gelation, respectively. Overall, for a given pectin concentration, SD rates were higher in hydrated pectin samples than in unhydrated samples. The difference between hydrated and unhydrated pectin samples can be seen very clearly in the 1% pectin samples (Fig. 4). In general, SD rates were higher at lower temperatures (Fig. 3,4).

Flory and Weaver (1960) studied collagen helical to random coil transformation. They pointed out that the effect of temperature on SD rate was analogous to a nucleated crystallization reaction. From consideration of the increase in minimum length required for thermodynamic stability at a given degree of super cooling $\Delta T = (T_m - T)$, where T_m is the equilibrium transformation (sol-gel transition) temperature, the rate of transformation was shown to follow:

$$SDR = \exp[-A/BT\Delta T] \quad (1)$$

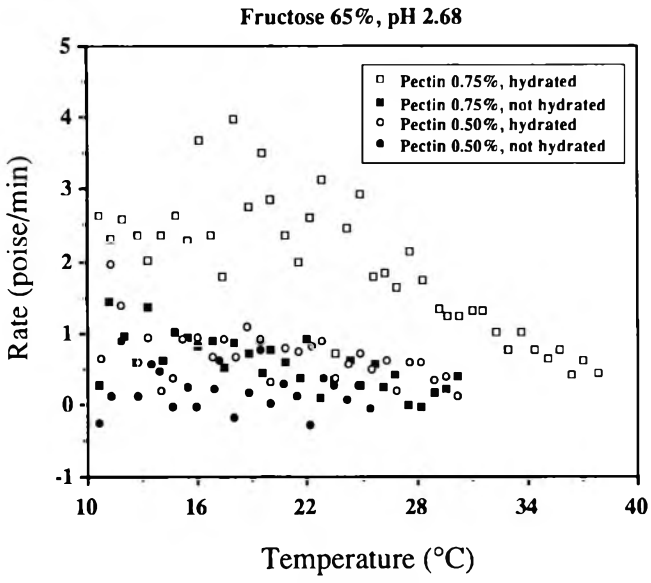

Fig. 3—Structure development rates in 0.5% and 0.75% pectin, and 65% fructose gels during cooling at 20°C/h. Complex viscosity (η) was used as measure of structure development.*

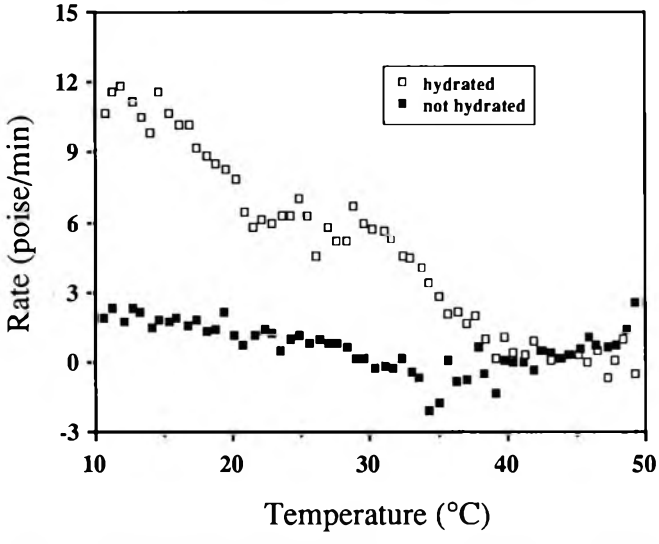

Fig. 4—Structure development rates in 1% pectin, and 65% fructose gel during cooling at 20°C/h.

where, SDR is structure development rate, also called gelation rate, T is gel temperature, and A and B are constants. It follows that the change in rate with temperature increases as T_m is approached. It also follows that Arrhenius plots: ln (SDR) vs. (1/T, °K) would be nonlinear.

For the SD rate-temperature (°K) data of 1% unhydrated pectin gel over the range 10°C to 30°C, the constants A and B (Eq. 1) were estimated by nonlinear regression analysis using the Optimization program in GENSTAT (Numerical Algorithms Group, Oxford, UK) on a computer (Model 9750, Prime Computer, Inc., Framingham, MA) to be −206 and 0.3, respectively, with $R^2 = 0.84$. The magnitude of T_m was taken as the gel state temperature (281°K) using the Winter and Chambon (1986) criterion based on DR tests. The experimental data were above T_m and the SD rate of the unhydrated pectin gel followed well the Flory-Weaver model (Fig. 5). However, the Flory-Weaver model prediction included a concave up shape, while the experimental data did not show such a trend.

For the hydrated pectin gel, T_m was about 300°K. Again,

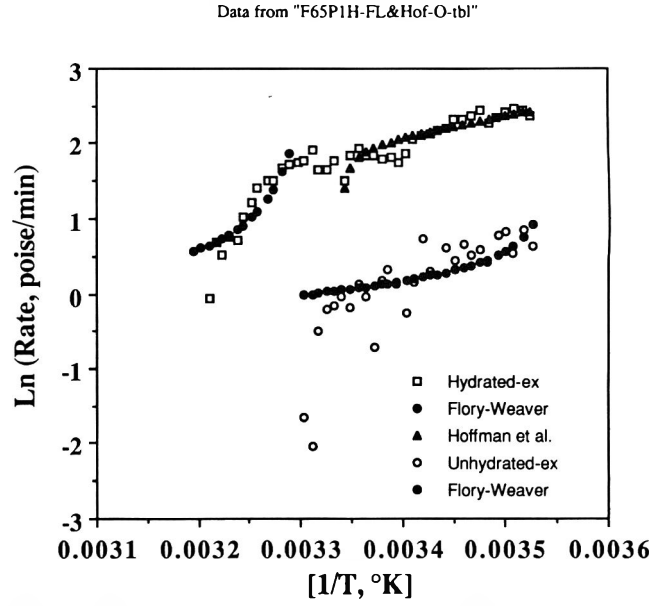

Fig. 5—Comparison of experimental structure development rates in 1% hydrated and unhydrated pectins, and predicted values from Flory-Weaver (1960) and modified Hoffman et al. (1975) models. Open symbols, experimental data. Closed symbols predicted values.

Eq. (1) predicted a concave up shape for SD rate of the gel above T_m (Fig. 5). For SD rate-temperature data below T_m, a modified form of the crystal growth model of Hoffman et al. (1975) described well the data (Fig. 5):

$$SDR = A^*\exp\left(\frac{B}{T}\right)^*\exp\left(\frac{-C}{T^*\Delta T}\right) \qquad (2)$$

where, T is temperature (°K) and $\Delta T = (T_m - T)$, and A, B, and C are constants. In comparison with Eq. (1), Eq. (2) contains an extra term. Magnitudes of A, B, C, and R^2 were 2.5×10^{-3}, 2.4×10^{3}, 1.89×10^{-3}, and 0.91, respectively. It is possible to fit the modified Hoffman model to segments of the entire SD rate-temperature data. However, it was developed specifically for fitting growth data below the gel temperature (Hoffman et al., 1975).

Long-term SD and SD rates

Over 22 days, magnitudes of G′, G″, and η* at 5 Hz of 0.5% hydrated pectin gel, pH 2.7, decreased when stored at 2°C and 11°C, remained virtually unchanged at 18°C, and increased at 25 and 38°C (Fig. 6). Calculated SD rates at 25°C were highest initially. They were 0.34, 0.20, and 0.23 poise/day after the first, second, and third days, respectively. Magnitudes of SD rates after the third day ranged between −0.05 and 0.12 poise/day. Again, the negative values are the result of breakage of bonds in the gel. In contrast to the pH 2.7 gel, magnitudes of G′, G″, and η* at 5 Hz of the pH 3.0 gel decreased at all storage temperatures (Fig. 7). Most of the decrease took place in the first three days. These results were attributed to hydrophobic interaction of the ester methyl groups that is strongly influenced by temperature, pH, and nature of cosolutes (Oakenfull and Scott, 1984). They suggest that pH and storage temperature must be carefully controlled in the study of pectin gel strength. While the importance of pH was well studied using sag tests (May and Stainsby, 1986), the importance and effects of storage temperature and time were not explored.

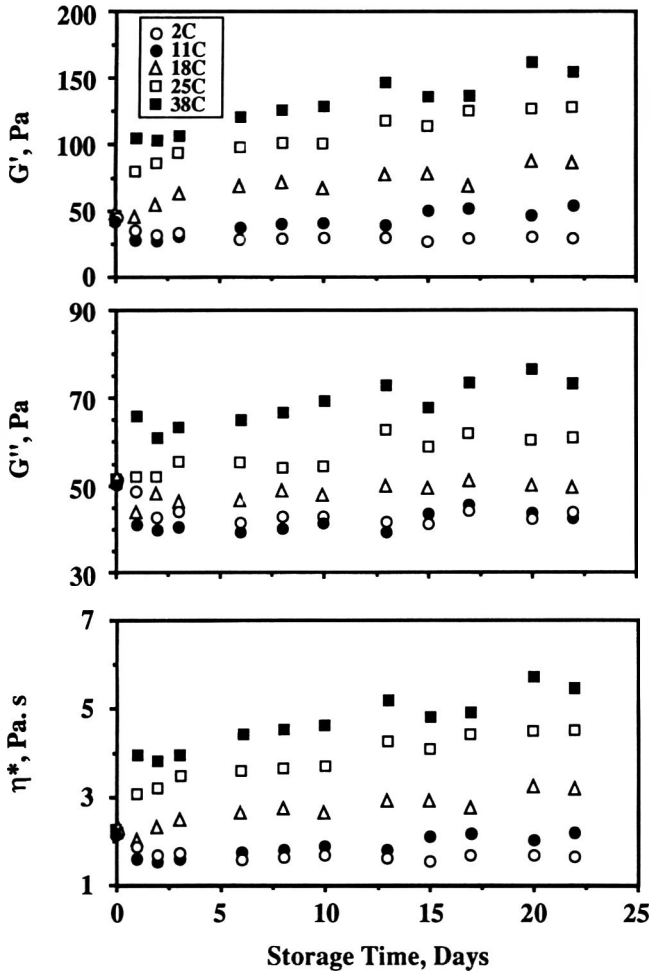

Fig. 6—Changes in G', G", and η in 0.5% hydrated pectin and 65% fructose gel, pH 2.7, stored for 22 days at different temperatures. Below 18°C rheological properties decreased with time.*

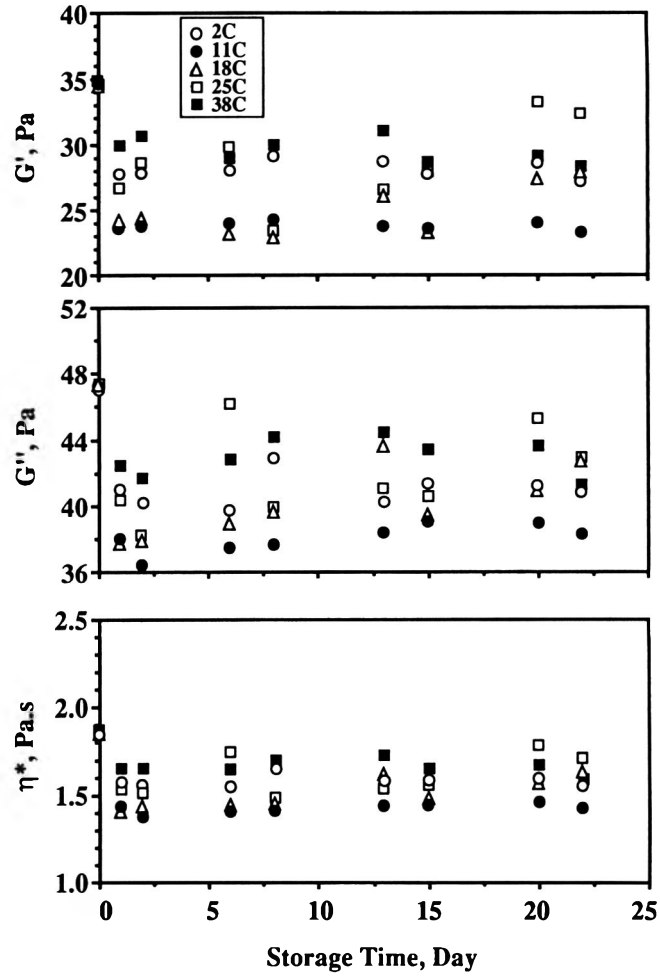

Fig. 7—Changes in G', G", and η in 0.5% hydrated pectin and 65% fructose gel, pH 3.0, stored 22 days at different temperatures. Rheological properties decreased with time at all temperatures.*

CONCLUSIONS

INCIPIENT SD rates in 0.5% and 0.75% pectin, 65% fructose gels were low. Negative SD rates may be due to breakage of bonds during gelation. Hydration of pectin for 16h increased SD rates and gel elasticity. The Flory-Weaver model described well the effect of temperature on SD rates below gelation temperature. Over 22 days SD was influenced strongly by temperature of storage and pH of the gels. SD rates of pH 2.7 stored gel were highest during the first three days and decreased subsequently. Viscoelastic properties of the pH 2.7 gels stored at temperatures higher than 18°C increased during storage, while the properties of gels stored at lower temperatures decreased. Because the viscoelastic properties of stored gels changed rapidly during the first three days, it would be advisable to conduct gel strength measurements after that time.

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Rheological Relationships Between Surimi Sol and Gel as Affected by Ingredients

B. YOO and C. M. LEE

ABSTRACT

Rheological properties of surimi sol showed that consistency index (K) was surimi concentration-dependent, as it decreased with increased level of ingredients except for carrageenan which increased K value. The flow behavior index and textural properties appeared to be a function of type and level of ingredients. Unlike starch and synergistic carrageenan, egg albumin, oil and i-carrageenan did not show a composite reinforcing effect. K values measured by rotational cylindrical spindle viscometry more closely correlated with textural properties ($r = 0.87$ for compressive force; $r = 0.87$ for shear force) than did other viscometries studied.

Key Words: surimi, rheology, composite system, consistency, flow behavior

INTRODUCTION

COMPOSITE SYSTEMS consisting of solid particulates dispersed in a polymeric matrix are frequently used in the plastics industry to enhance mechanical and thermal properties. The incorporation of particulates, however, may critically alter flow behavior characteristics of the system (Poslinski et al., 1988). Food systems are complex mixtures of many components, and may be considered as composites in which particulate ingredients are dispersed in a continuous matrix. The mechanical properties of solid food composites should behave in a manner similar to that predicted for polymers and plastics (Nielsen, 1974). Physical and structural principles for polymer composites have been applied in explaining the rheological behavior of food composite systems. Examples are the composite effects of glass particle size (Ross-Murphy and Todd, 1983; Langley and Green, 1989), fat droplets of different sizes and hardness (Kazantzis and Lee, 1983), and starch/protein (Lee and Kim, 1986; Kim and Lee, 1987). Surimi sol and gel containing various ingredients were expected to show rheological behavior as a composite.

Evaluation of the rheological properties of surimi sol would be useful not only in predicting textural properties but also in characterizing flow behavior and extrudability during fabrication. The rheological properties of surimi sol and gel are influenced by the composite characteristics determined by the type and level of ingredients as well as the dispersion and physical state of particulates (Lee and Kim, 1986). In our study, the composite characteristics were varied by dispersing biopolymeric ingredients in a surimi sol, as well as by altering the physical state of the dispersed phase through heating. Other studies concerning surimi have focused on the effects of ingredients on rheological properties of surimi or fish protein gel (Lee and Abdollahi, 1981; Burgarella et al., 1985a, 1985b; Lee and Kim, 1985; Wu et al., 1985; Kim and Lee, 1987; Hastings and Currall, 1988). However, no comprehensive studies have been reported regarding the effects of ingredients on rheological properties of both surimi sol and gel in the composite system.

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Our objectives were: to determine how composite characteristics influence rheological properties of surimi sol and gel, and to compare viscometric methods for correlating rheological properties of surimi sol with those of gel in the composite system.

MATERIALS & METHODS

SPRAY-DRIED egg albumin (Monark Egg Products, Kansas City, MO), starch (40% Melojel and 60% Frigex, National Starch, Bridgewater, NJ), Mazola corn oil (Best Foods, Englewood Cliffs, NJ), iota-carrageenan without (SD389) and with cation-donating synergists (mixture of K_2CO_3, Na_2CO_3 and $CaSO_4$) (ME621, formerly XP8009) (FMC, Philadelphia, PA) were used as representative ingredients in the surimi composite. Melojel is composed of unmodified 25% amylose and 75% amylopectin corn starch, while Frigex is hydroxypropylated modified 18–28% amylose and 72–82% amylopectin tapioca starch. The levels of each ingredient (egg albumin, 1–3%; starch, 4–8%; oil, 2–6%; Carrageenan-SD, 0.1–0.3%; Carrageenan-ME, 0.2–0.6%) were selected on the basis of commercial usage. Surimi prepared from Alaska pollock (*Theragra chalcogramma*) was supplied by Profish International (Seattle, WA).

Preparation of surimi sol and gel

Half-thawed surimi (−2°C) was chopped for 2 min with 1.5% salt (surimi weight basis) in a silent cutter, followed by additional chopping for 8 min with ingredients. Water was added to adjust final moisture of all formulas to 78% so results would reflect effects of ingredients. Immediately after preparation, the surimi sol (12 ± 1°C) was evaluated for its flow behavior before it gelled. For preparation of heat-induced surimi gels, molded samples were prepared by stuffing the remaining surimi sol into casings (30 mm diameter, Nojax cellulose; Viskase Sales Corp., Chicago, IL) and heating at 90°C in a steam cooker for 20 min, while fiberized samples were prepared by extruding surimi sol into a sheet, partially heat-setting, scoring, folding, and steam-cooking 15 min (Yoon and Lee, 1990). These heat-induced surimi gels were used for evaluating gel-forming ability.

Flow behavior of surimi sol

The flow behavior of surimi sol was evaluated by capillary and slit rheometry using an Instron testing machine (Model 1122) and rotational disc and cylindrical spindle viscometry using a Brookfield viscometer, respectively (Fig. 1). The capillary and slit extrusion cell, and the disc spindle were constructed at the Engineering Instrument Shop of the University of Rhode Island. From duplicate viscosity

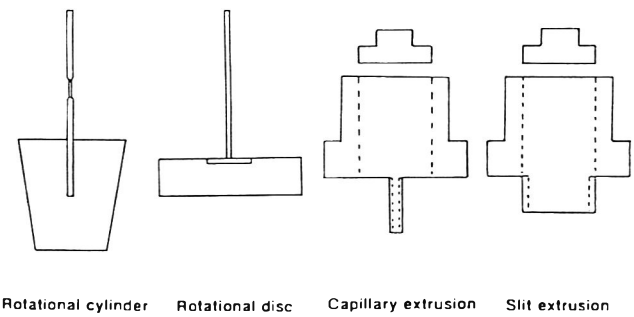

Fig. 1—Viscometric apparatus.

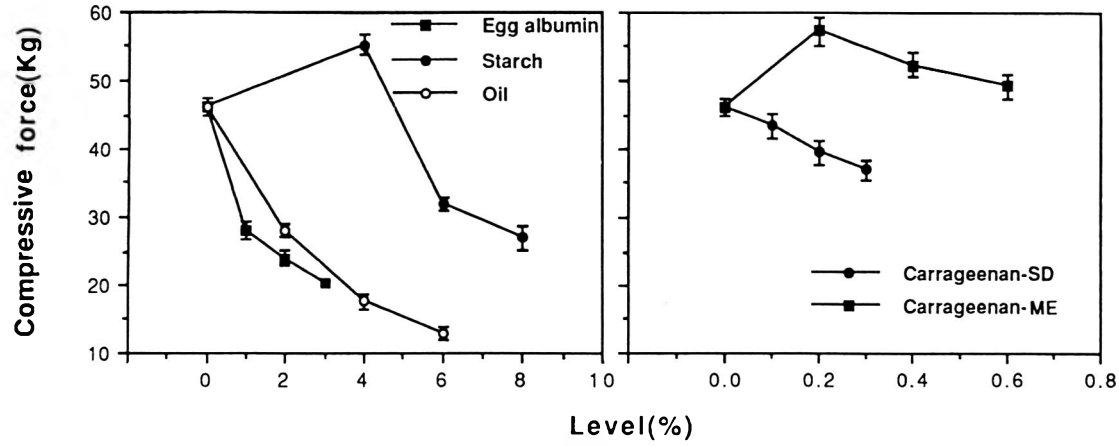

Fig. 2—Effect of various ingredients on compressive force of surimi gel.

Table 1—K values of surimi sols containing ingredients at varying levels by various viscometric methods

Sample	Level (%)	Consistency index (K) (dyne sec^n/cm^2) Capillary (× 10^6)	Slit (× 10^5)	Disc (× 10^4)	Cylinder (× 10^4)
Control		1.31	4.48	0.97	2.04
Egg albumin	1	1.18	4.10	0.90	1.90
	2	1.00	3.41	0.83	1.67
	3	0.88	3.09	0.76	1.63
Starch	4	0.98	3.88	0.81	2.02
	6	0.86	3.76	0.78	1.80
	8	0.76	3.08	0.71	1.63
Oil	2	0.91	3.76	0.94	1.75
	4	0.84	3.68	0.72	1.54
	6	0.71	2.92	0.66	1.43
Carrageenan (SD-389)	0.1	1.57	5.44	0.95	1.87
	0.2	1.55	4.93	1.02	2.15
	0.3	1.55	5.00	0.98	2.24
Carrageenan (ME-621)	0.2	1.34	4.63	1.01	2.46
	0.4	1.35	5.03	1.08	2.49
	0.6	1.41	5.62	1.02	2.51

measurements on two separate preparations, consistency index (K) and flow behavior index (n) were determined using a power law equation. The measurements were replicated two times at each speed for rheometry and at each rpm for viscometry. Using different viscometric methods, the flow behavior of surimi sol was determined as follows:

Capillary extrusion rheometer. The capillary extrusion cell was filled with surimi sol without inclusion of large air pockets. The measurement was made at crosshead speeds of 0.5, 1, 2, 5, 10 and 20 cm/min, while extruding a sol through a capillary (diameter 0.6 cm; length 8.4 cm). The extrusion force was obtained by subtracting the force measured at an orifice from that with a capillary attached (Mayfield et al., 1978). The shear stress (τ) and shear rate ($\dot{\gamma}$) were calculated using the following equations (Van Wazer et al., 1963):

$$\tau = \frac{r(F_c - F_o)}{2A\Delta L}; \quad \dot{\gamma} = \frac{4}{\pi r^3}Q$$

where r = radius of capillary (0.3 cm); F_c = force required to extrude the sample through the capillary; F_o = force required to extrude the sample without the capillary; A = cross-section area of capillary (0.28 cm^2); ΔL = length of the capillary (fixed at 8.4 cm); Q = flow rate (crosshead speed × cross section area of extrusion cell, 19.6 cm^2).

Slit extrusion rheometer. The measurement was made at crosshead speeds of 0.5, 1, 2, 5, 10 and 20 cm/min while extruding a sol through a rectangular flat opening (length 2 cm; width 3.9 cm; thickness 0.15 cm). The extrusion force was obtained by subtracting the force without a nozzle (L = 0) from that obtained with nozzle. The shear stress (τ) and shear rate ($\dot{\gamma}$) were calculated using the following equations (Dealy, 1982):

$$\tau = \frac{h(F_c - F_o)}{2A\Delta L}; \quad \dot{\gamma} = \frac{6Q}{wh^2}$$

where L = length of rectangular flat (2 cm); w = width (3.9 cm); h = thickness (0.15 cm); F_c = force to extrude the sample through the rectangular flat; F_o = force to extrude the sample through a slit at L = 0; A = cross-section area of slit (0.59 cm^2); Q = crosshead speed × cross-section area of extrusion cell (19.6 cm^2).

Rotational disc and cylindrical spindle viscometers. Small portions of the surimi sol were placed in a petri dish (8.8 cm dia × 1.4 cm h) and a 150 mL plastic cup for disc and cylindrical spindle viscometers, respectively. The speed of the Brookfield rotational viscometer was set at 1, 2.5, 5, 10, 20 and 50 rpm using a disc spindle (1.46 cm diameter and 0.15 cm thick) and a cylindrical spindle (0.32 cm diameter). Shear stress (τ) and shear rate ($\dot{\gamma}$) were determined using the following equations:

$$\tau = \frac{F\ \%M}{rA}, \quad \dot{\gamma} = \frac{2\pi r\ rps}{\delta},$$

where F = full torque capacity (57,496 dyne cm); M = torque reading; r = radius of spindle (0.73 or 0.16 cm); A = surface area in which a spindle contacted with sample (2.32 or 3.01 cm^2); rps = revolution per second; δ = gap where shear motion was involved (specified at 0.1 cm).

Textural properties

Textural properties were evaluated by measuring compressive force (cohesiveness), expressible moisture (water binding ability) for molded samples with cylindrical shapes of uniform geometry (22 mm diameter × 25 mm height) (Lee and Chung, 1989) and shear force for fiberized samples using an Instron testing machine. Force required to cut through the specimen was measured as shear force using a flat rectangular blade (0.1 cm thick × 5 cm wide × 5 cm long) (Yoon and Lee, 1990).

Statistical analysis

Correlation between rheological properties of surimi sol and gel in the composite system was determined using the Statistical Analysis System (SAS Institute Inc., 1985).

RESULTS & DISCUSSION

TYPICAL FLOW behavior curves of the surimi sol (control) from various viscometric methods (Fig. 2) showed pseudoplastic flow behavior was evident in all sol samples. K value of sols decreased with increases in levels of egg albumin, starch and oil, while no noticeable differences in K value were observed in carrageenan-incorporated surimi sols (Table 1). Starch-, egg albumin- and oil-incorporated surimi sols showed consistently lower K values than controls. This suggested that starch and egg albumin had a neutral effect on viscosity of surimi sol in the cold state. Such a decrease in K value with increased levels of starch and egg albumin was probably a result of interfered cohesion in the surimi sol matrix. The decreased K value of oil-incorporated surimi sol, may be ex-

Table 2—n values of surimi sols containing ingredients at varying levels by various viscometric methods

Sample	Level (%)	Flow behavior index (n) Capillary	Slit	Disc	Cylinder
Control		0.145	0.182	0.148	0.144
Egg albumin	1	0.151	0.189	0.151	0.133
	2	0.159	0.212	0.155	0.193
	3	0.169	0.224	0.155	0.193
Starch	4	0.152	0.203	0.183	0.161
	6	0.167	0.204	0.164	0.154
	8	0.173	0.208	0.154	0.175
Oil	2	0.171	0.188	0.131	0.117
	4	0.175	0.181	0.175	0.184
	6	0.181	0.215	0.168	0.189
Carrageenan (SD-389)	0.1	0.121	0.156	0.138	0.226
	0.2	0.124	0.173	0.147	0.192
	0.3	0.131	0.181	0.155	0.195
Carrageenan (ME-621)	0.2	0.140	0.197	0.176	0.165
	0.4	0.156	0.173	0.172	0.118
	0.6	0.159	0.180	0.177	0.124

plained by the lubrication effect of oil particles as well as by interference in sol cohesion by oil particles. Such reduction of K value after addition of starch, egg albumin and oil may also be explained by the dilution effect of the ingredients. However, carrageenan-incorporated surimi sol showed higher K values than controls, producing a thickening effect of carrageenan in the cold. Glicksman (1983) and Fennema (1985) suggested that the carrageenan anion reacted with protein to form a complex that could exist as a stable dispersion and increase viscosity and gel strength. Thus, K value was dependent on level of surimi in the composite system for all ingredients evaluated except carrageenan.

For flow behavior index (n), values of all sol samples showed the pseudoplastic flow behavior as indicated by n values from 0.118 to 0.226 (Table 2). The n values measured by capillary extrusion rheometry increased, showing that surimi sols became less pseudoplastic, as levels of ingredients increased. Surimi sol containing carrageenan (SD-389) or corn oil showed marked decreases, or increases of n value, respectively, indicating most and least pseudoplastic flow. However, in the slit extrusion rheometry and the rotational disc and cylindrical spindle viscometry, there was no discernible pattern of changes in flow behavior affected by ingredients. In the slit extrusion rheometry, carrageenan (SD-389)-incorporated surimi sol showed lower n values than controls. On the other hand, in rotational disc and cylindrical spindle viscometry higher n values of surimi sol were shown when mixed with carrageenan (ME-621) and (SD-389) irrespective of the level of ingredients. Changes in n value of composite surimi sols appeared to be affected by viscometric method and type and level of ingredients.

Table 3—Correlation coefficients between K values of surimi sols by various viscometric methods and textural properties of surimi gels in the composite system

Textural properties	Capillary extrusion	Slit	Rotational (disc)	Rotational (cylinder)
Compressive force (kg)	0.67	0.73	0.74	0.87
Shear force (g)	0.72	0.77	0.81	0.87
Expressible moisture (%)	−0.58	−0.65	−0.69	−0.73

The effects of various ingredients on compressive force and shear forces (Fig. 2 and 3) showed addition of egg albumin, corn oil and iota-carrageenan (SD 389) decreased compressive forces (gel cohesiveness) of molded samples and shear forces (firmness) of fiberized samples. We hypothesized that egg albumin reduced gel strength by interfering with the formation of the continuous matrix through retardation of cross-linking of myofibrillar protein (Okada, 1964; Lee and Kim, 1985, 1986) and by dilution effects on fish protein (Burgarella et al., 1985a; 1985b).

Vegetable oil is occasionally incorporated in surimi-based products to improve texture and freeze-thaw stability (Lee and Abdollahi, 1981). They found that addition of fat reduced freeze-induced rubberiness and chewiness. Hastings and Currall (1988) also reported that addition of corn oil decreased the compressive force and breaking force of surimi gels. The reduction of gel strength by addition of oil may be explained by the interference of oil with formation of the cohesive protein gel matrix (Liu and Lee, 1992).

Starch and synergist-containing carrageenan (ME-621), increased compressive force and shear force to maxima at 4% and 0.2%, respectively (Fig. 2, 3). Note that with addition of 4% starch compressive force increased while shear force decreased. A similar observation has been reported (Yoon and Lee 1990). Such discrepancy suggested that the properties measured were different between these two tests, namely, the compression test for cohesiveness and shear test for firmness. The starch level for maximum gel strength varies with type of starch as previously reported: 6% for native potato starch (Lee and Kim, 1986) and 8% for hydroxypropylated potato starch (Chen et al., 1993). The increased gel strength of the starch-(Lee and Kim, 1985, 1986; Kim and Lee, 1987; Yamazawa, 1991) and carrageenan-incorporated surimi (Bullens et al., 1990; Lavery and Lee, 1991) has been explained well by many studies of composite reinforcing effects. Lee and Kim (1986) reported that a maximum composite reinforcing effect of starch could not be achieved if the protein matrix was filled with too many starch globules as in the case of levels higher than 4% in our study.

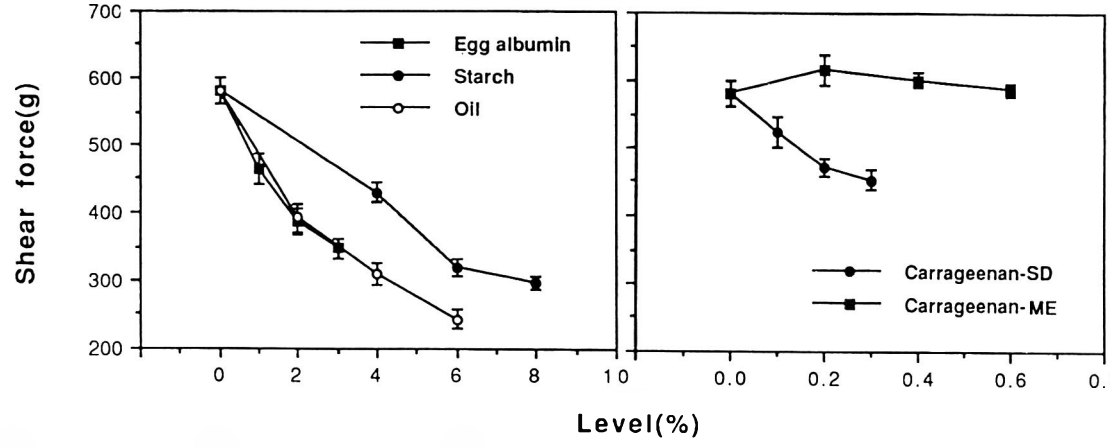

Fig. 3—Effect of various ingredients on shear force of surimi gel.

For the reinforcing effect of carrageenan, it was indicated that the synergist-containing sample (ME-621) enhanced the gel strength and water holding ability (WHA) of surimi gel by tightening the protein matrix (Bullens et al., 1990), as well as by increasing firmness and elasticity of carrageenan globules (Lavery and Lee, 1991). The synergists added to the i-carrageenan donate cations and form Ca^{2+} bridges that enhance cross-linking.

Results on composite surimi gel clearly suggested that changes in textural properties were a function of type and level of ingredients. Unlike starch and synergist-containing carrageenan (ME-621), egg albumin, oil and i-carrageenan did not show composite reinforcing effects by which the gel was strengthened.

Influence of various viscometric methods was studied on degree of correlation between textural properties of gel and K value of sol (Table 3) in the surimi composite system. Generally, moderate to good correlations ($r = 0.67$ to 0.87 for compressive force; $r = 0.72$ to 0.87 for shear force) were shown for various methods between K values and compressive and shear forces. Among them, K value measured by rotational cylindrical spindle viscometry correlated well with compressive force ($r = 0.87$) and shear force ($r = 0.87$) of surimi gel. The capillary extrusion rheometry showed lower correlation with compressive force and shear force. However, correlation coefficients of expressible moisture with K values were much lower than those of compressive and shear force. Thus, K values correlated well with compressive and shear forces of gels, but not with expressible moisture. The K value of surimi sol measured by rotational cylindrical spindle viscometry could be a good method for predicting textural properties of gels.

CONCLUSIONS

CHANGES in the textural properties of surimi gel in the composite system were a function of type and level of ingredients. Addition of egg albumin, corn oil, and iota-carrageenan weakened gel strength, while starch and synergist-containing carrageenan increased it through composite reinforcing effects. The higher K value of the carrageenan-incorporated surimi sol was due to a thickening effect of carrageenan in the cold. The K value of surimi sols prepared with starch, egg albumin and oil decreased with increases in levels of ingredients, showing that it was surimi concentration-dependent. All viscometric methods indicated that changes in flow behavior index (n) were a function of type and level of ingredient. Among those tested, the rotational cylindrical spindle viscometry gave the best prediction of gel texture from K values. Textural properties (compressive and shear force) of surimi gels clearly reflected the rheological properties of surimi sols.

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Thermal Conductivities of Starch Gels at High Temperatures Influenced by Moisture

JIANJUN WANG and KAN-ICHI HAYAKAWA

ABSTRACT

Thermal conductivities (k) of gelatinized starch gels and of starch gels with dissolved sucrose were determined at 80 to 120°C and at 39.6 to 75% moisture by a line heat source probe method. A special made sample holder prevented moisture evaporation during determination. Regression equations relating k to temperature and moisture were developed through stepwise regression analysis. Reliability of the developed equations was verified through experiments. The maximum error for regressed k values was 0.0085 *W/mK*.

Key Words: starch, gels, thermal conductivity, moisture content

INTRODUCTION

STARCH GELS are components of many foods. Their thermophysical data are key factors required for process design and optimization. Advances in computer technology have improved analytical and numerical accuracies for engineering analyses of heat transfer processes (Pham and Willix, 1989). There is need for accurate thermophysical property data, especially at temperatures > 80°C (temperatures for most thermal processes) at different moisture contents.

Thermal conductivities (k) of gelatinized starch gels below 70°C were reported by Maroulis et al. (1991). They observed that thermal conductivity values of gelatinized starch gels of 50 to 80% moisture at 30 to 70°C changed from 0.434 to 0.548 watt/meter Kelvin (W/mK). A regression equation relating k to temperature was developed by the same researchers. A proportional increase in thermal conductivity values for starch gels of 80 to 94% moisture was reported at 20 to 40°C by Drusas et al. (1986). There are no published k's of starch gels at temperatures > 100°C.

Many methods are available for thermal conductivity measurement. Among them, a line heat source probe method is most useful because of operational simplicity, quick response, and accuracy (Sweat, 1986). The basic theory and improvements for practical applications were discussed by Hooper and Lepper (1950), Blackwell (1954, 1956), Carslaw and Jaeger (1959), Nix et al. (1967), Sweat and Higgins (1973), and Murakami and Okos (1988).

The method has been applied to frozen foods (Sweat et al., 1973; Barrera, and Zaritzky, 1983), thermally processed foods (Baghe-Khandan and Okos, 1981), apple juice (Constenla et al., 1989), food powders (Murakami and Okos, 1986), fruits and vegetables (Sweat, 1974). The objectives of our study were to determine k's of starch gels with or without dissolved sucrose of different moisture contents at 80 to 120°C and to obtain reliable regression equations for estimating k's as a function of temperature and moisture.

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MATERIALS & METHODS

FIGURE 1 shows a cross-sectional view of the probe and sample holder assembly. The thermal conductivity probe sheath consisted of a 80-mm-long, 1.27 mm o.d. stainless steel tubing (D-HTX-18TW, Small Parts Inc., Miami, FL), meeting length/diameter > 25 suggested by Blackwell (1956). A stable and accurate, interchangeable miniature thermistor (Mitsubishi International Corp., Tokyo) was placed at the middle of the tubing for temperature measurement. A constantan wire (0.076 mm dia.) was a heat source, as used by many researchers (Sweat, 1974; Baghe-Khandan et al., 1981; Murakami and Okos, 1988; Rahman and Potluri, 1991). The void space inside the sheath was filled with a high thermal conductivity paste (OT-201, Omega

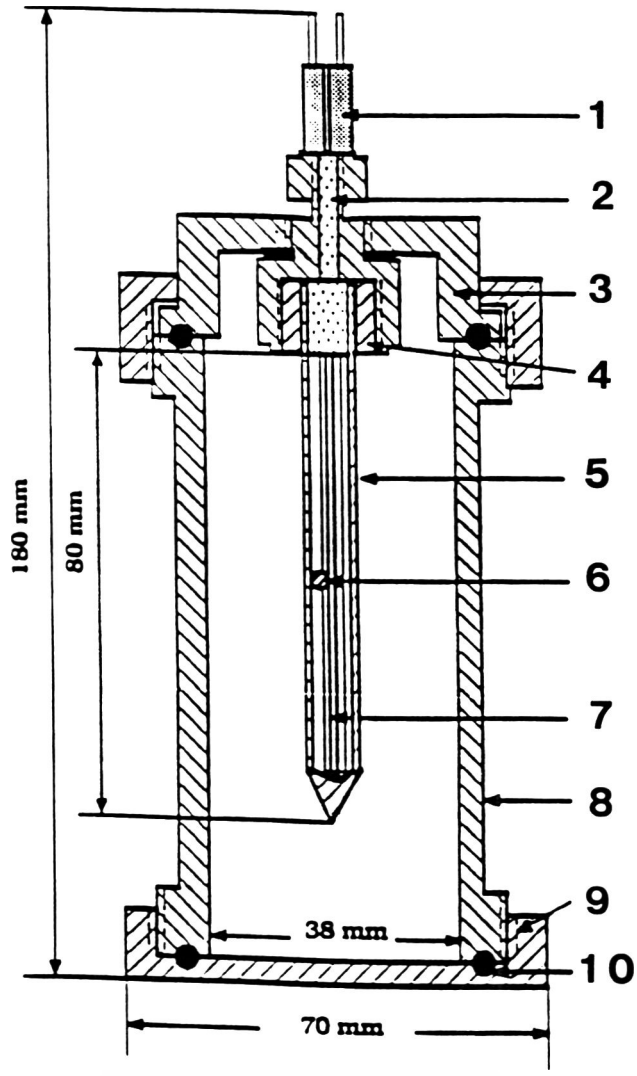

Fig. 1—A cross-sectional view of the probe and the sample holder assembly (probe diameter not in proportion) 1 = quick connector, 2 = Epoxy filler, 3 = upper lid of sample holder, 4 = Teflon insulation, 5 = 1.27 mm OD stainless steel tubing, 6 = thermistor, 7 = heater wire (constantan), 8 = sample holder body, q = bottom lid of sample holder, 10 = O-ring.

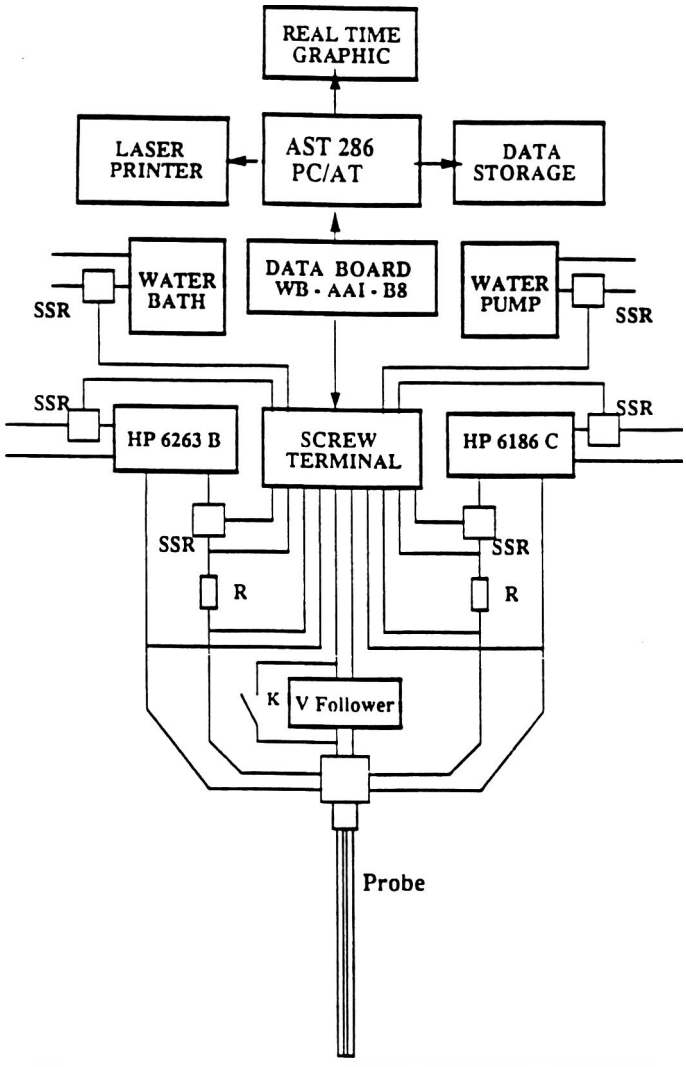

Fig. 2—System flow chart for thermal conductivity determination.

Engineering, Inc., Stamford, CT). A Teflon block (Small Parts Inc., Miami, FL) and epoxy resin filled a void space within a Swagelock fitting (R. S. Crum & Co., Mountainside, NJ) to reduce heat loss from the tubing to the sample holder eliminating metal-to-metal contact. The probe was fitted on the upper lid of the sample holder with the swagelock. The sample holder was made of aluminum alloy and the internal dimensions were based on analytical results on the minimum sample holder size (Vos, 1955). The sample holder was sealed at one end using a threaded flange-like ring to prevent relative movement between sample and probe and eliminate possible error. The other end was covered by a conventional turn-on lid. O-rings were used in all contacting surfaces to prevent moisture loss at high temperatures. The probe was calibrated using 0.5% agar gel and glycerin (99.5%), as commonly practiced (Sweat, 1986).

To facilitate data collection and analysis, a system similar to that of Murakami and Okos (1988) was developed (Fig. 2). Differences with our system were that it was usable for both thermocouple- and thermistor-based probes. It also used a high-resolution data acquisition board (WB-AAI-B8, Omega Engineering Inc., Stamford, CT), an accurate and stable voltage supplier for the heater constantan wire (HP 6263B, Hewlett Packard Co., Piscataway, NJ) and an accurate and stable current supplier to excite the thermistor (HP 6186, Hewlett Packard Co.). A voltage follower also compensated for the thermistor's sharp electrical resistance changes in the experimental temperature ranges. The follower could be bypassed when the thermocouple was used. A software program was developed to control all experimental procedures. The system was programmable, completely automated, and accomplished 0.01°C resolution temperature measurements when a thermistor was used in the probe (0.1°C resolution for a thermocouple-based system).

Table 1—Experimental thermal conductivity values of gelatinized starch gels

Temp (°C)	Moisture content (%)					
	49.18	52.12	54.23	60.44	69.97	74.97
	Thermal conductivity k (W/mK)					
120	0.4826	0.4879	0.4947	0.5074	0.5423	0.5656
110	0.4802	0.4881	0.4907	0.5039	0.5406	0.5627
100	0.4786	0.4844	0.4917	0.5062	0.5420	0.5650
90	0.4785	0.4855	0.4888	0.5044	0.5413	0.5667
80	0.4770	0.4814	0.4879	0.4999	0.5406	0.5667

Amioca starch powders containing 98% amylopectin (National Starch and Chemical Corp., Springfield, NJ) were mixed with distilled water at room temperature (≈23°C) to obtain desired moisture levels. Each sample was filled into the sample holder and gelatinized by heating 40 min in a well agitated 120°C oil bath (KS-6, Fisher Scientific, Inc., Springfield, NJ) as described by Maroulis et al. (1991). Starch gels with dissolved sucrose were prepared through similar procedures, except the sucrose was first dissolved in distilled water and kept at a starch-to-sucrose weight ratio of 4:1 for all moisture contents. The true moisture content of each sample, (average of three measurements), was determined upon completing k determination by a vacuum oven method (Helrich, 1990).

After gelatinization, samples were equilibrated to a selected oil bath temperature. The experiment began when temperature fluctuations over a 200-sec interval were < 0.02°C. The system produced a temperature response curve when plotting temperature increases over an initial level against ln(t) (t = time after heating the probe), identified the linear part of the curve, and estimated its slope by linear regression. A k value was determined by the equation $k = Q/4\pi S$ (Q is heat strength of the heat source, W/m; S = slope).

Empirical equations relating sample temperature (T) and moisture content (M) were obtained by analyzing experimental data through stepwise regression (Anonymous, 1985). To validate the equations experimentally, separate samples of different moisture contents were prepared. The k values of these samples were determined experimentally as described and compared with those estimated by empirical equations.

RESULTS & DISCUSSION

TABLES 1 and 2 show experimentally determined k values of gelatinized starch gels and gels with dissolved sucrose, respectively. Tabulated values are averages of four determinations with standard deviation < 0.008 W/mK. We observed clearly a strong moisture dependency and a weak temperature dependency of the k values. For example, there are >12% variations in k of starch gels in a moisture range of ≈ 50 to 75% and >19% k variations for gels with sucrose in a moisture range of ≈ 40 to 70%. As for temperature dependency, k variations of starch gels were < 1.5% for the range tested and those for the other gels < 1.2%

Table 2—Experimental thermal conductivity values of gelatinized starch gels with dissolved sucrose

Temp (°C)	Moisture content (%)							
	39.60	46.30	50.34	55.57	59.02	63.53	67.79	71.30
	Thermal conductivity k (W/mK)							
120	0.4416	0.4550	0.4633	0.4725	0.4854	0.5035	0.5227	0.5356
110	0.4414	0.4526	0.4628	0.4702	0.4835	0.5033	0.5215	0.5366
100	0.4416	0.4530	0.4594	0.4691	0.4832	0.5009	0.5201	0.5362
90	0.4396	0.4506	0.4624	0.4677	0.4819	0.5007	0.5183	0.5324
80	0.4363	0.4496	0.4587	0.4682	0.4807	0.5011	0.5170	0.5319

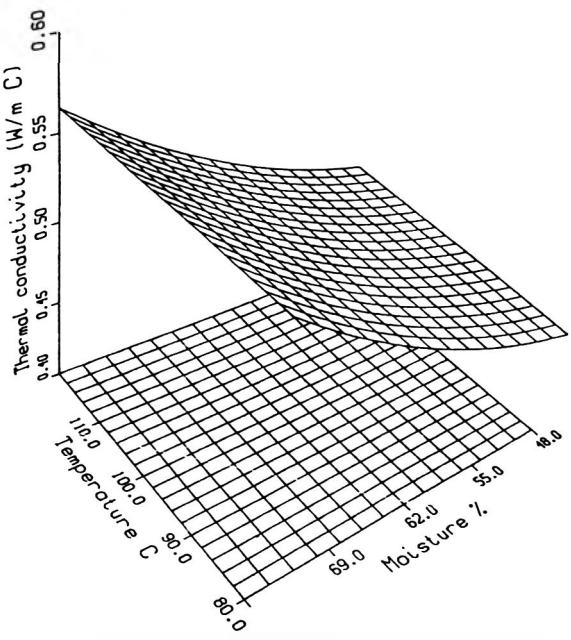

Fig. 3—Thermal conductivities of gelatinized starch gels as influenced by temperature and moisture content.

Table 3—Coefficients for regression Eq. (1) and (2)

Equation	Coefficient	Mean	Std[a]	r^2
(1)	A	0.41675583	0.004024	0.9975
	B	0.00066375	0.000106	
	C	0.00000036	0.0000001	
	D	0.00000950	0.0000013	
(2)	A	0.41829872	0.00433	0.9946
	B	0.00011312	0.000023	
	C	0.00001009	0.000003	
	D	0.00000043	0.0000004	

[a] Standard deviation

The following equations were obtained through regression analysis of determined k values:

Gelatinized starch gels

$$k = A + B * T + C * M^3 - D * T * M \quad (1)$$

Gelatinized starch gels with dissolved sucrose

$$k = A + B * T - C * M^2 + D * M^3 \quad (2)$$

where k is thermal conductivity (W/mK), T is initial temperature (°C), M is moisture content (wet base %) and A, B, C and D are statistically significant regression coefficients (Table 3). Eq. (1) is usable within a temperature range of 80 to 120°C and a moisture range of 40 to 71% and Eq. (2) within the same temperature range and a moisture range of 49 to 75%. These r^2 values were greater than those of similar reported equations, e.g., potatoes (Califano and Calvelo, 1991); starch gels, (Maroulis et al., 1991); vegetables and fruits (Vagenas et al., 1990). This was due to the accurate thermistor temperature sensor, stable power supply, and computerized operation and data collection.

Figures 3 and 4 are graphical representations of Eq. (1) and (2). Generally, k increased with increased sample moisture and/or temperature with greater influence of moisture on k. The overall temperature and moisture dependency of starch gel k's agreed with results of Maroulis et al. (1991) which covered 30 to 70°C sample temperatures and 50 to 80% moisture. Therefore, the k's of the starch gels could be estimated from room to thermal processing temperatures through combination of our results and those reported by Maroulis et al.

Tables 4 and 5 compare k's determined through separate experiments and those estimated by the regression equation.

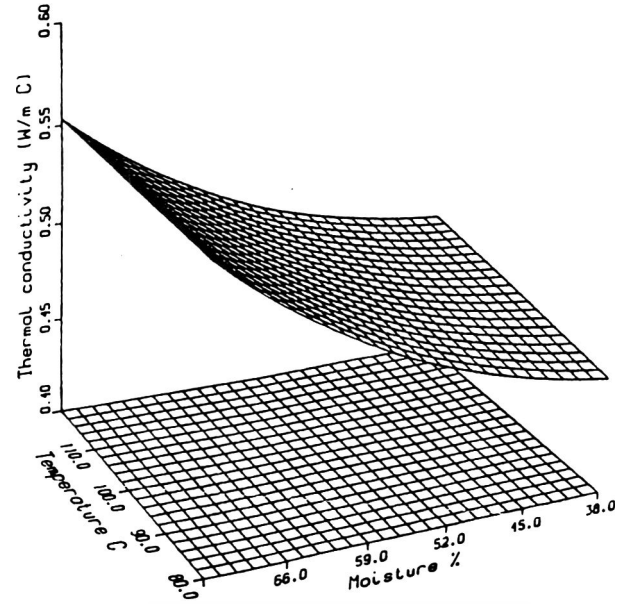

Fig. 4—Thermal conductivities of starch gels with dissolved sucrose as influenced by temperature and moisture content.

Table 4—Experimental verification of the regression equation for starch gels

Temp (°C)	Moisture (% wet base)	k (W/mK) Experiment	Eq. 1	%Error
120	74.95	0.5691	0.5626	0.66
120	63.10	0.5158	0.5149	0.36
120	60.28	0.5071	0.5065	0.06
110	74.95	0.5613	0.5630	0.17
110	63.10	0.5117	0.5142	0.25
110	60.28	0.5037	0.5056	0.19
100	74.95	0.5549	0.5634	0.85
100	63.10	0.5143	0.5136	0.07
100	60.28	0.5046	0.5047	0.01
90	74.95	0.5580	0.5639	0.59
90	63.10	0.5160	0.5130	0.03
90	60.28	0.5032	0.5038	0.06
80	74.95	0.5605	0.5644	0.39
80	63.10	0.5121	0.5123	0.02
80	60.28	0.5046	0.5029	0.3

Table 5—Experimental verification of regression equation for starch gels with dissolved sucrose

Temp (°C)	Moisture (% wet base)	k (W/mK) Experiment	Eq. (2)	%Error
120	57.28	0.4778	0.4795	0.17
120	62.24	0.4985	0.4965	0.2
110	57.28	0.4742	0.4784	0.28
110	62.24	0.4993	0.4953	0.4
100	57.28	0.4759	0.4773	0.14
100	62.24	0.4970	0.4942	0.28
90	57.28	0.4742	0.4762	0.2
90	62.24	0.4965	0.4931	0.34
80	57.28	0.4742	0.4751	0.09
80	62.24	0.4949	0.4919	0.3

Relative differences between both k's were <1%. Due to excellent reliability the developed equations could be used to analyze heat transfer in a starch gel undergoing high-temperature processing.

The k's of starch gels were compared with those of gels with dissolved sucrose at the same moistures and temperatures using Eq. (1) and (2). The starch gel k's were 2 to 5% greater than the latter k's. For example, the k's of starch gel and gel with sucrose were 0.4769 and 0.4559 W/mK, respectively, at 80°C and 50% moisture, and were 0.5400 and 0.5299 W/mK, respectively, at 120°C and 70% moisture.

The mobility of water molecules in a sucrose solution is

notably restricted according to a nuclear magnetic resonance study (Richardson et al., 1987). This implies that water molecules in a starch gel would be more mobile compared to those in a starch gel with dissolved sucrose. Therefore, lattice vibrations would likely occur more readily in the starch gel. This was probably the reason for the larger k's of the starch gels since lattice vibration is a major mechanism for heat conduction in a nonmetalic solid (Berman, 1976).

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*GELATINIZATION OF WHEAT STARCH. . .*From page 875

No significant change in length of this section was observed in the range conditions tested. The length of cooking zone was affected only by screw configurations and die sizes. Gelatinization of starch took place only in the cooking zone. Gelatinization initially followed a pseudo-second-order rate law, but it soon reverted to pseudo-first-order. The overall process could be approximated by a first order model to provide reasonably accurate data. The rate constant was a function of both shear stress and temperature.

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Determining Gelatinized Starch in a Dry Starchy Product

H.S. GURAYA and R.T. TOLEDO

ABSTRACT

Degree of gelatinization (DG) of starch in dry products was measured from the amount which dissolved in 0.2N KOH. The dissolved starch was complexed with 0.1N iodine and quantitated from absorption at 600 nm. When pregelatinized starch in KOH was the basis for the standard curve only 94% of added gelatinized starch mixed with raw starch was recovered. Recovery was 100% and coefficient of variation was <2% when absorbance of a blank was used to correct sample absorbance. The blank was prepared from the same mixture with only raw starch. The procedure adequately differentiated DG in several dry sweet potato-tapioca samples.

Key Words: Starch, gelatinized, sweet potato, tapioca

INTRODUCTION

STARCH rarely exists as raw granules in prepared foods. Gelatinized starch affects food texture by absorbing water, forming a gel or increasing viscosity of sols. The proportion of raw and gelatinized starch in ready to serve starchy products may be critical in determining acceptability. Texture of many foods such as, breakfast cereals, beverages, rice, noodles, pasta, and dried soups depends on gelatinized starch.

Some crispy snack foods are produced in two stages. The production of a dry intermediate known as a "half-product," followed by frying or baking to puff and impart crispiness. The half product is less bulky and more shelf stable than the crispy product. Texture and resistance to staling may depend upon the degree of gelatinization of starch. Such half-products may be produced by extrusion of semi-moist cereal flour or by shaping and drying dough from gelatinized moistened starch. Control of degree of gelatinization is important since raw starch, particularly from cereal grains, has low digestibility (Wooton and Chaudhry, 1980) and characteristic raw taste. Such starch reduces cohesiveness of a dry product and prevents expansion during puffing. Half-product for corn based crispy snacks may be produced by extrusion using cooled extruder dies to prevent expansion as the half product exits the extruder (Wilkinson and Short, 1989). Corn based half products have less acceptable flavors and thus other raw materials, particularly starchy vegetables, are alternatives.

Heating alters the structure of starch granules irreversibly during gelatinization. Although solubility, viscosity, and mechanical properties of gelatinized starch are slightly changed on dehydration or prolonged storage, such properties are distinct from those of raw starch. Time and temperature required to gelatinize starch depends on the source of the starch and other components such as moisture, protein and fat. Gelatinization of starch has been studied using the birefringence end point, viscosity relative to the maximal value, X-ray diffraction, and differential scaning calorimetry (Lund, 1984). Except for viscosity, these techniques are applicable only to purified fractions of starch and require costly instrumentation. They are not suitable for dry, processed starch mixtures.

Chiang and Johnson (1977) determined gelatinized starch in foods by measuring glucose released by digesting the product with glucoamylase using the *o*-toluidine color reaction. Interference from low molecular weight saccharides such as glucose, raffinose and sucrose was reported. Birch and Priestly (1973) found that gelatinized starch dissolved in 0.2N KOH but raw starch did not dissolve until concentration reached 0.5N KOH. The maximum absorbance of the iodine-amylose complex at 600 nm is constant in the presence of excess iodine at constant starch concentration (Banks and Greenwood, 1975). The amylopectin-iodine complex, on the other hand, absorbs maximally at 530–540 nm. Thus, starches which contain different amylose and amylopectin contents should give different absorbance values for the iodine blue complex at a given dissolved starch concentration.

Our objective was to develop a procedure for measuring the degree of gelatinization (DG) of starch in dry products containing mixed starches based on this hypothesis.

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MATERIALS & METHODS

Standard curve for gelatinized starch

Raw tapioca flour was vacuum oven dried. Gelatinized tapioca was obtained by cooking 20 min at 80% moisture and 100°C, drying 8 hr in a 2 mm thick layer in a tunnel dryer at 55°C, grinding in a Wiley mill through 0.5 mm screen and drying to constant weight in a vacuum oven. In all experimental mixtures containing sweet potato, it was pre-cooked, to inactivate indigenous amylases. Some starch in cooked sweet potato has been partially degraded by indigenous amylases during cooking. Cooked sweet potato powder was prepared by heating peeled cubed sweet potatoes 20 min in flowing steam at 100°C. After drying 2 hr in a tunnel dryer at 80°C and grinding in a Wiley mill through 0.5 mm screen, the powder was dried to constant weight in a vacuum oven. All powders were stored in air-tight containers in a desiccator over $CaCl_2$ after vacuum oven drying.

The standard curve for absorbance of the starch-iodine blue complex at 600 nm was developed separately for 0.2N KOH solubilized tapioca alone, and for KOH soluble fractions of cooked sweet potato, raw tapioca and cooked tapioca powder mixtures. The procedure was based on the amylose-iodine blue method described by Birch and Priestly (1973). A known weight (0.2g) of bone dry sample was added to 15 mL 0.2N KOH and stirred intermittently 15 min. The pH of the mixture was adjusted to 5.5 using 2N phosphoric acid and the volume made to 100 mL with distilled water. A 100 µL aliquot was transferred to a cuvette and diluted to 5 mL with distilled water. Fifty µL of 0.1N standard iodine solution (made according to section 50.018; AOAC, 1982) was added and absorbance at 600 nm was read in a spectrophotometer (Bausch and Lomb Spectronic-20). The instrument was standardized against a reference solution which contained all reagents except starch. Regardless of partial degradation by indigenous amylases of starch in cooked sweet potato, the iodine-tapioca-sweet potato starch complex had maximum absorption at 600 nm. The absorbance was constant within 15 min after iodine addition.

When samples contained only gelatinized tapioca powder, the standard curve was developed as absorbance at 600 nm as a function of quantity added. When the samples were mixtures of cooked sweet potato, raw tapioca and pre-gelatinized tapioca, the standard curve was based on absorbance of sample extract minus absorbance of a raw starch blank, as a function of quantity of gelatinized tapioca present in the sample. The spectrophotometer was standardized against a starch free reference solution prior to each measurement. The mixed starch samples contained 0.2 g each of cooked sweet potato powder and a mixture where the proportions of raw and gelatinized tapioca powders were varied. All analyses were conducted in triplicate and results are the means of 3 determinations.

Table 1—Percent starch gelatinization in sweet-tapioca half-product

	Initial moisture (%)								
	60%			70%			80%		
Temp.(°C)	100	110	120	100	110	120	100	110	120
Speed(rpm)									
1	76	71	71	86	86	86	57	74	85
	(3.5)[b]	(1.9)	(4.9)	(5.4)	(1.1)	(1.9)	(2.3)	(1.8)	(1.6)
6	60	77	79	67	92	91	86	95	100
	(4.0)	(0.7)	(1.4)	(7.2)	(6.4)	(0.3)	(1.6)	(5.1)	(2.7)
11	56	57	65	63	68	89	69	74	76
	(3.5)	(4.7)	(3.5)	(2.7)	(4.8)	(3.6)	(7.6)	(4.1)	(1.8)

[a] Averages of three replications
[b] Parenthesis, coefficients of variation

Table 2—ANOVA for degree of gelatinization

Source	DF	SS	
Moisture(M)	2	0.012734	**
Linear M	1	0.075264	**
Temperature(T)	2	0.012121	**
Linear T	1	0.107736	**
Speed(S)	2	0.013450	**
Linear S	1	0.044548	**
M×T	4	0.001046	**
T×S	4	0.002987	**
M×S	4	0.007675	**
M×T×S	8	0.005922	**
Error	54	0.002069	
Total	80		

** Significant at $p < 0.01$

Dry sweet potato-tapioca "half-product"

Samples for analysis were obtained by drum drying mashed cooked sweet potato and raw tapioca powder mixtures at different initial moistures, drum speed, and drum surface temperatures (Table 1). A double drum dryer (counter-rotating, 20.3 cm diameter by 28.5 cm long drum) was used. A paste containing 100 g cooked sweet potato solids and 100g raw tapioca powder and sufficient moisture to give the appropriate moisture content was applied on the space between the drums at one time. The paste was deposited as a thin layer with each rotation of the drum, and the process continued until all the paste was deposited, after which the sheet was peeled off the drum, cut into strips and air dried 1 hr at 80°C.

The conditions of the process resulted in the samples being exposed to different moisture and temperature conditions, resulting in varying degrees of starch gelatinization in the dried half-product. Each condition was replicated three times and the variability of the analytical results was estimated, by ANOVA analysis for significant treatment effects.

Gelatinized starch in dry sweet potato half product

The dry half product samples were ground through a 0.5 mm screen in a Wiley mill, dried 24 hr in a vacuum oven at 80°C and 600 mm Hg vacuum, and stored in air tight containers over $CaCl_2$ in a desiccator. Samples (0.2g) were analyzed as described for determination of the standard curve. A raw starch blank from a mixture of cooked sweet potato powder and raw tapioca powder was also prepared. The quantity of gelatinized starch was determined using a corrected absorbance (sample extract absorbance - raw starch blank absorbance) and the appropriate standard curve.

Standard curves and systematic error

The standard curve for absorbance at 600 nm (y_1) of dissolved starch-iodine complex vs g gelatinized tapioca powder (x) was linear. The regression equation was:

$$y_1 = 2.154x + 0.0208 \quad (1)$$

The correlation coefficient was 0.99 and systematic error (deviation of experimental points from expected value of dependent variable) was < 2%.

The standard curve for corrected absorbance at 600 nm (y_2) of dissolved starch-iodine complex from mixtures of sweet potato and tapioca powder vs g gelatinized tapioca in the mixture (x) was also linear. The regression equation was:

$$y_2 = 2.038x + 0.0097 \quad (2)$$

The correlation coefficient was 0.99 and systematic error was < 1%. Use of the corrected sample absorbance improved precision of the measurement.

RESULTS & DISCUSSION

EQUATION (1) was used to calculate the amount of gelatinized tapioca starch in solution from the corrected absorbance. Recoveries of known amounts of added gelatinized tapioca in samples were 93.11%, 95.88%, 94.34%, and 93.94% at added amounts of 0.05, 0.1, 0.15, and 0.2g respectively. The mean estimate was 94.1% of the amount present. However, when corrected absorbance was substituted for y_2 in Eq. (2), calculated x was 100% of amounts of gelatinized tapioca powder added to samples. The coefficient of variation was < 2%.

Gelatinized starch in sweet potato-tapioca half-product

Equation (2) was used to calculate gelatinized tapioca in half-product samples. Treatment means of percent gelatinization (Table 1) showed increasing gelatinization with increasing drum temperature at 80% initial paste moisture. However, at 70 and 60% initial moisture, gelatinization did not increase with increasing drum temperature. Both temperature and moisture content affect rate of gelatinization (Lund, 1984; Biliaderis et al. 1980) and our results confirmed this. The pattern of exposure of paste to heat in our system was such that the undeposited paste at the initial moisture level was exposed to heat received from the drums, but that already deposited was heated under reduced moisture conditions. Thus the time needed for complete deposition of the paste on the drums interacted with moisture reduction of deposited sheets to complicate relationships between gelatinization and conditions of the process. Gelatinization ranged from 56% at 60% initial moisture, 11 rpm and 100°C drum temperature to 100% at 80% initial moisture, 6 rpm, and 120°C drum temperature (Table 1). The only clear conclusion regarding effects of the process on gelatinization was that, at each drum temperature and speed, more gelatinization occurred with increasing initial moisture.

ANOVA of gelatinization data among the various half-product samples (Table 2) showed significant effects of all processing variables and their interactions. Thus the analytical procedure adequately differentiated degree of gelatinization of tapioca starch in differently processed mixtures. The procedure would permit a thorough analysis of the effects of the extent of gelatinization on mechanical properties of the half-product, the temperature dependence of the change in mechanical properties, and the puffing characteristics.

CONCLUSION

EXTRACTION with 0.2N KOH followed by determination of starch concentration in the extract by the iodine blue complex

—Continued on page 898

Extraction and Analysis of Metabolic Phosphates in Plants

DIANA F. SAYLER and PAUL J. GEIGER

ABSTRACT

We have developed a method for extraction and simultaneous detection and quantification of nucleotides and other phosphate compounds in various tissues. Analyses were based on modification of a method previously applied to animal tissue using an automated chromatographic analyser developed in our laboratory. Utilizing perchloric acid extracts of peas (fresh and frozen), tomatoes, and other plant tissues, we detected 19 organic phosphate compounds with the automated phosphate analyzer. The method is generally applicable to plant tissue.

Key Words: tomatoes, peas, phosphates, nucleotides, analysis

INTRODUCTION

WE STUDIED the feasibility of using an automated phosphate analyzer developed in our laboratory to explore the distribution of metabolic phosphates in plants (Bessman, 1974; Bessman et al., 1974; Geiger et al., 1980). The instrumentation has been used to study phosphorylated compounds in mammalian tissues regarding bioenergetics (Erickson-Viitanen et al., 1982a,b; Savabi and Bessman, 1986; Savabi and Kirsch, 1991; Zaidise, 1985). It has also been applied in a feasibility study to detect adulteration of orange juice (Lifshitz and Geiger, 1984).

An important area for botanicals and foodstuffs concerns the maturation, aging and changes in fruit and vegetable products. Phosphorylated compounds are important indicators of metabolic activity. Nucleotides are compounds by which energy may be transferred, and the distribution of sugar phosphates indicates the metabolic status of alternate pathways. In addition, sugar phosphates serve as precursors for synthesis of many metabolically active compounds including stored carbohydrates, nucleic acids, phospholipids, phosphoinositides, etc.

Phosphates are important in plants and foodstuffs. Nucleotides are used by the food industry as flavor enhancers (reviewed by Maga, 1983). Inorganic phosphates, sprayed on the undersides of leaves of cucumbers, have been used to protect the plant from anthracnose (Gottstein et al., 1989). Myoinositol hexaphosphate (phytate) forms insoluble complexes with zinc, iron and calcium in the human intestine. Such binding is important in calculating nutritional needs especially for those who consume very high fiber diets (Reddy et al., 1989; Oberleas et al., 1966).

Chromatographic methods have been applied for analysis of phosphorylated compounds. HPLC systems have been described for detection of vegetable nucleotide phosphates by Brown and Davis (1989) and Meyer and Wagner (1984). Their systems were limited to measuring nucleotides. HPLC analyses using UV detection may have interference from plant pigments and phenolics. Such UV absorbing substances must be removed before chromatography. HPLC systems do not detect non-nucleotide phosphates and separation of phosphate esters by paper chromatography (Bieleski and Young, 1962) is cumbersome and tedious.

In our system, many nucleotides and other phosphate containing compounds have been determined in a single run after a simple extraction procedure. Our system does not require removal of pigments and other phenolic base-containing compounds before chromatography as these are dry-ashed. Our objective was to study its application to plant-based foods.

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MATERIALS & METHODS

Sample selection

All fruits and vegetables were purchased from a local grocery store. Fresh frozen peas (*Pisum sativum*) were stored at −20°C until used. Fresh sugar snap peas were kept refrigerated in pods until assayed. Cherry tomatoes (*Lycopersicon lycopersicum*) were analyzed within one day of purchase.

Sample preparation

Tissue (≈ 5g) was homogenized in 10 mL ice-cold 0.6M $HClO_4$ containing 1 mM disodium EDTA using a Polytron (Brinkmann Insts., NY) at power setting 5 for 10 sec. Addition of EDTA provided a more reproducible extraction, probably helping to avoid problems with Ca^{++} and Mg^{++} ions, reported to precipitate and/or bind phosphate compounds (Lowry and Passonneau, 1972a). The homogenate was stored on ice for 30 min or longer and then centrifuged for 10 min at 1000 × *g*. At warmer temperatures standing in perchloric acid could destroy labile phosphate compounds but less than 30 min was not enough time for denaturation of certain enzymes, e.g. adenylate deaminase. The pellet was resuspended twice, using 5 mL of 0.6M $HClO_4$ and centrifuged as above. All three supernatant extracts were combined, centrifuged at 5000 × *g* for 10 min and passed through a 10 μm filter. The sample was then neutralized with KOH to pH 6.0–6.5 using Bromothymol blue as indicator, and final volume was recorded. The potassium perchlorate precipitate was packed by centrifugation, and the supernatant was kept frozen at −20°C until chromatographed. For recovery experiments, standards were added to tissue to 0.6M $HClO_4$ and EDTA just prior to homogenization. Based on previous experiments with frozen peas, standards were added to give three times the amount (in nmol/g fresh weight) of each compound found naturally in plant tissue, with exception of ATP. For example, 810 nmoles G6P standard were added per gram fresh weight since frozen peas contain approximately 270 nmol/g fresh weight G6P. Because ATP contains three phosphates, only two times the amount was added to prevent overloading the chromatographic system. For both experiments, the same amounts of standards were used.

Chromatographic separation

Ion-exchange chromatography was accomplished with a 0.3 × 15 cm analytical column (Altex Beckman, Berkeley, CA) packed in a flow of 0.6M ammonium chloride with the strong anion resin, AGMP-1 (Bio Rad Laboratories, Richmond, CA) sized to 18–25 μm. AGMP-1 is available at −400 mesh in bulk, but that was too heterogeneous to provide narrow peaks and the best resolution possible so we sized it further.

The sample was introduced into a pre-column 2.5 cm of the above resin topped with ≈0.6 cm of Celite 454 AW (Supelco Inc., Bellfonte, PA). This served both as a means for sample introduction and as a filter to protect the analytical column. Connections to the columns including a pressure gauge were made from the pump with 0.6 mm i.d. Teflon tubing. An ≈5-cm length between columns and a 50-cm length of 0.3 mm i.d. Teflon tubing from column exit to analyzer completed the chromatographic setup.

Linear gradient elution at 15 to 28 kg/cm^2 was accomplished with borate-containing ammonium chloride buffers at pH 8.5–9.0. Effluent from the analytical column was fed into the automatic analyzer for phosphate determination. This chromatographic procedure, utilizing an automated phosphate analyzer, permitted analysis of any phospho-

rus containing compound. The design of the instrument for dry ashing and its construction and operation were detailed previously (Geiger et al., 1980).

Briefly, the unit accepted column effluent or samples placed into collecting cups by hand at a position preceding the electric furnace. The furnace dried and ashed samples in Pyrex cups as they passed through it by means of a 40-place, rotating turntable. The turntable moved one cup position each 30 sec. Cups emerging from the furnace with the ashed samples were cooled for 2 min at room temperature (≈23°C) and acidic ammonium molybdate was added once every 30 sec. Blue color was allowed to develop for 2 min before the position of the turntable reached the point where solution was withdrawn from the cup and passed through the colorimeter. Next each cup was washed thoroughly before it was returned to the starting position where it received a small amount of oxidizing agent (3M HNO_3 with 0.01M $K_2B_4O_7$) just before it received more eluate.

Peaks were recorded at 820 nm and areas were calculated using a Shimadzu C-R3A integrating recorder attached to the colorimeter. The total phosphate of each peak was computed by comparing its area to the average area of peaks containing known amounts of inorganic phosphate standard added directly to the cups at the end of a run.

Compound identification

Peaks were identified by retention times and verifying positions by co-chromatography using authentic compounds from Sigma Co. (Saint Louis, MO). Peak identities were also substantiated in other ways as follows:

Samples were treated with acid washed Norit A (Fisher Scientific Co., Pittsburgh, PA) to remove nucleotides and phytic acid. Before addition of charcoal samples were adjusted to pH 1–2 with perchloric acid to minimize binding of sugar phosphates. After the charcoal procedure samples were neutralized to pH 6–6.5 and placed on the column. Re-chromatography confirmed nucleotide removal.

Samples were subjected to strong acid hydrolysis using 0.6M $HClO_4$, heated to 100°C for 15 min, cooled and neutralized. This treatment removed beta and gamma phosphates from nucleotides. Following re-chromatography, the homogeneity of certain peaks was thus further confirmed. Samples were also hydrolyzed with Dowex-50W (Sigma #50×4-200R) and on re-chromatography showed the same results as $HClO_4$ hydrolysis above. ATP was confirmed by treatment of samples with hexokinase (Lowry and Passonneau, 1972b). Re-chromatography confirmed proportional increases in ADP and glucose 6-phosphate (G6P). Dihydroxyacetone phosphate (DHAP) and/or glyceraldehyde 3-phosphate (GAP) were removed by increasing pH of the sample to 10 and allowing to stand at room temperature (≈33°C) for 20–30 min. Re-chromatography verified the positions of these compounds.

RESULTS & DISCUSSION

PLANT TISSUES we studied, included commercial frozen peas, fresh sugar snap peas and cherry tomatoes. The chromatogram (Fig. 1) of a typical peak profile from an extract of fresh sugar snap peas was recorded. Compared to frozen peas, the fresh peas had higher levels of many metabolic phosphates including glucose 1-phosphate (G1P), an increase of 119%, dihydroxyacetone phosphate (DHAP) 238%, fructose 6-phosphate (F6P) 69%, G6P 193%, 3-phosphoglycerate (3PGA) 154%, uridine 5′-diphosphoglucose (UDPG) 166%, guanosine 5′-monophosphate (GMP) plus inosine 5′-monophosphate (IMP) 110%, ATP (including uridine 5′-triphosphate, UTP, and guanosine 5′-diphosphate, GDP) 603% and an increase of 275% in phytic acid (PA). Both kinds of peas had similar amounts of nicotinamide adenine dinucleotide (NAD) and ADP. Cytidine 5′-triphosphate (CTP) was present in sugar snap peas but was minimal or absent from frozen peas.

The level of ATP in the fresh sugar snap peas (stored at 0–5°C) was higher than that of frozen peas presumably because the fresh peas continued to metabolize during storage. In several samples of frozen peas, ATP was essentially missing probably due to processing changes. Representative compound data for frozen peas (Table 1) illustrate typical precision from chromatographing the same extract of three different days. Results were comparable to those reported for mammalian tissue (Geiger et al., 1980; Zaidise, 1985). Precision routinely achieved with

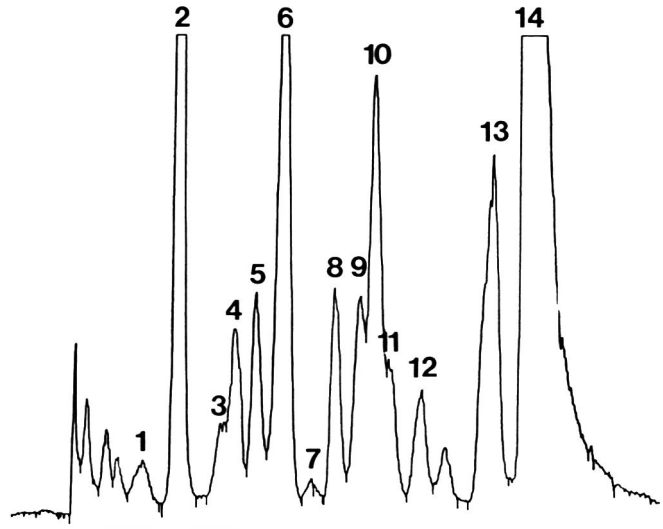

Fig. 1—Peaks of phosphorylated compounds from sugar snap peas. Peak 1, G1P;2, Pi (inorganic phosphate); 3, NAD; 4, DHAP; 5, F6P; 6, G6P; 7, AMP; 8, 3PGA; 9, UDPG; 10, GMP–IMP; 11, ADP; 12, CTP; 13, ATP+UTP+GDP; 14, PA. Peaks prior to #1 are phospholipids.

standards chromatographed in neutral, aqueous solution varied from 2–5% standard error. Natural extracts showed a wider variation. Small peaks up to about 10 nmols showed the least and those > 30 nmols showed best precision. Being a function of peak broadness precision would improve with other anion exchange resins (smaller sized packings and higher pressures). This would require further development as no available HPLC columns are compatible with our system. Estimates for the analyzer were based on replicates of four or five samples.

In cherry tomatoes, preliminary data showed that AMP was the predominant nucleotide compared to frozen or fresh peas. The predominance of AMP in tomatoes has also been reported by Inaba et al. (1980). This observation probably did not result from errors in experimental technique as recovery of added authentic ATP was reproducible at about 83%. Furthermore, ADP did not increase. Hence, degradation of ATP was not the source of large amounts of AMP in the tomatoes.

Fresh sugar snap peas seemed to have minimal or undetectable amounts of AMP. Except for AMP, cherry tomatoes had lesser amounts of all phosphorylated compounds than fresh snap peas. Compared to frozen peas, cherry tomatoes contained about two times the amount of G1P but about the same amount of DHAP, 3PGA, ATP, and PA and smaller amounts of the other metabolic phosphates. Fructose 1, 6-diphosphate and guanosine 5′-triphosphate have also been detected in other plant tissues.

The peaks we identified may not represent all phosphate compounds since other small peaks could be hidden. However,

Table 1—Phosphorylated intermediates in frozen peas[a]

Compound	Mean ± SEM
NAD[b]	123 ± 11.5
F6P	135 ± 2.3
G6P	273 ± 12.1
AMP	141 ± 5.8
3PGA	104 ± 1.7
UN[c]	35 ± 1.7
UDPG[b]	130 ± 8.1
GMP[b]	51 ± 3.5
ADP	82 ± 7.5
ATP[b]	27 ± 3.5
PA[b]	407 ± 30.0

[a] A single, typical extract was chromatographed on three different days of the week. Data shown as nmol/g fresh weight. (n = 3).
[b] Contains an amount of unidentified compound or compounds.
[c] Unidentified but well separated.

Table 2—Recovery of selected standards of phosphorylated compounds subjected to extraction and analysis[a]

	% Recovery	
	Sample	
Compound	1	2
F6P	107	94
G6P	91	82
AMP	85	84
UDPG	85	90
ADP	102	96
ATP	82	84

[a] Standards were addec to the extraction medium of sugar snap peas just before homogenization.

the reported peaks were the major metabolic phosphates in the tissues. The compounds in our chromatograms confirmed overall those from earlier work Bieleski (1962) identified by paper chromatography of potato extracts. Phospholipids and precursors appeared in our typical chromatogram ahead of G1P and inorganic phosphate (Pi). Such compounds require further study and additional identification methods.

The extraction procedure used on the peas and tomatoes was simple, with few steps. Three extractions of the sample were adequate. A fourth yielded only 1% or less additional metabolic phosphates from precipitated material. Other researchers have stated that $HClO_4$ extraction of plant tissues was not as effective as other methods. Recovery was either not quantitative or not reproducible. Brown and Davis (1989) asserted that recovery was not as high as with their formic acid method. Bieleski and Young (1962) observed loss of 11% of total phosphate on neutralization of perchloric acid. However, with addition of EDTA, to protect against interference of calcium and possibly magnesium (Lowry and Passonneau, 1972a) we obtained good results without complex, time-consuming procedures. Recovery experiments with authentic compounds showed notable losses were avoided during extraction. In addition, no deterioration of phosphate compounds was noted over 2 wk when samples were stored at −20°C. Assays of frozen peas kept at −20°C using different samples from within the same package also showed no significant measurable changes in phosphorylated intermediates over 41 days. The percentage recovery was high (Table 2) for selected authentic samples of important metabolic phosphates. These were added to different batches of sugar snap peas before homogenization as described. Recovery of individual standards was within the range expected and confirmed the range of recoveries reported by Brown and Davis (1989).

CONCLUSION

THE EXTRACTION method developed in our laboratory is simple and direct requiring few steps. With the automated phosphate analyzer we could separate and quantify some major metabolic phosphates of plant tissues and foodstuffs. This method should be highly useful in studying changes such as maturation, aging, and decay, as well as effects of genetic engineering on fruit and vegetable products.

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Carbonated Water Lexicon: Temperature and CO_2 Level Influence on Descriptive Ratings

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ABSTRACT

A lexicon for describing the sensory perception of carbonated water was developed by a trained panel. It included: salty, sour, bitter, cooling, astringency, bubbly, bubble size, bubble sound, gas expansion feeling, bite, burn, and numbing. Four CO_2 levels (noncarbonated, and 1.69, 2.75, and 4.63 volumes) and two temperatures (3 and 10°C) were tested. Ratings of all descriptors, except cooling, increased significantly as CO_2 level increased. Bubble size and bubble sound were rated higher for 10°C samples while cooling, bite, burn, and numbing were rated higher for 3°C samples. The descriptors were classified into four groups (cooling, taste, trigeminal, and mechanoreception descriptors) based on principal component analysis.

Key Words: carbonation, lexicon, acceptability, sensory, descriptive ratings

INTRODUCTION

CARBONATED beverages are popular with all age groups—especially young people. In addition to soda pop, other carbonated beverages, especially carbonated mineral water, are regularly consumed. Most such drinks are flavored, but some, e.g. mineral waters, are not. Beverage industries develop flavor formulas to produce desired flavors or mouthfeel. Soft drink and mineral water industries need information about how flavor and mouthfeel are affected by different levels of CO_2 in a beverage, since carbonation may influence these properties.

Such beverages are consumed generally in one of two ways. The beverage is consumed at refrigerated temperature or it is poured over ice. Hence, it is important to understand the effect of temperatures within this range on perceived CO_2 level as well as the effect of temperature on sensory characteristics.

Yau and McDaniel (1990) investigated the effect of increasing levels of CO_2 in water on increased carbonation perception. Carbonation perception was defined as the overall perception including stinging, burning, cooling, irritation, etc.; the feelings associated with stimulation of nerve endings of the trigeminal nerve. They found power functions of 2.79 for swallowing and 2.65 for expectorating carbonated water. The functions represented a sharp increase of perceived carbonation with increasing CO_2 concentration.

Yau and McDaniel (1990) also studied the effects of temperature on the perception of overall carbonation intensity. They reported that carbonation intensity was perceived, by both untrained and trained panels, to be greater at lower temperatures than at higher temperatures. This temperature effect was carbonation level dependent, and temperature differences were more notable at higher carbonation levels (Yau and McDaniel, 1991).

A few studies also investigated the effect of added CO_2 on specific attributes such as sourness and sweetness. Lederer et al. (1991) found that sourness, bitterness, and astringency were enhanced by high levels of CO_2 in flavored, carbonated milk beverages. The sensory rating of sweetness was also enhanced by carbonation of a blueberry flavored, carbonated milk beverage (Yau et al., 1989).

Development of a complete descriptive lexicon for carbonated beverages which includes flavor, sound, and factors associated with the trigeminal nerve system has not been reported. A major objective of our research was to develop descriptors for carbonated water using a trained panel, develop precise definitions for such descriptors, and validate the use of the descriptors. In addition we also investigated how use of descriptors was influenced by different carbonation levels and temperatures.

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MATERIALS & METHODS

Samples

Commercial, bottled water (Aqua Cool, Portland, OR) was used to produce the samples. Aqua Cool filtered the water (chlorinated Cascade Mountains water) using a series of filters to remove large particles, followed by activated carbon and polymeric resins to remove color, odors, chlorine and other chemical contaminants. The water was disinfected using ozone. According to Aqua Cool, samples contained an average of 1.5 ppm calcium, 0.31 ppm magnesium, 0.7 ppm sodium and 1.0 ppm chloride.

The water was carbonated with commercial carbon dioxide (Industrial Welding, Albany, OR) in a Zahm and Nagel 18.9 L, stainless steel carbonator (Zahm and Nagel Co., Buffalo, NY). Four levels were produced [noncarbonated, 1.69 volume (vol) (SD = 0.05), 2.75 vol (SD = 0.058), and 4.63 vol (SD = 0.064)]. The 1.69 vol CO_2 samples were produced using water, bottles, and CO_2 maintained at 21°C. The 2.75 and 4.63 vol CO_2 samples were produced using 21°C CO_2 and 1°C water, with bottles and carbonator immersed in ice. Batches of 18.9 L for each level were produced. All samples were bottled in 828 mL green, glass bottles and stored at 1°C until evaluated.

Sample pH was 6.04 for noncarbonated, 3.71 for 1.69 vol CO_2, 3.65 for 2.75 vol CO_2, and 3.51 for 4.63 vol CO_2. The pH was measured at 22°C with a Corning 125 pH meter using a Sensorex epoxy-body, sealed-reference combination electrode (S200C). The pH meter was calibrated with buffers of pH 3 and 7 (Microessential Laboratory, Brooklyn, NY).

Carbonation levels were measured using a Zahm and Nagel piercing device (Zahm and Nagel Co., Buffalo, NY). Sample temperatures and headspace pressures were measured after agitation of bottles. Temperature and pressure readings were converted to "volumes CO_2/volume water" using a conversion table (Zahm and Nagel Co., Buffalo, NY). One volume was defined as that amount of CO_2 dissolved in water at equilibrium, at 15.56°C, 1 atm pressure.

Trained panel

Training. Panelists (seven females and one male), all students or faculty of the Dept. of Food Science & Technology at Oregon State Univ., participated in training and subsequent evaluation of carbonated samples. Panelists were consumers of carbonated beverages. Descriptors and definitions were developed over 18 1-hr training sessions. Reference standards were used in training for taste descriptors, astringency and chalkiness (Table 1).

Testing. Testing was conducted in the Sensory Science Laboratory at Oregon State University, Corvallis, OR, in individual booths. Carbonation levels (noncarbonated, and 1.69 vol, 2.75 vol, and 4.63 vol) were tested at 3°C and 10°C. The 3°C samples were kept cold by packing in ice before and during the test. The 10°C samples were tempered by placing in a plastic foam cooler filled with 10°C water. Samples were presented monadically in 86 mL plastic cups (Sweet-

Table 1—Reference standards for carbonation descriptors

Descriptors	wt/wt[a]	Source
Salty	.2%	NaCl (Morton International, Inc., Chicago, IL)
Sweet	1%	sucrose (C & H Sugar Col, Concord, CA)
Sour	.03%	citric acid (Haarman and Reimer Co., Elkhart, IN)
Bitter	.04%	caffeine (Fisher Scientific, Fairlawn, NJ)
Astringency	.08%	alum (McCormack, Baltimore, MD)
Chalky	1%	cornstarch (Best Foods, CPC International, Inc., Englewood Cliffs, NJ)

[a] All standards prepared using commercial, bottled water (Aqua Cool, Portland, OR).

heart, Maryland Cup Co., Owings Mills, MD) coded with three digit, random numbers. Reference standards, as described in training, were provided and a warm-up sample of noncarbonated spring water was presented 5 min prior to beginning the test to provide similar testing conditions for the first sample and subsequent samples. Panelists were instructed to swallow each sample three times consecutively before rating. This procedure most closely simulated normal consumption of carbonated beverages (as evaluated in an informal study). Samples were presented at intervals of 5 min to facilitate accurate testing and to allow receptor recovery.

To avoid fatigue, descriptors were divided into two sets on separate ballots. The first set of descriptors included cooling, salty, sweet, sour, bitter, astringency, and chalky. The second set included bubbly, bubble size, bubble sound, gas expansion feeling, bite, burn, and numbing. The two sets of descriptors were evaluated in separate tests. Each set was tested on three consecutive days with one replication tested each day. All eight samples (carbonation by temperature treatment) were tested at each session.

Each descriptor was rated using a 16-point intensity scale (0= "none," 1= "just detectable", 3= "slight," 5= "slight to moderate," 7= "moderate", 9= "moderate to large", 11= "large", 13= "large to extreme," and 15= "extreme"). For the descriptor bubble size, the same 16-point scale was used but 1= "extremely small," 3= "small", and 15= "extremely large."

Experimental design and statistical analysis

A randomized, balanced, complete block design was used with four carbonation levels, two temperature levels, and three replications (Cochran and Cox, 1957). An assumption in this design was that panelists maintained the same sensory sensations/perceptions when evaluating the separate descriptor sets.

Analysis of variance (ANOVA), principal component analysis (PCA), and correlations were carried out using *Version 6.03 SAS Statistical Package* (SAS Institute, Inc., Cary, NC). Panelist, replication, temperature, CO_2 level effects, and interaction effects were testing using ANOVA. A mixed effects F-test model was used with treatment effects fixed and all other effects (including panelist) considered random (Anderson and Bancroft, 1952). The data of nonperceivers (persons giving zero ratings for all temperature and CO_2 levels for a particular attribute) were not included in data analysis, because inclusion could obscure potential differences found by perceivers.

Panelist-by-treatment interactions were visualized by line graphs. Panelist ratings (y-axis) were plotted vs each treatment (x-axis) to search for systematic inconsistencies among panelists contributing to variation. Treatment differences of descriptors with significant ($p \leq 0.05$) panelist-by-treatment interactions were interpreted cautiously.

Fisher's least significant difference (LSD) test ($p \leq 0.05$) was used to compare treatment differences. The formula and degrees of freedom were constructed according to the formula provided by Anderson and Bancroft (1952). Using this formula, a few cases were encountered where there was a significant F-value, but differences among treatment means could not be specified because the LSD value was too large. For such cases, a t-test, with degrees of freedom determined by formula (Anderson and Bancroft, 1952), was used to test for significant differences among treatments. PCA of replication mean scores for descriptors was conducted. Chalky and sweet were not analyzed because of lack of panel agreement.

RESULTS & DISCUSSION

Lexicon development

Eight samples were presented to the panel for training during each of 18 sessions. During the first three sessions, panelists independently listed terms to describe the taste, mouthfeel, and sound of the samples. Twenty-two descriptors were generated from these sessions. During the following sessions, the list was narrowed as redundant terms of those with broad, ambiguous meanings such as "fresh" or "refreshing" were eliminated. Terms were eliminated after discussion among panelists and panel leader, and agreement that there were remaining terms to describe the sensations represented.

Table 2—Carbonation descriptors and definitions developed by trained panel

Descriptor	Definitions
Salty	the taste stimulated by NaCl in water.
Sweet	the taste stimulated by sucrose in water.
Sour	the taste stimulated by an acid such as citric acid or lactic acid in water.
Bitter	the taste stimulated by caffeine in water.
Cooling (physical)	the sensation of reduced temperature experienced as a result of exposure to thermally cold substances.
Astringency	the complex of tactile sensations due to shrinking, drawing, or puckering of the oral epithelium as a result of exposure to such substances as alums or tannins.
Chalky	the perception of particles in the mouth experienced between the tongue and the upper palate, teeth, or sides of the mouth.
Bubbly	the feeling of bubbles mechanically coming in contact with the oral epithelium including the feeling of the bubbles' movement, and/or bursting, and popping.
Bubble size	the perception of the size of the bubbles in the mouth.
Bubbly sound	the sound of bubbles bursting in the mouth.
Gas expansion feeling	the release of CO_2 from solution upon introduction into the mouth resulting in the feeling of fullness or expansion of the mouth.
Bite (chemical)	the stinging experienced primarily in the oral cavity as a result of exposure to CO_2.
Burn (chemical)	the perception of increased temperature and irritation resulting from the exposure to CO_2. The sensation lingers after the stimulus is removed.
Numbing	the perception of loss of feeling, or an anesthetized feeling within the oral cavity.

Examples of this elimination process were the descriptors bite and burn. Originally, pain, pricking, fizzy, tingle, sting, and bite were listed as mouthfeel terms. After several sessions, panelists agreed that these terms described either intensity or location of a particular sensation which could be described by the term bite. All of the terms except bite were then dropped. The descriptor burn evolved in a similar manner. Initially, several terms including abrasiveness, harshness, warmth, pain, and burn were used. Through discussion and training, they were narrowed to the term burn. Another complex and difficult descriptor was the term salty. Initially, mineral, baking soda, and salty were suggested as separate terms. Through discussion and training, they were included as part of the term salty.

Through discussion and training the panel agreed upon 14 descriptors and corresponding definitions for the flavor, mouthfeel and sounds associated with carbonated water (Table 2). Taste descriptors included salty, sweet, sour, and bitter. Mouthfeel descriptors were more extensive, including cooling (physical), astringency, chalky, bubbly, bubble size, gas expansion feeling, bite (chemical), burn (chemical), and numbing. Bubbly sound was also included. Any applicable definitions for terms in ASTM standard terminology were used (ASTM, 1991).

After agreement on terms and definitions, some panelists who understood the definition did not always use a particular descriptor. Such terms were predominantly the taste descriptors, but also included astringency and gas expansion feeling. Bitter, salty, sweet, sour, and astringency solutions were presented to panelists in varying concentrations to assure they understood the terms and definitions. Panelists have been occasionally confused by sour and bitter (Robinson, 1970), sour and salty (Meiselman and Dzendolet, 1967), and sour and sweet (Meiselman and Dzendolet, 1967), especially at lower con-

Table 3—Significance levels[a] for main factors (temperature, CO_2 level, panelist, and replication) and all interactions for descriptors

Source of variance	Descriptors											
	Cooling	Salty	Sour	Bitter	Astringency	Bubbly	Bubble size	Bubbly sound	Gas expansion	Bite	Burn	Numbing
Temperature	***	ns	ns	ns	ns	ns	**	***	ns	*	*	*
CO_2 Level	ns	***	***	**	***	***	ns	***	***	***	***	***
Temp × CO_2	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns	ns
Panelist	***	***	***	***	***	***	***	***	***	***	***	***
Replication	ns	ns	ns	ns	ns	ns	ns	ns	*	*	ns	ns
Pan × Rep	*	***	ns	ns	ns	**	ns	***	ns	ns	***	ns
Temp × Pan	***	ns	ns	ns	ns	ns	ns	ns	ns	ns	***	***
Temp × Rep	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
CO_2 × Pan	ns	***	***	ns	***	**	***	***	***	***	***	***
CO_2 × Rep	ns	ns	ns	ns	ns	ns	ns	ns	*	ns	ns	ns
Temp × CO_2 × Pan	ns	ns	ns	ns	ns	ns	ns	**	*	ns	ns	ns
Temp × CO_2 × Rep	*	ns	ns	ns	ns	ns	ns	*	ns	ns	ns	ns
Temp × Pan × Rep	ns	*	ns	ns	ns	ns	ns	ns	ns	*	ns	ns
CO_2 × Pan × Rep	*	ns	*	ns	ns	*	ns	***	*	ns	ns	ns

[a] ns not significant at $p \leq 0.05$.
*,**,*** refers to significance a: $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.

Table 4—Mean ratings[z], standard deviations (SD), significance levels, and LSD values for descriptors by carbonation level averaged over temperature

Descriptor	Non-carbonated	Carbonation level (vol) 1.69	2.75	4.63	Sig.[y]	LSD
Bubbly	0.0[a]	1.1[a]	5.0[b]	8.3[c]	***	1.28
	(0.0)	(1.3)	(2.4)	(2.5)		
Bubble size	0.0[a]	1.0[a]	4.1[b]	6.6[c]	p = .06	1.43
	(0.0)	(1.2)	(2.2)	(2.7)		
Bubbly sound	0.0[a]	0.2[a]	3.8[b]	7.0[c]	***	1.42
	(0.0)	(0.6)	(2.2)	(2.6)		
Gas expansion feeling	0.0[a]	1.3[a]	4.8[b]	8.5[c]	***	1.69
	(0.0)	(1.5)	(2.6)	(2.4)		
Bite	0.4[a]	3.2[b]	6.3[c]	9.4[d]	***	1.79
	(1.2)	(2.0)	(2.2)	(3.1)		
Burn	0.4[a]	2.0[ab]	3.6[b]	5.6[c]	***	1.63
	(1.3)	(2.1)	(2.3)	(3.0)		
Numbing	0.7[a]	2.1[ab]	3.5[bc]	4.9[c]	***	1.66
	(1.1)	(1.6)	(2.3)	(3.1)		
Astringency	0.0[a]	1.3[b]	2.0[bc]	2.5[c]	***	.70
	(0.0)	(1.2)	(1.1)	(1.4)		
Cooling	8.4	8.7	8.9	8.8	ns	–
	(2.4)	(2.5)	(2.4)	(2.3)		
Salty	0.0[a]	2.4[b]	2.3[b]	2.3[b]	***	1.22
	(0.2)	(2.1)	(2.0)	(1.9)		
Sour	0.2[a]	2.5[b]	3.4[b]	3.7[b]	***	1.27
	(0.6)	(1.5)	(1.8)	(2.0)		
Bitter	0.0[a]	1.8[b]	2.2[b]	2.1[b]	**	.95
	(0.0)	(1.6)	(1.3)	(1.3)		

[z] Sixteen-point intensity scale (C = none, 15 = extreme).
[y] ns not significant at $p \leq 0.05$.
*,**,*** refers to significance at $p \leq 0.05$, $p \leq 0.01$, and $p \leq 0.001$, respectively.
[a-d] Means within a row followed by the same letter are not significantly different at $p < 0.05$.

centrations. These could be corrected by defining taste adjectives and providing appropriate taste comparisons (O'Mahoney et al., 1979). All panelists perceived these descriptors, but some did not perceive them at the low levels where they occurred in the test samples. Those were the same panelists classified as nonperceivers in analysis of testing results.

Lexicon use

One way we validated the lexicon was by examining use of descriptors by the panel. During training some panelists did not use certain terms. This was also true during testing. Salty was never detected by one panelist, astringency by two panelists, sourness by three panelists, bitterness by four panelists and two of them never used the term "gas expansion feeling." Only one panelist gave scores for chalky and two used the term sweet. This was unexpected since a greater number of panelists (not always the same ones), had used those descriptors during training. Evidence of such inconsistency working with very low concentration solutions was discussed by other researchers (O'Mahoney et al., 1979). Chalky may have been confused with astringency as the particulate feeling in the mouth is similar to that induced by precipitation of proteins from astringent compounds. Nonzero scores given by panelists for sweet were predominantly for non-carbonated samples. Bartoshuk et al. (1964) found the inherent perception of water is not necessarily tasteless, but after adaptation to acids, may seem to taste sweet (Bartoshuk, 1974). Perhaps panelists were affected by this adaptation effect, as sourness was a component of the profile. Chalky and sweet were dropped as indicated. All other descriptors were utilized by the majority of panelists consistent with their importance in describing the perception of carbonated water.

We questioned whether all descriptors were relevant to the descriptive profile of carbonated water. Possibly panelist ratings could reflect overlapping use of descriptors. A concerted effort was made during training to clearly delineate descriptors and definitions to ensure that not more than one descriptor was used for a particular perception. This was accomplished using standards and extensive panel discussion. All definitions were distinctly different (Table 2).

ANOVA revealed significant treatment differences by temperature level and CO_2 level for several attributes (Table 3). All descriptors were significant for panelist effect (Table 3). Panelist effect is commonly found in ANOVA results of trained panel data, especially when intensity standards are not used. The significant panelist effect indicated they were using different parts of the intensity scale for rating attributes. However, this did not interfere with treatment effects.

There were no significant temperature × CO_2 interactions and there was only scattered significance for all other interactions with exception of panelist × CO_2 and panelist × replication (Table 3). The raw data, using line graphs, indicated minor inconsistencies throughout which contributed to significant interaction effects. Such inconsistencies were not systematic and were not confined to single panelists. Individual panelist standard deviations revealed few scattered uncharacteristically large standard deviations, which were not confined to any one panelist.

Much significant panelist related variation seemed to result from the sensitivity of the test, to the few panelists who scored in slightly different ways from each other. This was most noticeable when some panelists rated both the non-carbonated and 1.69 vol. samples zero while other panelists rated the same samples as different. Deletion of data from an inconsistent panelist would be expected to reduce significant interaction effects. We did not identify any panelist who was systematically inconsistent and therefore did not eliminate any panelist data from analysis.

We also tried to validate the lexicon by examining the ratings for interrelationships among descriptors. However, we could not determine whether the panel was using descriptors independent of each other by examination of their ratings. Bub-

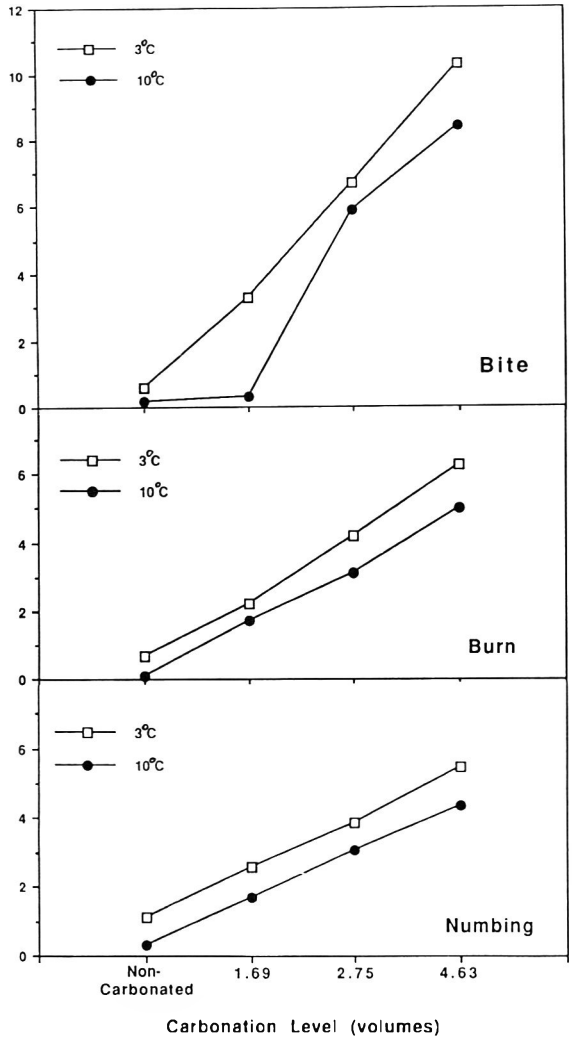

Fig. 1—Relations between sensation of bite, burn, and numbing, carbonation level ano temperature. Scale ratings from 0 = none to 16 = extreme.

Table 5—Mean ratings[a], standard deviations (SD), and t-test sig of significant descriptors by temperature level averaged over CO_2 level

	Cooling	Bite	Burning	Numbing	Bubble size	Bubble sound
3°C	10.4	5.2	3.3	3.3	2.6	2.5
	(1.7)	(4.3)	(3.3)	(2.8)	(3.0)	(3.2)
10°C	7.0	4.4	2.5	2.4	3.3	3.1
	(1.7)	(3.7)	(2.6)	(2.5)	(3.4)	(3.5)
Sig.[b]	***	*	*	*	**	***

[a] Sixteen-point intensity scale (0 = none, 15 = extreme).
* p≤0.05, ** p≤0.01, *** p≤0.001.

bly and gas expansion had similar ratings for CO_2 level averaged over temperature (Table 4). Burn and numbing had similar ratings for the samples at each carbonation level (Fig. 1). The sensations represented by bubbly and gas expansion feeling are dissimilar and would not be expected to cause confusion. Burn and numbing may represent sensations which overlapped because both definitions pertain to a sensation which lingers. Burn is characterized by irritation and numbing by loss of feeling.

Effect of CO_2 levels on carbonation perception

All descriptors tested, with exception of cooling and bubble size, were significant for carbonation level averaged over temperature (Table 4). Two basic patterns were associated with changes in carbonation level. The first was the increase in

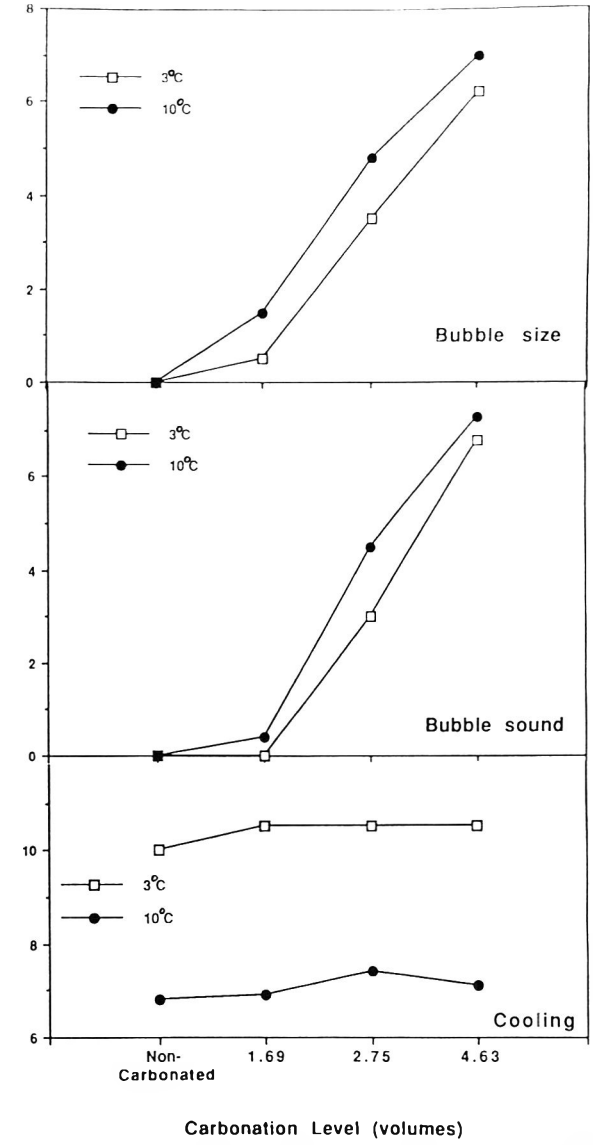

Fig. 2—Relations between bubble size, bubble sound, cooling, carbonation level, and temperature. Scale ratings from 0 = none to 16 = extreme.

rating of intensity of a descriptor as CO_2 level increased. This was evident for most non-taste attributes. Each incremental level of carbonation was significantly different from the preceding level. For bubbly, gas expansion feeling, and bubble sound, the non-carbonated samples and the lowest level of carbonation were not significantly different (Table 4). However, those levels were lower compared to the middle level, and the highest level was higher compared to the others. For bite and, to a lesser degree, burn, numbing, and astringency, higher carbonation levels resulted in higher intensity ratings at each level of carbonation (Table 4). This was consistent with the sharp increase of perceived CO_2 magnitude reported with increasing concentration found by Yau and McDaniel (1990), as well as the results on nasal pungency reported by Garcia-Medina and Cain (1982) and Cain and Murphy (1980).

The second pattern was associated with the taste descriptors of salty, bitter, and sour and was quite different compared to nontaste descriptors. Ratings for the 3 samples with CO_2 added were not significantly different from each other, however, they were significantly higher than the non-carbonated sample (Table 4). Sourness ratings, did not significantly increase as carbonation levels increased. Lederer et al. (1991) found sourness, astringency and bitterness ratings were enhanced at higher car-

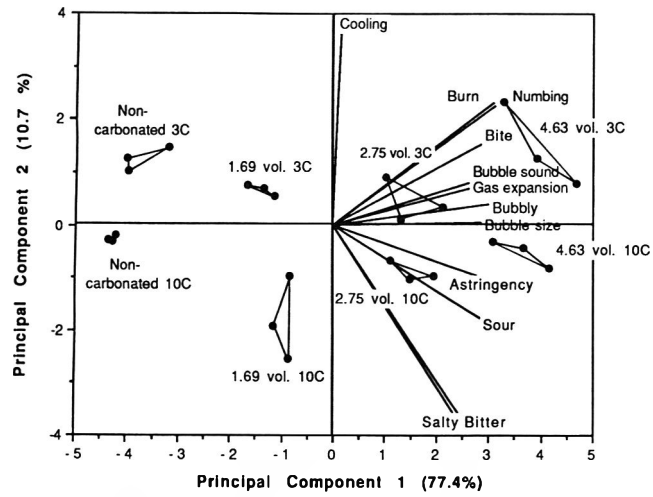

Fig. 3—Principal components 1 vs 2 intensity ratings for carbonated water descriptors for the eight samples. Connected points for each sample represent three replications across eight panelists.

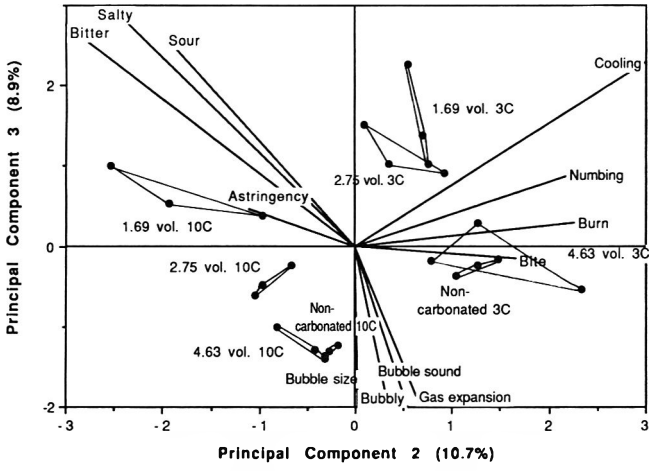

Fig. 4—Principal components 2 vs 3 intensity ratings for carbonated water descriptors for the eight samples. Connected points for each sample represent three replications across eight panelists.

bonation levels, although their highest level of carbonation (1.42 vol) was lower than our lowest level (1.69 vol).

Effect of temperature on carbonation perception

Six descriptors changed with temperature change averaged over carbonation level. Cooling, bite, burn, and numbing were higher in the 3°C samples, while bubble size and bubble sound were higher in the 10°C samples (Table 5). An interesting aspect was the difference between mouthfeel and taste components with respect to temperature. The majority of mouthfeel components were significant, conversely, none of the taste components changed significantly within the range of temperatures tested. Graphing intensity ratings for significant descriptors demonstrated that (except for cooling) they changed not only with temperature but also with CO_2 level (Fig. 1 and 2). Bite, for example, consistently had higher intensity ratings for carbonated samples which were at 3°C compared to 10°C. This same pattern was evident for burn and numbing. For bubble size and bubble sound, the pattern was the same as the carbonation level changed, but the temperature relationship was reversed. Cooling ratings were very different for the two temperature levels with the 3°C samples much higher. However, these ratings were not affected by CO_2 level.

Our results were consistent with the report of Yau and McDaniel (1991), who found carbonation intensity was perceived higher at lower temperatures than at higher temperatures. However they were contrary to the findings of Green (1990) that highlighted lowering of ratings for "oral irritation" with decrease in temperature for capsaicin, piperine, ethanol, and NaCl. The range of temperature (24–46°C) in that study, however, was quite different.

Principal component analysis

The PCA model of descriptors was explained almost entirely by the first three principal components (97%). PC1 explained 77.4%, PC2 accounted for 10.7%, and PC3 accounted for 8.9% of the model. Graphs (Fig. 3 and 4) of PCA loadings gave a clear picture of the goodness of replications and how descriptors related to each sample, carbonation level, and temperature level. Distinct separation between the samples as well as the tight grouping of the three replications for each sample indicated that panelists could readily differentiate between samples and evaluate them in a highly consistent manner. The graphs showed a noticeable difference between samples based on carbonation level and temperature level. PC1 scores for samples increased dramatically with increasing carbonation as evidenced by spacing between carbonation levels and orientation of the plotted samples from left to right (Fig. 3). The sample plottings were oriented from the lower left to the upper right (Fig. 4) showing that the 3°C samples had higher scores for PC2 and PC3 compared to the 10°C samples.

PC1 could be defined as overall carbonation impact, as all terms except cooling had high loadings on PC1. PC2 could be defined as cooling, numbing, burn, and bite. This explained the major sensory differences between temperature levels after accounting for effects of carbonation. These were primarily trigeminal responses (mouthfeel). The PC2 by PC3 plot noted a third separation in the data. The lower carbonation levels had a different perception of tastes after accounting for overall carbonation and mouthfeel.

Correlation indicated all descriptors, except cooling, were significantly, though not strongly, correlated. However, descriptors could be grouped to more clearly reflect interactions with each other. The tastes, saltiness, bitterness, astringency and sourness, were closely related, as were bite, burn and numbing (trigeminal descriptors). Descriptors related to mechanoreception (bubbly, bubble size, bubble sound and gas expansion feeling) were very closely related, while cooling was not associated with the other attributes.

CONCLUSION

OVERALL carbonation perception in carbonated water could be divided into a lexicon of descriptors which more accurately described this complexity of sensations. The CO_2 and temperature level greatly influenced intensity ratings for each descriptor, which, except cooling, increased as CO_2 levels increased. Bubble size and bubble sound were higher at 10°C while cooling, bite, burn, and numbing were higher at 3°C. PCA revealed all descriptors, except cooling, were correlated and the descriptors could be divided into 4 groups (cooling, taste, trigeminal, and mechanoreception descriptors) to more clearly reflect interactions. Understanding the rating responses for descriptors in carbonated water systems could facilitate prediction of flavor profiles of formulated, flavored, carbonated beverages such as the interrelationships of sourness and sweetness in a beverage to which CO_2 was added.

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GELATINIZED STARCH DETERMINATION. . .From page 889

provided an accurate technique for quantifying the amount of gelatinized starch in dry mixtures. Interference of other compounds was avoided by a correction on measured sample absorbances by subtracting values of an appropriate raw starch blank.

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Hydrophilic Edible Films: Modified Procedure for Water Vapor Permeability and Explanation of Thickness Effects

T. HABIG McHUGH, R. AVENA-BUSTILLOS, and J.M. KROCHTA

ABSTRACT

The ASTM E96 Standard Method for determining water vapor permeability (WVP) was modified for hydrophilic edible films. Accurate measurement of relative humidity conditions and maintenance of 152 m/min air speeds were essential outside the test cups. The WVP Correction Method was developed to account for the water vapor partial pressure gradient in stagnant air layer of the test cup. Errors were as high as 35% without this correction. Applying these guidelines explained commonly observed thickness effects on WVP values of hydrophilic films. Relative humidity was the cause of observed thickness effects.

Key Words: hydrophilic, edible films, water vapor permeability, WVP

INTRODUCTION

CONSUMERS demand higher quality and longer shelf-life in foods while reducing disposable packaging materials and increasing recyclability. Such concerns have caused increased interest in edible film research. Edible films, by regulating water, oxygen, carbon dioxide, and lipid movement in food systems, provide potential solutions to such concerns. Researchers are investigating protein, polysaccharide and lipid films, alone and in combination, for their effectiveness as mass transfer barriers. Potential applications and properties of edible films were reviewed by Kester and Fennema (1986), Guilbert (1986) and Krochta (1992).

Edible films often contain hydrophilic components, such as proteins or polysaccharides (Banker et al., 1966; Lieberman and Gilbert, 1973; Schwartzberg, 1986; Kester and Fennema, 1986). Modeling transport through such films is extremely complex, due to nonlinear water sorption isotherms and water-content-dependent diffusivities (Schwartzberg, 1986). Water vapor flux through hydrophilic films varies non-linearly with water vapor partial pressure gradient. Therefore, permeability is not an inherent property of hydrophilic films (Crank, 1975). Consequently, numerous anomalies have been reported, such as thickness effects on permeability properties of hydrophilic films (Hauser and McLaren, 1948; Schultz et al., 1949; Barrer, 1951; Banker et al., 1966; Hagenmaier and Shaw, 1990). No clear explanation of such effects has been presented. This is partially due to the lack of reliable methodologies for determination of true partial pressure gradients experienced by the films during water vapor permeability (WVP) tests.

Reliable methodologies are extremely important in that permeability values are commonly used in product shelf-life predictions, as well as in tailoring film permeability properties for specific food applications. The most commonly used method for determining WVP is the ASTM E96 method (1980). This gravimetric method involves sealing a test film in a cup partially filled with water, saturated salt solution or desiccant, leaving an air gap beneath the film. The test cup is then placed in a controlled temperature and relative humidity and the weight change over time is measured to determine the steady-state flux of water vapor through the film The flux is divided by the partial pressure difference at the film during testing and multiplied by its thickness to calculate film WVP values.

The ASTM E96 (1980) method is designed to determine WVP properties of synthetic hydrophobic polymer packaging films. Consequently, it makes assumptions which may not apply to hydrophilic film systems. The ASTM method assumes that resistances to mass transfer are negligible in gas phases on both sides of the test film mounted in the cup. This is true of the mass transfer resistance above the cup, provided convection is induced by a fan moving air at greater than 152 m/min, as recommended by ASTM and confirmed in this study. However, natural convection within the cup is usually negligible. Large errors in WVP can occur due to the partial pressure gradient within the stagnant air layer between the mounted film and the water, saturated salt solution or desiccant in the cup when film resistance is small, as with hydrophilic films of proteins and/or polysaccharides. The conventional ASTM E96 (1980) method does not account for this partial pressure gradient.

The first objective of our study was to determine conditions necessary for accurate determination of WVP of hydrophilic films and to develop a WVP Correction Method to account for the partial pressure gradient in the stagnant air layer of the test cup. Our second objective was to utilize the resulting accurate water vapor partial pressure and WVP values to explain commonly observed thickness effects on permeability properties of hydrophilic edible films.

MATERIALS & METHODS

Materials

The sodium caseinate used to make films was Alanate 110 from New Zealand Milk Products, Inc. (Santa Rosa, CA). Due to homogeneous structure, sodium caseinate films were chosen for examination of thickness effects on film WVP properties. BiPRO whey protein isolate for films was supplied by Le Sueur Isolates (Le Sueur, MN). The beeswax incorporated into emulsion films was purchased from Aldrich Chemical Company, Inc. (Milwaukee, WI). Sorbitol and glycerin plasticizers were obtained from Fisher Scientific, Inc. (Fair Lawn, NJ). Lithium chloride, magnesium chloride, potassium carbonate, sodium bromide and sodium chloride salts used for saturated salt solutions were purchased from Fisher Scientific, Inc. (Fair Lawn, NJ). Commercial cellophane films were obtained from the Mann Laboratory, University of California, Davis. Polyester (Mylar) and High Density Polyethylene (HDPE) films used in the 1985 ASTM E96 round robin test were donated by Murray Toas of CertainTeed Corporation (Blue Bell, PA).

Film formation

Aqueous solutions of 8% (w/w) sodium caseinate were made for casting films. A vacuum was applied to solutions prior to casting to prevent formation of air bubbles in the films. Aqueous solutions of 10% (w/w) whey protein isolate (WPI) were prepared and heated at 90°C for 30 min in an oil bath (Haake Model No. N4B, Catalog No. 13-874-119C, Fisher Scientific, Inc., Fair Lawn, NJ). Solutions were cooled to room temperature (≈23°C) and a vacuum was applied to remove air. A weight of glycerin (GLY) or sorbitol (S) equal to the weight of WPI was added as plasticizer for casting 50% WPI/50% GLY or 50% WPI/50% S films. Whey protein solutions (10% w/w)

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for emulsion films were prepared as described. A weight of sorbitol equal to 28% or 50% of the original WPI was added as plasticizer. The whey protein/sorbitol solutions were then reheated to 75°C and a weight of beeswax (BW) equal to 50% or 28% of original WPI was added. After allowing the beeswax to melt, the mixture was homogenized using an Ultra-Turrax homogenizer (Ultra-Turrax, Model T25, IKA-Works, Inc., Cincinnati, OH) for 1 min at 13,500 rpm, followed by 4 min at 20,500 rpm. Emulsion films had compositions of 56% WPI/ 16% S/ 28% BW and 56% WPI/ 28% S/ 16% BW, total solids basis.

Different thickness caseinate films were cast by pipetting 10–40 mL of 8% protein solution onto a 14.7 cm internal diameter, rimmed, smooth, polymethylmethacrylate (Plexiglas) plate on a leveled granite surface. Whey protein solutions and emulsions were applied at 2.625g total solids/plate. Six films were prepared for each type film. Solutions were spread evenly with a bent glass rod and allowed to dry for about 18 hr at room temperature (≈23°C). Dried films could be peeled intact from the casting surface.

Film thicknesses

Thicknesses of films were measured with a micrometer (L.S. Starrett Co., Series 436, Catalog No. T436RL-1, Athol, MA.) to the nearest 0.0025 mm at five random positions around the film. Individual film thickness measurements varied up to 5%. Average values of five thickness measurements/film were used in all WVP calculations.

WVP determination

Motors (Bodine Motor, Model No. 574, Minarik Electric Co., Fresno, CA) were installed with variable speed controllers (Motor Master, Series 20000, Minarik Electric Co., Fresno, CA) and fans (Refrigeration Supply House, Model No. 607601-01, Sacramento, CA) into plastic desiccating cabinets (Fisher Scientific, Inc., Catalog No. 08-647-28, Fair Lawn, NJ) by the Food Science & Technology machine shop at the Univ. of California, Davis. The cabinets were placed in a 25°C controlled temperature room. Air speeds were measured using an anemometer (Solomat, Model No. 127MS, Stamford, CT). Fan speeds were set to achieve various air velocities up to 183 m/min in the cabinets. The effect of air speed on film WVP properties was examined using cellophane films. Each cabinet contained a hygrometer (Airguide, Model No. 605, Chicago, IL) to monitor relative humidity. Prior to each experiment, cabinets were equilibrated to 0% relative humidity using calcium sulfate desiccant (Drierite, Fisher Scientific, Inc., Fair Lawn, NJ).

Circular test cups were made of polymethylmethacrylate (Plexiglas) in the Food Science & Technology machine shop at the Univ. of California, Davis. The external cup dimensions were 8.2 cm diameter and 1.25 cm tall. The area of the cup mouth was 78.5 cm^2, and the cup well depth was 1.1 cm. Deeper cups, with identical external diameters and cup mouth areas, were made to examine effects of stagnant air layer height on film WVP. These cups were 2.4 cm and 3.4 cm tall, with 2.2 cm and 3.2 cm cup well depths, respectively. Cup walls were sufficiently thick to render the cup impermeable to water vapor. Silicon sealant (High Vacuum Grease, Dow Corning, Midland, MI) and four screws, symmetrically located around the cup circumference, were used to seal films into test cups.

Deionized water or equivalent amounts of saturated salt solutions were placed in the bottoms of the test cups to expose the film to a high relative humidity inside the test cups. For examination of effects of stagnant air layer gap height on film WVP values, the 2.4 cm tall cups were filled with 12 mL or 18 mL of deionized water and the 3.4 cm tall cups were filled with 10 mL, 20 mL or 30 mL of deionized water. All other experiments used the 1.25 cm tall cups containing 6 mL deionized water or equivalent amounts of saturated salt solutions.

Next, films were mounted in the cups. The distance between the solution and the film was determined both before and after each experiment to the nearest 0.025 mm using a micrometer (Lufkin Rule Co., Model No. 515, Saginaw, MI.). Average stagnant air gap heights were calculated. All thickness effect experiments were performed between 7.7 mm and 8.5 mm average stagnant air gap heights with caseinate films. Effects of air gaps between 7.7 mm and 30.3 mm on caseinate film WVP were also examined to validate use of the WVP Correction Method.

After assembly, the test cups containing films were inserted into the pre-equilibrated 0% RH desiccator cabinets. Within 2 hr, steady state had been achieved; five weights were then taken for each cup at > 3 hr intervals. Four replicates of each film were tested.

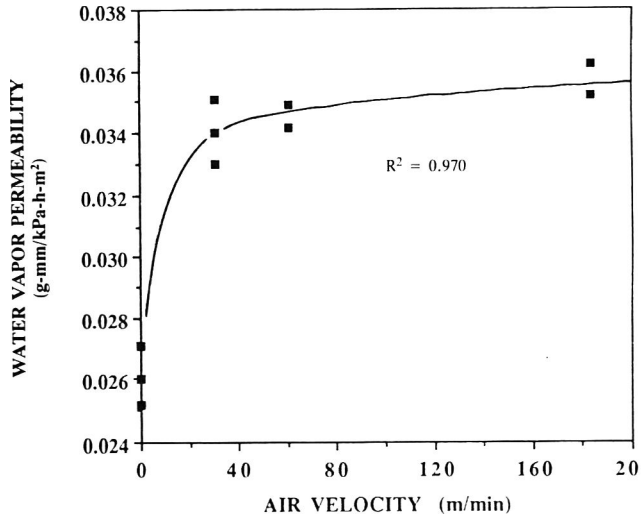

Fig. 1—Effect of cabinet air velocity on cellophane film water vapor permeability values at 25°C.

Water vapor transmission rate (WVTR) calculations

WVTR was determined using Eq. (1) (ASTM E96 method, 1980). Regression analysis of weight loss as a function of time was performed to insure that accurate steady state slopes were obtained. Regression coefficients were >0.998 at $p<0.001$. The area of film exposed in the test cup was 78.5 cm^2.

$$WVTR = \frac{Slope}{Film\ area} \quad (1)$$

where Slope = Weight loss vs. Time.

Water vapor permeability (WVP) correction method

Using a classical method for calculating diffusion of water vapor through air, mass transfer through the stagnant air layer in the test cup was accounted for (Bird et al., 1960; Krochta, 1992). Equation (2) enables calculation of the corrected water vapor partial pressure at the film inner surface (p_2) for hydrophilic films.

$$WVTR = \frac{P * D * Ln[P - p_2)/(P - p_1)]}{R * T * \Delta z} \quad (2)$$

where P = Total pressure; D = Diffusivity of water through air at 25°C; R = Gas law constant ($82.057*10^{-3}$ m^3-atm/kgmol-K); T = Absolute temperature (298 K); Δz = Mean stagnant air gap height (zoriginal + zfinal)/2; p_1 = Water vapor partial pressure at solution surface; p_2 = Corrected water vapor partial pressure at film inner surface in cup.

Water vapor permeance could then be calculated using Equation (3). The corrected partial pressure at the inner surface of the film, p_2 was employed in Eq. (3) to calculate true water vapor permeance.

$$Permeance = \frac{WVTR}{p_2 - p_3} \quad (3)$$

where p_3 = Water vapor partial pressure at film outer surface in the cabinet.

Equation (4) could then be used to calculate true WVP of the film, by multiplying the corrected permeance and average film thickness. This procedure, using Eq. (2), (3) and (4), was defined as the WVP Correction Method.

$$Permeability = Permeance * Thickness \quad (4)$$

RESULTS & DISCUSSION

Air velocity effects

The effect of air velocity on WVP for cellophane films (Fig. 1.) showed air speeds in excess of 152 m/min. resulted in constant WVP. Below that speed, WVP decreased exponentially with decreasing air speeds, indicating the importance of

Table 1—Effect of stagnant air gap height on water vapor permeability calculated using the ASTM E96 method for sodium caseinate films at 25°C

Air gap height (mm)	Film thickness (mm)	RH[a] (%)	WVP[b] (g-mm/kPa-h-m²)
8.08	0.081	100	1.18
12.60	0.079	100	0.88
30.34	0.071	100	0.56

[a] Relative humidity values refer to ASTM assumed conditions at the film underside during testing.
[b] Water vapor permeability.

Table 2—Comparison of the ASTM E96 method and the WVP Correction method for calculation of film inner surface relative humidity and water vapor permeability values at 25°C.

	RH[a] (%)		WVP[b] (g-mm/kPa-h-m²)	
Film Type	ASTM	WVP correction	ASTM	WVP correction
Hydrophilic films				
50%WPI/50%GLY[c]	100	65	3.35	5.16
Sodium Caseinate	100	80	1.18	1.48
56%WPI/16%S/ 28%BW[d]	100	94	0.80	0.85
Synthetic films				
HDPE[e]	100	100	0.0012	0.0012
Polyester	100	100	0.0091	0.0091

[a] Relative humidity values refer to conditions at the film underside during testing.
[b] Water vapor permeability.
[c] 50% WPI/50%GLY is a film having total solids of 50% whey protein isolate and 50% glycerin.
[d] 56% WPI/28%BW/16%S films have 56% whey protein isolate, 28% beeswax and 16% sorbitol solids.
[e] HDPE abbreviates high density polyethylene films.

air circulation. Schultz et al. (1949) reported that relative humidity at the outer film surface was maintained near the equilibrium of the solution or desiccant in the bottom of the desiccator when the fan was run at 850 rpm. This was not generally applicable since rpm does not consistently define air speed. Therefore, 152 m/min was a better reference for accurate maintenance of relative humidity, as well as for elimination of mass transfer resistance outside the test cups. Failure to adhere to these air circulation conditions could result in large errors in WVP values.

WVP correction method

WVP values were calculated using experimental WVTR data using different stagnant air gap heights and similar film thicknesses (Table 1). The ASTM E96 method is based on the assumption that the resistance to mass transfer within the stagnant air layer is negligible; therefore, the relative humidity under the film is assumed to be 100% when water is inside the test cup. Thus, the WVP values for films tested using different air gap heights should be equal, according to the ASTM method. The different WVP values obtained at different stagnant air gap heights (Table 1) prove that the ASTM assumption of 100% RH at the film underside was inappropriate for examination of hydrophilic film WVP properties. The WVP Correction Method accounts for the water vapor partial pressure gradient in the stagnant air gap, alleviating the effects of air gap height on film WVP values.

The WVP Correction Method was essential when examining the WVP properties of hydrophilic edible films (Table 2). Up to 35% errors were found when the WVP correction method was not employed. The effect of the WVP Correction Method decreased as WVP values of films decreased and the relative humidity at the inner surface of the film in the cup approached 100%. Whey protein emulsion films containing 56% WPI, 16% S and 28% BW (weight percentage dry solids) maintained 94% RH at the film inner surface. However, 50% WPI and 50% GLY films were less resistant to mass transfer, resulting in 65% RH at the film inner surface.

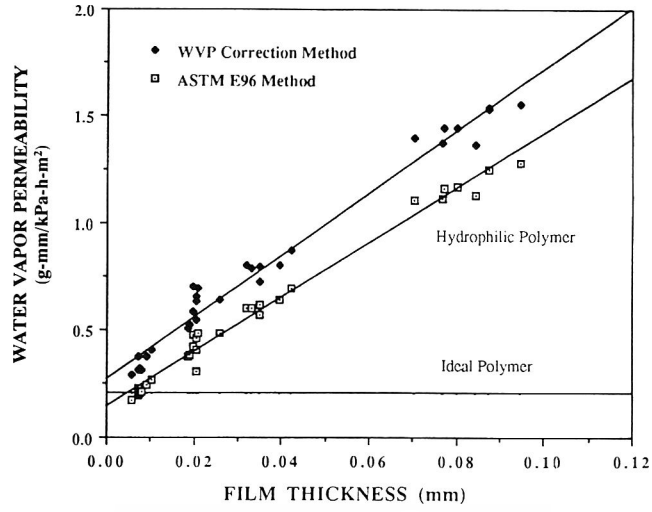

Fig. 2—Relationship between film thickness and water vapor permeability for hydrophilic sodium caseinate films and ideal polymer films at 25°C.

The WVP Correction Method was not necessary for synthetic polymer films (Table 2) such as high density polyethylene (HDPE) and polyester (Mylar), due to their low WVPs. High resistances to moisture transfer resulted in maintenance of the assumed 100% RH at the film underside within the test cup for those films.

Thickness effects

Due to their homogeneous structure, sodium caseinate films were chosen for examination of thickness effects on film WVP properties. Many edible films are hydrophilic in nature, resulting in characteristic thickness effects (Fig. 2) for sodium caseinate films. Ideal polymeric films exhibit no thickness effect on WVP; however, hydrophilic films often exhibit positive slope relationships between thickness and water vapor permeability. The WVP values for sodium caseinate films calculated using the WVP Correction Method were compared with those using the ASTM E96 method. It was apparent (Fig. 2) that use of the WVP Correction Method did not eliminate thickness effects.

Previous studies have indicated similar relationships between film thickness and permeability properties in hydrophilic film systems (Hauser and McLaren, 1948; Schultz et al., 1949; Barrer, 1951; Banker et al., 1966; Swarbrick and Anton, 1968; Hagenmaier and Shaw, 1990). Several explanations have been provided for such anomalous thickness effects. Hauser and McLaren (1948) attributed the observed thickness effect in cellulose films to different structures being formed at different thicknesses. Schultz et al. (1949) attributed them to equilibrium moisture relationships at film-air interfaces differing from test cup solution equilibrium conditions; however, they failed to define such relationships. Barrer (1951) and Banker et al. (1966) extended the Hauser and McLaren (1948) explanation by attributing thickness effects to film swelling as a result of attractive forces between films and water. Such film swelling could result in varying film structures. Hagenmaier and Shaw (1990) reported thickness effects in hydroxymethylcellulose films, yet provided no explanation. The WVP Correction Method provides a means for explaining such thickness effects.

The effect of film thickness on the corrected percent relative humidity conditions at the underside of the film in the test cup (Fig. 3) indicated an exponential relationship between these factors. Without the WVP Correction Method to calculate the true relative humidity conditions at the film underside, this

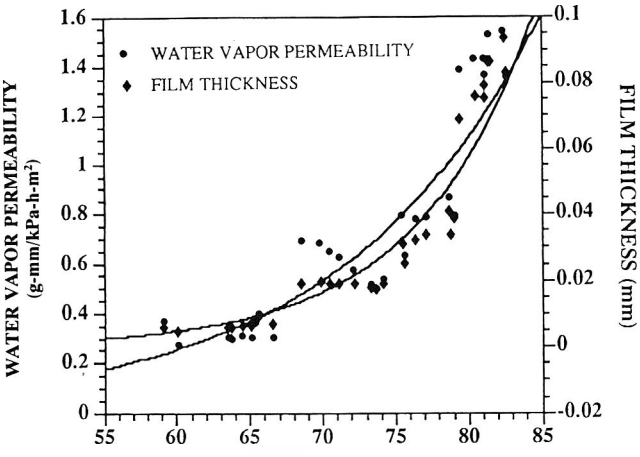

Fig. 3—Relations between percent RH at underside of film and WVP and film thickness for sodium caseinate films at 25°C.

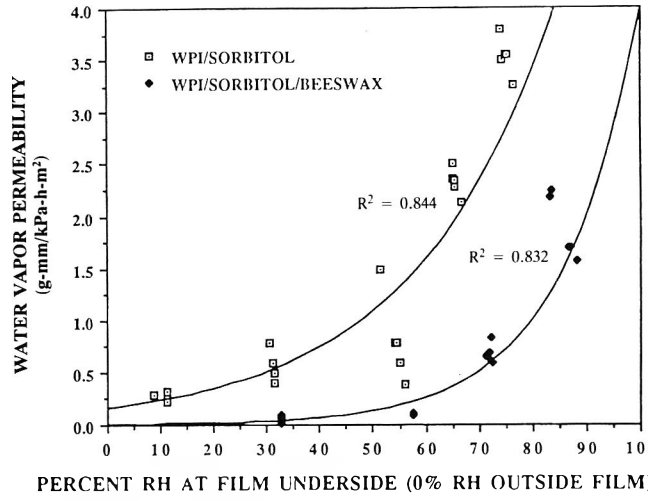

Fig. 4—Effect of relative humidity conditions under the film on the water vapor permeability properties of 50% WPI/50%S and 56% WPI/28%S/16%BW films at 25°C.

Table 3—Effect of stagnant air gap height on WVP values calculated using the WVP Correction method for sodium caseinate films at 25°C

Air gap height (mm)	Film thickness (mm)	RH[a] (%)	WVP[b] (g-mm/kPa-h-m²)
8.08	0.038	74.9	1.08
12.50	0.079	75.2	1.30
8.08	0.010	65.7	0.41
30.34	0.071	66.3	0.84

[a] Relative Humidity values refer to calculated conditions at the film underside during testing.
[b] Water Vapor Permeability.

relationship was not apparent. As film thickness increased, the film provided an increased resistance to mass transfer across it; consequently, the equilibrium water vapor partial pressure at the inner film surface increased.

An exponential relationship existed between the corrected relative humidity conditions at the underside of the film and the film WVP values for caseinate (Fig. 3) and other types of hydrophilic films (Fig. 4). These curves are characteristic of all hydrophilic films, even those containing lipids, as can be observed for the 56%WPI/28%S/16%BW emulsion films.

An exponential relationship between relative humidity and WVP in hydrophilic film systems has been reported in other studies. De Leiris (1986) found that WVP of an oriented polyamide hydrophilic film varied exponentially with the outer relative humidity conditions. The inner relative humidity was held constant at 0% RH. Myers et al. (1962) observed similar exponential relationships between water vapor transmission and RH for Nylon 6-6 films. These relationships were also reported by Karel et al. for cellulose films (1959). Hauser and McLaren (1948) reported exponential relationships between permeability and relative humidity for polyvinyl alcohol, cellulose acetate, and nylon hydrophilic films. Lieberman et al. (1972) observed similar effects for collagen based edible films. They reported that both water sorption and carbon dioxide permeability varied exponentially with RH conditions of the test. Hagenmaier and Shaw (1991) reported the same relationships between oxygen permeability and RH for shellac coatings. Hydrophobic films such as polyethylene were shown by Myers et al. (1962) to have water vapor transmission rates independent of RH.

The true cause of thickness effects shown in Fig. 2 is apparent from Fig. 3. The exponential relationships between WVP and RH overlap with those between film thickness and RH (Fig. 3). Hydrophilic films exhibit increased WVP values at increasing film thicknesses due to the increased water vapor partial pressure conditions to which the underside of the film is exposed within the cup. Thus, the true cause of thickness effects shown in Fig. 2 was relative humidity. RH increased due to increased film thicknesses resulting in higher WVP values because of the non-linear nature of hydrophilic film sorption isotherms.

Development of curves depicting the relationship between WVP and 0 - X% RH conditions enables prediction of film WVP properties under any RH gradient. Hauser and McLaren (1959) developed an extremely useful model to predict WVP values for hydrophilic film systems under any RH conditions, using plots such as those shown in Fig. 3 and 4. This method allows modeling of mass transfer through coatings on food systems exposed to any water activity gradient. McHugh and Krochta (1993) showed that the Hauser and McLaren model enabled accurate prediction of film WVP under any RH gradient.

Stagnant air layer effects on WVP correction method

In order to explore the applicability of the WVP Correction Method, the effect of the stagnant air gap height on the relationship between WVP and calculated inner surface RH was examined. Several characteristic results are listed (Table 3) and a complete plot of all the data allowed comparison of relationships between them (Fig. 5). The stagnant air gap height affected the relationship between calculated RH and WVP (Table 3), and the use of the WVP Correction Method accounted for this effect. The effect of film thickness on WVP was earlier shown to result from variations in the water vapor partial pressure at the underside of the film during testing. Under equivalent RH conditions, we concluded that film WVP values should be equivalent, independent of film thickness. Comparison of selected film RH and WVP values at 8.08 and 12.5 mm stagnant air gap heights using the WVP Correction Method, showed that at similar RH (74.9% and 75.2%) WVP was within ± 8%. This difference was slight, considering the exponential effect of RH on film WVP values. Thus, there was little or no effect of stagnant air gap height < 12.5 mm gaps on film WVP values. Calculated RH at 8.08 and 30.34 mm stagnant air gaps was similar for the films we used (65.7% and 66.3%). Therefore, film WVP values were expected to also be within ±8%, assuming convection within the stagnant air gap was negligible. However, WVP values differed by a factor of 2, suggesting convection existed within the stagnant air layer for large air gaps.

At stagnant air gap depths of ≈14 mm (1/depth = 0.071/

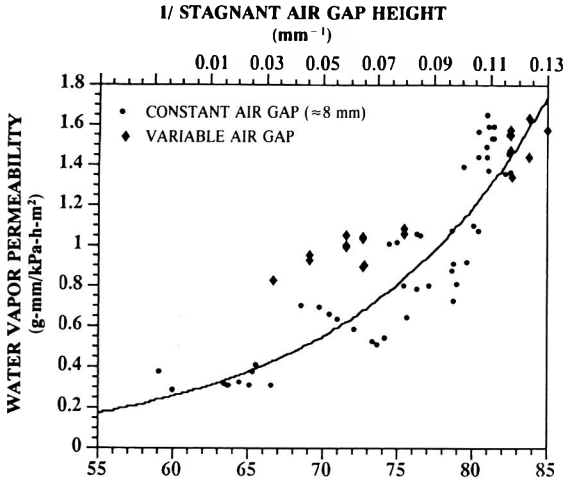

Fig. 5—Effect of stagnant air gap height on the relationship between calculated relative humidity under the film and water vapor permeability of sodium caseinate films at 25°C.

mm) convection within the cup (Fig. 5) was neglible due to wall effects. At < 14 mm stagnant air gaps, WVP values corresponded with the relationship between WVP and RH obtained for different film thicknesses at ≈8 mm air gap. However, at >14 mm stagnant air gaps, some convection was present within the cups, probably due to evaporative cooling. Low levels of convection are responsible for the higher than expected WVP values when stagnant air gaps were >14 mm. We recommend that stagnant air gap heights of <14 mm should be employed for accurate determination of film WVP using the WVP Correction Method.

CONCLUSIONS

The WVP Correction Method can be used to obtain corrected WVP values and film underside RH conditions. These values, as well as film thicknesses, should be reported in edible film studies. Only with accurate WVP values and knowledge of true relative humidity test conditions can barrier properties of such films be assessed for possible food applications and compared with other films. Cabinet air speeds should be maintained >152 m/min to ensure accurate external film RH and minimize mass transfer resistance at the film interface.

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Water Vapor Permeability of Caseinate-Based Edible Films as Affected by pH, Calcium Crosslinking and Lipid Content

R.J. AVENA-BUSTILLOS and J.M. KROCHTA

ABSTRACT

Edible films were cast from solutions of sodium or calcium caseinate and from emulsions of these proteins with acetylated monoglyceride, beeswax, and stearic acid. The water vapor permeabilities of the films were evaluated at 25°C using the ASTM E96-80 method, modified to calculate the % relative humidity at the film underside. Adjustment to pH 4.6 (isoelectric point), calcium ion crosslinking and combined effects of calcium ascorbate buffer (pH 4.6) reduced water vapor permeability of sodium caseinate films by 36%, 42%, and 43%, respectively. Calcium caseinate-beeswax emulsion films had water vapor permeabilities up to 90% lower than pure sodium caseinate films. Water vapor permeability varied by a factor of two depending on emulsion film orientation, indicating nonisotropic structure.

Key Words: casein, edible film, crosslinking, permeability

INTRODUCTION

DEVELOPMENT of casein-based coatings and films can utilize the excellent nutritional value and increase potential for rapid acceptance and widen use of this dairy product (Fox, 1989). Caseins as emulsifiers can create stable casein-lipid emulsions that can be easier to apply as coatings on foods than lipids alone with possibilities for films with applicable water vapor and gas permeabilities. Caseins, being amphipathic, are very surface-active and readily locate at oil-water interfaces (Leman and Kinsella, 1989). The hydrophilic nature of proteins incorporated into edible films induces interaction with water, causing swelling (Barrer, 1951) and apparent thickness effects (Banker et al., 1966; Swarbrick and Amann, 1968; Biquet and Labuza, 1988; McHugh et al., 1993), and deviation from Fick's law (Barrer, 1951; Crank, 1975).

Casein has been studied for making films and was reported to be an ineffective moisture barrier, as expected for hydrophilic materials (Guilbert, 1986). However, some industrial applications of crosslinked caseins are related to their capacity to reduce gas and water permeabilities, such as in leather finishes, wood glues and paper coatings (Barrer, 1951; Southward, 1989; Anonymous, 1990). Krochta et al. (1990) took advantage of the emulsifying capabilities of casein to create casein-lipid emulsion films with improved moisture-barrier properties. In those emulsion films, the lipid material provided resistance to moisture movement, while the casein provided structural cohesion, bound the film to wet surfaces, and reduced the waxy appearance. Kamper and Fennema (1984) reported that water vapor permeability of emulsion films varied greatly with film composition and orientation of molecules. Also, lipids seem to be the most effective barrier to movement of water through edible films.

Moisture and gas barrier properties of casein-based films may be enhanced by calcium crosslinking of casein and by adjusting to the isoelectric point to optimize protein-protein interactions. These treatments could possibly reduce protein solubility and reduce water vapor and gas permeabilities. Kester and Fennema (1986) indicated that treatment with calcium ions had no effect on water-barrier properties of protein films, but acid treatments improved resistance to water vapor diffusion in protein films. Southward (1989) reported that calcium crosslinking of casein resulted in water insolubility for glue manufacture. Krochta et al. (1990) reported that the moisture-barrier properties of emulsion films were improved with calcium-ion crosslinking of casein and by adjusting casein-lipid films to the casein isoelectric point. Relative water vapor transmission rates were compared, but permeabilities were not measured. It was assumed that ionic crosslinking reduced protein polymer segmental mobility, as well as protein solubility in water, thus reducing water vapor and gas permeability through the protein matrix. Likewise, adjustment to the isoelectric point modifies molecular configuration and could influence film mass-transfer properties.

The objectives of our study were to determine the effects of pH adjustment and calcium ion crosslinking, different concentrations of acetylated monoglycerides, stearic acid and beeswax, and film orientation on water vapor permeability of caseinate-based films.

MATERIALS & METHODS

Film emulsion composition

Solutions of sodium and calcium caseinate and their emulsions with different lipid materials at 8% (w/w) total solids in water were made for casting films. One sodium caseinate and one calcium caseinate were studied: Alanate-110™ and Alanate-310™, respectively (New Zealand Milk Products, Santa Rosa, CA). Myvacet 5-07™ (Eastman Chemical Products, Inc., Kingsport, TN), an acetylated monoglyceride which earlier demonstrated good film formation properties (Krochta et al., 1990), was used. Australian white beeswax (National Wax Co., Skokie, IL) and 95% stearic acid (Aldrich Chem. Co., Inc., Milwaukee, WI) were also used.

Solutions were prepared by first adding the selected amount of sodium or calcium caseinate to 184g distilled water at room temperature (≈23°C) in a 500-mL vacuum flask. For making emulsion, the solution was warmed to 50°C or 68°C on a hot-plate stirrer and the selected amount of Myvacet 5-07, or beeswax and stearic acid, respectively, was added to prepare caseinate-based emulsions up to a total of 16g solids in a 200-g solution. After melting, the mixture was homogenized at 13,500 rpm for 1 min in an Ultra-Turrax T-25 homogenizer (IKA-Works, Inc., Cincinnati, OH) with a 25-F probe. Vacuum was then applied to remove bubbles that could become pinholes after film drying.

Film casting

A film was cast by pouring 20 mL of a solution or emulsion onto a 14.7-cm (i.d.) rimmed, smooth Plexiglas™ poly-(methyl methacrylate) plate on a level surface. Six films were prepared for each solution or emulsion. The solution or emulsion was spread evenly with a bent glass rod and allowed to dry overnight for ≈18 hr at room temperature (≈23°C). The resultant film could be peeled intact from the casting surface.

Calcium crosslinking and pH adjustment

A calcium chloride solution (pH 9.6) and different buffers at pH 4.6 (average isoelectric point of caseins) were used to soak dried

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Table 1—Effect of calcium crosslinking and isoelectric point adjustment on water vapor permeability of sodium caseinate (Alanate-110) films at 25 °C. A calcium caseinate (Alanate-310) film is also reported for comparison[a]

Parameter	Sodium caseinate (A-110)	Calcium caseinate (A-310)	A-110 Treated with sodium acetate buffer (pH 4.6)	A-110 Treated with sodium ascorbate buffer (pH 4.6)	A-110 Treated with calcium chloride (pH 9.6)	A-110 Treated with calcium ascorbate buffer (pH 4.6)
Water vapor permeability (g-mm/KPa-hr-m²)	1.53 ± 0.12[a]	1.17 ± 0.09[b]	1.04 ± 0.13[c]	0.93 ± 0.05[c,d]	0.88 ± 0.07[d]	0.87 ± 0.11[d]
Thickness (μ = mm × 10^3)	83 ± 7[a]	82 ± 9[a]	70 ± 10[b]	72 ± 10[b]	58 ± 12[c]	72 ± 10[b]
% RH at film underside	81.2 ± 2.0[a]	84.8 ± 0.9[c]	84.3 ± 1.6[c]	85.6 ± 1.2[c,d]	82.6 ± 1.1[b]	86.4 ± 1.5[d]

[a] Values reported are means and standard deviations. Superscript letters indicate significant difference at $p<0.05$ by Duncan's multiple comparison tests. 1 mil = 0.001 in = 0.0254 mm. 1 g-mm/KPa-hr-m² = 125.97 g-mil/mmHg-day-m².

Table 2—Effect of composition of sodium caseinate (A-110)/acetylated monoglyceride (M-507) emulsion films on water vapor permeability at 25 °C (shiny side down)[a]

% Sodium caseinate (A-110) in emulsion film	Water vapor permeability (g-mm/KPa-hr-m²)	Film thickness (μ = mm × 10^3)	% RH at film underside
100	1.53 ± 0.12[a]	83 ± 7[c]	81.2 ± 2.0[a]
80	1.06 ± 0.11[b]	94 ± 8[a]	88.1 ± 0.8[c]
50	0.92 ± 0.02[c]	88 ± 5[b]	88.0 ± 0.4[c]
20	0.66 ± 0.16[d]	40 ± 8[d]	83.5 ± 2.6[b]

[a] Values reported are means and standard deviations. Superscript letter indicate significant difference at $p<0.05$ by Duncan's multiple comparison tests. 1 mil = 0.001 in = 0.0254 mm. 1 g-mm/KPa-hr-m² = 125.97 g-mils/mmHg-day-m².

sodium caseinate films and sodium caseinate/acetylated monoglyceride emulsion films. One-minute soaks were used, instead of the 3-min soaks reported earlier (Krochta et al., 1990). Excess buffer solution was removed by immersing treated films in distilled water for 1 min. Resulting films were allowed to dry overnight.

Film thickness

Initial and final thicknesses of the films, before and after each experiment, were measured with a micrometer at five random positions by slowly reducing the micrometer gap until the first indication of contact.

Water vapor permeability

The ASTM E96-80 (ASTM, 1989) "Water Method", modified to estimate the % relative humidity (% RH) at the film underside (Krochta, 1992; McHugh et al., 1993), was used to measure water vapor permeability with poly-(methyl methacrylate) test cells. Distilled water (7 mL) was placed in each test cell to expose the film to high % RH on one side through a circular opening of 5 cm diameter. Four 6.5-cm-diameter films of each formulation were sealed in the test cells using a silicone sealant (High Vacuum Grease, Dow Corning, Midland, MI) to avoid vapor leaks through cell joints. The side of the film facing the high % RH was noted. Cast films were shiny on the side facing the casting plate surface and dull on the side facing the drying air during casting. After assembly, test cells with films were weighed on an analytical balance with ±0.0001g precision. Twelve cells, corresponding to three different film treatments, were placed randomly in cabinets containing anhydrous calcium sulfate (W.A. Hammond Drierite Co., Xenia, OH). The cabinets were equipped with fans for high air movement to ensure 0% RH throughout. This provided a strong driving force across the film for water vapor diffusion. Unsteady-state periods as short as 5 min for thin caseinate films were demonstrated in preliminary tests. Weights of test cells were recorded for 24 hr at steady-state over 6 periods of time, not less than 3 hr apart, in an incubator room at 25°C. By simple linear regression, the slope of weight loss vs. time was calculated to determine the water vapor transmission rate in g/hr. Linear regression analysis gave very significant correlation ($p<0.001$, with $r = 0.995$) as mean value for all test films. Water vapor flux was calculated by dividing water vapor transmission rate by the test cell mouth area. Permeance was calculated using the vapor pressure of liquid water at 25°C (Perry and Chilton, 1973) and the % RH difference, as estimated from the % RH at the film underside (Krochta, 1992; McHugh et al., 1993). Water vapor permeability was calculated by multiplying permeance by the mean final film thickness (ASTM, 1989).

RESULTS & DISCUSSION

Calcium crosslinking and pH adjustment

Adjustment to pH 4.6, calcium ion crosslinking and combined effects with calcium ascorbate buffer reduced water vapor permeability (Table 1) of sodium caseinate films by 36%, 42%, and 43%, respectively. Calcium caseinate had a lower permeability than sodium caseinate. However, the calcium caseinate permeability was not as low as those sodium caseinate films modified with calcium chloride solution or calcium ascorbate buffer. We concluded that the calcium caseinate film and the calcium ion-modified sodium caseinate films had lower water vapor permeability than sodium caseinate films, due to calcium crosslinking. However, treatment of sodium caseinate films with calcium ion appeared to produce more effective reduction of film water vapor permeability, compared to those films made directly from calcium caseinate. We further concluded that modification of sodium caseinate films by adjustment to the casein isoelectric point (pH 4.6) reduced water vapor permeability, most likely due to increased protein-protein interaction.

To compare water vapor permeabilities of edible hydrophilic films requires knowledge of the film thickness and % RH at each film underside (Krochta, 1992; McHugh et al., 1993). The thicknesses of the treated films were reduced by the buffer and calcium chloride soaking followed by water rinsing (Table 1). Buffer treatment followed by rinsing reduced thickness an average of 14%, and calcium chloride treatment reduced thickness 31%. We hypothesized that chemical treatments tightened the molecular structure of the caseinate films. The % RH at the film underside was higher for modified sodium caseinate films. Thus, treatments reduced water vapor permeability of sodium caseinate films, in spite of subsequent exposure to higher relative humidity than that for untreated sodium caseinate films.

Caseinate/lipid emulsion films

Introducing acetylated monoglyceride to sodium caseinate film had significant effects on reducing water vapor permeability (Table 2). Increasing acetylated monoglyceride content further reduced permeability. Water vapor permeability of 20% caseinate/80% acetylated monogylceride was 43% that of neat sodium caseinate film.

Calcium ascorbate buffer treatment did not affect water vapor permeability of sodium caseinate/acetylated monoglyceride emulsion films, in contrast to effects on pure sodium caseinate films. Integrity was a problem with buffer treatment of emulsion films. Our buffer treatment results were contrary to those reported by Krochta et al. (1990), who found a reduction in water vapor permeability of sodium caseinate/acetylated mono-

Table 3—Effect of composition of sodium caseinate (A-110)/beeswax emulsion films on water vapor permeability at 25 °C (shiny side down)[a]

% Sodium caseinate (A-110) in emulsion film	Water vapor permeability (g-mm/KPa-hr-m²)	Film thickness (μ = mm × 10³)	% RH at film underside
100	1.53 ± 0.12[a]	83 ± 7[a]	81.2 ± 2.0[a]
87.5	0.99 ± 0.05[b]	92 ± 8[b]	87.7 ± 0.8[b]
75	0.41 ± 0.04[c]	104 ± 7[c]	95.2 ± 0.6[c]
62.5	0.40 ± 0.03[c]	103 ± 5[c]	95.2 ± 0.9[c]

[a] Values reported are means and standard deviations. Superscript letters indicate significant difference at $p < 0.05$ by Duncan's multiple comparison tests. 1 mil = 0.001 in = 0.0254 mm. 1 g-mm/KPa-hr-m² = 125.97 g-mils/mmHg-day-m².

Table 4—Effect of film orientation and formulation on water vapor permeability of calcium caseinate (A-310)/beeswax emulsion films at 25 °C[a]

% Calcium caseinate (A-310) in emulsion film	Water vapor permeability (g-mm/KPa-hr-m²)	Film thickness (μ = mm × 10³)	%RH at film underside
(Shiny side down)			
100	1.17 ± 0.09[a]	82 ± 9[a]	84.8 ± 0.9[a]
72.5	0.55 ± 0.06[b]	80 ± 12[a]	91.6 ± 1.0[b]
62.5	0.34 ± 0.07[c]	82 ± 8[a]	94.8 ± 1.4[c]
(Dull side down)			
100	1.17 ± 0.09[a]	82 ± 9[a]	84.8 ± 0.9[a]
72.5	0.28 ± 0.05[c]	75 ± 8[a]	95.5 ± 0.9[c]
62.5	0.15 ± 0.07[d]	75 ± 2[a]	97.4 ± 0.1[d]

[a] Values reported are means and standard deviations. Superscript letters indicate significant difference at $p < 0.05$ by Duncan's multiple comparison tests. 1 mil = 0.001 in = 0.0254 mm. 1 g-mm/KPa-hr-m² = 125.97 g-mil/mmHg-day-m².

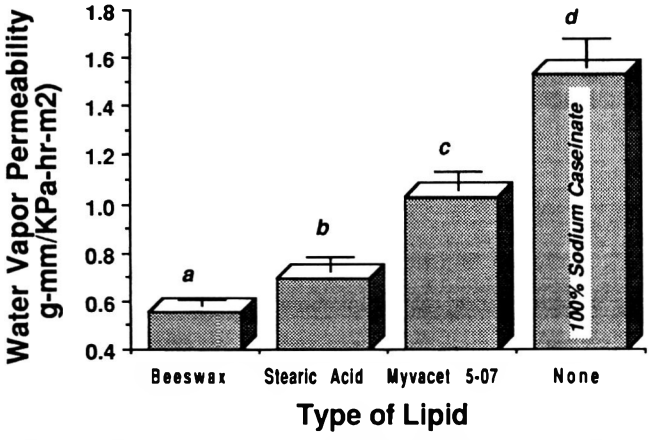

Fig. 1—Effect of lipid type on water vapor permeability in an 80% sodium caseinate (A-110)/20% lipid emulsion film at 25°C (films oriented shiny side down). Means and standard deviations. Letters indicate significant difference at $p < 0.05$.

glyceride emulsion films with buffer treatment to pH 4.6. They probably reflect differences in extent of film modification or in film handling procedures. The longer film treatment times by Krochta et al. (1990) may have produced more extensive protein-protein interaction and/or crosslinking. Chaumette (1991) reported considerable variability in water vapor transmission of sodium caseinate/acetylated monoglyceride emulsion films that were crosslinked with peroxidase. While maximum crosslinking reduced water vapor transmission, partially crosslinking caseinate-based films reportedly caused an increase in water vapor transmission.

Water vapor permeability of sodium caseinate film was reduced more effectively by adding beeswax (Table 3) to give a sodium caseinate-based emulsion film. Adding more than 25% beeswax did not further reduce water vapor permeability, perhaps because it was not dispersed effectively in the sodium caseinate emulsion. Nonetheless, permeability dropped to 26% of that for neat sodium caseinate film. These results most likely reflect the greater hydrophobicity and crystallinity of beeswax, compared to acetylated monoglyceride.

The % RH at the film underside also increased (Table 3) as sodium caseinate content was reduced. In spite of the fact that % RH at the film underside increased as % beeswax increased, water vapor permeability was reduced at low sodium caseinate levels. Also, film thickness increased as relative content of beeswax increased.

Film orientation

Film orientation (toward the water surface in the testing cups) had no effect (Table 4) for pure calcium caseinate films. However, addition of beeswax produced orientation effects, and differences in water vapor permeability due to film orientation increased as amount of lipid increased. The 72.5% calcium caseinate/27.5% beeswax film and the 62.5% calcium caseinate/37.5% beeswax film had 49% and 56% reductions in permeability, respectively, with the dull side down compared to the shiny side down. These results were in spite of the fact that the % RHs at the underside of the dull-side-down films were greater than that for the shiny-side-down film (Table 4). Also, film thickness was not different for differently oriented films, although there was a tendency for more pronounced swelling for films oriented with shiny side down. Other data showed water vapor permeability was higher when the shiny side faced the high % RH, independent of type of caseinate or lipid in emulsion films.

We hypothesized that the lipid concentrated in the dull side of the film facing the air, and that the caseinate concentrated in the shiny side facing the casting plate surface, as expected based on density differences. We also hypothesized that the protein content was swollen by the water-protein interaction, as demonstrated by greater swelling in films with high caseinate concentrations at comparable RH, resulting in greater water permeability. Emulsion films with shiny side down showed a tendency to be thicker than those with dull side down. Kester and Fennema (1989) indicated that swelling probably affected the structural integrity and barrier properties of the lipid component in the emulsion films. However, films oriented dull side down cracked more frequently, as probably the caseinate layer exposed to 0% RH was more brittle for lack of water as plasticizer. These findings indicate the need to report direction of water vapor flow through multicomponent films, as recommended by ASTM (1989). Also, one must consider the influence of molecular orientation of film constituents on film properties.

Type of lipid

Beeswax incorporation into sodium caseinate film was more effective in reducing water vapor permeability (Fig. 1) than stearic acid and acetylated monoglyceride. Also, the sodium caseinate film permeability was higher ($p < 0.001$) than permeability of the caseinate-formulated emulsion films. The beeswax-sodium caseinate film had ≈ half the permeability of the acetylated monoglyceride-sodium caseinate film. Addition of acetylated monoglyceride to a sodium caseinate/beeswax emulsion film system increased water vapor permeability, compared to a sodium caseinate/beeswax film at the same total lipid content. Acetylated monoglycerides are frequently added to wax-based coating formulations for pliability (Kester and Fennema, 1989). According to Kester and Fennema (1989), water vapor permeability is related to lipid morphology. The crystalline structure of beeswax films is related to ability to reduce water vapor permeability more effectively.

CONCLUSIONS

DESIRABLE functional properties of caseinate-based films and potential for water vapor permeability reduction by formulation and protein modification provide caseinates advantages for edible coating applications. Water vapor permeabilities of pure caseinate films were reduced by pH adjustment, calcium cross-

linking, and incorporation of lipid materials. Casein-formulated edible films have the advantage that casein forms aqueous emulsions with acetylated monoglyceride and moderate levels of stearic acid and beeswax. This can facilitate coating application to food products at room temperature, while acetylated monoglyceride, stearic acid and beeswax alone require heating for application. Also, casein-formulated films are nearly invisible, adhere well to wet surfaces, and have a bland flavor that will not interfere with the sensory attributes of uncoated products. Beyond control of moisture loss, such edible films have potential for controlling oxygen, ethylene and carbon dioxide content in fruit and vegetable tissue. Such control could reduce product respiration and extend produce shelf-life, as an alternative modified atmosphere treatment.

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Solid Food Thermal Conductivity Determination at High Temperatures

J. P. GRATZEK and R. T. TOLEDO

ABSTRACT

Thermal conductivity of carrots and potatoes was measured using a line heat source probe adapted for measurements to 130°C. Fastest response and rapid attainment of linearity in temperature rise vs *ln* time plots, were obtained with a silicone oil-filled probe with an uninsulated thermocouple junction. Custom designed electronics to amplify thermocouple output, and computer control of measurements, data acquisition and analysis resulted in 0.4% data repeatability (two standard deviations) for calibration standards. Thermal conductivity of carrot and potato vs temperature agreed well with the published volume fraction model for multi-component foods.

Key Words: thermal conductivity, carrots, potatoes, rapid determination

INTRODUCTION

THERMAL PROPERTY data for foods at high temperature conditions are necessary for development of continuous sterilization and aseptic packaging processes for low acid foods containing particulates. Since direct temperature measurement in moving particles is not possible, the U.S. Food & Drug Administration has accepted mathematical simulation to prove safety of thermal processes (Dignan et al., 1989). Reliability of heat transfer models that predict microbial lethality in continuously processed suspended particles must be based on accurate values of the particle thermal properties which include thermal conductivity. A system that enables rapid measurement of thermal conductivities of solids at aseptic processing temperatures and pressures is needed.

Little work has been reported on thermal properties of solid foods at elevated temperatures. Tung et al. (1988) used a quasi-steady state technique to measure thermal diffusivity of foods at 60–100°C. That work demonstrated increasing values of thermal diffusivities with increasing temperatures for vegetable, starch, and meat products. Wadsworth et. al. (1969) demonstrated a similar trend for sweet potatoes undergoing immersion heating. Those findings were consistent with the properties of water — the major component of foods considered in the studies— which has increasing thermal conductivity with increasing temperature. Chang and Toledo (1990) by trial and error simultaneously determined heat transfer coefficients and effective thermal diffusivities during unsteady state heating of carrots at UHT conditions in a packed bed. They reported significant elevation of effective thermal diffusivity and that the elevation was the result of internal convective heat transfer due to penetration of heating fluid into the particle. Heat transfer coefficients between particles and fluid flowing concurrently have been reported by Sastry et al. (1989) and Mwangi et al. (1993). Good agreement was reported between measured values of heat transfer coefficients and values obtained from published correlation equations for calculating heat transfer coefficients from fluid properties and conditions of flow. However, the true conditions of flow for suspended particles, free to change positions and interact with surrounding particles while flowing in the pipe, were not well defined. Therefore, if simulation of microbial lethality in a flowing particle is possible, validation of the calculated process using microbial inoculation would be required to prove safety.

Average heat transfer coefficients between a flowing fluid and particles may be estimated from microbial lethality or time/temperature changes induced by heat within a holding tube of an aseptic processing system. Such methods have the advantage of utilizing food particles of the size and density used in practice. Accurate thermal conductivity data will be needed when estimating convective heat transfer coefficients from quantitation of microbial lethality in continuously processed inoculated particles. Multiple pairs of heat transfer coefficients and thermal conductivity could give similar time and temperature profiles in particles undergoing transient heating. Thus, an error in thermal conductivity values would cause over or underestimation of heat transfer coefficients.

The objective of our study was to develop a system for measuring thermal conductivity in the range 20–130°C. We hypothesized that the change in thermal conductivity with temperature would parallel that of the volume averaged multi-component model of Choi and Okos (1987). If water penetrated the particle during sample heating, the increase in moisture content should affect the thermal conductivity vs temperature profile.

MATERIALS & METHODS

Theoretical basis for thermal conductivity measurements

We measured thermal conductivity using the line heat source method, also known as the probe method. The temperature rise in a cylindrical probe surrounded by the food sample initially at thermal equilibrium was measured after a step input of thermal energy generation within the probe. This method was used because of its relative simplicity, ability to measure thermal conductivity directly, and short measurement times, all important advantages over steady-state techniques.

In measurements with a constant line heat source, heat is transferred radially to the surrounding sample only by conduction. Nonideal end effects are minimized by using a probe with a large length/diameter ratio. The temperature rise at the line source resulting from the energy input was monitored with time. This time-temperature history and the level of energy input were used to calculate thermal conductivity.

The governing differential equation describing this time dependent heat transfer is:

$$\partial T/\partial t = \alpha \nabla^2 T, \qquad (1)$$

and is subject to the boundary conditions:

$$\begin{aligned} &\text{at } t \leq 0 \text{ and any } r && \Delta T(r,t) = 0, \\ &\text{at } r = 0 \text{ and any } t \geq 0 && \lim_{r \to 0} (r \partial T/\partial r) = -q/2\pi k, \\ &\text{at } r = \infty \text{ and any } t \geq 0 && \lim_{r \to \infty} \Delta T(r,t) = 0, \end{aligned}$$

where, q is the magnitude of power applied per unit length to the linear, constant heat source; α is the thermal diffusivity; and, $\Delta T(r,t) = T(r,t) - T_o$, where T_o is the initial equilibrium temperature of the fluid.

Thermal diffusivity is defined as follows:

$$\alpha = k/\rho c_p,$$

where k = thermal conductivity, ρ = density, and c_p = specific heat.

For sufficiently large times for a heat source with a non-zero radius,

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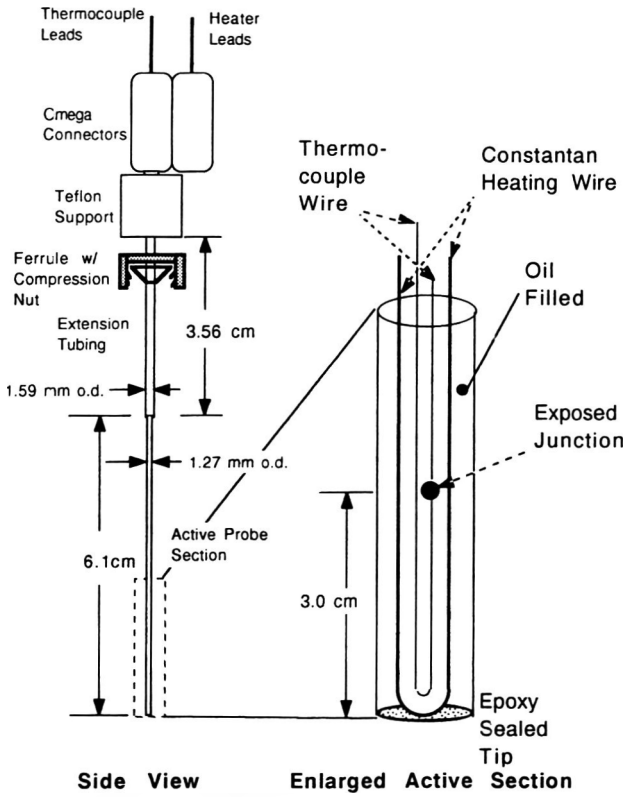

Fig. 1—Thermal conductivity probe.

and assuming that k, ρ, and c_p do not vary over the length of the test, an approximate solution to the governing differential equation (Eqn. 1) is (Healy et al., 1976):

$$\Delta T(a,t) = q/4\pi k\, ln(4\alpha t/a^2 c), \quad (2)$$

where c = 1.781. . . and a = radius of the heat source.

Equation (2) indicates a linear relationship between ΔT and *ln* time and is the basis for the thermal conductivity measurements using the line heat source method. From (2),

$$k = q \Big/ \left(4\pi \frac{d\Delta T(a,t)}{d\, ln(t)}\right). \quad (3)$$

The linear ΔT vs *ln*(t) relationship allows for convenient monitoring of the reliability of the measurement. When other modes of heat transfer, namely convection, and non-equilibrium situations exist, the anomaly can easily be detected by departure of the ΔT vs. *ln* time from linearity (Healy, et al., 1976).

Probe construction

A schematic of the probe is shown (Fig. 1). It consisted of 4 sections: miniature connectors (Omega Engineering), Teflon support, extension tubing, and the active probe section. Teflon insulated Constantan heater wire (0.076 mm wire diameter, 0.076 mm PFA insulation) and Teflon insulated Copper-Constantan thermocouple wire (0.076 mm wire diameter, 0.076 mm PFA insulation) were enclosed in the 6.9 cm long 18 gage (1.27 mm o.d.) stainless steel active probe section. Both wires were cemented into the tip of the probe with high temperature epoxy (Omega Bond 200). The uninsulated thermocouple junction was 3.0 cm. from the probe tip. The active section was filled with silicone oil and sealed at the top with epoxy. The active section's outer diameter fit snugly into the inside of the extension tubing (1.59 mm o.d., 1.32 mm i.d.). The two were epoxied together with the smaller active section inserted 7.6 mm into the extension tube. The exposed active tube section was then 6.1 cm, giving a length/diameter ratio of 48. The heater wire and thermocouple wire were enclosed by the extension tubing and terminated in a miniature connector.

The extension tubing was fitted with a ferrule and compression nut to allow placement of the active section into high pressure environments while the probe leads remained at ambient conditions. The

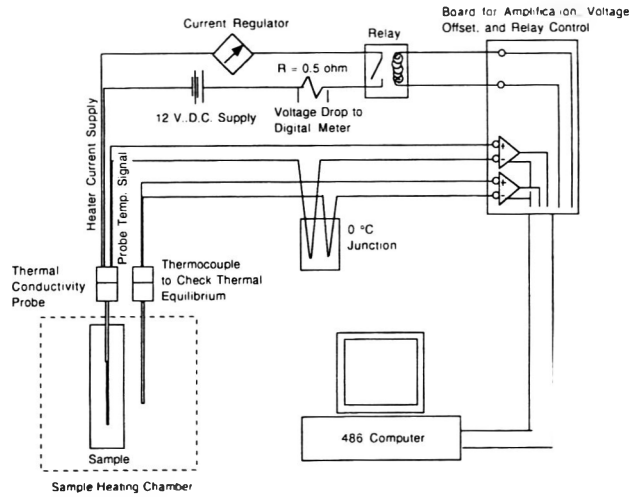

Fig. 2—Instrumentation arrangement.

Teflon™ outer support served as a probe handle as well as a means for joining the miniature connectors and the extension tube. The Teflon support completely encircled the upper end of the extension tubing. The two were pressure fitted together and sealed with epoxy. The end of the Teflon support was machined to fit tightly into a square gap in the miniature connector. A hole drilled in the connector allowed the heater wire to pass through to the heater leads.

Thermal conductivity measurement

A schematic of the instrumentation for thermal conductivity measurements is shown (Fig. 2). A software controlled 12 bit high speed internal A/D converter (Metrabyte Dash-8) installed in a 486 personal computer was used to control the thermal conductivity measurement. After the sample was loaded into the heating chamber with the probe inserted along the axis of the cylindrical sample, the temperature of both the surrounding fluid and the sample were monitored until they were identical. The pre-calibrated thermocouple signals were amplified (gain = 1480) and offset by −5 volts with a custom made electronic circuit board so that the entire range of the 12 bit −5 V to +5 V A/D converter could be used. Accuracy of temperature measurements was probably no greater than 0.1°C, while measurement resolution was < 0.1°C. When the sample and surrounding fluid were at the same temperature, the measurement was started. The computer controlled relay triggered flow of a pre-set constant current into the probe heater wire. The current level was determined by measurement of the voltage drop across a high precision (0.05%) 0.5 ohm shunt resistor with a digital voltmeter. The calibration current level of 0.140 amps was used for all tests. The temperature signal was measured at a sampling frequency of 200 Hz with time averaging each 0.15 sec. Tests were usually run for 30 sec. Time and temperature information were recorded.

Calibration

Probes were calibrated with glycerin and 0.4% agar gel at room temperature (≈25°C). Use of thickeners such as agar to inhibit convection is an accepted practice (Sweat, 1986). Thermal conductivity was determined from the following:

$$k = C2I^2R \Big/ \left(4\pi \frac{d\Delta T(a,t)}{d\, ln(t)}\right), \quad (4)$$

where I = the probe heater input current, R = the resistance/length of Constantan heating wire, and C = the calibration factor. The numerator is multiplied by 2 because the wire doubled back in the probe. ΔT was the temperature rise from the equilibrium temperature as recorded by the probe thermocouple. The calibration factor accounted for uncertainties in heating wire resistance and heating wire input current, and any nonideal geometry effects. After an initial transient period ΔT vs *ln* time approached linearity. The calibration factor C was chosen to set k equal to the published thermal conductivity of 0.4% agar gel. Values of C for the probes were between 1.02 and 1.04. The glycerin was used to verify the validity of the calibration

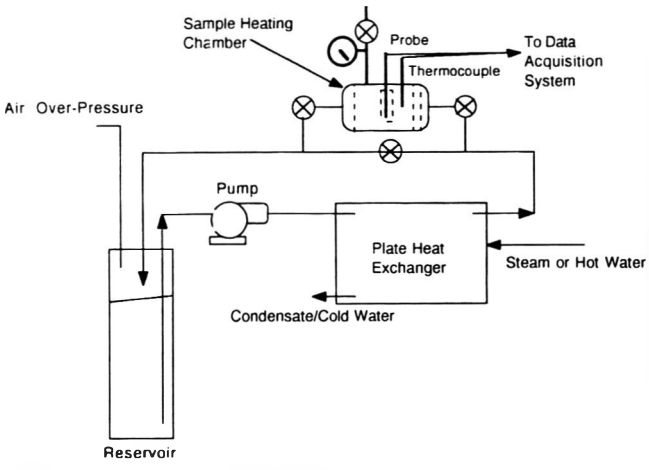

Fig. 3—Recirculating heating system.

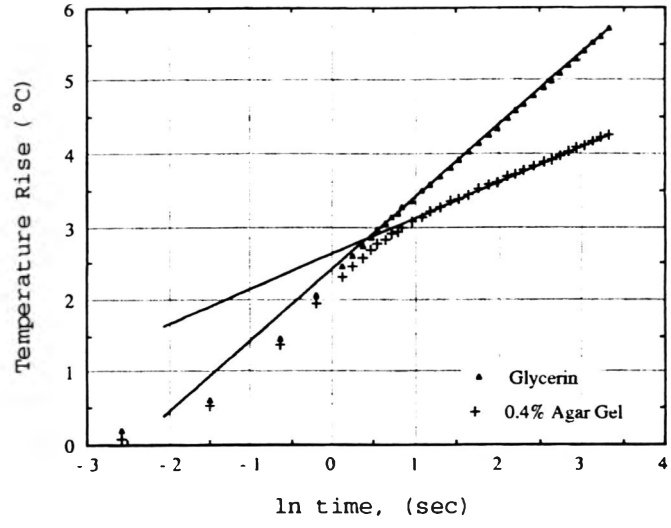

Fig. 4—Typical experimental data obtained during probe calibration.

factor determined using agar gel on a sample having different thermal conductivity.

Sample heating

Two heating systems were used. One used a recirculating temperature controlled fluid for heating (Fig. 3). This heating circuit consisted of a water reservoir, pump, plate heat exchanger, and sample heating chamber. It was necessary to apply air pressure to the reservoir to prevent boiling at > 100°C. Water could be pumped through the sample chamber or the chamber could be bypassed by adjusting appropriate valves. Samples 6.6 cm long and 1.65 cm in diameter were placed in a cylindrical wire mesh frame fastened in the center of the sample heating chamber. The cylindrical sample heating chamber (7.62 cm i.d., 15.24 cm long) had two layers of perforated plate at the inlet side and one layer of perforated plate at the exit side. The plate nearest the entrance had a baffle placed directly downstream from the entrance in order to spread the flow. After loading the sample, its heating chamber was filled with water at room temperature (≈25°C) and low pressure. Thermal conductivity measurements at room temperature were made upon reaching temperature equilibrium. The sample chamber was isolated from the heating water circuit while preheating. When the water reached measurement temperature, it was routed through the chamber. The water and sample temperatures were monitored until equilibrium was reached, at which time a thermal conductivity measurement was initiated. After a pause to reestablish equilibrium, another measurement was made at the same temperature. After completing measurements at a particular temperature, the sample chamber was isolated and water was heated and the procedure repeated at the next temperature level, or the sample was removed for moisture determination. The ability of the system to isolate the sample chamber made it possible to simulate fast heating conditions inherent in a continuous aseptic processing system, preheating water already at the measurement temperature suddenly rerouted to establish rapid temperature equilibration of the sample. No radial variation of heating fluid temperature within the chamber was observed after steady state was achieved.

The second system was a closed pressure vessel which enclosed the sample and probe entirely and was heated from outside. Probe heater wires and thermocouple wires passed through a hermetic sealed pipe fitting (Omega) in the top of the vessel. A thermocouple was inserted into the top of the vessel through a compression fitting to measure temperature of the fluid surrounding the sample. After loading the sample with inserted probe, the vessel was closed and pressurized. Heating was carried out in an ethylene-glycol constant temperature bath. This closed vessel had the disadvantage that the sample was heated more slowly to the target temperature than in the open system. The time for the closed vessel to reach equilibrium at measurement temperature was about three times longer than in the continuous system. Potato and carrot samples (1.8 cm diam) heated by direct contact with the water at the measurement temperature required about 14 min to reach equilibrium at 70°C from initial 28°C. Similar samples in the closed vessel required about 45 min to reach equilibrium at 70°C. The single advantage of the closed system was that it offered more accurate temperature control than the open heating system.

Fluid temperature variation over the length of measurements for both systems was generally < 0.1°C at all temperatures used. Occasionally, changes in the steam supply pressure caused variation in recirculating fluid temperature causing false measurements. Proper temperature control of the heating fluid was essential for accuracy and repeatability. Temperature variation during a measurement resulted in thermal conductivity deviations much wider than in experiments where fluid temperature was well controlled.

Moisture determination

Sample moisture was determined by weighing samples prior to and after vacuum drying at 65°C for 18 hr. (Pomeranz, 1987)

RESULTS & DISCUSSION

Probe design

The use of a silicone oil filled probe resulted from comparison of five probe designs, of identical geometry but with different filling materials; silicone oil, mercury, and air. The thermocouple junctions of the oil and air filled probes were either electrically insulated or uninsulated, but the mercury probe necessitated an insulated junction. The oil filled probe with an uninsulated junction had the best time response in a preliminary study. The time required for its ΔT vs *ln* time plot to become linear was the least among the probe designs.

Probe calibration

Accuracy of thermal conductivity measurements required proper calibration. At the apparent linear segment of the ΔT vs *ln* time plot (Fig. 4), slopes determined by simple linear regression varied depending on the time span selected. Apparent linearity was exhibited about 2.5 sec after the probe was energized. For glycerin (Fig. 5A), the slope determined from data between 1.12 and 25 sec increased slightly with increasing initial time; the final time in all cases was 25 sec. For the agar gel (Fig. 5B), the slope decreased with increasing initial time. When larger initial times were used in the regression, R^2 values decreased, yet the slope approached a steady value. This observation indicated that the lessening in R^2 values was due to resolution and noise limitations in temperature measurement and not to increasing curvature of the trace. For the glycerin calibration data, residual plots (Fig. 6A & 6B) indicated random scatter for the regression fit using initial and end regression times of 10 and 25 sec ($R^2 = 0.9993$), while a distinct bend was seen for the fit of data from 2.6 to 25 s ($R^2 = 0.9997$). When power input was constant, temperature rise/time interval between measurements decreased with increasing time from

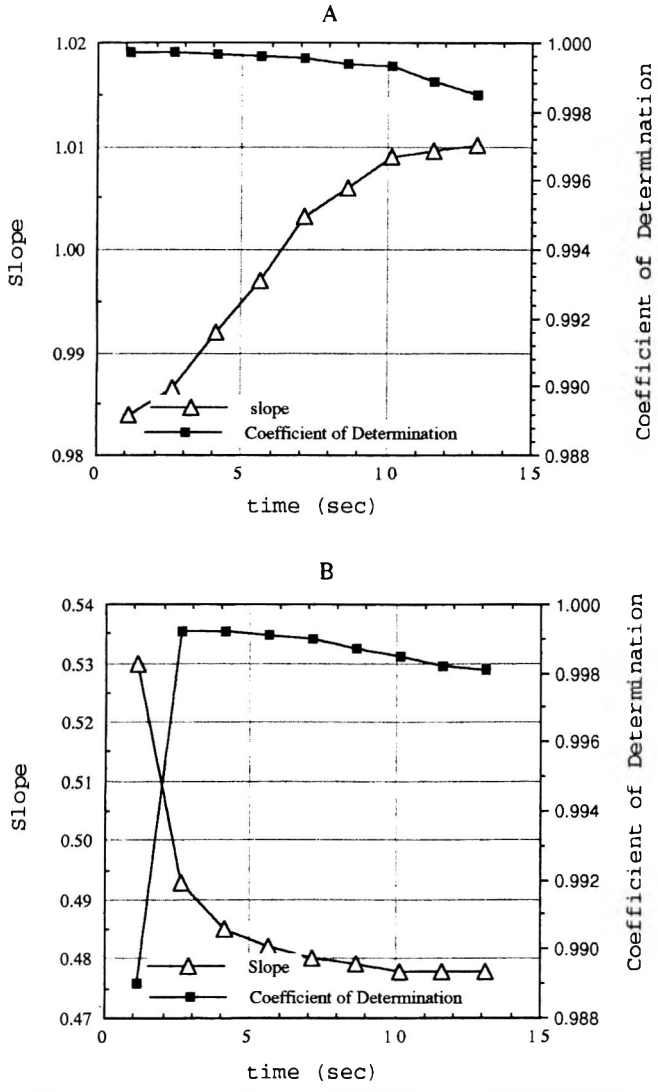

Fig. 5—Effect of the starting time on slope and coefficient of determination of the linear regression when 25 sec was used as the end time; (A) glycerine (B) 0.4% agar gel.

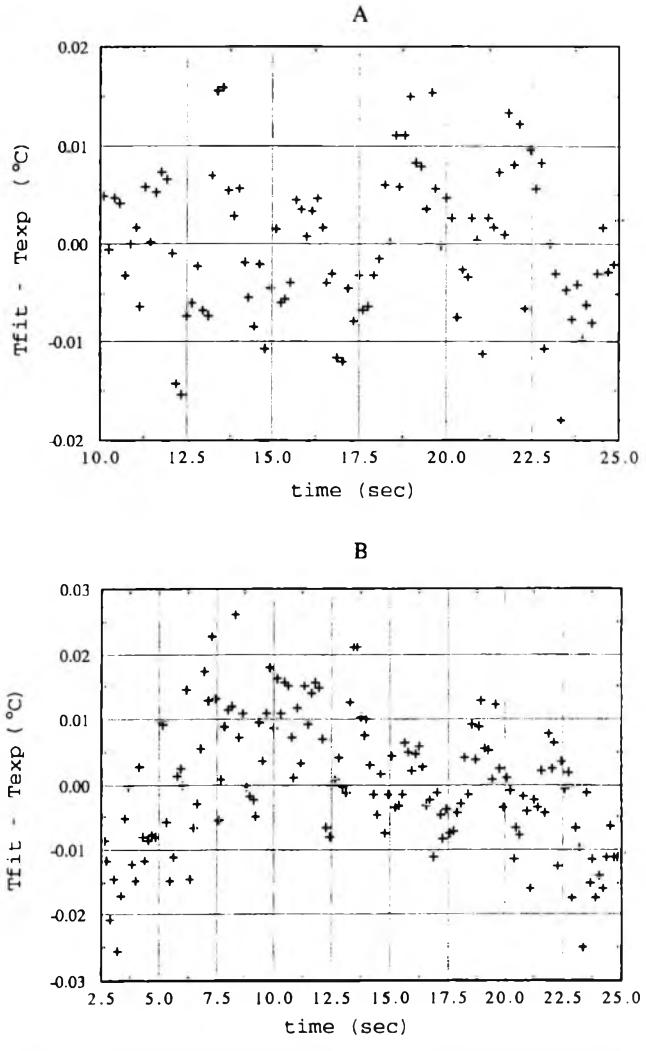

Fig. 6—Residuals (Fitted values of T – Experimentally measured T) in the indicated time range when fitted values were calculated from regression constants obtained from data at the 10–25 sec interval (A, R^2 = 0.9993) or 2.5–25 sec interval (B, R^2 = 0.9997).

initial energization of the probe. The temperature rise approached the resolution limits of the instrument increasing the signal/noise ratio. Though the region increased in linearity at time intervals farther from initial energization, R^2 values decreased.

Our observation of the change in slope depending on time span used was predicted by Karwe and Tong (1992) in their analysis of a simplified composite probe by a finite difference technique. As in Fig. 5A, they predicted for glycerin a gradual increase of slope over the "linear" region, with slope differences decreasing as initial time was farther from initial measurement time. This deviation from the performance of a true line heat source as analyzed by Healy et al. (1976) was, in large part, due to the probe finite radius, thermal conductivity, and heat capacity. All these are unavoidable and important aspects of probes for measuring solid foods. However, the deviation was dependent on probe geometry and construction rather than material surrounding the probe, therefore the effect could be accounted for in the calibration of a particular probe. To minimize measurement variability the calibration constant for each probe was determined from data in the time interval 10 to 25 sec. In that interval the slope on data from both calibration standards stabilized while a R^2 value of $\geq$ 0.998 was maintained. All subsequent measurements were evaluated in the 10 to 25 sec interval. R^2 values for all measurements were $\geq$ 0.996. Inconsistent choice of time interval would result

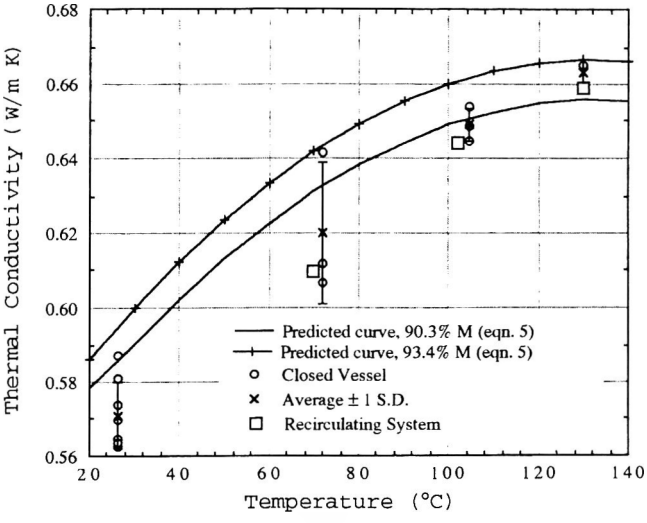

Fig. 7—Carrot thermal conductivity.

in error. For example the thermal conductivity reading with a probe calibrated with 0.4% agar gel using the interval 10 to 25 sec would be 3.0% lower when the interval chosen was 2.6 to 25 sec though the R^2 value for the latter was higher.

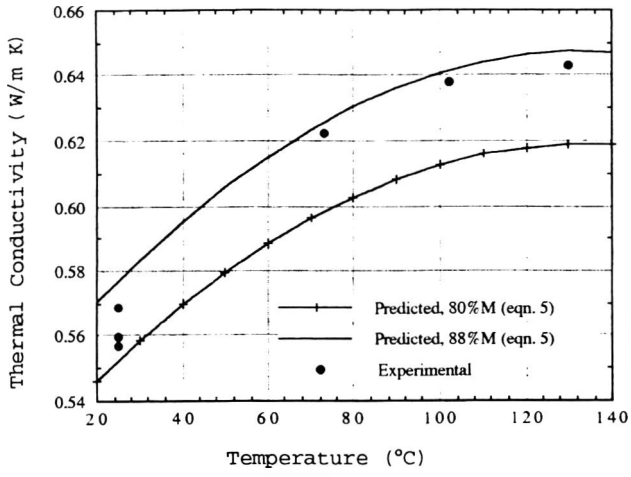

Fig. 8—Potato thermal conductivity.

The standard deviation of the slope (n = 4) for agar gel at 24°C was 0.2% of the mean (standard deviation on thermal conductivity = 0.2%). For glycerin the standard deviation of slope (n = 4) was also 0.2% of the mean. The calibration factor attained using agar gel was validated using glycerin. The measured value for glycerin was 2.0% higher than published values. The reason for this difference is unknown, but it was probably due to a nonlinearity inherent in the probe response. The calibration nevertheless was more reliable than single point calibration in which no measurement time interval is specified. More analytical/numerical work is required if probe accuracy is to be further improved. We conservatively assumed probe accuracy to be ≤ 2% for thermal conductivity values between those of glycerin and water, although repeatability of a well controlled measurement at room temperature was ± 0.4% of the mean (2 standard deviations). Since the foods used had thermal conductivities nearer that of water (agar gel) than glycerin, the calibration factors based on 0.4% agar gel were used.

Thermal conductivity of potato and carrots

Thermal conductivities of carrot and potato in the range 30°C to 130°C were determined (Fig. 7 and 8). Predicted values using Choi and Okos correlation (1987) for multicomponent food systems are shown for comparison. The correlation is of the type:

$$k = \Sigma X_{vi} k_i \qquad (5)$$

where X_{vi} is the volume fraction of components—water, protein, carbohydrate, fiber, lipid, and ash; k_i is the conductivity of the particular component. k_i and component densities are temperature dependent. The values predicted using Choi and Okos' correlation equation were based on means of initial and final moisture contents of the samples and the proximate analysis of samples (*USDA Agricultural Handbook No. 8*, Watt and Merrill, 1982). To ensure similar moisture pickup by all samples, 2.8 kg/cm² was superimposed in all measurements.

For carrots three replications each using the closed vessel heating system were conducted at 70, 100, and 130°C. At each replication 3 separate measurements were done. Initial thermal conductivity at room temperature (≈25°C) was measured for every sample. No increase in thermal conductivity at room temperature was observed as a result of pressurizing the vessel. In addition, one replication was done at each temperature using the recirculating fluid heating system. Moisture gain and thermal conductivity values were similar for both closed vessel and recirculating fluid systems, though come-up times for heating were markedly different. Heating to 130°C in the recirculating system caused a 2.9% moisture gain (90.8% initially to 93.7% post measurement) over 20 min exposure, while in

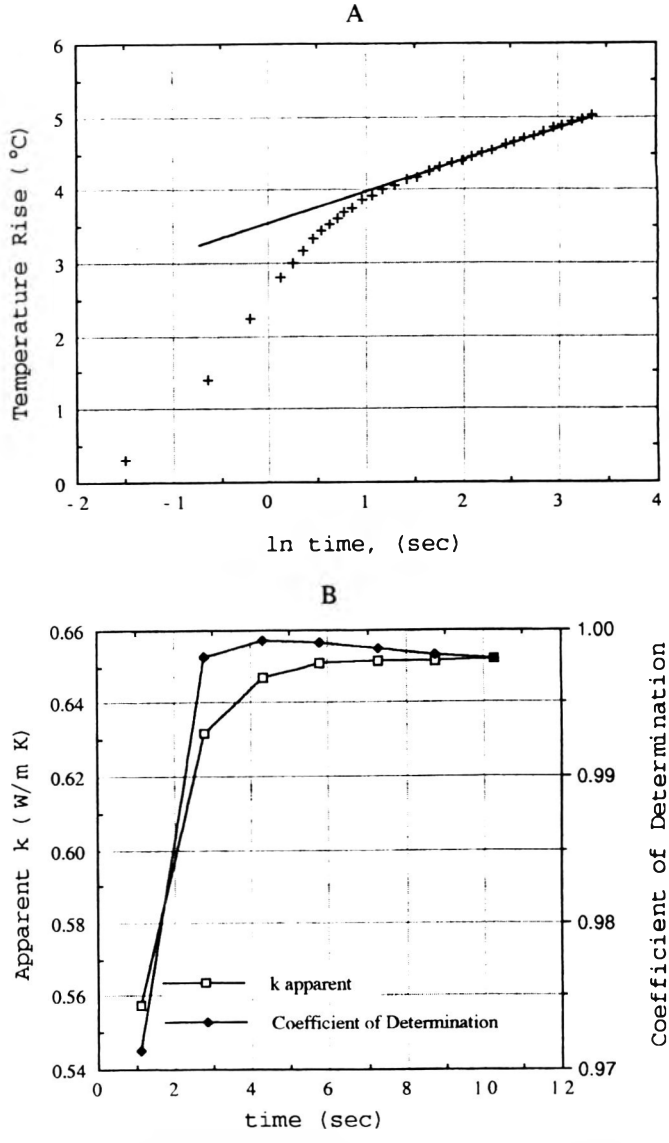

Fig. 9—Typical data for measurements on carrots at 130°C (A), and calculated values of k and R² values of the regression analysis (B) when time span used to obtain data for the regression analysis ranged from the indicated time to 25 sec.

the closed vessel the gain was 3.2% (90.2%, s.d. = 0.8%, initially to 93.4%, s.d. = 0.6%, post measurement) over 70 min exposure. In the closed vessel, a 40 min exposure at 70°C caused a gain in moisture of 0.8%(s.d. = .2%) while a 60 min exposure at 100°C caused a 2.3%(s.d. = 0.4%) moisture increase. Thermal conductivity values were nearly identical with results for the recirculating system within one s.d. of the average for the closed system. No advantage was observed in use of the recirculating system on carrots, but this may not be the case for some foods such as beef, in which breakdown of connective tissue may occur over long holding times altering thermal conductivity. The predicted curves (from Choi and Okos' correlation) for multi-component foods at both initial moisture content and at the highest end moisture content, were calculated for carrot. The increase of experimental values with temperature did not parallel the predicted curves for carrots. This suggested that moisture changes and structural changes during heating are important in altering thermal conductivity. k values at room temperature were lower than the predicted value for identical moisture. However, at 130°C k values were nearly the same for the predicted value at the same moisture. This suggested that the change induced by heating was not

only moisture related but also structural. Choi and Okos' correlations were developed with homogeneous liquid systems in which structure was insignificant. Data for carrot at 130°C were recorded (Fig. 9A). The apparent thermal conductivity values changed when the anchor for the time interval for data analysis changed from 1 to 10 sec, and a constant end in the interval of 25 sec was used (Fig. 9B). k approached a constant when the interval started after about 7 sec. These data further supported use of the time interval of 10 to 25 sec in calibration.

One replication at each temperature of 70, 100, and 130°C was done for potato using the closed system. Initial moisture was 80% (s.d. = 2%). Moisture gain at 100°C and 130°C were 6% and 8%, respectively, with treatment times for both about 90 min. Experimental values fell within predicted values of k at initial and final moisture values (Fig. 8). k values were parallel to the predicted curve at 88% moisture. The effect of heating systems on values of k was not determined for potatoes. The increase in measured thermal conductivity with temperature exhibited by carrot and potato samples was expected. k for potato paralleled values predicted by the multi-component model (Eq. 5) based on final moisture. The divergence of k values for carrot from those predicted by Eq. (5) may have been due to structural and moisture changes induced by heating.

To determine the relative importance of the observed thermal conductivity changes in thermal processing, a MATLAB computer program was written to evaluate microbial lethality in cylindrical particles undergoing isothermal heating. The program evaluated heating for constant and time-varying thermal conductivity models. Particle properties were assumed as follows: height = 0.76 cm, radius = 0.32 cm, c_p = 4200 J/ kg K, p = 1000 kg/m^3, h = 1000 W/ m^2 K. Heating was simulated at 132.2°C from a uniform particle temperature of 30°C. The calculated inactivation for temperature varying k, using a 3rd order polynomial based on data for potato, was 35% higher than the inactivation when the k value for potato at room temperature was used. This alone may not be notable due to the conservative nature of thermal process design. However, in estimating fluid to particle heat transfer using microbial markers and lethality measurements to determine heat transfer coefficients, an error in estimating k over the range of process conditions would induce error in the calculated heat transfer coefficient. For the process described, the heat transfer coefficient would be overestimated by 27% if room temperature thermal conductivity instead of the appropriate temperature dependent value were used.

CONCLUSION

THE LINE probe method was effective in measuring thermal conductivity of food solids at temperatures between 30 and 130°C. The published correlation equation for thermal conductivity based on composition alone was in general agreement with measured values for carrots and potatoes. The increase in k of carrots did not parallel the change in predicted k with temperature indicating moisture ingress and possible structural changes were important factors. For potatoes the values measured at the elevated temperatures closely paralleled predicted values based on moisture of the potato measured after the test. Ignoring thermal conductivity changes with temperature would cause errors in estimating heat transfer coefficients in continuous sterilization systems based on microbial survival.

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Numerical and Statistical Methodology to Analyze Microbial Spoilage of Refrigerated Solid Foods Exposed to Temperature Abuse

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ABSTRACT

Numerical and statistical procedures based on pseudo-zero for the lag and first order reaction kinetics for the exponential growth phase were developed to analyze non-isothermal microbial spoilage. Arrhenius model parameters and their accuracy were estimated for a mixture of *Pseudomonas fluorescens, Staphylococcus aureus* and *Achromobacter lwoffi* growing in a seafood model. Linear regressions used with isothermal experiments generated initial values for nonlinear estimations of the frequency (K_0) and activiation energy (E_a) constants. An optimization technique was used to minimize the square difference between experimental and estimated values while parameter accuracy was assessed using a bootstrap method. E_a and $\ln(K_0)$ were 109 ± 3.4 and 48.3 ± 1.5 kJ/mole for the exponential, and 152 ± 4.0 and 64.4 ± 1.7 kJ/mole for the lag phase, respectively. The Mann-Whitney-Wilcoxon rank sum test showed no significant differences between parameters generated by two different temperature profiles (5% significance level).

Key Words: seafood, microbes, statistical methods, spoilage, kinetics

INTRODUCTION

COMMERICAL INTEREST in refrigerated foods has expanded rapidly in response to consumer demand for convenient and fresh-like products. Development of technologies to design foods with extended refrigerated shelf-life is limited by wide temperature ranges as food marketing extends from producer to consumer. Reduced shelf-life in a food product is usually evaluated by the measurement of one or more quality factors. Such factors can be physical, chemical, microbiological or sensory indices (Taoukis and Labuza, 1989). In most situations, the reactions involved follow first or pseudo zero order kinetics. The temperature dependence of most reactions, including microbial growth can be described by the Arrhenius model (Labuza and Riboh, 1982; Labuza and Kamman, 1983; Fu et al., 1991). Processors of refrigerated products are interested in models for the extent of the microbial lag phase as its end reflects the possibility of a pathogen beginning to multiply in a given food, thus increasing consumer risks. They are also interested in the multiplication of spoilage microorganisms in the exponential phase which would reduce product quality. They are generally not interested in models for the stationary phase as this situation corresponds to products exceeding an acceptable loss of quality. A different situation is the production of fermented foods which would require models including the stationary phase (e.g., Grazier et al., 1993).

Microbial product stability is largely determined at any given time by the cumulative effect of fluctuating temperature throughout previous handling. The temperature fluctuation effect on microbial stability can be quantitatively studied by computer supported techniques which have been recently used to evaluate effectiveness of measures to control and prevent temperature abuse (Almonacid-Merino and Torres, 1993). The exponential phase of microbial growth is usually described by a first order reaction while the lag phase can be approached using a "marker" indicator reflecting cell physiology status (Srivastava and Volesky, 1990). For example, it has been shown that exponential phase cells have a relatively higher RNA content than lag-phase cells. Also, RNA concentration changes linearly during the lag phase and reaches a maximum value when entering the exponential phase (Herbert, 1961; Srivastava and Volesky, 1990).

Most kinetic studies are carried out isothermally. This is a well documented procedure for shelf-life determinations (Hill and Grieger-Block, 1980; Labuza and Riboh, 1982; Labuza and Kamman, 1983; Lai and Heldman, 1983; Haralampu et al., 1985; Cohen and Saguy, 1985; Arabshahi and Lund, 1985; Nunes et al., 1991). In general, it consists of estimating the rate constant at different but constant temperatures within the range of interest. Kinetic parameters and their accuracy are estimated by linear regressions based on the Arrhenius model. This method is known as the two-step method (Nunes et al., 1991). An alternative is the estimation of kinetic parameters by nonlinear regression. The minimum for the sum of square differences between experimental and calculated values can be used to determine which kinetic parameters best describe data generated under non-isothermal conditions. The minimum is found using numerical or analytical optimization techniques. Among the advantages of this approach are reductions in time and experimental labor. In addition, nonisothermal experiments more closely simulate spoilage situations since foods are subjected to a range of temperature conditions. Furthermore, constant temperature tests do not detect transient thermal history effects which could introduce errors in shelf-life estimations for changing temperature storage. Nonisothermal tests can detect thermal history effects, although they would be difficult to incorporate into shelf-life prediction models.

Nonisothermal experiments are not practical if the estimation of parameter variability requires a large number of experiments and normal distribution assumption. Non-parametric statistics do not rely upon normal distribution assumptions and could be used to assess this variability (Efron, 1979b; Efron and Tibshirani, 1986; Nasri et al., 1993). The "bootstrap" is a non-parametric free-distribution method to assess variability using a small number of experiments and standard error estimations (Efron, 1979a,b, 1981a,b; Efron and Tibshirani, 1986; Efron, 1988). Our objective was to apply this method for nonisothermal microbial growth. The experimentation included determination of the lag and exponential phase for growth in pollack surimi of a mixture of *Pseudomonas fluorescens, Staphylococcus aureus* and *Achromobacter lwoffi*. These microorganisms have been reported to be associated with the microbial spoilage of seafood (Jay, 1978).

MATERIALS & METHODS

Microbial growth

Isothermal tests. *P. fluorescens* ATCC 15456 and *A. lwoffi* ATCC 17925 were grown at 26°C in 300 ml side arm flasks containing 30 ml nutrient broth (8 g/L) (Difco Laboratories, Detroit, MI). *S. aureus*

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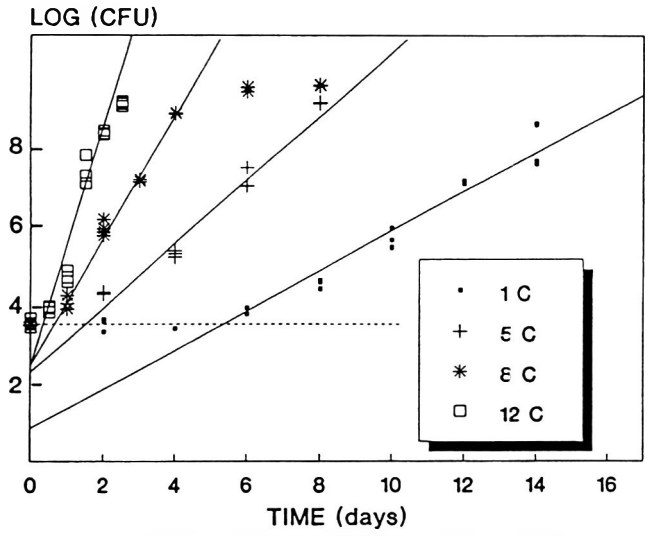

Fig. 1—Growth in a seafood model of a Pseudomonas fluorescens, Staphylococcus aureus *and* Achromobacter lwoffi *mix incubated at 1, 5, 8 and 12°C. Microbial counts in the stationary phase were not used in estimations of kinetic parameters.*

Table 1—Rate constants, isothermal experiment

	Temperature,°C			
	1	5	8	12
Lag phase, days	5.37	1.51	0.63	0.44
Marker rate constant, unit/day	0.19	0.66	1.59	2.27
Specific rate constant, 1/day	1.21	1.79	3.65	7.26

Table 2—Kinetic parameters, isothermal experiment

	E_a kJ/mole	$\ln(K_0)$
Lag phase	151 ± 26[a]	64.8 ± 11.1
Exponential phase	109 ± 12	48.0 ± 5.3

[a] Variability estimated as standard error

Table 3—Initial parameter set and limits, explicit complex constraints

	Initial solution	Lower limit	Upper limit
E_a', kJ/mol	151	131	177
$\ln(K_0')$	64.8	53.7	75.9
E_a, kJ/mol	109	97.0	121
$\ln(K_0)$	48.0	42.7	53.3

ATCC 13565 was grown in the same medium but at 37°C. Mid-exponential growth cells were recovered by centrifugation (3,000 x *g*, 10 min, SS-34 rotor, Sorval Superspeed RC2-B, Norvalk, CT), resuspended in 30 ml nutrient broth containing 16% glycerol (M778-09, J.T. Baker Inc., Phillipsburg, NJ) and stored at −80°C. Just prior to use, the suspension was thawed at room temperature and counted on plate count agar (Difco Laboratories) as a post-inoculation verification that cells had survived the preparation procedure. The final inoculum was a mixture of *P. fluorescens, A. Lwoffi,* and *S. aureus* with each bacteria in the same proportion and obtained from single stock cultures.

Heat-sealed pouches filled with 10 g commercial pollack surimi (Lot No. 16392, 70% moisture content, Unisea Cold Storage, Redmond, WA) mixed in a 3:1 proportion with distilled water were frozen at −40°C and then radappertized (5 Megarad) at the Oregon State University Radiation Center. After thawing at room temperature, the pouches were opened under sterile conditions, inoculated with 1 ml of microbial mixture (10^3–10^4 cells/g), homogenized for 2 min using a Stomacher (Model STO-400, Tekmar Co., Cincinnati, OH). Two pouches incubated at 1, 5, 8, and 12°C were removed from the incubator at time intervals depending on storage temperature. From each pouch, two samples were used to determine in triplicates microbial counts on total plate count agar. Initial dilutions were prepared in the pouch with the sample and mixed using the Stomacher.

Nonisothermal tests. Pouches prepared as described were incubated for 9 days in two temperature-controlled chambers. Each was programmed with a base temperature of 1–2°C superimposed with different and arbitrary temperature steps of a few hours between 5 and 14°C. Temperature inside a pouch in each chamber, as well as chamber temperature, were monitored using an electronic datalogger (Model 21X, Campbell Scientific Inc., Logan, UT). Preliminatry experiments showed no temperature difference between different locations in the small food sample in each individual pouch (≈ 15 cm x 10 cm). Duplicate total counts were determined from two pouches removed from each chamber at 24 h intervals. Again, initial dilutions were prepared in the pouch using the Stomacher. Other experimental conditions were the same as those used for isothermal tests.

THEORETICAL CONSIDERATIONS

Microbial Growth Model

Exponential phase. First order reactions kinetics for microbial growth with an Arrhenius model for the temperature dependency for the growth constant can be described as:

$$K = K_0 e^{-E_a/RT} \tag{1}$$

This leads to the following expression for growth under non-isothermal conditions:

$$\frac{N}{N_0}(\theta) = \mathrm{EXP}\left[K_0 \int_0^{\theta} e^{-E_a/RT(\theta)} d\theta\right] \tag{2}$$

The kinetic parameters (E_a, K_0) can be evaluated from experiments made at different but constant temperatures. At each temperature, K was computed from the slope of the plot ln(N) as a function of time. The Arrhenius plot was then used to obtain E_a and $\ln(K_0)$ and used in Eq. (2) to evaluate growth under non-isothermal conditions. The integral expression in Eq. (2) was solved numerically using the trapezoidal rule (Singh, 1983).

$$I = \int_0^{\theta} e^{-E_a/RT(\theta)} d\theta = \int_0^{\theta} f(\theta) d\theta \approx \frac{\Delta\theta}{2}\left[f(\theta_0) + f(\theta_f) + 2\sum_{k=1}^{n-1} f(\theta_k)\right] \tag{3}$$

Lag phase. The lag phase was estimated on a semilogarithm plot as the time corresponding to the intersection of the horizontal line passing through the population at time 0 and the straight line describing the exponential growth phase. The lag phase duration was modeled assuming a key intracellular component reaches a certain maximum concentration when the lag phase is completed. The concentration increase of this component, frequently reported to be RNA (Herbert, 1961; Srivastava and Voleskey, 1990), was assumed to follow pseudo zero order kinetics. Kinetic parameters for the lag phase were estimated by assuming that the production of this intracellular chemcial or "marker" could be described as follows:

$$\frac{d[\mathrm{MARKER}]}{d\theta} = \text{constant} \tag{4}$$

Replacing the marker concentration by the fraction $[\mathrm{MARKER}]/[\mathrm{MARKER}]_{max}$ the rate constant became $K' = 1/\theta_L$ where θ_L is lag time at that given temperature and Eq. (4) could be rewritten as:

$$\frac{d\dfrac{[\mathrm{MARKER}]}{[\mathrm{MARKER}]_{max}}}{d\theta} = K' \tag{5}$$

with

$$\frac{[\mathrm{MARKER}]}{[\mathrm{MARKER}]_{max}} = 0 \quad \text{at time} = 0 \tag{6}$$

$$\frac{[\mathrm{MARKER}]}{[\mathrm{MARKER}]_{max}} = 1 \quad \text{at time} = \theta_L \tag{7}$$

The following expression is obtained by integration and used to analyze a non-isothermal lag phase:

$$\frac{[\mathrm{MARKER}]}{[\mathrm{MARKER}]_{max}} (\text{at time} = \theta) = \int_0^{\theta} K_0' e^{-E_a/RT(\theta)} d\theta \tag{8}$$

Again, the integral expression in this equation was solved by a nu-

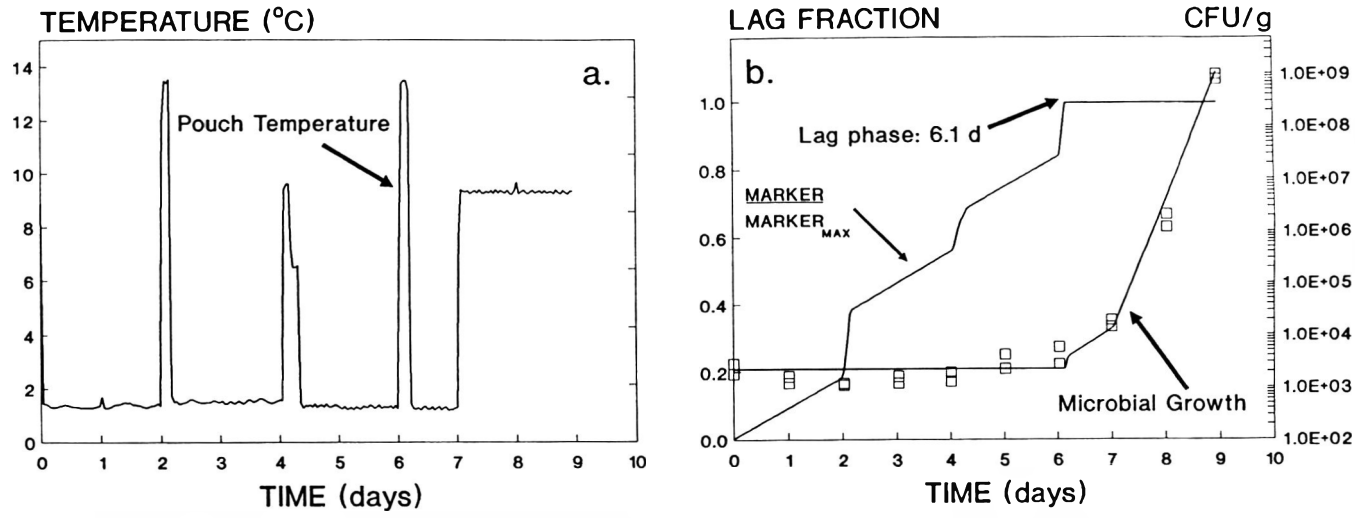

Fig. 2—(a) Pouch temperature as affected by stepwise fluctuation of the incubation chamber temperature, profile #1. (b) Experimental (□), and simulated growth and degree of lag phase completion for a Pseudomonas fluorescens, Staphylococcus aureus *and* Achromobacter Iwoffi *mix growing in a seafood model incubated non-isothermally. Lag phase is expressed as a fraction of the maximum "marker" concentration and was completed when that fraction was 1.*

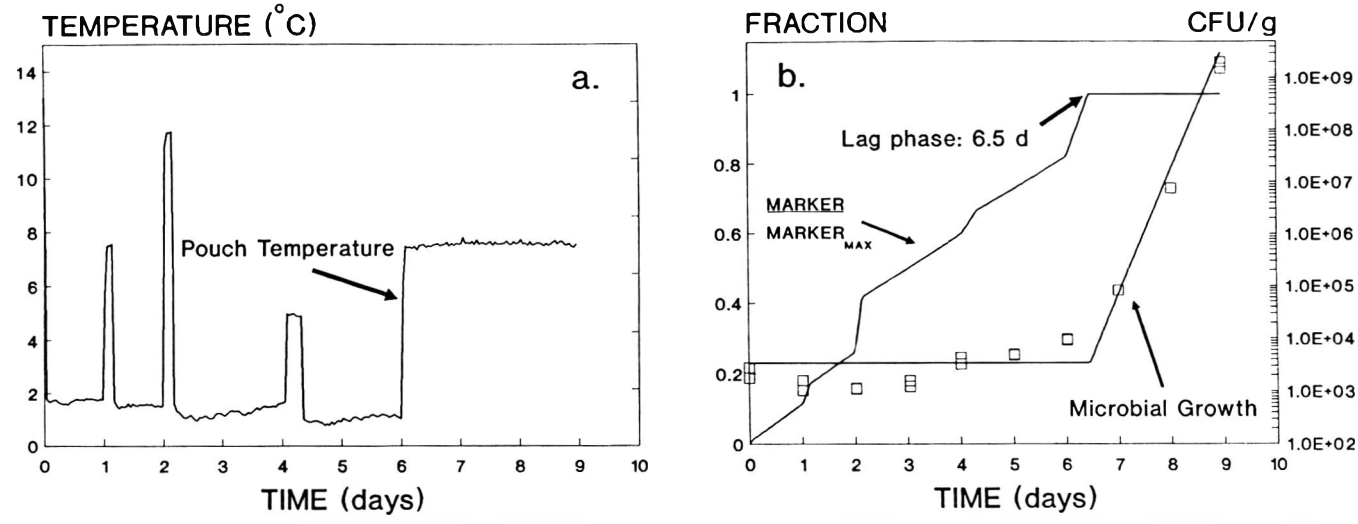

Fig. 3—(a) Pouch temperature as affected by stepwise fluctuation of the incubation chamber temperature, profile #2. (b) Experimental (□), and simulated growth and degree of lag phase completion for a Pseudomonas fluorescens, Staphylococcus aureus *and* Achromobacter Iwoffi *mix growing in a seafood model incubated nonisothermally. Lag phase is expressed as a fraction of the maximum "marker" concentration and was completed when that fraction was 1.*

merical method. The complete microbial model for any time-temperature record consisted then of the two following processes occurring in series:

$$d\frac{[MARKER]}{[MARKER]_{max}} = K' \text{ for } 0 < \theta \leq \theta_L$$

$$\text{i.e. } 0 < \frac{[MARKER]}{[MARKER]_{max}} \leq 1 \quad (9)$$

and

$$\frac{dN}{d\theta} = KN \qquad \text{for } \theta \geq \theta_L \quad (10)$$

In general, nonisothermal growth may be described as:

$$N = f(E_a, K_0, E'_a, K'_0, T(\theta)) \quad (11)$$

or,

$$N = f(\beta, T(\theta)) \quad (12)$$

where β is the set of unknown parameters, and $T(\theta)$ is a known time-temperature profile.

Statistical analysis

In isothermal experiments, β and its accuracy was estimated by linear regression and using Arrhenius plots. In nonisothermal experiments, β was estimated from Eq. (12) by fitting the best curve to nonisothermal data, i.e. by choosing values minimizing the following objective function:

$$OBJ = \sum_{i=1}^{n} [N_{obs} - N(\beta_i, T(\theta))]^2 \quad (13)$$

with β_i a set of parameters to be tested. The estimators obtained by minimizing Eq. (13) are considered unbiased and did not overestimate or underestimate systematically the true parameters. The minimum could be found numerically and sometimes analytically if the regression model were not a complicated mathematical function. In the case of the microbial model here presented the "Complex" method (Spendley et al., 1962; Box 1965; Beveridge and Schechter, 1970) was used to systematically evaluate Eq. (13) using different parameter sets β_i until the one minimizing this equation was found. Given a multivariable function $V = v(x_1, x_2, \ldots x_m)$, the minimum could be approached using two constraint types. If ℓ_i and u_i are upper and lower limits for the variable x_i, an explicit constraint could be expressed as $\ell_i \leq x_i \leq u_i$ $(i = 1, 2, \ldots m)$. Although our case involved only explicit

Table 4—Bootstrap kinetic parameters

Sample #	E_a', kJ/mol	$\ln(K_0')$	E_a, kJ/mol	$\ln(K_0)$
a. Temperature profile #1				
1	156	65.8	106	46.7
2	147	62.1	108	47.4
3	151	64.1	111	48.8
4	149	63.1	113	49.3
5	151	63.9	112	49.1
6	158	66.6	113	49.9
7	159	67.1	105	46.2
8	151	63.8	112	48.9
9	154	65.1	113	49.4
10	152	64.3	106	46.1
11	146	61.5	111	49.1
12	150	63.2	114	50.1
13	148	62.7	108	47.4
14	148	62.5	107	47.3
15	144	60.8	107	46.9
16	151	63.8	110	48.5
17	157	66.2	112	49.1
18	156	66.3	107	46.6
19	148	62.7	111	49.1
20	150	63.5	108	47.5
21	159	67.1	108	47.8
22	154	65.3	107	46.8
23	152	64.4	105	46.2
24	153	64.6	103	45.2
25	151	63.8	117	51.8
b. Temperature Profile #2				
1	157	66.5	105	45.9
2	154	65.1	111	48.4
3	144	60.7	109	48.3
4	154	65.1	112	49.3
5	156	66.1	108	47.1
6	156	65.8	111	48.4
7	152	64.3	109	48.3
8	160	67.6	116	50.6
9	144	60.9	116	50.8
10	148	62.7	105	45.8
11	154	65.3	111	49.1
12	154	65.1	116	51.1
13	148	62.4	108	47.1
14	149	62.6	111	48.4
15	149	63.1	109	48.3
16	149	63.2	111	48.5
17	152	64.3	109	48.1
18	159	67.1	103	45.2
19	144	61.1	114	49.8
20	150	63.3	106	46.9
21	152	64.3	112	49.2
22	152	64.3	103	45.1
23	151	64.1	103	45.2
24	152	64.3	111	49.1
25	154	65.1	111	49.7

constraints an implicit constraint could have been expressed as $G_j \leq 0$ with $G_j = g_j(x_1, x_2, \ldots, x_m)$ for $j = 1, 2, \ldots n$.

An initial point must be provided in the Complex method and all constraints must be satisfied. The calculation procedure began by the search for 2m-1 additional feasible solutions using the initial feasible point and random numbers. The next step was a systematic and iterative search for an improvement on the worst feasible solution. The convergence criterion to stop this iteration process is a negligible improvement in the objective function (Eq. 13) specified as follows:

$$\frac{V_{worst} - V_{best}}{V_{best}} \leq 0.001 \tag{14}$$

where V_{worst} and V_{best} were selected from the 2m objective function values available at a given iteration point. When the parameter set minimizing Eq. (13) was found, Eq. (12) was rewritten in terms of estimators as follows:

$$\hat{N} = f(\hat{\beta}, T_j(\theta)) \tag{15}$$

Accuracy evaluation of parameters obtained with the Complex method was done using a bootstrap procedure (Efron 1979a,b, 1981a,b; Efron and Tibshirani, 1986; Efron, 1988). In our case, a set of observations $(N_{obs}, \theta)_j$ was obtained from an unknown probability model P. The statistics of interest $\hat{E}_a$, $\hat{K}_0$, $\hat{E}'_a$, and $\hat{K}'_0$ had been calculated from the minimization of Eq. (13) and we wanted to assess their accuracy. The accuracy of the parameters could be estimated by obtaining independently generated replicates of $\hat{\beta}$, say $[\hat{E}_a(1), \hat{K}_0(1), \hat{E}'_a(1), \hat{K}'_0(1)]$; $[\hat{E}_a(2), \hat{K}_0(2), \hat{E}'_a(2), \hat{K}'_0(2)]$; ... $[\hat{E}_a(n), \hat{K}_0(n), \hat{E}'_a(n), \hat{K}'_0(n)]$. However, this option was an inconvenient procedure as it required a large number of experiments.

Assuming additive errors independent of sampling time (θ_i), the bootstrap method allowed us to estimate the unknown probability model P which could be described as:

$$(N_{obs})_i = N_i + \epsilon_i \tag{16}$$

$$\text{with } N_i = f(\beta, T(\theta_i)) \tag{17}$$

with ϵ_i an independent error with an unknown probability distribution F for an observation $(n_{obs})_i$. The minimization of Eq. (13) gave estimated mean values:

$$\hat{N}_i = f(\hat{\beta}, T(\theta_i)) \tag{18}$$

and estimated errors (residuals) given by:

$$\hat{\epsilon}_i = (N_{obs})_i - \hat{N}_i \qquad (i = 1, 2, \ldots, n) \tag{19}$$

This allowed us to obtain an estimated version of F:

$$\hat{F}\text{: probability } \frac{1}{n} \text{ on } \hat{\epsilon}_i \qquad i = 1, 2, \ldots, n \tag{20}$$

Bootstrap data sets $(N^*)_i$ were then generated according to

$$(N^*)_i = \hat{N}_i + \epsilon_i^* \qquad i = 1, 2, \ldots, n \tag{21}$$

with $\epsilon_1^*, \epsilon_2^*, \ldots, \epsilon_n^*$ independent observations drawn randomly from $\hat{F}$. The data sets $(N^*)_i$ were used to generate a bootstrap least square estimate of $\hat{\beta}_i^*$ calculated using Eq. (13). The process was repeated B times for each of the two time temperature records $T(\theta_i)$. A relatively small bootstrap size (B = 25) has been reported to give acceptable standard error estimations (Efron and Tibshirani, 1986). The populations of 25 parameter sets for the experimental temperature records $T_j(\theta)$ can be represented as follows:

$$\begin{matrix} E_{a_1}, & K_{0_1}, & E'_{a_1}, & K'_{0_1} \\ E_{a_2}, & K_{0_2}, & E'_{a_2}, & K'_{0_2} \\ \vdots & \vdots & \vdots & \vdots \\ \vdots & \vdots & \vdots & \vdots \\ E_{a_{25}}, & K_{0_{25}}, & E'_{a_{25}}, & K'_{0_{25}} \end{matrix} \tag{22}$$

The estimated variability for each parameter could be computed as follows:

$$\hat{\sigma}_{\hat{E}_a^*} = \frac{\sum_{b=1}^{B} [\hat{E}^*_{a_{(b)}} - \hat{E}^*_a(\bullet)]^2}{B} \tag{23}$$

where

$$\hat{E}^*_a(\bullet) = \frac{\sum_{b=1}^{B} \hat{E}^*_{a_{(b)}}}{B} \tag{24}$$

Analogous calculations are made to obtain the variablity of the other three parameters.

A nonparametric procedure was used to test for significant differences between the estimated parameters generated by the two independent temperature records, $T_1(\theta)$ and $T_2(\theta)$. The lack of a significant difference would suggest the absence of thermal history effects on microbial growth. An appropriate procedure was the Mann-Whitney-Wilcoxon rank sum test which could be summarized as follows (Daniel, 1978). The M (= $n_1 + n_2$, in our case $n_1 = n_2 = 25$) bootstrap-generated values were assigned a ranking number (R_j). The sum of ranks assigned to one of the populations was:

$$W = \sum_{j=1}^{n} R_j \tag{25}$$

A large sample approximation ($n_i \geq 20$) (Daniel, 1978) for a two-side test of the null hypothesis H_0 (i.e., the two samples come from identical populations) vs the alternative hypothesis H_a (i.e., population 1

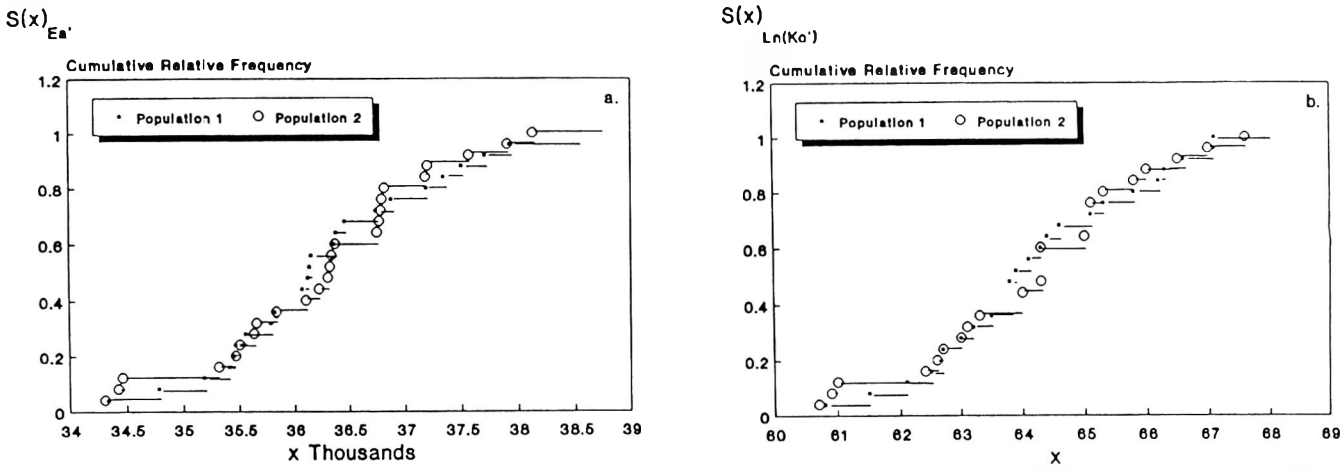

Fig. 4—Cumulative relative frequency comparison of lag phase parameters generated by temperature profiles #1 (°-) and #2 (·-). (a) activation energy (E_a'); (b) ln(K_o').

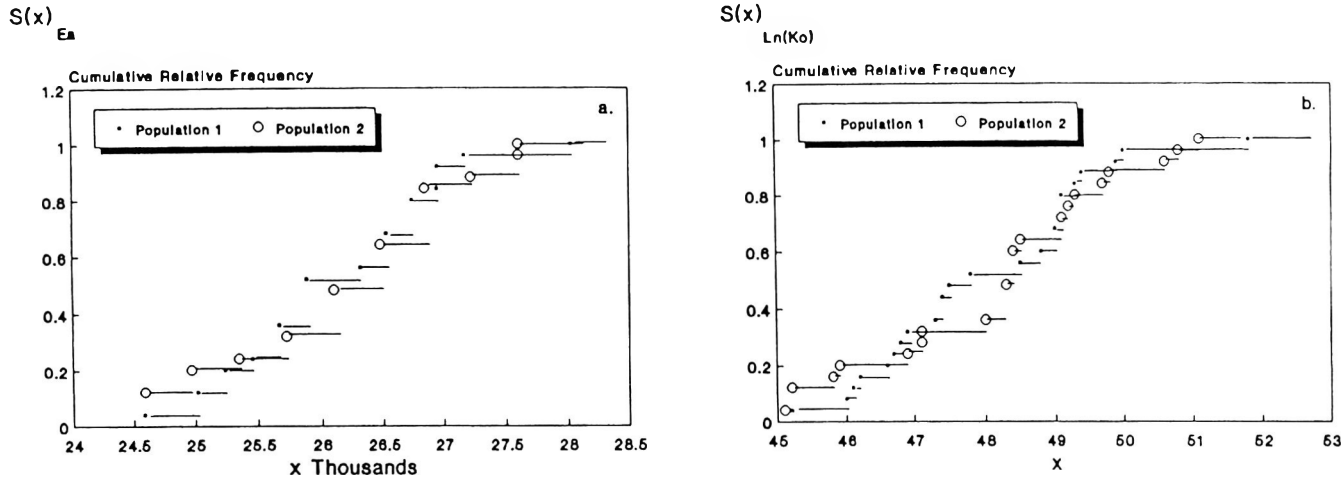

Fig. 5—Cumulative relative frequency comparison of exponential phase parameters generated by temperature profiles #1 and #2 obtained. (a) activation energy (E_a); (b)ln(K_o).

Table 5—Test of hypothesis, Mann-Whitney-Wilcoxon rank sum test

Rank sum				W'				
E_a'	$\ell n(K_0')$	E_a	$\ell n(K_0)$	E_a'	$\ell(K_0')$	E_a	$\ell n(K_0)$	z (0.05)
645	642	657	652	0.15	0.09	0.38	0.28	1.96

parameters differ from those for population 2) was used to define W':

$$W' = \frac{W - E_C(W)}{[var_0(w)]^{1/2}} = \frac{W - \left[n_1 \frac{(n_2 + n_1 + 1)}{2}\right]}{\left[n_1 n_2 \frac{(n_1 + n_2 + 1)}{12}\right]^{1/2}} \qquad (26)$$

At a significant level α, W' could be tested using z_α values obtained from normal distribution tables, N(0, 1) as follows:

$$\begin{aligned} &\text{reject } H_0 \quad \text{if} \quad W' \geq z_\alpha \\ &\text{accept } H_0 \quad \text{if} \quad W' < z_\alpha \end{aligned} \qquad (27)$$

Implementation of statistical procedures. A temperature profile and the corresponding data set were fed into the model, Eq. (10) and (11). The isothermal kinetic parameters were used as initial solution in the Complex method optimization, and the standard error for these parameters became the lower and upper limit for each parameter. The lag phase duration, i.e. when $[RNA]/[RNA]_{max}$ reaches 1, was determined first. Microbial counts up to that point were averaged and this value became N_0 for the exponential phase simulation establishing a connection and dependence between both phases of the growth model. Sample temperatures were read every 2 min which was also the numerical integration time increment. Finally, minimization of Eq. (13) generated the kinetic parameter estimates.

The same procedure was applied for the second temperature profile. Using the estimated parameters and the experimental microbial data, the residuals were evaluated using Eq. (19). Bootstrap data sets (25) were generated by evaluation of Eq. (21) and used to find estimated bootstrap parameters following the same procedures used for the original experimental data. Finally, bootstrap accuracy was evaluated using Eq. (23).

RESULTS & DISCUSSION

Isothermal experiments

Isothermal growth experiments at 1, 5, 8, and 12°C are shown in Fig. 1. Lag phase duration and specific growth rate constant at each temperature were recorded (Table 1). Note that this experiment was conducted to obtain initial values for the kinetic parameters (E_a and ln K_0) for the microbial population mixture and that microbial counts in the stationary phase were excluded from linear regression calculations. Incubation chambers were frequently open for sampling purposes and had on-off temperature controls; therefore temperature control was no better than ± 1°C. Kinetic parameters obtained by linear regressions (Table 2) were the initial values needed for the Complex method used for the nonlinear least square parameter estimation. Parameter accuracy information was used to select lower and upper limits for these (Table 3).

E_a and ln(K_0) values could not be chosen independently at

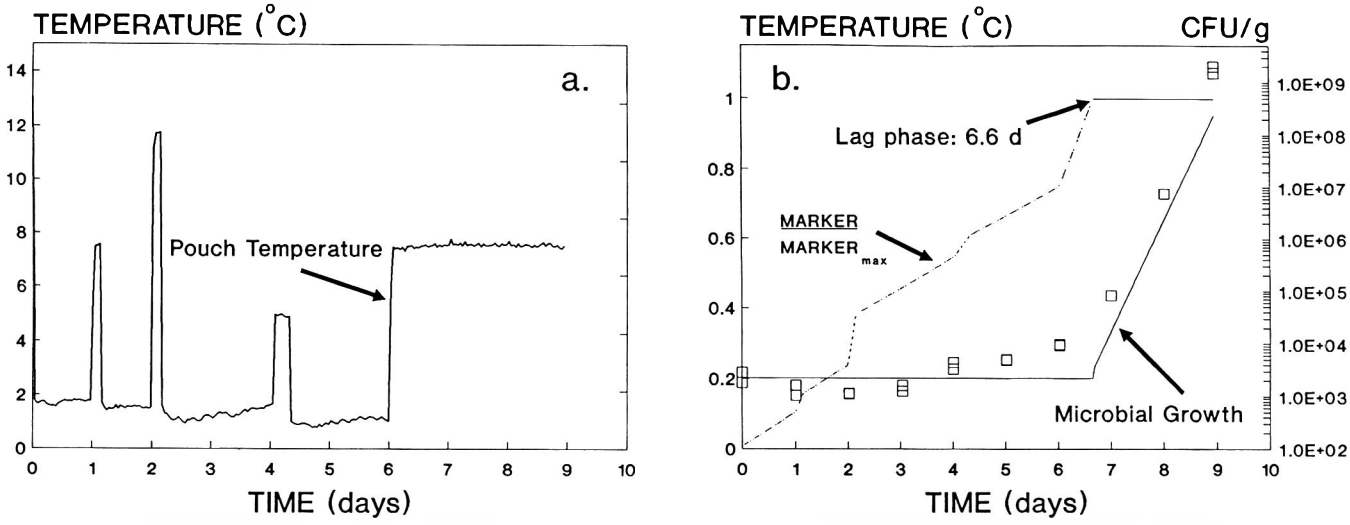

Fig. 6—(a) Pouch temperature as affected by stepwise fluctuation #2 of the incubation chamber temperature. (b) Experimental (□) growth for stepwise fluctuation #2 for a Pseudomonas fluorescens, staphylococcus aureus *and* Achromobacter lwoffi *mix growing in a seafood model incubated nonisothermally. Simulated growth and degree of lag phase completion were obtained using parameters estimated from temperature profile #1 (Fig. 2a). Lag phase is expressed as a fraction of the maximum "marker" concentration and was completed when that fraction was 1.*

Table 6—Kinetic parameters, nonisothermal experiment

Profile	E_a', kJ/mole	$\ell n(K_0')$	E_a, kJ/mole	$\ell n(K_0)$
1	152 ± 4.0	64.4 ± 1.7	109 ± 3.4	48.3 ± 1.5
2	152 ± 4.2	64.2 ± 1.8	109 ± 3.9	48.5 ± 1.7

random which was the procedure used in the Complex method to minimize the square difference. The distribution of rate constants is fixed by upper and lower bounds that differ from those associated with a normal distribution with constant standard deviation (Lenz and Lund, 1977). In the Arrhenius plot, the rate constants are normally distributed for the average activation energy. However, at higher activation energies, the mean reaction rate constant is larger and the distribution is skewed toward higher values of K at the given temperature. Since the optimization technique chooses E_a and $\ln(K_0)$ at random, improper combinations of parameters could occur. These combinations would result in high values of the objective function (Eq. 13) and were rejected by the minimization process.

Nonisothermal experiments

Pouch temperature as affected by stepwise fluctuations of the incubation chamber temperature and the experimental microbial counts were compared (Fig. 2 and 3). The degree of lag phase completion was described on a 0–100% maximum marker concentration scale. Both temperature profiles resulted in lag phase durations over 6 days longer than the value observed at constant 1°C (5.4 days). A comparison of lag phase values obtained by isothermal and nonisothermal experiments requires incubators with a precise temperature control and the removal of samples with the utmost care. The latter was not necessary to fulfill our research objectives, i.e. the application of non-parametric statistics and numeric techniques to develop procedures to evaluate nonisothermal microbial growth. The isothermal experiments were needed only to generate initial values to search for kinetic parameters obtained from nonisothermal experiments. In actual practice, a user may skip the isothermal experiments and select seed values for nonisothermal procedures on the basis of previous experience for similar food products.

Table 4 shows the two sets of 25 bootstrap parameters calculated from each temperature profile. Visual comparisons of the cumulative relative frequency graphs for these two sets showed no apparent differences for the lag phase (Fig. 4) and exponential phase (Fig. 5) parameters. These observations were confirmed by the Mann-Whitney-Wilcoxon test which found also no significant differences ($\alpha = 0.05$) (Table 5). Finally, Fig. 6 shows again experimental and simulated values for temperature profile #2. In that case the simulated values were obtained using the kinetic parameters generated from temperature profile #1 instead of #2. A comparison of simulations showed that lag phase was 6.5 days (Fig. 3) and 6.6 days (Fig. 6) and time to reach 10^8 cells/g was 7.4 days (Fig. 3) and 7.6 days (Fig. 6). Similar observations could be made when experimental counts for temperature profile #1 were compared with simulated values using the kinetic parameters generated from temperature profile #2 (data not shown). These simulations were consistent with the Mann-Whitney-Wilcoxon test. These experimental observations indicated that the 2 temperature profiles used did not detect a thermal history effect. Further studies using other microorganisms and more widely differing temperature profiles are needed to evaluate the frequency and severity of thermal history effects on microbial activity estimations, particularly on kinetic parameters used to predict the lag and growth phase.

Table 6 shows estimated parameters and their bootstrap accuracy. The standard error for parameters estimated non-isothermally were smaller than those obtained from isothermal experiments (Table 2). Isothermal experiments required more experimental work since rate constants were estimated first and then used to obtain Arrhenius parameters. The isothermal parameter estimation assumed a constant temperature which was only approximately true experimentally. Temperature fluctuated around the selected value and if the fluctuation had been taken into account the experiment would have been non-isothermal.

CONCLUSIONS

Nonisothermal parameter estimations and bootstrap accuracy determinations simplified experimental work and eliminated data analysis assumptions. The growth model generated from rather simple and few experimental measurements could be used to analyze a variety of commercial storage and distribution problems. The effect of package size, packaging material, temperature abuse profiles and many other effects could be readily simulated to reduce the tests needed to establish the safety of a refrigerated product. Two important assumptions

were made to evaluate microbial kinetic data. Experimental errors were assumed additive and independent of sampling time. The latter assumption could be revised and group errors for samples handled identically, i.e. samples counted after the same number of dilutions. Although the lag growth phase was simulated successfully under variable temperature by assuming a "marker" indicator reflecting cell physiology, the growth model could be improved by including an acceleration phase. The bootstrap method allowed determination of kinetic parameter variability which could be used in estimating the uncertainty of shelf-life predictions.

NOMENCLATURE

B	number of bootstrap samples
E_a	activation energy (first order kinetics), kJ/mole
E_a'	activation energy (pseudo zero order kinetics), kJ/mole
F	probability distribution model
G	arbitrary function
H_0	null hypothesis
H_a	alternative hypothesis
i	counter
I	numerical integral
j	counter
K	specific growth rate constant (first order kinetics), d^{-1}
K'	reaction rate constant (pseudo zero order kinetics), units/day
K_0	frequency factor (first order kinetics), d^{-1}
K_0'	frequency factor (pseudo zero order kinetics), units/day
ℓ	lower limit
m	integer number
M	integer number
n	integer number
N	microbial counts, CFU/g
n_1	number of elements in population 1
n_2	number of elements in population 2
N_{obs}	observed microbial counts, CFU/g
OBJ	objective function
P	probability distribution model
R	universal gas constant, kJ/mole K
T	temperature, °C or K
u	upper limit
V	arbitrary function
W	Mann-Whitney-Wilcoxon statistic
x	variable
z_α	critical value at significant level α (normal distribution)
β	set of kinetic parameters
ϵ	residuals
θ	time
θ_0	initial time
θ_f	final time
θ_k	indicator time for each time interval
θ_L	lag time duration
σ	standard error
*	indicates a value generated by bootstrap
$\wedge$	indicates an estimated variable

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OSU Agricultural Experiment Station Publication No. 10,013.

Solid-Phase Acid Extraction Improves Thiobarbituric Acid Method to Determine Lipid Oxidation

S. RAHARJO, J.N. SOFOS, and G.R. SCHMIDT

ABSTRACT

Samples (110g) of raw (17.2–22.6% fat) and cooked 12.6–16.4% fat) ground beef in plastic cups were stored aerobically at 4 ± 1°C. Lipid oxidation was measured by four versions of the thiobarbituric acid (TBA) test, including aqueous acid extraction-C_{18} (TBA-C_{18}), direct heating, distillation, and unmodified aqueous acid extraction; and by sensory evaluation of rancid odor after 0, 2, 4, 6, and 8 days storage. The TBA-C_{18} method was more specific ($P<0.05$) and its limit of determination was 20 times lower than the other methods in detecting malonaldehyde. Results correlated ($r=0.856$ to 0.883 in raw, and $r=0.936$ to 0.981 in cooked meat) with sensory evaluation scores.

Key Words: beef, TBARS, lipid, peroxidation, malonaldehyde

INTRODUCTION

SPECTROPHOTOMETRIC detection of the malonaldehyde-thiobarbituric acid (TBA) complex has been widely used for measuring lipid oxidation in meat products. The TBA test can be conducted in meat by: (a) directly heating the samples in the presence of TBA, followed by separation of the red pigment by centrifugation (Uchiyama and Mihara, 1978; Pokorny and Dieffenbacher, 1989); (b) distillation of the sample, followed by reaction of the distillate with TBA (Tarladgis et al., 1960; Rhee, 1978; Ke et al., 1984; Hoyland and Taylor, 1989); (c) extraction of the lipid portion of the sample with chloroform-methanol and reaction of the extract with TBA (Witte et al., 1970; Salih et al., 1987). The direct heating and distillation TBA methods may form additional malonaldehyde and other TBA-reactive substances (TBARS) through degradation of polyunsaturated fatty acid hydroperoxides during analysis (Hoyland and Taylor, 1991). A major concern with the TBA method on extracted material is that much of the preformed malonaldehyde, naturally present in the aqueous phase of a meat sample, may remain undetected (Schmedes and Holmer, 1989). Heating the lipid portion (containing different levels of polyunsaturated fatty acids) with the TBA solution could also generate variable levels of TBARS including malonaldehyde (Gutteridge and Quinlan, 1983). Thus, that method may also provide information on susceptibility of different lipids to autoxidation.

The aqueous acid extraction TBA method uses milder conditions than the other tests. No heating is applied to the sample which minimizes formation of additional malonaldehyde and other TBARS. The procedure, however, is still not specific for detection of malonaldehyde, because other TBARS in the extract could have the same absorbance as the malonaldehyde-TBA complex (Squires, 1990; Draper and Hadley, 1990). We developed an aqueous acid extraction TBA-C_{18} method (Raharjo et al., 1992) with the potential of overcoming this interference problem. However, the analytical reliability of that method needs to be compared with other TBA methods.

Our objectives were to evaluate the specificity and limit of determination of the TBA-C_{18} method compared to other tests. They involved direct heating, distillation and aqueous acid extraction. We also determined the correlation between results of the TBA-C_{18} analysis and other TBA methods. Finally, we evaluated the correlation between sensory evaluation of rancid odor and TBA numbers from the four TBA methods.

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MATERIALS & METHODS

Sample preparation

Frozen ground beef from a local grocery store, was thawed at 4°C overnight. A portion (700g) of the ground beef in a beaker was cooked in water bath (National Appliance Co., Portland, OR) of 94 ± 1°C for 20 min to internal temperature 70°C, measured by thermocouple (Atkins Technical Inc., Gainesville, FL). Samples (110g) of raw (17.2–22.6% fat) and cooked ground beef (12.6–16.4% fat) were placed in plastic cups covered with caps (Solo Cup Co., Urbana, IL), and stored aerobically at 4 ± 1°C. The extent of lipid oxidation was measured after 0, 2, 4, 6, and 8 days storage by 4 different TBA tests and rancid odor development was assayed by sensory evaluation.

TBA methods

Direct heating. The direct heading TBA method was carried out as described by Uchiyama and Mihara (1978). Prior to homogenization, 0.15% of butylated hydroxytoluene (BHT) (Sigma Chemicals Co., St. Louis, MO) based on fat content, was added to each sample to prevent autoxidation during analysis (Pikul et al., 1983). The same amount of BHT was also added before homogenization to all samples analyzed by all other TBA procedures.

Distillation. The distillation TBA procedure was performed as described by Tarladgis et al. (1960).

Aqueous acid extraction. The aqueous acid extraction TBA test was performed as described by Salih et al. (1987), except that 5% (w/v) aqueous trichloroacetic acid (TCA) (Mallinckrodt, Paris, KY) was used for the extraction solvent.

Aqueous acid extraction TBA-C_{18}. Ground beef samples (10 g) were homogenized with 40 mL of 5% (w/v) aqueous TCA in an Osterizer blender (Sunbeam Corp., Milwaukee, WI) at room temperature (≈23°C) for 1 min. The meat homogenate was centrifuged at 10,000 × *g* for 5 min and the supernatant was filtered through a Whatman micro fiber glass filter grade C (Whatman, Hillsboro, OR) into a 50 mL volumetric flask. Filtrate volume was adjusted to 50 mL using 5% (w/v) aqueous TCA. A portion (5 mL) was reacted with 5 mL of 80 mM TBA in a test tube with a screw cap, while heating in a water bath of 94 ± 1°C for 5 min. The pH of the formed red pigment was adjusted to ≈7 with 5N NaOH (Mallinckrodt, Paris, KY) and 0.2 mL of 3% (w/v) phosphate buffer of pH 7.2 (Becton Dickinson and Co., Cockeysville, MD) prior to pumping through a solid phase extraction Sep-Pak® C_{18} cartridge (Waters, Milford, MA).

Prior to its use, the C_{18} cartridge had been washed with 10 mL of absolute methanol (Mallinckrodt), followed by 10 mL of distilled water at ≈20 mL/min. The flow rate was measured by loading the solution into a 12-mL syringe (Becton Dickinson and Co.), connected to a C_{18} cartridge, then manually pumped through the cartridge with a plunger for a specified period of time. The sample (10 mL) was loaded to the syringe and passed through the treated C_{18} cartridge at ≈5 mL/min to allow adequate time for the C_{18} matrix to bind the red colored malonaldehyde and other TBARS complexes. The eluted solution from the cartridge was discarded. Unreacted TBA solution and other components were removed by eluting the loaded sample with 10 mL of distilled water at ≈10 mL/min. The eluted solution from the cartridge was discarded. The malonaldehyde-TBA complex was recovered and separated from other TBARS by eluting the cartridge with 10 mL of absolute methanol at ≈10 mL/min. The absorbance of the methanol

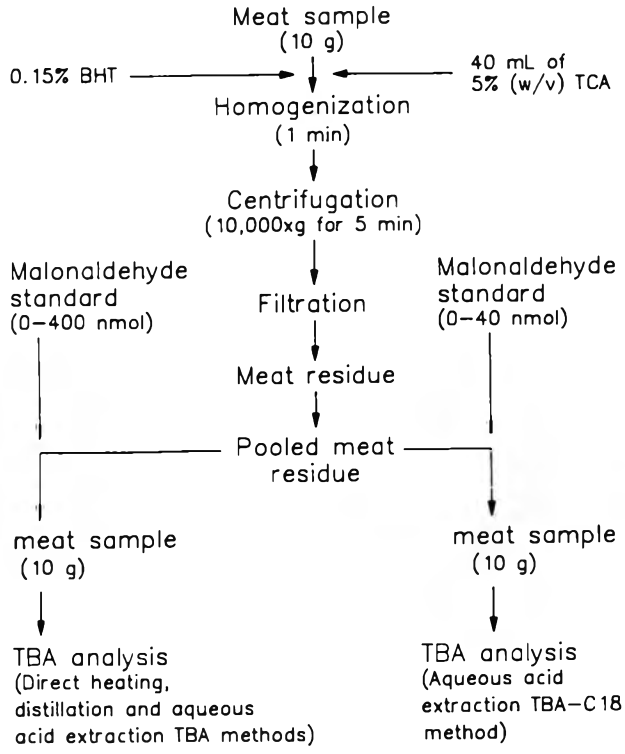

Fig. 1—Experimental procedures to determine recoveries and limits of determination of malonaldehyde. BHT: butylated hydroxytoluene, TCA: tricnloroacetic acid; TBA: thiobarbituric acid.

cluent containing the malonaldehyde-TBA complex was measured at 525 nm using a spectrophotometer. The malonaldehyde-TBA complex was confirmed by spotting the red complex from meat samples and the red complex from malonaldehyde standard on the same TLC-C_{18} plate. This involved heat-evaporating a 5-mL portion of methanol cluent in a water bath at 30°C for 40–60 min to a final volume of 0.5 mL. The sample and standard concentrated colored solutions were spotted on TLC-C_{18} (octadecylsilane bonded) plates (5 cm × 20 cm, 200 μm thick) with fluorescence at 254 nm (Whitman). The plates were developed with a mixture of absolute methanol:distilled water (60:40, v/v) until the solvent front reached 2.5 cm from the top edge. The plate was removed and dried at room temperature (≈23°C) and spots were observed under ultraviolet light (254 nm).

Determination of interference

The level of interfering substances in each TBA method was determined by subjecting the red pigment, after the malonaldehyde-TBA reaction, to solid phase extraction using the C_{18} cartridge to separate the malonaldehyde-TBA complex from other TBARS complexes. The difference between absorbance of total TBARS complexes (A_{TBARS}) prior to C_{18} treatment and the absorbance of the malonaldehyde-TBA complex (A_{MA}) after C_{18} treatment was designated as interference. The relative percentage of interference was calculated as $[(A_{TBARS}-A_{MA})/A_{TBARS}] \times 100\%$.

Blank, extraction recovery and limit of determination

Sample blanks, for correcting the TBA numbers, were prepared by subjecting pre-extracted ground beef residues to the TBA methods. Preparation of pre-extracted ground beef residues and determination of extraction recovery of malonaldehyde are described in Fig. 1. In the aqueous acid extraction TBA-C_{18} test, the malonaldehyde-TBA complex (100 mL), from the reaction of 50 mL of meat extract with 50 mL of 80 mM TBA, was passed through a Sep-Pak® C_{18} cartridge at ≈10 mL/min. The malonaldehyde-TBA complex was then recovered by elution with 5 mL absolute methanol.

Recovery values from the four TBA methods on raw and cooked ground beef were, respectively, as follows: direct heating (52.2% and 40.5%), distillation (70.6% and 68.7%), aqueous acid extraction (69% and 70%), and aqueous acid extraction TBA-C_{18} (74.7% and 76.7%). Each of these recovery values were subsequently used to calculate conversion factors (K) as described by Pikul et al. (1989). The TBA numbers (mg malonaldehyde equivalents/kg meat) were subsequently calculated by multiplying the absorbance by its corresponding K value. The four TBA methods had K values in the range of 6.3–11.8. The limits of determination in all methods were calculated according to procedures of Rudolph and Steinhart (1987); Currie (1968); and Thier and Zeumer (1987).

Sensory evaluation

Prospective panelists (8) were screened using threshold and difference tests (Amerine et al., 1965) for rancid odor. Six panelists were selected based on consistency of judgements. These selected panelists were trained in four sessions. In each session, (≈ 10 min), each panelist was served two sets of raw or cooked ground beef. Each set consisted of five individual meat samples with varying degrees of rancidity, prepared by mixing appropriate amounts of fresh and rancid ground beef. Preparation of rancid meat and serving samples followed procedures described by Rajarjo et al. (1989). The panelists were asked to rank samples from the lowest to highest degree of rancid odor by smelling.

Randomly coded samples of raw or cooked ground beef (10g) were placed in plastic cups with caps (Solo Cup Co.). These samples had been stored at 4 ± 1°C for 0, 2, 4, 6, and 8 days before analysis. At each evaluation day a freshly thawed raw and freshly cooked ground beef sample (10g) were also used as references. No samples were preheated and all were served in one session at room temperature (24–25°C). The six panelists were asked to evaluate the samples by smelling and score them on a 5-point scale, where 1 indicated no rancid odor, and 5 signified very pronounced rancid odor. To minimize odor cross-contamination among samples, panelists were instructed to briefly open the cap near their nose, and to reclose it.

Statistical analyses

Each experiment was replicated four times. The TBA numbers and amounts of malonaldehyde interfering substances originating from meat samples were analyzed by linear regression. Correlations between TBA numbers by aqueous acid extraction TBA-C_{18} method and TBA numbers resulting from the other methods tested were analyzed by Fisher transformation (Edwards, 1984). The same analysis was also performed to evaluate correlations between sensory evaluation scores and TBA numbers from the four methods.

RESULTS & DISCUSSION

Effect of TBA method variation on TBA numbers

The TBA numbers by the four methods showed a linear increase ($P<0.01$) in both raw (slope 0.16–0.59) and cooked (slope 0.15–1.06) ground beef with storage up to 8 days at 4 ± 1°C (Fig. 2). The direct heating, distillation and unmodified aqueous acid extraction TBA methods resulted in higher ($P<0.01$) slopes of TBA numbers in raw (2.2–3.7 times) and cooked (2.8–7.0 times) samples compared to those of the TBA-C_{18} method. Both, the direct heating and distillation methods involved heating at 94 ± 1°C (for 40 and 15 min, respectively). Heat treatment of unsaturated fatty acids in the presence of fatty acid hydroperoxides and TBA solution produced more TBARS than heating unsaturated fatty acids and TBA solution only (Gutteridge and Quinlan, 1983). Thus, additional TBARS including malonaldehyde could be produced during analysis itself. Pikul et al. (1989) evaluated three different TBA methods (distillation, aqueous acid extraction and lipid extraction) on raw chicken breast meat. They reported that the distillation TBA method produced TBA numbers 1.35 times higher than the aqueous acid extraction method.

The aqueous acid extraction method resulted in higher ($P<0.01$) TBA numbers (2.2 to 2.8 times) than those of the TBA-C_{18} method. Since no heat treatment was applied to the meat sample by either method, the formation of additional TBARS during analysis would be minimal. However, the unmodified aqueous acid extraction procedure, as well as the direct heating and distillation methods, do not specifically measure malonaldehyde in meat samples (Draper and Hadlery,

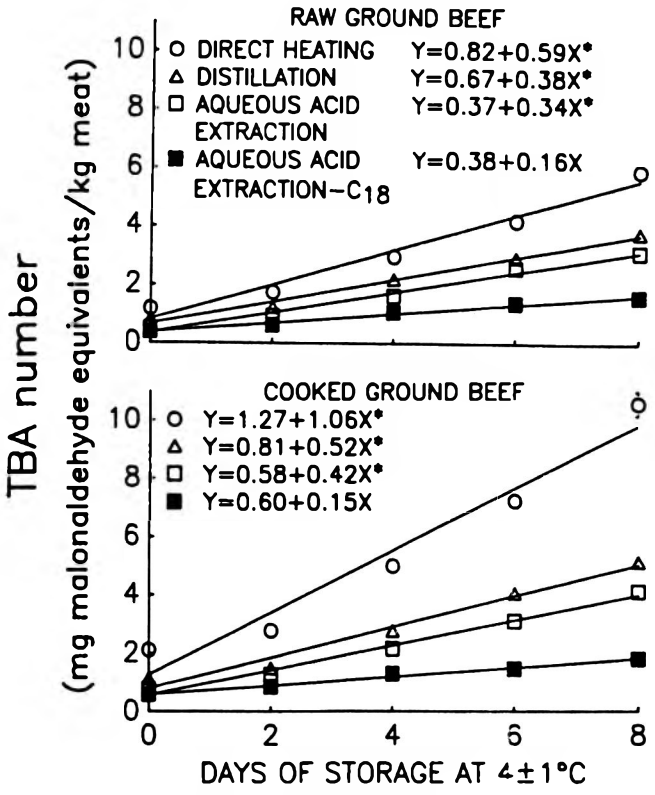

Fig. 2—Thiobarbituric acid (TBA) numbers of raw and cooked ground beef samples during aerobic storage at 4±1°C up to 8 days. (*) Slope of linear equation was significantly (P<0.05) different from slope of the linear equation of the aqueous acid extraction TBA-C_{18} method. All intercepts were not significantly (P>0.05) different.

1990; Squires, 1990). Other aldehydes, from degradation of lipid peroxides, have been reported to produce the same red-colored complex after reaction with TBA solution (Kosugi et al., 1989). Such TBARS would interfere with the red malonaldehyde-TBA complex. Use of the Sep-Pak® C_{18} cartridge could remove such interference.

Correlation between values from the TBA-C_{18} and other methods

The correlations between TBA numbers from the TBA-C_{18} and the other methods were evaluated in raw and cooked beef samples to determine the reliability of the new procedure (Table 1). All correlation coefficients (r) were different (P<0.05) from zero (0.967–0.993). When r values from the three regression lines in the raw ground beef were compared to corresponding r values in the cooked ground beef they were found not significantly (P>0.05) different. These results suggested that the TBA-C_{18} method would have the same degree of correlation with the other methods studied. Therefore, the newly developed TBA-C_{18} method is more specific than the others.

Correlation between sensory evaluation scores and TBA numbers

Consumers judge rancidity in meat products by smelling or tasting. It is important to evaluate the correlation between TBA numbers by any given TBA method and sensory scores for rancid odor. Our results indicated that TBA numbers from all TBA methods tested significantly (P<0.05) correlated (r = 0.856–0.981) with sensory scores for rancid odor in raw or cooked ground beef (Table 2). When r values from the four regression lines in raw ground beef were compared with corresponding values from cooked ground beef no difference

Table 1—Linear regression correlation between aqueous acid extraction TBA-C_{18} and other methods for measuring lipid peroxidation[a]

Comparison	Linear equation	Correlation coefficient (r)
Raw ground beef		
DH × C_{18}	Y = −0.53 + 3.66X	0.975[b]
DS × C_{18}	Y = −0.22 + 2.37X	0.993[b]
AE × C_{18}	Y = −0.44 + 2.15X	0.991[b]
Cooked ground beef		
DH × C_{18}	Y = −2.78 + 6.89X	0.967[b]
DS × C_{18}	Y = −1.20 + 3.41X	0.979[b]
AE × C_{18}	Y = −1.03 + 2.75X	0.984[b]

[a] DH: direct heating TBA method, DS: distillation TBA method, AE: aqueous acid extraction TBA method, and C_{18}: newly developed aqueous acid extraction TBA-C_{18} method.
[b] r values with same superscript not significantly (P>0.05) different. The r values were significantly (P<0.05) different from zero.

Table 2—Linear regression correlation between sensory analysis scores and TBA methods[a]

Comparison	Linear equation	Correlation coefficient (r)
Raw ground beef		
S × DH	Y = 1.77 + 0.26X	0.883[b]
S × DS	Y = 1.71 + 0.41X	0.874[b]
S × AE	Y = 1.83 + 0.44X	0.856[b]
S × C_{18}	Y = 1.63 + 0.96X	0.863[b]
Cooked ground beef		
S × DH	Y = 1.61 + 0.17X	0.981[b]
S × DS	Y = 1.55 + 0.34X	0.961[b]
S × AE	Y = 1.56 + 0.43X	0.974[b]
S × C_{18}	Y = 1.15 + 1.16X	0.936[b]

[a] S: sensory evaluation score of rancid odor, DH: direct heating TBA method, DS: distillation TBA method, AE: aqueous acid extraction TBA method, and C_{18}: newly developed aqueous acid extraction TBA-C_{18} method.
[b] r values with same superscript not significantly (P>0.05) different. The r values were significantly (P<0.05) different from zero.

(P>0.05) was found. Thus the TBA-C_{18} method had the same degree of correlation with sensory scores as the other TBA methods. Previous studies reported sensory evaluation scores significantly correlated with TBA results (Igene et al., 1985; Poste et al., 1986; Salih et al., 1987).

Interfering substances found in various TBA methods

The TBA-C_{18} method was used as a reference for comparison because it was more specific than the other three methods tested. When analyzed by the direct heating TBA method on day-0, samples had a higher (P<0.05) percentage of interfering substances (46.9-50.3%) than those detected by distillation (6.3–8.4%) or unmodified aqueous acid extraction TBA methods (21.9–24%) (Fig. 3). In addition, the percentage of interfering substances formed by the direct heating method increased (P<0.05) by 2.5% per day during refrigerated storage of cooked meat samples. The percentages of interfering substances were not (P>0.05) changed during refrigerated storage of either raw or cooked meat samples. The percentage of interfering substances found in the aqueous acid extraction method increased (P<0.05) by 3.5–4.2% per day during refrigerated storage of both raw and cooked meat samples. The percentage of interfering substances (6.3–8.4%) formed by the distillation TBA method did not change (P>0.05) during refrigerated storage of samples. The low percentage of interference in the distillation TBA method indicated that less malonaldehyde interfering substances were present.

Limit of determination

The sample blank values were used for correcting results of TBA analysis and for calculating the limit of determination according to Rudolph and Steinhart (1987) and Currie (1968). In addition, the procedure of Thier and Zeumer (1987) was also used to calculate the limit of determination of the four

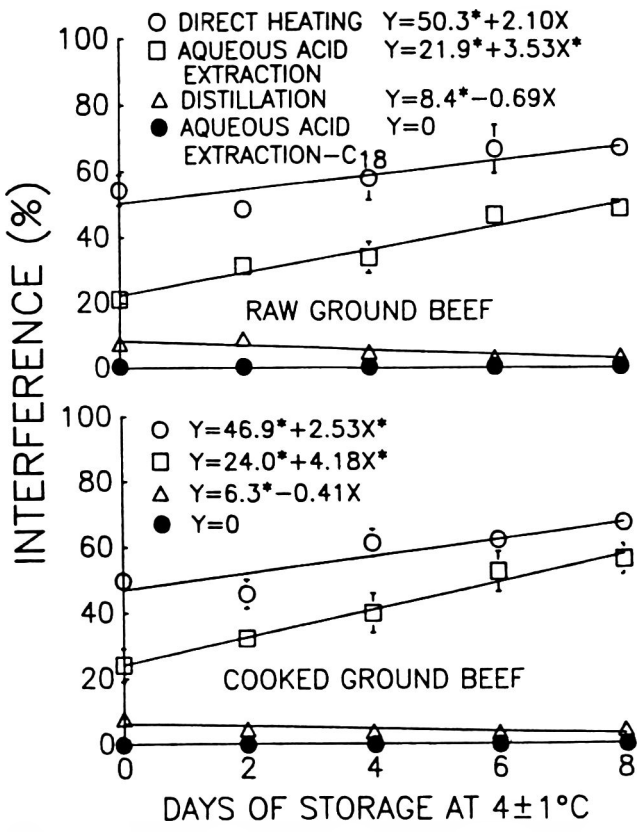

Fig. 3—Percentage of interfering substances originating from raw and cooked ground beef during storage at 4±1°C up to 8 days. Interference expressed as percentage of absorbance of TBA-reactive substances (TBARS) other than malonaldehyde divided by absorbance of total TBARS including malonaldehyde. Intercept or slope with asterisk (*) significantly (P<0.05) greater than zero.

methods. The direct heating, distillation, and aqueous acid extraction methods had similar limits of determination (≈1.33–2.04 nmol of malonaldehyde equivalents/mL meat extract) when calculated by each of the 3 methods (Table 3). Therefore, we could reasonably conclude that the limit of determination of the 3 methods was 2 nmol of malonaldehyde equivalents/mL meat extract. The total volume of meat extract (50 mL) obtained by each of these methods was derived from 10g of meat sample. Thus, the 10 g meat sample contained 100 nmol (or 7.2 μg) of malonaldehyde equivalents. This is identical with 0.72 mg malonaldehyde equivalents/kg meat or a TBA number of 0.72. No significant ($P>0.05$) differences were found between limits of determination of raw and cooked ground beef.

The use of a Sep-Pak™ C_{18} cartridge in the TBA-C_{18} method improved the limit of determination from 2 nmol to 0.1 nmol malonaldehyde equivalents/mL meat extract. Thus the TBA-C_{18} method had a limit of determination ≈20 times lower (0.036 mg malonaldehyde equivalents/kg meat) than the other methods.

CONCLUSIONS

THE PROPORTION of malonaldehyde interfering substances produced during TBA analysis depended on the state of the meat (raw or cooked) and on the type of TBA method used. The use of Sep-Pak™ solid phase extraction C_{18} cartridge improved the specificity and limit of determination from 0.72 mg to 0.036 mg malonaldehyde equivalents/kg meat. The TBA-C_{18} method resulted in lower TBA numbers because it did not involve sample heating and removed interfering substances.

Table 3—Limit of determination of malonaldehyde by four TBA methods (nmol malonaldehyde equivalents/mL meat extract), by three procedures

TBA method	Ground beef	Sample blank mean[a] (std dev)	Limit of determination[b] Procedure 1	Procedure 2	Procedure 3
Direct heating	Raw	1.03 (0.12)	1.75	1.69	2.00
	Cooked	1.32 (0.12)	2.04	1.69	2.00
Distillation	Raw	0.71 (0.14)	1.55	1.97	2.00
	Cooked	0.90 (0.13)	1.68	1.83	2.00
Aqueous acid extraction	Raw	0.61 (0.12)	1.33	1.69	2.00
	Cooked	0.66 (0.14)	1.50	1.97	2.00
Aqueous acid extraction-C_{18}	Raw	0.04 (0.01)	0.10	0.14	0.10
	Cooked	0.05 (0.01)	0.11	0.14	0.10

[a] Sample blank mean and standard deviation used for calculating limit of determination.

[b] Procedure 1: limit of determination calculated as mean plus 6 × standard deviation (Rudolph and Steinhart, 1987). Procedure 2: limit of determination calculated as 14.1 × standard deviation (Currie, 1968). Procedure 3: limit of determination determined by adding the smallest level of pure malonaldehyde to the meat samples which could be detected by the TBA method at 99% confidence of difference from blank (Thier and Zeumer, 1987).

The TBA-C_{18} method had the same degree of correlation with sensory evaluation scores as the other methods.

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Malondialdehyde Oxidation by Hydrogen Peroxide and by Light-Excited Riboflavin in Model Systems

ZHANYUAN DU and WILLIAM J. BRAMLAGE

ABSTRACT

Oxidation of malondialdehyde (MDA) by H_2O_2 was affected by concentrations of MDA or H_2O_2, pH and solvent. The reaction displayed an approximate 1:1 ratio of H_2O_2/MDA consumption and probably produced malonaldehyde as the oxidation product in moderate conditions. Oxidation of MDA by light-excited riboflavin produced superoxide anion, and the amount of superoxide anion generated was MDA concentration-dependent.

Key Words: Hydrogen peroxide, malondialdehyde, riboflavin, superoxides

INTRODUCTION

MALONDIALDEHYDE (MDA), a product of lipid peroxidation, has been reported extensively in foods (Siu and Draper, 1978; Gray, 1978) and in living systems (Janero, 1990; Dhindsa et al., 1981). The significance of MDA in human health (Shamberger et al., 1974; Mukai and Goldstein, 1976) and in association with plant senescence (Kar and Mishra, 1976) is of increasing interest. The toxicity of MDA to living systems is attributed mainly to its ability to alter or cross-link a variety of biomolecules such as proteins and enzymes (Buttkus, 1967; Crawford et al., 1967; Chio and Tappel, 1969;), lipoproteins (Fogelman et al., 1980), DNA (Nair et al., 1984; Summerfield and Tappel, 1981), amino phospholipids (Bidlack and Tappel, 1973), and amino acids (Lewis and Wills, 1962; Nair et al., 1981) via conjugated Schiff bases having the characteristic N−C=C−C=N fluoromorphic system (Janero, 1990). However, the potential of MDA to modify biomolecules *in vivo* may be affected by the balance of MDA formation and MDA degradation. Potential pathways of MDA catabolism through an aldehyde dehydrogenase-mediated pathway (Marnett et al., 1985) or through a peroxidase-mediated pathway (MacDonald and Dunford, 1989) have been proposed. Several *in vivo* metabolites of bound MDA with amino acids or amino residues of proteins were reported (Draper et al., 1986, 1988).

The high chemical reactivity of MDA is a concern regarding both its toxicity and the reliability of the thiobarbituric acid (TBA) assay for MDA. For example, the MDA-nitrite interaction may make the TBA assay unusable in meat systems where nitrite is in large excess (Kolodziejska et al., 1990). Also the instability of MDA in the presence of H_2O_2 may not only affect TBA assay, but also the true level of MDA *in vivo*, resulting in alteration of its biological effect (Kostka and Kwan, 1989). This is because both MDA and H_2O_2 exist in systems such as senescing plant tissues (Brennan and Frenkel, 1977; Dhindsa et al., 1981; Ferguson et al., 1983), and some lipid peroxidation-promoting systems (Pradham et al., 1990; Farouk et al., 1991). Our objective was to determine whether MDA could be oxidized directly by both H_2O_2 and light-excited riboflavin in model systems. This can help in understanding possible effects of MDA in food systems.

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MATERIALS & METHODS

MDA was prepared by hydrolyzing malonaldehyde bis(dimethyl acetal) in 1N HCl, and final pH was adjusted by 1N NaOH to pH 6-7. The reaction of MDA and H_2O_2 was investigated in 0.05M tris buffer (pH 7.0) with varying concentrations of MDA and H_2O_2 at room temperature (≈23°C) unless indicated. The reaction course was monitored by measuring the disappearance of MDA and/or H_2O_2 at intervals. To measure the pH or solvent dependency of the reaction, MDA and H_2O_2 were incubated in tris buffers (pH 10.4 or 7.0), monophosphate-hydroxide buffer (pH 6.0) or 0.5N HCl (pH 0.3) or 2N HCl. pH change was measured at intervals in nonbuffered aqueous reaction mixtures, the initial pH of which was adjusted by diluted HCl or NaOH solutions. Absorbance spectra were recorded from a spectrophotometer.

The oxidation of MDA by light-excited riboflavin was investigated in 0.05M phosphate buffer (pH 7.8) at room temperature. The reaction was monitored by measuring the superoxide anion generated by reoxidation of MDA-reduced riboflavin in the presence of O_2 using a modification of the method of Dhindsa et al. (1981). The reaction mixture contained 0.05M phosphate buffer (pH 7.8), 75 μM nitro blue tetrazolium (NBT), 2 μM riboflavin, and varying concentrations of MDA or MDA and 10 mM methionine. Reaction mixtures (3 mL) in uniform transparent tubes were shaken and placed 50 cm below a light bank consisting of eight 15 W fluorescent lamps. The reaction was started by switching on the light and allowed to proceed for 10 min. The reaction was stopped by switching off the light, and the absorbance by the reaction mixture at 560 nm was read as a measure of superoxide anion generated. An irradiated reaction mixture without MDA and methionine developed only slight color and served as control. The inhibition of NBT reduction by superoxide anion was demonstrated in the presence of 0 to 240 units of bovine liver superoxide dismutase (SOD) in the above reaction mixtures.

MDA concentrations were determined by TBA assay according to Wilbur et al. (1949). Two mL of 20% tetrachloric acid (TCA) aqueous solution and 1 mL of 0.67% TBA aqueous solution were added to 2 mL of the properly diluted reaction mixture in tubes. The tubes then were heated in a boiling water bath for 15 min, cooled quickly with running tap water and centrifuged at 15,000 rpm for 15 min. The absorbance at 532 nm of the clear supernatant was recorded. MDA concentrations were calculated by using a molar absorbance coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$.

H_2O_2 concentrations were determined according to Brennan and Frenkel (1977), modified as follows: 1 mL properly diluted reaction mixture and 4 mL water were added to 1 mL titanium reagent (20% titanic tetrachloride in concentrated HCl, v/v). After mixing and standing 5 min, the absorbance was determined at 415 nm vs a blank without H_2O_2. Concentrations of H_2O_2 were obtained by comparison with a standard curve.

To determine the reducing capacity of H_2O_2-MDA reaction mixture, 1 mL of the mixture was titrated vs 0.01M $KMnO_4$ in 15 mL of 2% (v/v) sulfuric acid until a faint purple color persisted for at least 15 sec. A control was run without the reaction mixture.

Data in tables were recorded as means ± standard deviation, unless otherwise indicated, of at least three replications for individual points obtained in a typical experiment. In the graphs, error bars indicate standard errors. No error bar indicates the magnitude of standard error was within the symbols.

Malonaldehyde bis(dimethyl acetal) was purchased from Aldrich and hydrogen peroxide was from Mallinckrodt. Other chemicals were from Sigma.

RESULTS & DISCUSSION

MDA and H_2O_2 both decreased proportionally with time when 2 mM H_2O_2 and 10 mM MDA were incubated together in a

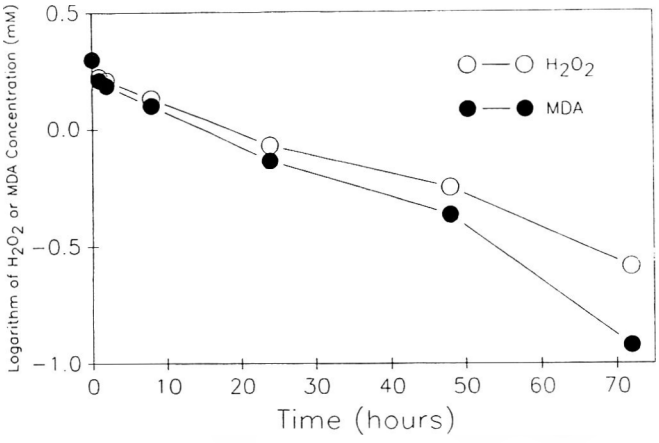

Fig. 1—Changes in MDA and H_2O_2 concentrations during the H_2O_2-MDA reaction. The reaction mixture contained 0.05M Tris buffer (pH 7.0), 2 mM H_2O_2 and 10 mM MDA. Standard error bars are within symbols.

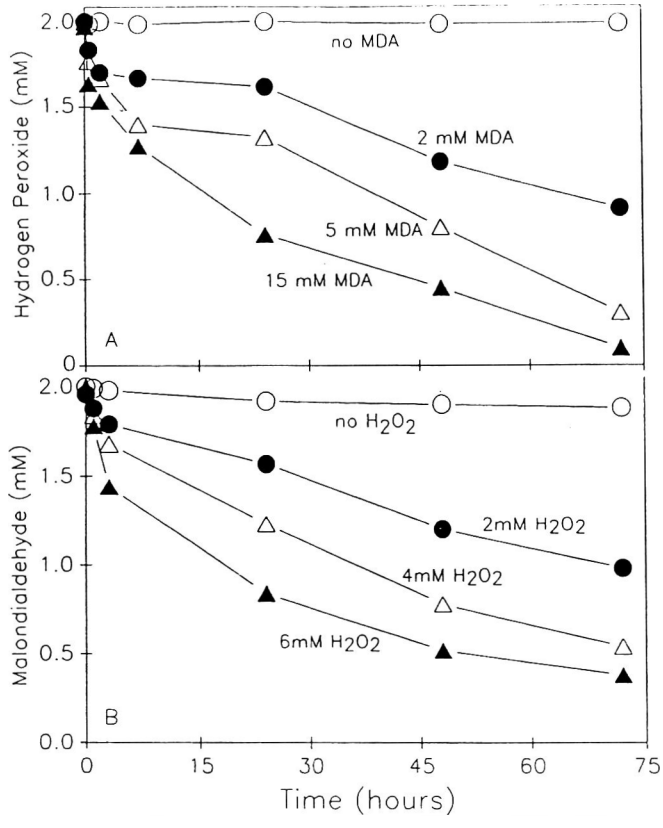

Fig. 2—Effects of MDA or H_2O_2 concentrations on decreases of H_2O_2 or MDA in the reaction mixture in Tris buffer (pH 7.0). Standard error bars are within symbols.

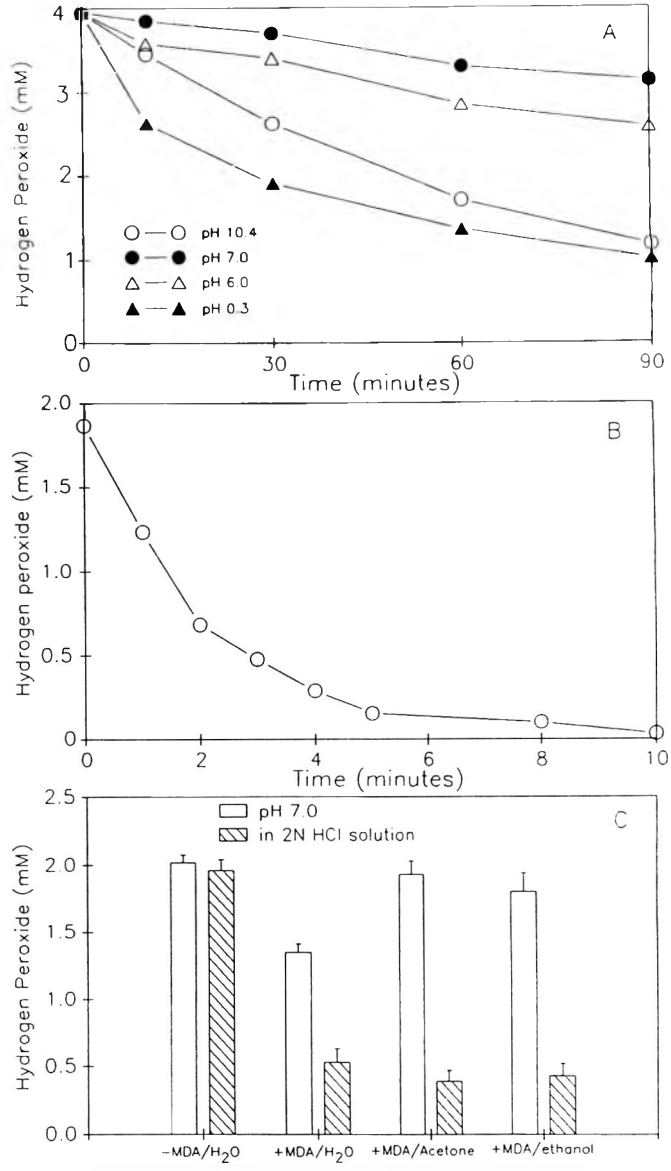

Fig. 3—Effects of pH and solvent on H_2O_2-MDA reaction as measured by decreases of H_2O_2 in the reaction mixture.(A): reaction of 4 mM H_2O_2 and 4 mM MDA in four different pH solutions; (B): reaction of 2 mM H_2O_2 and 10 mM MDA in 2N HCl solution; (C): reactions of 2 mM H_2O_2 and 2 mM MDA in water, 60% acetone or ethanol aqueous solutions. The H_2O_2 concentrations were measured after 48 and 0.25 hr of reaction at pH 7.0 and 2N HCl solution, respectively. No error bar indicates error within symbols.

tris buffer (pH 7.0) at room temperature (Fig. 1). Rates of decrease were fastest during the first few hours of reaction. Similar results also were obtained when 2 mM MDA and 10 mM H_2O_2 were incubated (data not shown). Higher concentrations of MDA or H_2O_2 resulted in faster rates of decrease of either H_2O_2 (Fig. 2A) or MDA (Fig. 2B). However, an incomplete hydrolysis of malonaldehyde bis(dimethyl acetal) (e.g. 1 hr in 0.01N HCl) may greatly reduce the rate of decrease of either MDA or H_2O_2. Free MDA may undergo slow self-condensation in aqueous solutions (Kwon and Watts, 1964). Kostka and Kwan (1989) first reported the instability of MDA in the presence of H_2O_2, however they did not simultaneously demonstrate the decomposition of H_2O_2 in the presence of MDA.

The instability of MDA in the presence of H_2O_2 could be interpreted in two ways: (1) H_2O_2 accelerates the MDA decomposition as a catalyzing factor in the system; or (2) H_2O_2 reacts with MDA to form a new compound. Results strongly suggest that a reaction between MDA and H_2O_2, i.e. oxidation of MDA by H_2O_2, occurred in the systems, since MDA and H_2O_2 decreased proportionally.

The reaction between MDA and H_2O_2 was greatly affected by pH and solvent in the reaction mixture. Both strongly acidic and alkaline conditions enhanced the reaction, as compared to the reaction in near neutral conditions (Fig. 3A). With 2 N HCl in the reaction mixture, the reaction went nearly to completion within minutes, as measured by the decrease of H_2O_2 (Fig. 3B). However, little decrease of H_2O_2 was observed if the reaction was conducted in 60% acetone or ethanol at pH 7.0. In the presence of 2 N HCl, the reaction was greatly enhanced both in pH 7.0 aqueous solution and in 60% acetone

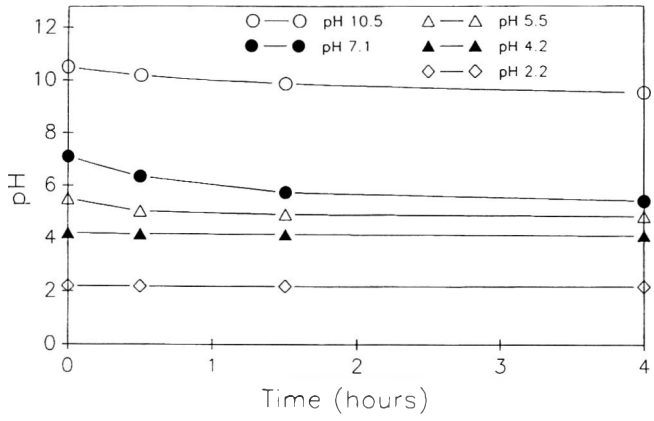

Fig. 4—pH changes of the non-buffered reaction mixtures in the course of H_2O_2-MDA reaction. The initial pH of the reaction mixture containing 4 mM H_2O_2 and 4 mM MDA was adjusted by diluted HCl or NaOH to values indicated. Standard error bars are within symbols.

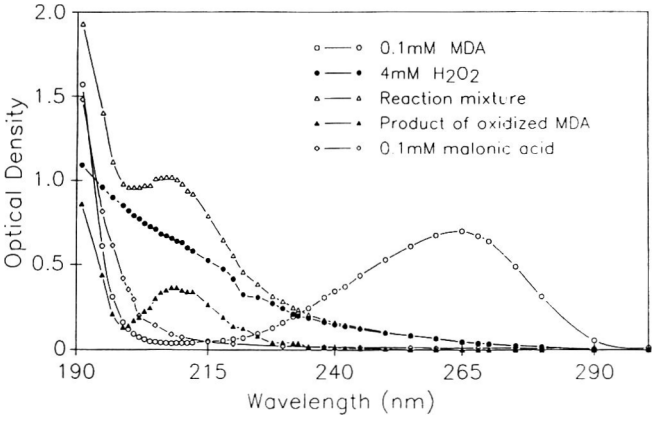

Fig. 5—UV absorbance spectra of components indicated. Reaction mixture contained 0.1 mM MDA and 4 mM H_2O_2 and its UV absorbance spectrum was recorded after 72 hr reaction at room temperature. The spectrum of the product of H_2O_2-oxidized MDA was estimated by subtracting the spectrum of 4 mM H_2O_2 from that of the reaction mixture.

Table 1—Consumption of 0.01M $KMnO_4$ in titration of solutions of H_2O_2, MDA or MDA-H_2O_2 reaction mixture[a]

Solutions	0.01M $KMnO_4$ consumed (ml)	Normality ratio $KMnO_4$: reductant
1 mL of 30 mM H_2O_2 (A)	3.0 ± 0.07	1 : 1.00
1 mL of 30 mM MDA (B)	5.8 ± 0.21	1 : 1.93
A + B (titrated immediately)	8.7 ± 0.24	1 : 2.93
A + B (titrated after 120 hr)	3.1 ± 0.17	1 : 1.13

[a] The product mixture was prepared from the reaction of 30 mM H_2O_2 and 30 mM MDA for 120 hr. Data are means ± SD of three duplications.

or ethanol solution (Fig. 3C). The reaction appeared to be promoted more by aqueous solutions than by organic solvents such as acetone or ethanol.

A slow pH drop occurred during oxidation of MDA by H_2O_2 at relatively high pH conditions (Fig. 4). This suggested that MDA was oxidized either into carboxylic compound, i.e. malonaldehydate or malonic acid by H_2O_2. The proportional disappearance of both MDA and H_2O_2 (Fig. 1) suggested that the oxidation of MDA by H_2O_2 was a 1:1 reaction. The small difference between the rates of decrease for MDA and H_2O_2 (Fig. 1) could be due to the self-condensation of MDA, as shown in Fig. 2B. The ratio of H_2O_2/MDA, however, remained near unity (0.97 ± 0.02) after a 120-hr reaction regardless of concentrations of H_2O_2 or MDA in the systems, as in

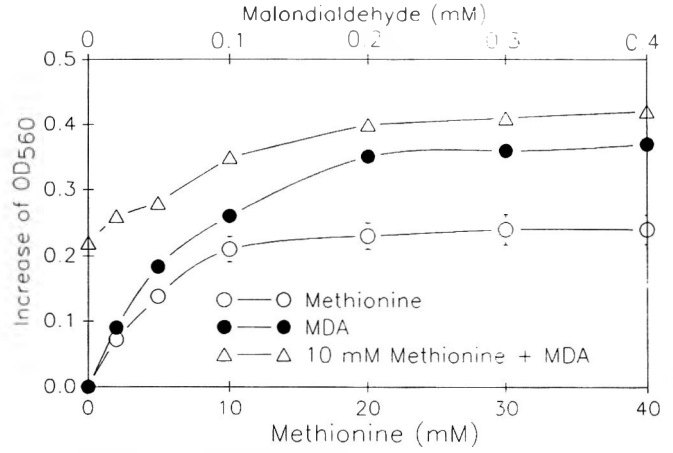

Fig. 6—Oxidation of MDA by light-excited riboflavin in the MDA-riboflavin and MDA-methionine-riboflavin systems, as measured by the ability to reduce NBT, i.e. the increase of OD_{560} of superoxide anion generated in the presence of O_2 in the systems. The methionine-riboflavin system is shown for comparison.

the reactions shown (Figs. 1 and 2). When equal amounts of 30 mM H_2O_2, MDA, or mixture of H_2O_2 or H_2O_2-oxidized MDA were titrated with 0.01M $KMnO_4$, we observed that MDA consumed twice the amount of 0.01M $KMnO_4$ as H_2O_2 alone. Also a mixture of equal amounts of MDA and H_2O_2 consumed almost three times the amount of $KMnO_4$ when titrated immediately after mixing, compared to H_2O_2 at the same concentration. The H_2O_2-oxidized MDA, i.e. mixture of equal amounts of MDA and H_2O_2 after 120-hr reaction at room temperature, consumed only about one-third as much 0.01M $KMnO_4$ compared to the mixture titrated immediately after mixing (Table 1). This indicated that half the aldehyde groups in MDA remained unchanged after the H_2O_2-MDA reaction. Thus, the data support the hypothesis that the MDA was oxidized into malonaldehydate in the model systems.

Comparing UV absorbance spectra further indicated malonaldehydate formation during MDA oxidation by H_2O_2. MDA had a single maximum at 265 nm (Fig. 5), close to the reported maximum at 267 nm (Janero, 1990), while malonic acid had no absorbance maximum in the wavelength range 190–300 nm. The UV spectrum of H_2O_2-oxidized MDA was estimated by subtracting the absorbances of 4 mM H_2O_2 from the absorbances of the mixture of 0.1 mM MDA and 4 mM H_2O_2. The UV spectrum of H_2O_2-oxidized MDA (maximum about 210 nm), was clearly different from that of either MDA or malonic acid. We concluded that MDA was oxidized by H_2O_2 into malonaldehydate in the system reported here.

Reduction of light-excited flavins has been reported when proper electron donors such as EDTA or methionine were present, and superoxide anion could be generated when the reduced flavins are reoxidized in the presence of O_2 (Beauchamp and Fridovich, 1971; Vargas and Maurino, 1987). The ability to reduce dyes by superoxide anion generated in the above system affords a measure of the reduction-oxidation-reduction cycle of flavins. This system became a routine assay of SOD activity using methionine as electron donor and NBT as the final electron receptor (Beauchamp and Fridovich, 1971). MDA, alone or combined with methionine, was oxidized as an electron donor by light-excited riboflavin in this system. Furthermore MDA was much more effective (about 150 times) than methionine as an electron donor in terms of the proper concentrations needed to generate superoxide anion (Fig. 6). The ability of the MDA-riboflavin or MDA-methionine-riboflavin system to generate superoxide anion in the presence of O_2 was determined by inhibiting the NBT reduction with bovine liver SOD (Fig. 7). However, H_2O_2-oxidized MDA alone exhibited less

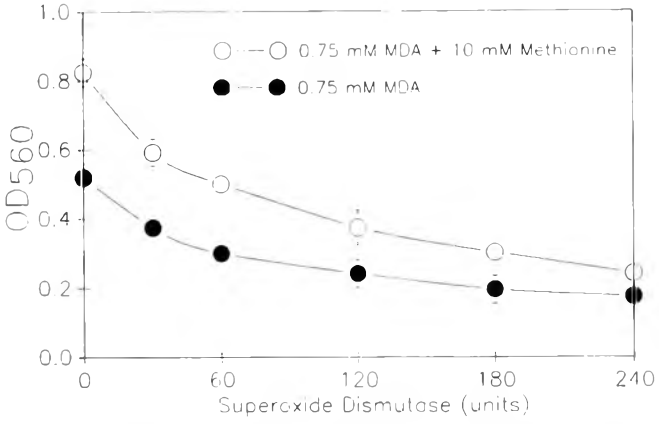

Fig. 7—Inhibition by bovine liver SOD of NBT reduction by superoxide anion generated in the MDA-riboflavin or MDA-methionine-riboflavin system.

than 10% of the activity of MDA alone. Whether the residual activity of H_2O_2-oxidized MDA was due to an incomplete oxidation of MDA by H_2O_2, or to the product of H_2O_2-oxidized MDA, is not known.

Both MDA and H_2O_2 are toxic to living cells due to their high chemical activity, and they exist *in vivo* at concentrations similar to those tested in our system (Dhindsa et al., 1981; Brennan and Frenkel, 1977). Toxicity of H_2O_2 may be from its oxidative ability, as reported by Chevalier et al. (1990) and Kell and Steinhart (1990). H_2O_2 also was reported to be associated with responses to physiological processes such as senescence (Chin and Frenkel, 1977; Takahama, 1989; Trippi et al., 1989) and stresses (Rich et al., 1976; Murphy and Huerta, 1990; Apostol et al., 1989) in plants. Although H_2O_2 is catabolized by catalase and peroxidases, it accumulates under some conditions such as stresses (Tanaka et al., 1982). Oxidation of MDA by H_2O_2 *in vivo* might reduce toxicity of both MDA and H_2O_2. However it may more greatly reduce the toxicity of MDA because the concentrations of H_2O_2 are usually much higher than those of MDA *in vivo* (Dhindsa et al., 1981; Brennan and Frenkel, 1977). Thus, the direct oxidation of MDA by H_2O_2 may contribute greatly to the degradation of MDA *in vivo*. MDA is produced by lipid peroxidation associated with biological membranes where hydrophobic conditions usually exist. This hydrophobicity may retard the oxidation of MDA by H_2O_2, because the oxidation of MDA by H_2O_2 is favored by strong hydrophilic or ionized conditions. Thus, the distribution of MDA in cells also may affect its degradation. MDA may react *in situ* with membrane lipids or proteins before it is released to cell saps, and also could be oxidized by other strong oxidants *in vivo*. The MDA-H_2O_2 interaction may similarly exist in many foodstuff systems, because both MDA and H_2O_2 (or other peroxides) can be produced simultaneously in the lipid peroxidation process (Gray, 1978; Pompella et al., 1987).

Both MDA and flavins exist in living cells. Recently, flavin-mediated photoinactivation of nitrate reductase was reported in spinach to involve superoxide radicals (Vargas and Maurino, 1987). Whether the MDA-riboflavin or other similar systems exist *in vivo* is not known. If they exist, this system also may contribute to the toxicity of MDA *in vivo* because of the toxicity of superoxide anion (Winston, 1990). The toxicity of MDA *in vivo* is probably very complex, and may depend on its formation, distribution, degradation including oxidation by H_2O_2 or light-excited flavins, and interactions with other compounds. Since our data were obtained from model systems, further experiments are needed to evaluate biological effects of MDA oxidation in living tissues or food materials. In addition, caution must be observed when a TBA assay is conducted in systems where H_2O_2 and riboflavins exist.

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Simplified Enzymatic-gravimetric Method for Total Dietary Fiber In Legumes Compared with a Modified AOAC Method

BETTY W. LI and MARIA S. CARDOZO

ABSTRACT

Two methods (an AOAC and a simplified enzymatic-gravimetric method) were used to analyze seven types of canned legumes and eight cooked legumes. Total dietary fiber (TDF) of the canned products ranged between 17% and 23% (dry basis) for chick peas, Great Northern beans, kidney beans, pinto beans, pork & beans, vegetarian beans in tomato sauce, and 27–31% for wax beans. These values were comparable for both methods. However, results on cooked legumes were very different between methods. TDF values for several types of beans and peas were higher and ranged between 31% and 55% by the AOAC/Tris-Mes buffer method as compared to 17% and 29% using the simplified method. Chemical analysis of dietary fiber residues showed the major difference between the methods was in the extent of starch removal as affected by starch gelatinization.

Key Words: legumes, beans, dietary fiber, analysis

INTRODUCTION

LEGUMES are second to cereals as important sources of dietary fiber (DF), protein and starch. Some potential health benefits are attributable to consumption of foods high in dietary fiber in general and legumes in particular; e.g. lowering of serum cholestrol (Anderson et al., 1984, 1990; Shutler et al., 1989), improvement of glycemic response (Jenkins et al., 1980, 1984; Thorne et al., 1983) and possibly reducing colon cancer risk (Correa 1981). In nationwide samplings of grocery stores, canned and packaged dry beans, peas, and lentils constituted 18% of purchase volume of all processed-vegetables between 1984 and 1988, with an increase to 18.5% in 1989. Meanwhile, legumes have also gained popularity in restaurants (Morrow 1991).

With increasing consumer interest in fiber-rich foods, and the demand for more nutrient data, there is a need for information on dietary fiber content of the many varieties of legumes consumed. Analysts (Anderson et al., 1988; Mongeau et al., 1989; Englyst and Kingman, 1990a; Marlett, 1992) have reported values for dietary fiber in a few selected legumes using different methods.

The objective of our study was to compare a variety of canned and dried beans (national and store brands) for total dietary fiber content using two enzymatic-gravimetric methods: an official AOAC method (Lee et al., 1992) and a simplified method (Li and Andrews, 1988).

MATERIALS & METHODS

Materials

Canned or dried legumes were purchased within the Washington, D.C. area from local retail outlets. Products were selected based on sales volume as determined by 1988 Nielsen Scantract Data (Nielsen, A.C. Company, Northbrook, IL). Three brands each of eight dry legumes (1 lb bag) and seven canned legumes (16 oz) were purchased during June and July 1991.

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Sample preparation

Canned beans. Beans were drained (except for kidney beans and beans in sauce) and homogenized using a food processor (Cuisinart Model DLC-8, Cuisinarts, Inc. Greenwich, CT). Portions were removed for moisture determination and rest of the samples were lyophilized. Dried samples were then ground in a Wiley Mill to pass through a 30 mesh screen. Samples were further dried under vacuum at 60°C and stored at room temperature (~25°C) in air tight containers in a desiccator until analyzed.

Dried beans. Samples (~200g) were weighed and deionized water (1L) was added to 4-L pot for quick soaking as described on the package, i.e. after addition of water, samples were brought to a boil and boiled for 2 min, removed from heat, covered and left to stand 1 hr. Cooking was resumed with occasional stirring. Testing for doneness was determined after 30 min cooking by crushing beans between the thumb and forefinger. Beans were considered done when they crushed easily. Beans were tested each 10 min after the first 30 min of cooking. Most beans were done between 45–60 min. Aliquots were removed for moisture determination and then treated as described for canned beans.

Determination of moisture and dietary fiber

Moisture was determined by drying preweighed cooked or canned homogenized samples under vacuum at 60°C for 3.5 hr. Total dietary fiber was determined using either an AOAC/Tris-Mes buffer method, AOAC #991.43 (Lee et al., 1992, a modification of AOAC #985.29) or a simplified total dietary fiber method (Li and Andrews, 1988) with some modifications as follows; samples were autoclaved at 130°C instead of 121°C, and amyloglucosidase (3 mg/sample) from Boehringer-Mannheim (#208-469) was used. With this enzyme no blank correction was necessary. Main differences between the two enzymatic-gravimetric methods were:

(1) The AOAC Method used an amylase to solubilize and an amyloglucosidase to hydrolyze starches, while the simplified method incorporated an autoclaving step for solubilization followed by incubation with an amyloglucosidase. (2) The simplified method omitted a protease treatment. (3) The simplified procedure did not require pH adjustment.

Determination of neutral sugars and uronic acids

Efforts were made to account for discrepancies in values obtained using the two methods and different gelatinization temperatures in the simplified procedure. Neutral sugars and uronic acids were determined on dietary fiber residues of two bean samples which had large discrepancies in dietary fiber values between the two methods. Samples were also analyzed for total, soluble and insoluble dietary fiber by the two methods with or without dimethyl sulfoxide (DMSO) treatment before enzyme incubation. Residues were analyzed chemically according to a procedure described by Englyst et al., (1990b), which included a primary hydrolysis step to solubilize cellulose with 12M H_2SO_4 at 35°C and a secondary hydrolysis step to depolymerize all polysaccharides in 2M H_2SO_4 at 100°C. The resulting neutral sugars were then quantitated as alditol acetates by gas liquid chromatography (Hewlett Packard 5840 equipped with a fused silica capillary column). Uronic acids in the acid hydrolyzates were determined by a colorimetric procedure also modified by Englyst et al. (1990b).

RESULTS & DISCUSSION

IN MOST LEGUMES analyzed, differences were observed in the total dietary fiber (TDF) content between brands. Thus,

Table 1 – Total dietary fiber by AOAC/Tris-Mes and Simplified Methods in canned legumes

Legume	Brand[d]		MethodA[a] g/100 g dry weight	MethodB[b] g/100 g dry weight	Method A g/100 g wet weight	Method B g/100 g wet weight
Chick peas	A		18.8 ± 0.92[c]	19.5 ± 0.92	6.99 ± 0.34	7.22 ± 0.35
	B		15.5 ± 0.35	21.5 ± 0.00	4.79 ± 0.11	6.62 ± 0.00
	C		17.6 ± 0.21	20.9 ± 0.42	5.03 ± 0.57	5.96 ± 0.12
		Mean	17.4 ± 1.56	20.6 ± 1.05	5.60 ± 1.09	6.60 ± 0.63
Great Northern beans	B		22.0 ± 0.21	19.8 ± 0.07	6.13 ± 0.06	5.52 ± 0.02
	D		21.2 ± 0.21	20.1 ± 0.28	5.16 ± 0.05	4.88 ± 0.06
	E		25.5 ± 1.27	24.2 ± 0.71	6.43 ± 0.32	6.09 ± 0.18
		Mean	22.9 ± 2.26	21.4 ± 2.44	5.91 ± 0.66	5.49 ± 0.61
Kidney beans	A		22.8 ± 0.14	20.1 ± 0.07	5.29 ± 0.03	4.68 ± 0.01
	C		24.6 ± 0.71	26.8 ± 0.49	5.04 ± 0.14	5.50 ± 0.11
	F		20.6 ± 1.56	20.3 ± 0.00	4.76 ± 0.36	4.69 ± 0.00
		Mean	22.7 ± 2.00	22.4 ± 3.83	5.03 ± 0.27	4.96 ± 0.47
Pinto beans	G		20.4 ± 0.21	18.0 ± 0.07	4.49 ± 0.04	3.97 ± 0.01
	H		21.5 ± 0.35	18.7 ± 0.14	4.87 ± 0.08	4.23 ± 0.04
	I		21.2 ± 0.99	17.8 ± 0.49	5.34 ± 0.25	4.49 ± 0.13
		Mean	21.1 ± 0.56	18.2 ± 0.44	4.90 ± 0.43	4.23 ± 0.26
Pork and beans	A		18.2 ± 0.07	18.0 ± 0.14	5.01 ± 0.02	4.95 ± 0.04
	J		17.4 ± 0.57	17.8 ± 1.10	4.64 ± 0.29	4.65 ± 0.14
	K		17.3 ± 1.06	18.8 ± 0.07	4.23 ± 0.25	4.59 ± 0.01
		Mean	17.7 ± 0.51	18.2 ± 0.56	4.63 ± 0.39	4.73 ± 0.19
Vegetarian beans in tomato sauce	J		15.2 ± 0.07	16.3 ± 0.42	4.03 ± 0.02	4.30 ± 0.11
	L		20.1 ± 0.35	17.7 ± 0.00	5.66 ± 0.09	4.97 ± 0.00
	M		15.0 ± 0.49	17.0 ± 0.21	4.20 ± 0.14	4.75 ± 0.06
		Mean	16.8 ± 2.89	17.0 ± 0.70	4.63 ± 0.81	4.67 ± 0.34
Wax beans	A		30.9 ± 0.14	28.1 ± 0.28	2.17 ± 0.00	1.97 ± 0.02
	D		31.5 ± 0.85	28.2 ± 0.00	2.77 ± 0.07	2.48 ± 0.00
	N		30.1 ± 0.49	25.1 ± 0.21	2.12 ± 0.04	1.76 ± 0.01
		Mean	30.8 ± 0.68	27.1 ± 1.73	2.35 ± 0.36	2.07 ± 0.37

[a] Method A - AOAC/Tris-Mes Buffer Method
[b] Method B - Simplified Method
[c] Mean value of two replicates ± standard deviation
[d] Brand Code Information available from authors on request.

the TDF values for individual brands of seven canned legumes using the AOAC/Tris-Mes buffer method and the simplified method are summarized in Table 1. Since each legume sample was analyzed only in duplicate, no statistical analysis was attempted. However, for both methods, the average TDF value (as percent dry matter) ranged between 17% and 23% for six of the canned legumes, and 27–31% for wax beans. Overall, the two methods gave comparable results for canned legumes. For dry legumes cooked according to package instructions, except for lima beans, results were very different. TDF values were much higher (double in most cases) and ranged between 31% and 55% according to the AOAC/Tris-Mes method as compared to 17% to 29% using the simplified method (Table 2). Larger variability between the 3 brands of each legume was also observed for the AOAC method. This could be partly accounted for by day to day variability using the AOAC method for this type of sample, from repeated analyses of some samples on several different days (data not shown here), while the simplified method tended to give more consistent values. The major difference between the two methods is in the extent of starch removal as affected by starch gelatinization. The AOAC method uses a heat-stable amylase at 100°C, while the simplified method requires autoclaving the samples in water at 130°C. Preliminary studies indicated that autoclaving at 121°C did not solubilize all starch in some legumes. Compositions of dietary fiber polysaccharides (DFP) of the gravimetrically isolated residues of cooked kidney beans and chick peas are shown in Tables 3A and 3B. Hydrolysis of kidney bean residues from gelatinization at 130°C and 121°C with the simplified method resulted in a total DFP content of 22.6% and 38.1%, respectively. The absolute differences could be accounted for in the glucose content: 14.2% vs 30.9%. This indicated that the lower temperature used for gelatinization was not sufficient to hydrolyze all the starch in kidney beans. However, if the beans were pretreated with DMSO for the simplified method at 121°C or the AOAC method at 100°C, the hydrolysis data indicated much lower glucose, similar to that from beans gelatinized at 130°C without DMSO.

Fractionation of the legume fiber into soluble and insoluble components (Lee et al., 1992; Li and Cardozo, 1990) revealed that resistant starch was mainly in the insoluble fraction. For example, in the case of kidney beans, hydrolysis of the insoluble dietary fiber fraction from the AOAC method, yielded a DFP content of 43.6% as compared to 21.6% for residue from sample which had been treated with DMSO. The sum of DFP of the soluble and insoluble fractions from DMSO treated sample (AOAC procedure) also compared favorably with the value obtained by the simplified procedure (24.9% vs. 22.6%).

A similar trend was observed for chick peas where the lower 121°C gelatinization temperature for the simplified method resulted in overall higher total DFP due mainly to incomplete starch removal. However, for that sample, the DMSO treatment seemed to have hydrolyzed more starch (lower glucose value) than the 130°C temperature, resulting in a total DFP of 9.76% versus 13.43% at 130°C without DMSO. The former agreed with that from the DMSO treated residue of the AOAC method. This could indicate that for certain samples, DMSO may not solubilize starch exclusively as has been assumed. Total DFP contents of the soluble fraction with and without DMSO treatment, for both methods, were similar. However, there appeared to be a difference in partitioning of the various sugar components by the simplified method. For kidney beans and chick peas, the TDF and insoluble DF fractions from AOAC method as compared to the simplified method, with or without DMSO, all showed slightly higher arabinose. The same fractions from kidney beans had lower mannose content.

The data suggest that the exceptionally high values for some cooked beans by the AOAC/Tris-Mes method could be explained by incomplete removal of starch at the temperatures used. Starch in most legumes has been difficult to remove by

Table 2—Total dietary fiber by AOAC/Tris-Mes and Simplified Methods in cooked legumes

Legume	Brand		Method A[a]	Method B[b]	Method A	Method B
			g/100 g dry weight		g/100 g wet weight	
Black beans	F		51.4 ± 0.00[c]	24.8 ± 0.07	15.4 ± 0.00	7.46 ± 0.02
	G		49.0 ± 3.90	24.8 ± 0.28	15.7 ± 1.25	7.96 ± 0.09
	O		50.0 ± 0.71	23.6 ± 0.00	15.5 ± 0.22	7.32 ± 0.00
		Mean	50.1 ± 1.18	24.4 ± 0.71	15.5 ± 0.17	7.58 ± 0.34
Chick peas	G		33.1 ± 0.71	16.4 ± 0.14	13.7 ± 0.29	6.79 ± 0.06
	H		30.5 ± 0.21	17.3 ± 0.49	12.5 ± 0.09	7.06 ± 0.20
	P		30.1 ± 2.47	16.9 ± 0.21	13.3 ± 1.09	7.46 ± 0.09
		Mean	31.3 ± 1.60	16.9 ± 0.43	13.1 ± 0.62	7.10 ± 0.34
Great Northern beans	F		48.1 ± 0.57	24.8 ± 1.20	15.9 ± 0.19	8.17 ± 0.39
	G		41.4 ± 1.13	21.1 ± 0.07	14.9 ± 0.41	7.59 ± 0.03
	O		37.9 ± 0.78	23.1 ± 0.49	11.3 ± 0.23	6.89 ± 0.15
		Mean	42.5 ± 5.16	23.0 ± 1.80	14.0 ± 2.39	7.55 ± 0.64
Kidney beans	F		53.1 ± 0.14	28.5 ± 0.21	16.9 ± 0.05	9.11 ± 0.07
	G		58.4 ± 0.40	30.2 ± 0.78	18.7 ± 0.14	9.68 ± 0.25
	O		55.1 ± 0.21	28.8 ± 0.28	17.4 ± 0.07	9.10 ± 0.09
		Mean	55.5 ± 2.67	29.2 ± 0.92	17.7 ± 0.90	9.29 ± 0.32
Large Lima beans	F		27.8 ± 0.07	26.8 ± 0.49	7.77 ± 0.02	7.87 ± 0.14
	G		24.6 ± 0.09	32.1 ± 0.71	6.93 ± 0.03	9.02 ± 0.19
	O		30.1 ± 1.13	28.2 ± 0.42	8.39 ± 0.32	7.87 ± 0.12
		Mean	27.5 ± 2.74	29.0 ± 2.73	7.69 ± 0.73	8.25 ± 0.66
Navy beans	F		47.3 ± 0.85	22.0 ± 0.64	15.4 ± 0.28	7.17 ± 0.21
	G		56.8 ± 0.92	28.2 ± 0.14	18.8 ± 0.30	9.33 ± 0.05
	O		59.6 ± 0.71	27.6 ± 0.49	19.3 ± 0.32	8.96 ± 0.16
		Mean	54.6 ± 6.45	26.0 ± 3.40	17.8 ± 2.15	8.49 ± 1.15
Pink beans	G		45.1 ± 1.27	21.6 ± 0.21	16.0 ± 0.45	7.69 ± 0.08
	H		40.2 ± 0.71	23.6 ± 0.14	13.4 ± 0.24	7.88 ± 0.05
	O		45.4 ± 0.14	21.5 ± 0.35	16.4 ± 0.05	7.80 ± 0.13
		Mean	43.6 ± 2.92	22.3 ± 1.15	15.3 ± 1.63	7.79 ± 0.09
Pinto beans	F		46.3 ± 0.78	22.0 ± 0.28	17.6 ± 0.29	8.34 ± 0.11
	G		32.7 ± 0.92	23.5 ± 0.21	11.5 ± 0.32	8.27 ± 0.07
	O		45.4 ± 0.57	22.0 ± 0.35	15.5 ± 0.19	7.54 ± 0.12
		Mean	41.5 ± 7.59	22.5 ± 0.88	14.9 ± 3.09	8.05 ± 0.44

[a] Method A - AOAC/Tris-Mes Buffer Method
[b] Method B - Simplified Method
[c] Mean value of two replicates ± standard deviation

Table 3—Composition of dietary fiber (DF) fractions of kidney beans (A) and chick peas (B)

Method Fractions	Treatment	Rhamnose	Arabinose	Xylose	Mannose	Glucose	Galactose	Uronic Acids	Total DFP[a]
				g/100 g dry weight					
(A) Kidney beans									
Simplified									
Total DF	130°C	–	4.53	2.06	0.42	14.22	1.00	3.20	22.63
	121°C	–	5.09	1.98	0.52	30.86	1.11	3.19	38.05
	121°C, DMSO	–	5.24	2.03	0.60	13.81	1.17	3.12	22.23
Insoluble DF	121°C	–	2.36	1.25	0.42	31.28	0.55	1.65	33.37
	121°C	–	2.32	1.11	0.50	14.56	0.51	1.61	18.34
Soluble DF	121°C	0.05	2.50	0.68	0.09	0.08	0.63	1.65	4.89
	121°C, DMSO	0.05	2.64	0.83	0.09	0.12	0.64	1.64	5.19
AOAC									
Total DF	100°C	–	5.93	2.08	0.08	33.25	1.20	3.41	40.90
	100°C, DMSO	0.11	5.87	2.08	0.33	8.20	1.17	3.12	18.59
Insoluble DF	100°C	0.09	5.33	1.47	–	39.25	1.00	1.89	43.64
	100°C, DMSO	0.11	4.36	1.18	0.10	15.17	0.68	2.69	21.61
Soluble DF	100°C	0.04	0.81	0.41	0.29	0.06	0.29	0.71	2.32
	100°C, DMSO	0.04	1.60	0.71	0.29	0.16	0.53	0.35	3.27
(B) Chick peas									
Simplified									
Total DF	130°C	0.09	3.52	0.43	–	8.62	0.39	2.04	13.43
	121°C	–	3.90	0.47	0.18	12.64	0.57	2.00	17.59
	121°C, DMSO	0.09	3.81	0.46	0.18	3.98	0.56	1.93	9.76
Insoluble DF	121°C	–	2.17	0.33	0.12	11.94	0.37	1.44	14.56
	121°C, DMSO	0.08	2.34	0.37	0.19	4.63	0.37	1.61	8.53
Soluble DF	121°C	0.04	1.42	0.12	0.04	0.04	0.22	0.55	2.13
	121°C, DMSO	0.02	0.59	0.09	0.05	1.18	0.09	0.38	2.13
AOAC									
Total DF	100°C	–	4.55	0.51	0.10	17.45	0.59	2.21	22.62
	100°C, DMSO	0.08	4.39	0.51	0.33	3.42	0.58	1.98	10.05
Insoluble DF	100°C	0.09	4.38	0.45	–	20.39	0.44	1.39	24.15
	100°C, DMSO	0.15	4.22	0.45	0.17	5.38	0.41	1.56	10.98
Soluble DF	100°C	0.04	0.33	0.03	0.29	0.05	0.18	0.30	1.09
	100°C, DMSO	0.03	0.33	0.03	0.28	0.05	0.18	0.28	1.05

[a] Total DFP = (neutral sugars + uronic acids) × 0.89.

enzyme hydrolysis (Neilson and Marlett, 1983; Marlett et al., 1989). This was attributed to entrapment of legume starch in parenchyma cells which leads to incomplete swelling during cooking (Wursch et al., 1986). It may also be due to the high amylose content of legumes, 30-40% as compared to 25-30% for most other starchy foods (Wolever, 1990). However, during the canning process, legumes are subjected to high temperature treatment, i.e. blanching in water for 5–10 min at 93–99°C and retorting for 40 min at 130°C (Phillips, 1990). This may explain why the starch in canned beans could be readily hydrolyzed even under conditions in the AOAC method. With the simplified method, the 130°C gelatinization temperature resulted in dietary fiber residues with minimum contamination of resistant starch. Pretreatment with DMSO for the AOAC/Tris-Mes method could prevent resistant starch from being included as dietary fiber. Until a general agreement is reached regarding inclusion of resistant starch as dietary fiber and the reliability of DMSO pretreatment to solubilize starches, DMSO should be used only with reservation.

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