6 November-December 1979
Volume 44 : Number 6

Coden: JFDSAZ 44(6) 1573–1822 (1979)
ISSN: 0022–1147

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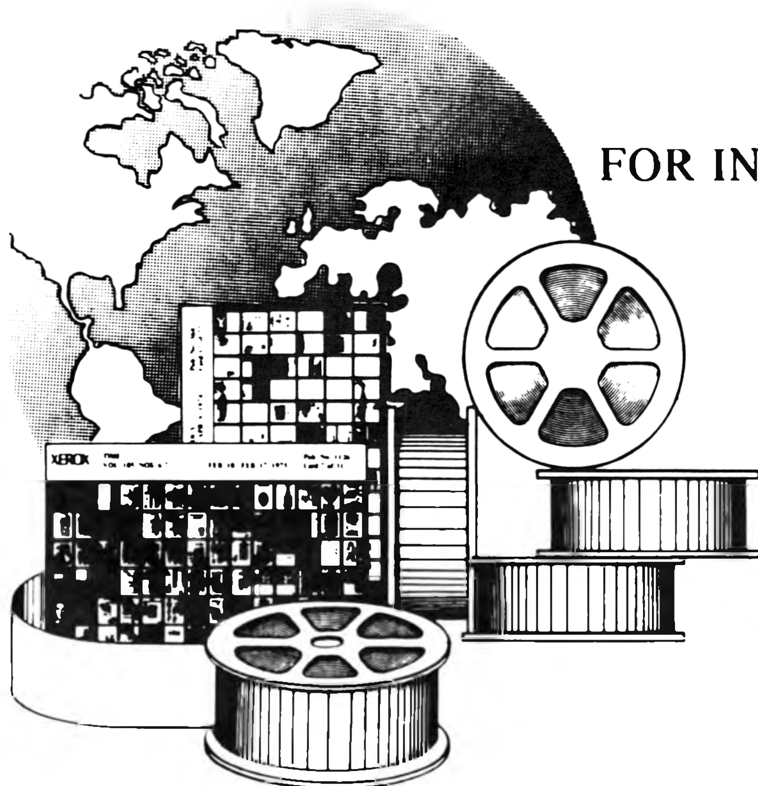

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November-December 1979
Volume 44: Number 6

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Memo *FROM THE SCIENTIFIC EDITOR*

During 1979, 675 manuscripts were submitted for publication in the *Journal of Food Science,* a slight decrease from the number submitted in 1978; 470 papers were published in the six issues in 1979, a total of 1856 pages.

In general, the review process is working well and I especially appreciate the attitude of reviewers when the large volume of manuscripts arrives following the Annual Meeting. Publication time remains at about nine months. Many delays are due to slow revision by the authors; the amount of time needed for typesetting and printing also contributes to the delay.

If readers have concerns about published manuscripts, the best approach is a Letter to the Editor which along with a response from the authors will be published in a later issue of the Journal.

Again I need to recognize the excellent support for the Journal from Anna May Schenck, John Klis, Barney Schukraft, and Gladys Anderson. They are responsible for the final form and rapid publication schedule for the Journal.

The Editorial Board has been busy, as usual, in monitoring the quality of the Journal. Again I want to express my thanks to the retiring Board members and welcome the new Board members starting three-year terms.

Editorial Board Changes for 1979

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ESTIMATION OF OXIDATIVE DETERIORATION OF OILS AND FOODS BY MEASUREMENT OF ULTRAWEAK CHEMILUMINESCENCE

R. USUKI, T. KANEDA, A. YAMAGISHI, C. TAKYU and H. INABA

ABSTRACT

The present study was undertaken to evaluate the oxidative deterioration of oils and foods by using the single photoelectron counting system, which was designed for the measurement of ultraweak chemiluminescence. Thermally oxidized soybean oils were prepared and their chemical characteristics and chemiluminescence were measured. The results showed that the increase of emission intensity was closely correlated to the oxidative deterioration of oils, instant Chinese noodles and milk powder. This new method has a great advantage in measuring the quality of food in a short period of time by a nonexpert without any prior treatment.

INTRODUCTION

IN THE FIELD of food analysis and food technology, one might expect that the quality of various foods could be easily estimated in a short time. One group of authors have been investigating the measurement of ultraweak chemiluminescence (CL) for more than a decade, and have designed the novel optical electronic system, the single photoelectron counting system, which detects the ultraweak CL at about 10^{-15} W or less and analyzes its emission spectrum (Inaba et al., 1969, 1975; Shimizu et al., 1973). Since then we have devised a new analytical method to evaluate the quality of food by the measurement of ultraweak CL, which radiates from food, mainly when in its deteriorated state (Usuki et al., 1978a).

As it is commonly known, the oxidation of fats, oils and foods is a very important problem, especially with regard to food quality, flavor and hygiene. It has been shown that the oxidation of organic materials is accompanied by the emission of weak CL (Vassil'ev and Vichutinskii, 1962; Gundermann, 1965). But it was very difficult to detect this emission quantitatively before the development of photon counting technique. As a result of the measurement of CL from several oxidized oils and some deteriorated foods, we found the availability of CL for food evaluation and quality control as described in the following.

EXPERIMENTAL

Apparatus

Two systems were designed and constructed, one for practical evaluation through the detection of CL (Yamagishi et al., 1978), and the other for its spectral analysis (Inaba et al., 1969; 1975). Figure 1 shows a schematic diagram of the sample container and the sophisticated photon counting system manufactured by Tohoku Electronic Industries Co. Ltd. for field work.

Two quartz cells (50 × 10 × 43 mm) are put in an oven to compare quantitatively the emission intensity from the two samples. Usually one cell is the sample to be tested and the other is a standard sample used as a reference. The weak CL from the two samples

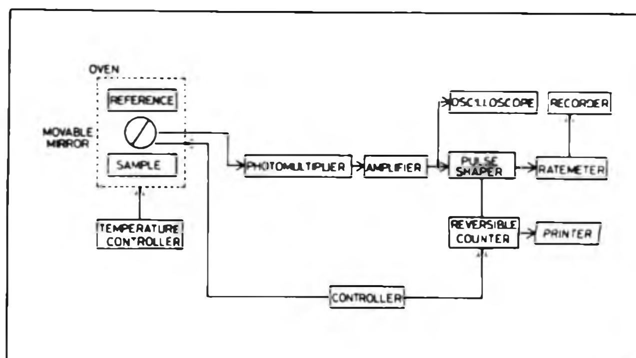

Fig. 1—Schematic diagram of the single photon counting system for the measurement of ultraweak chemiluminescence.

are alternately detected through a rotatable mirror placed between the two cells by a cooled photomultiplier. The output pulses of the photomultiplier, the number of which corresponds to the emission intensity, are processed by several electronic circuits and then measured by an electronic container followed by a digital printer or a recorder for the display.

Determination of chemical characteristics

Acid value (AV), peroxide value (POV), iodine value (IV), and conjugated fatty acid content were estimated by the Japan Oil Chemist's Society's Standard Method for Analysis of Fats and Oils. Carbonyl value (COV) was determined by Kumazawa and Ōyama's method (1965) and TBA value by the modified method of Asakawa et al. (1975). Optical density at 440 nm was used to designate the brown color of the sample.

RESULTS & DISCUSSIONS

Measurement of chemiluminescence

The emission intensity is affected by the temperature of the cell oven, with or without bubbling oxygen during the measurement. Figure 2 shows the change of emission intensity of commercial salad oil with time variation of the sample temperature. The emission intensity was found to in-

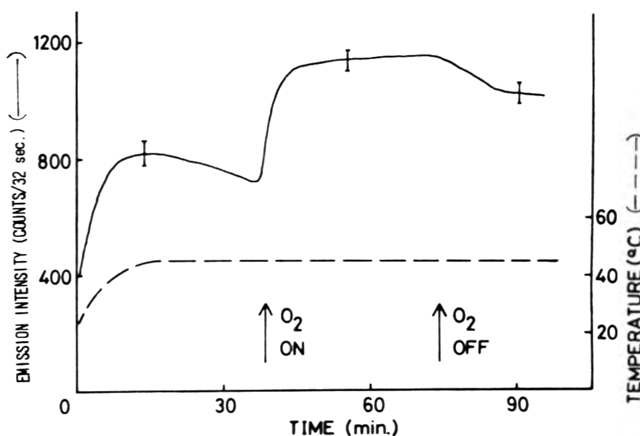

Fig. 2—Changes of emission intensity of commercial salad oil with or without bubbling oxygen at 45° C.

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0022-1147/79/0006-1573$02.25/0
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Table 1—Chemiluminescence and chemical characteristics of thermally oxidized soybean oils with different heating times

Heating time at 180°C (hr)	AV	POV	COV	IV	Conjugated unsaturated fatty acid (%) Diene	Conjugated unsaturated fatty acid (%) Triene	Brown color[a]	Emission intensity (counts/32 sec)
0	0.046	0.9	3	129	0.34	0.055	0.056	728
1	0.048	3.6	7	128	0.53	0.080	0.096	655
2	0.049	5.4	16	128	0.68	0.146	0.122	667
4	0.073	5.1	38	126	1.55	0.148	0.175	719
6	0.094	5.7	37	125	1.35	0.147	0.157	991
8	0.129	6.3	70	122	1.54	0.156	0.247	1361
14	0.309	8.1	87	120	1.90	0.128	0.404	2464
24	1.85	8.7	302	103	3.78	0.125	1.99	12193
32	2.01	9.6	304	96	4.45	0.116	4.41	22193

[a] Brown color was represented by the optical density of oils at 440 nm.

crease with the temperature up to a maximum value, then decrease gradually with the exhaustion of oxygen from the sample. When oxygen was bubbled into the sample after this period, as shown with a vertical arrow, the intensity increased again and reached a constant as seen in the figure. This constant emission intensity was independent of the amount of initial oxygen retained in the sample. Accordingly, the experiment thereafter was carried out with bubbling oxygen.

Table 2—Changes of POV and COV of sample oils during the measurement of chemiluminescence

Heating time at 180°C (hr)	POV Before measurement	POV After measurement Without O_2	POV After measurement With O_2	COV Before measurement	COV After measurement Without O_2	COV After measurement With O_2
0	0.91	1.41	1.54	3.3	3.1	3.4
1	3.6	4.4	4.4	7.7	7.0	8.7
2	5.4	6.2	4.5	15.8	15.3	14.1
6	6.4	5.8	5.7	37.4	36.0	35.0
14	8.1	7.9	7.8	87.0	84.6	80.1
24	8.7	—	8.3	302.0	—	314.3
32	9.5	—	8.6	304.3	—	296.2

Chemiluminescence of thermally oxidized soybean oil

2.6 kg additive-free soybean oil put in a 5-L round bottom flask was heated at 180°C with blowing air, and at intervals of several hours about 100g of the heated oils were taken out for the measurement of CL and chemical characteristics. The results are shown in Table 1.

Emission intensity was rather low in oil heated for a short time, because the emitting species were exhausted by bubbling with heating. After heating for 6 hr, emission intensity increased gradually, and finally, in oil heated for 32 hr, which seemed to be highly oxidized according to its chemical characteristics, it reached about 30 times that of nonheated oil. Thus, in the case of thermally oxidized oil, CL may increase with heating time at least in the edible range.

Some chemical characteristics and CL are illustrated graphically in Figure 3. The increase of emission intensity was closely correlated to the decrease of iodine value and secondly to the increase of AV and COV. From these results we noticed the correlation between the CL and thermally oxidative deterioration of oils. Also a similar relationship was observed in the case of thin film heating such as pan-frying of cabbage with soybean oil.

Changes of oils by oxygen during the measurement

In order to investigate the effect of oxygen on the sam-

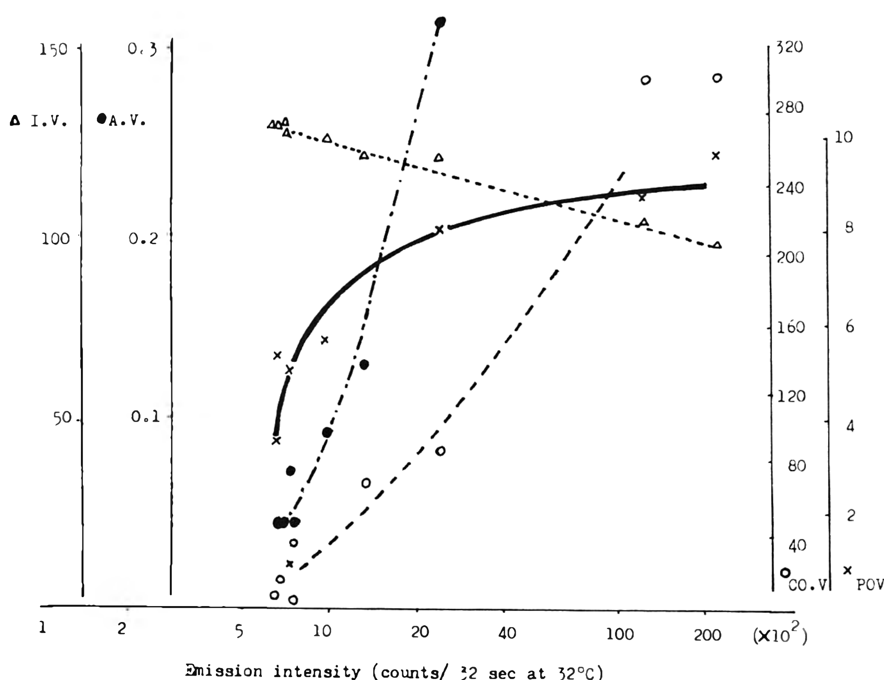

Fig. 3—Relationship between the chemiluminescence and the chemical characteristics of thermally oxidized soybean oils heated at 180°C for different heating time.

Table 3—Chemiluminescence and chemical characteristics of potato fried soybean oils with different frying times

Frying time at 180°C (hr)	AV	COV	IV	Conjugated unsaturated fatty acid (%)		Brown color	Emission intensity[a] (counts/30 sec)
				Diene	Triene		
0	0.063	0.6	130	0.53	0.020	0.033	30
1	0.078	12	130	0.71	0.025	0.128	200
2	0.078	15	130	0.75	0.026	0.167	250
3	0.099	17	129	0.77	0.026	0.198	370
4	0.122	23	128	1.02	0.034	0.253	370
5	0.128	25	128	1.06	0.034	0.313	420
6	0.136	26	128	1.11	0.042	0.414	680
7	0.151	25	127	1.19	0.042	0.410	720
8	0.159	31	127	1.29	0.043	0.425	1370

[a] Emission counts were measured without bubbling of oxygen.

ple oils. POV and COV were determined before and after the measurement of CL (Table 2). The result showed that any effect of oxygen on oils was not observed during the measurement of CL.

Chemiluminescence of potato frying oil

10 kg of sliced potatoes were fried at 180°C in 2 kg of soybean oil for 8 hr. As shown in Table 3, the CL increased with the oxidative deterioration of the frying oil. However, the deterioration of this frying oil was not severe like the heated oil described above.

Several kinds of frying oil were collected from food manufacturers, and the different emission intensities were observed (Usuki et al., 1978b). These results indicate that, in the case of frying, using different kinds of oil, by different heating procedures, the deteriorated frying oils reveal different emission intensity and so it is difficult to compare them quantitatively. But we believe this method of measuring CL can be adapted adequately for the quality control of oils, in the case of the same frying oil using the following process. That is, the same frying oil, heated at the same temperature in the same container.

Chemiluminescence of autoxidized soybean oil

2 kg of soybean oil were left by a window of our laboratory, and at intervals of several days about 50g autoxidized oils were taken out for the measurement of the CL, AV and POV (Table 4). Emission intensity of autoxidized soybean oil increased with the progress of oxidation. After irregular increase, emission intensity decreased conversely in the oil with maximum POV (1065). Therefore, the measurement of CL in autoxidized oil can be adapted to estimate the slightly autoxidized stage of oils.

The emissions from autoxidized lipids have been detected in methyl oleate (Vassil'ev and Vichutinskii, 1962), n-butyl oleate (Höfert, 1964), sodium linoleate (Mizuno et al., 1974), sodium linoleate-lipoxigenase (Inaba et al., 1975; Nakano and Sugioka, 1977), and linoleic and docosahexenoic acid film (Slawson and Adamson, 1976; Adamson and Slawson, 1976.), but no quantitative measurements were carried out. We believe that this is the first report which has made clear the relationship between the oxidative stage and emission intensity.

Applications to some deteriorated foods

To apply this method to foods, instant Chinese noodles, a most popular instant food among Japanese, were measured (Table 5). Crushed noodles were used for the measurement of CL, while POV and AV were determined in oils obtained by the extraction with ethyl ether from noodles.

In the case of the samples which were left in the laboratory, the increase of emission intensity was found to correspond to POV and AV, and in irradiated samples, emission intensity corresponded to the irradiation period of the sterilamp, while the changes of POV and AV were not observed.

Another application was carried out on modified milk powder for infants, packaged with nitrogen gas. As shown in Table 6, the irradiation of the sterilamp caused the increase of TBA value and POV, which would be attributable to the oxidative deterioration of milk lipids. At the same

Table 4—Chemiluminescence of autoxidized soybean oils

Period of autoxidation[a] (days)	POV	AV	Emission intensity (counts/32 sec)
0	0.86	0.021	2200
7	16.6	0.025	6400
9	25.9	0.037	5700
20	48.7	0.043	5400
31	64.9	0.055	8000
42	108.6	–	12000
148	1065	4.019	1300

[a] At room temperature

Table 5—Chemiluminescence of autoxidized instant Chinese noodles

Sample	POV	AV	Emission intensity (counts/32 sec)
Left in lab for 70 days	14.6	0.16	2200
Left at windowside for 43 days	43.3	0.22	4200
Irradiation of sterilamp for			
0 min	9.8	0.14	2200
30	10.9	0.15	2200
60	9.7	0.16	2350
90	9.4	0.15	2500
120	9.6	0.18	3100
180	9.4	0.15	4000
240	8.8	0.14	4600

Table 6—Chemiluminescence of irradiated milk powder

Sample	TBA value	POV[a]	Odor[b]	Emission intensity (counts/32 sec)
No irradiation	0.25	2.1	Not offensive	750
Irradiation for				
1 hr	0.30	–	Not offensive	3000
4	0.32	5.0	Stimulous: x, Sulphureous: +~++	4300
8	0.34	5.8	Stimulous: x, Sulphureous: +~++	4800
24	0.51	–	Stimulous: +, Sulphureous: ++	5400
36	0.49	7.8	Stimulous: +, Sulphureous: ++	4400
60	0.51	8.4	Stimulous: ++, Sulphureous: ++	5000
10 min	0.36	2.6	Not offensive	2800
30	0.38	2.4	Not offensive	3500

[a] Only POV were determined on the extracted oils from milk powder.

[b] Using a profile sheet, intensity of odor was designated on an intensity scale of five as follows: 0: undetectable; x: very slightly; +: slightly; ++: moderate: +++: strong.

Table 7—Effect of oxygen on emission counts of heated oils during the measurement of chemiluminescence

Heating time at 180°C (hr)	Emission intensity (counts/32 sec)		
	With bubbling oxygen	Without bubbling oxygen	Difference
0	728	525	203
1	655	606	49
2	667	566	101
4	719	582	137
6	991	801	180
8	1361	903	458
14	2464	1215	1249
24	12329	3538	8791
32	22193	4437	17756

time, the increase of emission intensity was observed during the irradiation of the sterilamp only for a short time (10 min.).

CL emits only from the surface of foods. So by measuring CL, we can detect the slight changes of food quality even in a small amount, which cannot be determined by any other chemical method.

Mendenhall (1977) measured the CL of breakfast cereals and soda crackers by accelerated aging tests with heat and also with light, and pointed out the possibility of estimating these food qualities by comparing the emission patterns. Although our measurements were carried out at a constant temperature, other information could be obtained by the same measurement at elevated temperatures or with the addition of a luminescence sensitizer.

In other experiments, we detected different emission intensity in each of the foods and oils we tested. In some cases, emission intensity conversely decreased during the deterioration of foods. Thus at the present stage of this study it is not clear what is being measured. The conditions for measurement of CL should be selected carefully if this method is used in any other application.

Emitting species in deteriorated oils

It is very interesting to clarify what kinds of substance would be responsible for CL. Many researchers have made various conjectures concerning emitting species; for example, excited ketone (Nathan et al., 1975; Adamson and Slawson, 1976), singlet oxygen (Kasha and Khan, 1970; Arneson, 1970; Nakano et al., 1975, 1976; Adamson and Slawson, 1976; Foote, 1976), and superoxide anion (Arneson, 1970). We suppose there are several emitting species in deteriorated oils.

The results shown in Table 7 seem to be suggestive for an elucidation of this problem. It shows the different emission intensity of thermally oxidized soybean oils during the measurement of CL with or without bubbling oxygen. Large differences of emission intensity were observed in the oils heated for 24 and 32 hr. Furthermore, as mentioned previously, we noticed that any other change of POV and COV was not observed in the oils when the CL was measured with or without oxygen. These results may suggest that the bubbling oxygen would be converted to activated oxygen as it is an emitting species, such as singlet oxygen, superoxide anion, hydroxyl radical, and hydroperoxy radical.

A filter spectral analyzer was used for testing ultraweak CL of oxidized oils as a second system of our measuring apparatus. We have confirmed that there were different spectral characteristics between the autoxidized and thermally oxidized soybean oils. Moreover, the addition of β-carotene, known as a singlet oxygen quencher (Foote and Denny, 1968), caused the disappearance of 370 nm and 470 nm in the peaks of the emission spectra in autoxidized oil (Yamagishi et al., 1978). Although there seemed to be various emitting species in thermally oxidized oil, singlet oxygen would be responsible in part for CL emission in autoxidized oil. Detailed elucidation of the emission spectra in connection with emitting species will be reported later.

In conclusion, this method has a great advantage in measuring the quality of food in a short period of time by a nonexpert, without prior treatment. Accordingly, we expect to use this method in other foods, food additives, and antioxidants.

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Ms received 12/21/78; revised 4/28/79; accepted 5/5/79.

The authors are grateful for the collaboration of Dr. A. Saeki, president of Tohoku Electronic Industries Co. Ltd., throughout this work.

SCREENING OF HIGH β-GALACTOSIDASE-PRODUCING FUNGI AND CHARACTERIZING THE HYDROLYSIS PROPERTIES OF A SELECTED STRAIN

G. M. PASTORE and Y. K. PARK

ABSTRACT

Extracellular β-galactosidase-producing fungi were screened from soil, and one strain of Scopulariopsis was selected for further study because of high productivity of the enzyme. The strain of Scopulariopsis accumulated large amounts of the enzyme extracellularly on wheat bran medium (Koji process) during incubation. The enzyme was produced constitutively, and actively hydrolyzed lactose in acid whey at 55–60°C. Thus, half-hydrolysis of lactose occurred after 3 hr of incubation when the enzymatic activity was 250 units per 100 ml of acid whey (pH 4.5) at 55°C. After 50 hr of incubation, approximately, 95% of lactose was hydrolyzed and paper chromatograms indicated no detectable lactose in whey. Scopulariopsis lactase is highly thermostable and very active at acid pH as compared to yeast lactase.

INTRODUCTION

THE USE OF lactase (β-galactosidase, β-D-galactoside galactohydrolase E.C. 3.2.1.23) in the dairy industry is one of the most promising applications of enzymes to food processing (Pomeranz et al., 1962; Pomeranz, 1964; Wendorff et al., 1971).

The lactose fermenting yeasts such as *Saccharomyces fragilis, Zygosaccharomyces lactis,* or *Candida pseudotropicalis* are the most obvious sources of the enzyme and have commonly been used for its preparation (Young and Healy, 1957; Myers and Stimpson, 1956). Wierzbicki and Kosikowski (1973a,b), examined 23 species of molds, yeasts and bacteria for their lactase potential and found the largest amount of lactase in the molds to be produced by *Neurospora crassa* ATCC 18419. *Saccharomyces fragilis* C_{17} produced the most lactase of the four yeasts examined, and the bacterial strain *Lactobacillus helveticus* C_{11} was best. Sorensen and Crisan (1974) also screened thermophilic fungi from soil, and examined for thermostable lactase. Lactases from the microorganisms mentioned are located in the cells. Some microorganisms liberate lactase extracellularly during growth in culture medium. Lactases from *Aspergillus foetidus* (Borglum and Sternberg, 1972) and *Aspergillus oryzae* (Park et al., 1979) are extracellular enzymes which are used commercially for food processing.

The object of this study was to isolate extracellular β-galactosidase-producing fungi from soil and to examine the enzyme preparation from a selected strain of *Scopulariopsis sp.*, to elucidate enzymatic properties.

MATERIALS & METHODS

Isolation of microorganisms.

One gram of soil sample was added to 10 ml of sterilized water in a test tube and the supernatant liquid was streaked onto a potato dextrose agar plate with a wire loop. After incubation at 30°C for 3–5 days, well isolated colonies were transferred to potato dextrose agar slants.

Methods for selection of strains capable of producing extracellular β-galactosidase. A spore suspension was prepared by adding 10 ml of sterilized water to slant culture and the surface gently rubbed with a sterilized wire loop to give suspension of spores. One ml of the spore suspension from each strain was used as the inoculum for 20g of sterilized wheat bran medium (mixture of one part of wheat bran and one part of water) in 500-ml Erlenmeyer flasks. Incubation was at 30°C for 5 days. After incubation, the wheat bran was mixed with 200 ml of deionized water and agitated for 1 hr at approximate room temperature. The mixture was filtered and the filtrate was examined for enzymatic activity.

Preparation of enzyme concentrate

Enzymes in the filtrate were concentrated by precipitation with ethyl alcohol (70% v/v) at 4°C. The precipitate was freeze dried.

Assay of β-galactosidase activity

The enzymatic activity was measured with the substrate, o-nitrophenyl-β-D-galactopyranoside (ONPG) in sodium acetate buffer as described by Park et al. (1979). Lactose hydrolyzing activity was also determined by adding 4 ml of 1% lactose in 0.1M sodium acetate buffer (pH 5.0) to 1 ml of an appropriate dilution of an enzyme sample to be tested. The mixture was incubated at 60°C for 15 min. After incubation, the amounts of glucose liberated during hydrolysis were measured by a glucose oxidase-peroxidase-chromogen procedure as described by Dahlquist (1964).

Preparation of whey

Whey was prepared by adjusting pH of commercial cow's milk to 4.5 with hydrochloric acid and coagulated casein was removed by filtration. The lactose concentrations in whey were determined by the method described in AOAC (1965).

Paper chromatography

Paper chromatograms of lactose hydrolysates in whey were developed by the ascending technique at room temperature using Whatman Chromatography Paper 1 (18 × 44 cm). The following solvent systems were used: n-butanol:pyridine:water (6:4:3). The sugars were detected by spraying with Aniline phthalate reagent (Merck).

Table 1—Extracellular lactase activity of screened fungi. Lactase activities were examined at pH 5.0 and 6.5.

Microorganisms	Extracellular lactase activity at pH 5.0 μmole/min/ml	Extracellular lactase activity at pH 6.5 μmole/min/ml
Scopulariopsis sp.	1.40	0.42
Spicaria sp. 1	1.13	0.11
Spicaria sp. 2	1.22	0.18
Aspergillus niger 1	0.41	0.17
Aspergillus niger 2	0.25	0.10

RESULTS

Isolation and screening

1067 strains of fungi were isolated from soil by the plate culture method using potato dextrose agar and examined for extracellular lactase. As shown in Table 1, five strains were found to demonstrate high enzyme activity at pH 5.0, and one strain, Scopulariopsis, was selected for further study because of highest productivity of the enzyme.

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0022-1147/79/0006-1577$02.25/0
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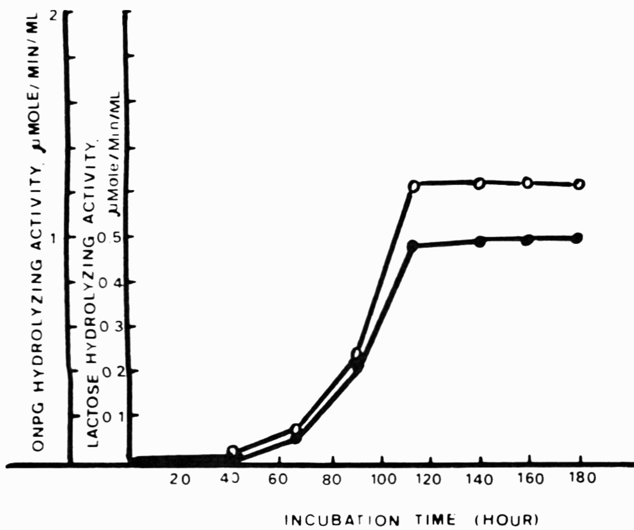

Fig. 1—Time course of the lactase production in wheat bran culture by Scopulariopsis sp. ○——○ *ONPG hydrolyzing activity;* ●——● *Lactose hydrolyzing activity.*

Production of the enzyme

Enzyme production of a wheat bran culture of Scopulariopsis as a function of time is illustrated in Figure 1. Maximum enzymatic activity is reached after 110 hr of incubation, and productivity of the enzyme appears not to be altered by the addition of 1% of lactose (as inducer) to wheat bran medium. Therefore, the extracellular lactase from Scopulariopsis is produced constitutively in wheat bran culture. Activity of 1 mg of the enzyme concentrate is 1 unit.

Characteristics of the enzyme

Maximum hydrolysis of lactose by the enzyme occurred at pH 4 and 5, and for ONPG, was at pH 3,6 to 5, as shown in Figure 2. Figure 3 illustrates the effect of temperature on the enzymatic activity. Optimum temperature for hydrolysis of lactose was 50–65°C and maximum hydrolysis of ONPG was 65°C.

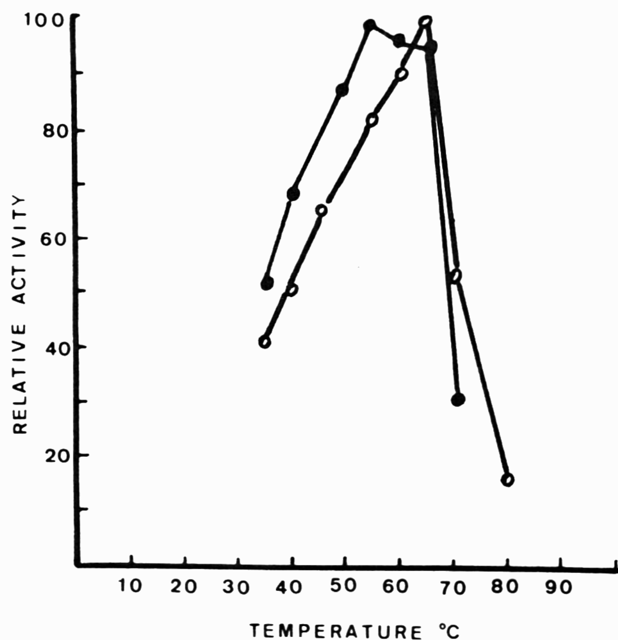

Fig. 3—Effect of temperature on the hydrolysis of ONPG and lactose: ○——○ *ONPG;* ●——● *Lactose.*

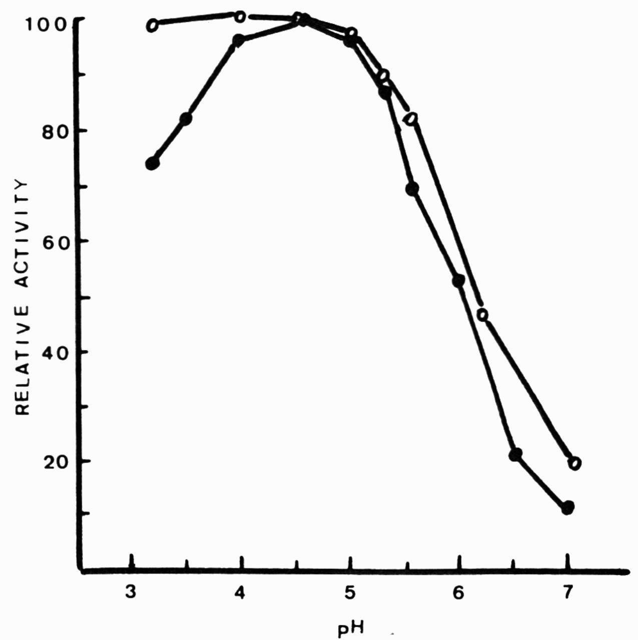

Fig. 2—Influence of pH on the hydrolysis of ONPG and lactose: ○——○ *ONPG;* ●——● *Lactose.*

Hydrolysis of lactose in whey

A mixture of 100 ml of whey containing 5.26g of lactose and 100 units of the enzyme material was incubated at various temperatures. Periodically, samples were taken and the amounts of glucose liberated were measured by a glucose oxidase-peroxidase-chromogen procedure. The results are shown in Figure 4. Time function of the enzymatic hydrolysis was approximately 87% at 55°C after 50 hr of incubation. At 60°C, lactose was actively hydrolyzed during the initial phase of incubation although final hydrolysis (approx 77%) was found to be lower than 55°C incubation trials. Thus, half-hydrolysis of lactose in whey was reached after 7 hr of incubation at 60°C. Effect of the enzyme concentration on hydrolysis of lactose in whey was

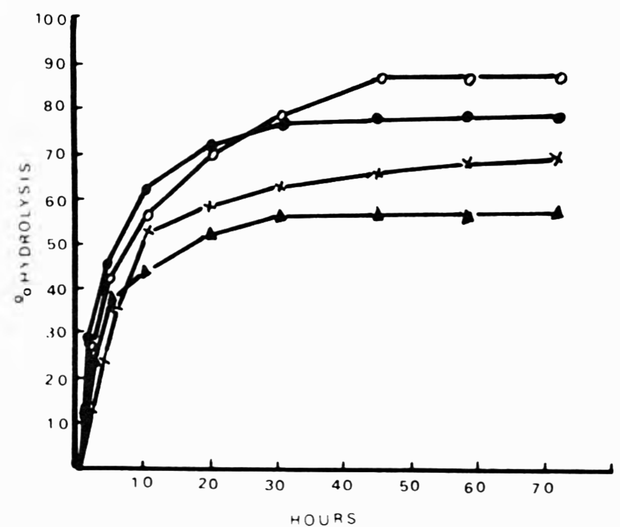

Fig. 4—Effect of temperature on the hydrolysis of lactose in whey: ×——× *50°C;* ○——○ *55°C;* ●——● *60°C;* ▲——▲ *65°C.*

Table 2—Comparison of Scopulariopsis lactase with commercial enzymes

Microorganism	Optimum pH	Optimum temp (°C)	Location of enzyme	References
Aspergillus oryzae	4–5	55–60	Extracellular	Park et al. (1979)
Aspergillus foetidus	3.5–4.0	66–67	Extracellular	Borglum & Sternberg (1972)
Aspergillus niger	3.5–4.5	55	Not reported	Wallerstein Co. (1974) Wierzbicki & Kosikowski (1973b)
Kluyveromyces fragilis (S. fragilis)	6.3–6.5	35–45	Cell bound	Wierzbicki & Kosikowski (1973a)
Scopulariopsis	3.6–5.0	50–65	Extracellular	

examined by incubating a mixture of 100 ml of whey and various amounts of the enzyme preparation (90, 150, 200, and 250 units, respectively) at 55°C for 50 hr and the amounts of glucose were determined. As shown in Figure 5, the rate of enzyme-catalyzed reaction depends directly on the concentration of the enzyme, as is true for any catalyst. Half-hydrolysis of lactose occurred after 3 hr of incubation when the enzymatic activity was 250 units. After 50 hr of incubation, approximately, 95% of lactose was hydrolyzed and paper chromatograms of the samples indicated that the enzyme hydrolyzed lactose almost completely to glucose and galactose (Fig. 6).

DISCUSSION

MICROBIAL LACTASES are an important industrial enzyme for food processing. Most microbial lactases, which were investigated previously, are cell bound enzyme (Cohn, 1957; Anema, 1964; Citti et al., 1965; Rohlfing and Crawford, 1966; Wierzbicki and Kosikowski, 1973a; Sorensen and Crisan, 1974). However, Borglum and Sternberg (1972) and Park et al. (1979) had investigated purification and characterization of an extracellular lactase from *Aspergillus foetidus* and *Aspergillus oryzae* respectively. During the course of isolation and selection of microorganisms, it was found that many strains of fungi produce lactase extracellularly on wheat bran culture. Among 5 strains of fungi which were selected from a large group using wheat bran medium, Scopulariopsis appears to be a potential enzyme producer, and the enzyme appears to be the most useful for industrial processing because of ease of producing the enzyme and its excellent hydrolysis property of lactose in whey. Furthermore, there is a possibility to increase yield of the enzyme from Scopulariopsis by artificial mutation process. It is known that suitable microbial mutants sometimes produce many times the yield of enzyme obtainable from the parent culture.

A comparison of Scopulariopsis lactase with existing microbial lactases for dairy processing is shown in Table 2. Scopulariopsis lactase is highly thermostable and very active in an acid pH range as compared to a yeast lactase. This is also true for other fungal lactases. The combination of high temperature and acid pH for optimum activity of lactase is desirable for acid whey processing although low pH is not suitable for treating milk. Therefore, it is apparent that Scopulariopsis lactase is applicable to a cottage cheese operation and does offer a potential solution to a serious problem of acid whey disposal. These experimental conditions also can limit microbial growth during hydrolysis.

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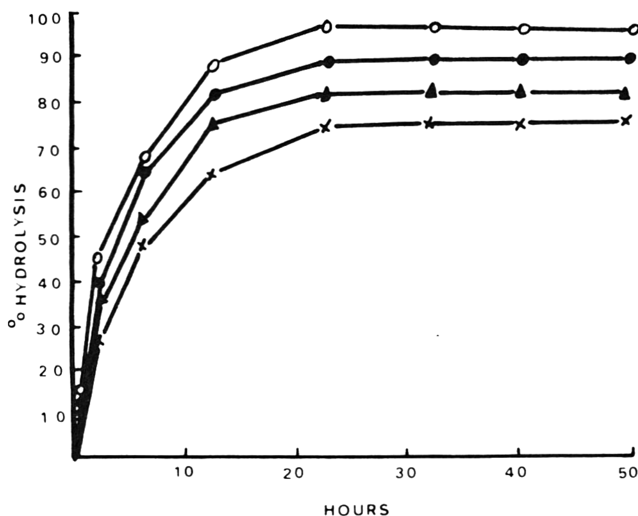

Fig. 5—Effect of the enzyme concentration on the hydrolysis of lactose in whey: x——x 90 units/100 ml of whey; ▲——▲ 150 units/100 ml of whey: ●——● 200 units/100 ml of whey; ○——○ 250 units/100 ml of whey.

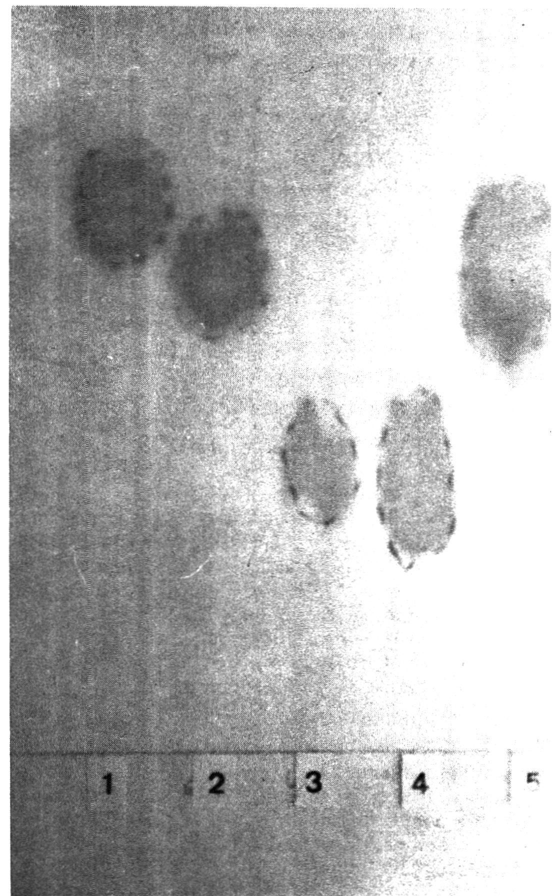

Fig. 6—Paper chromatograms of lactose hydrolysates by Scopulariopsis lactase: (1) glucose; (2) galactose; (3) lactose; (4) whey without enzyme; (5) whey with enzyme.

BARLEY PROTEIN CONCENTRATE FROM HIGH-PROTEIN, HIGH-LYSINE VARIETIES

Y. VICTOR WU, KENNETH R. SEXSON and JAMES E. SANDERSON

ABSTRACT

An alkaline extraction procedure was developed to produce protein concentrate and starch fraction from ground barley. Optimum extraction was at pH 11.2 in 0.045–0.06N sodium hydroxide with a barley-to-solvent ratio of 1:10. After centrifugation, the alkaline extract was adjusted to pH 5.3–5.4 to yield a precipitate and a supernatant. Protein content (nitrogen × 6.25) of the concentrates (dried precipitate) varied between 72 and 84%, and the concentrates accounted for 51–72% of the total barley proteins. The concentrates had from 2.9–5.0g lysine and 2.1–4.2g total sulfur amino acids per 16g of nitrogen. The concentrates were insoluble in water between pH 5 and 6; solubility was 42–47% at pH 2.2 and 39–82% at pH 9.6. All protein concentrates had good hydration capacity (2.7–3.7).

INTRODUCTION

NORMAL BARLEY, like most cereals, is deficient in lysine and is used mainly for brewing and for animal and poultry feed. The recent availability of high-protein, high-lysine barley varieties makes it more attractive as a food. The first high-lysine barley, Hiproly, was selected from 2000 samples of the world barley collection (Munck et al., 1970); it has a high protein as well as a high lysine content. A high-lysine mutant, Risø 1508, was established by treating a normal variety with ethyleneimine (Ingversen et al., 1973).

Earlier work on alkaline extraction of cereal grains to make protein concentrates include oats (Cluskey et al., 1973), wheat (Wu and Sexson, 1975), corn (Wu and Sexson, 1976), triticale (Wu et al., 1976), and sorghum (Wu, 1978). This paper describes an alkaline extraction process for preparing a protein concentrate and starch fraction from ground barley of high protein and high lysine contents. Because food potential of these products depends on both composition and functional properties, we have determined protein, starch, fat, ash and amino acid composition of both barley protein concentrate and by-products, as well as nitrogen solubility, hydration capacity, emulsifying activity and emulsion stability of the concentrate only.

MATERIALS & METHODS

Barley

CI 4362 is a hulless high-protein barley of Ethiopian origin. Hiproly is a hulless high-protein, high-lysine barley. Risø 1508 is a hulled high-lysine barley. Each barley was ground four times in a hammer mill equipped with a screen containing 1/16 in. diameter holes; 76, 76, and 83% of the ground CI 4362, Hiproly and Risø 1508 barleys passed through a 100-mesh screen, respectively. The particle size of ground barley is included here for reference only, and all the ground barley is used for extraction.

Determination of optimum conditions

Ground Hiproly barley was mixed with a number of solvents at a specified weight-to-volume ratio, stirred for 25 min at room temperture and then centrifuged 10 min at 3300 × G. A portion of the supernatant was analyzed for nitrogen in duplicate by micro-Kjeldahl, and a portion of the remaining supernatant was freeze-dried.

An alkaline extract (7 ml) of Hiproly barley (obtained from extraction with solid-to-solvent ratio of 1:10 at pH 11.2) was pipetted into each of eight centrifuge tubes, and hydrochloric acid solution was added dropwise to each tube until pH of 3.5, 4.0, 4.5, 5.1, 5.5, 6.1, 6.6, and 7.1 were attained. The mixture in each tube was stirred for 25 min and then centrifuged at 3300 × G for 10 min. A portion of each supernatant after centrifugation was analyzed for nitrogen, and the amount of protein precipitated at each pH level was calculated.

Fractionation procedure

Ground barley (120g) and 1200 ml of NaOH (0.06N for Hiproly and 0.045N for CI 4362 and Risø 1508) were stirred for 25 min at room temperature, and the slurry pH was adjusted to pH 11.2 by addition of hydrochloric acid or sodium hydroxide as needed (Fig. 1). The slurry was centrifuged at 3300 × G for 15 min, and the supernatant was decanted and adjusted to pH 5.3–5.4 with 6N hydrochloric acid to precipitate almost all the protein. The mixture was centrifuged at 3300 × G for 15 min to separate the precipitate from supernatant; they were freeze-dried separately and designated first precipitate (protein concentrate) and first supernatant.

The alkaline solid from the initial centrifugation was redispersed with 720 ml of water, and sodium hydroxide was added as needed so that the slurry pH was close to 11.2 (Fig. 1). This slurry was stirred for 25 min and passed through 100-mesh bolting cloth to remove bran. The slurry that passed through the cloth was centrifuged at 3300 × G for 15 min to give a starch layer (bottom), a solids layer above starch, and supernatant. The supernatant was adjusted to pH 5.3 by addition of 6N hydrochloric acid to precipitate most of the protein. The mixture was centrifuged at 3300 × G for 15 min to yield a precipitate and a supernatant, and they were freeze-dried separately to give a second precipitate and a second supernatant. The starch was separated from the layer above the starch by removal of the latter with a spatula. The starch, the layer above the starch and the bran were each neutralized with 6N hydrochloric acid and freeze-dried.

Determination of composition

Protein, fat and ash were determined as described by procedure numbers 46-13, 30-26 and 08-16 of AACC Approved Methods (1971), respectively. Moisture was determined by heating samples at 105°C to constant weight in duplicate. Starch was measured by a polarimetric method (Garcia and Wolf, 1972).

For amino acid analysis, each sample was hydrolyzed for 24 hr by refluxing in constant-boiling hydrochloric acid. The hydrolyzed sample was evaporated to dryness, and the residue was dissolved in pH 2.2 citrate buffer. A portion of the acid hydrolyzate was analyzed in a Beckman Spinco Model 121 amino acid analyzer, and the data were computed automatically (Cavins and Friedman, 1968).

Determination of properties of protein concentrate

Nitrogen solubility was measured by mixing 0.1g of protein concentrate with 10 ml of water, and either hydrochloric acid or sodium hydroxide solution was added dropwise to obtain the desired pH values from 2.2–10. The mixture was stirred for 25 min and centrifuged at 1300 × G (or 27,000 × G if needed) for 20 min to separate solid and supernatant satisfactorily. The supernatant was analyzed for nitrogen, and the percentage of soluble nitrogen was calculated at each pH value. Hydration capacity was determined by procedure number 56-20 of AACC Approved Methods (1971). Emulsifying activity and emulsion stability were measured by the method of Yasumatsu et al. (1972) for a simple system, where the concentrate, soybean oil and water were emulsified in a Virtis homogenizer at 10,000 rpm and then centrifuged (for emulsifying activity) or heated and centrifuged (for emulsion stability).

RESULTS & DISCUSSION

Optimum pH of extraction

Various aqueous solutions were used to extract ground

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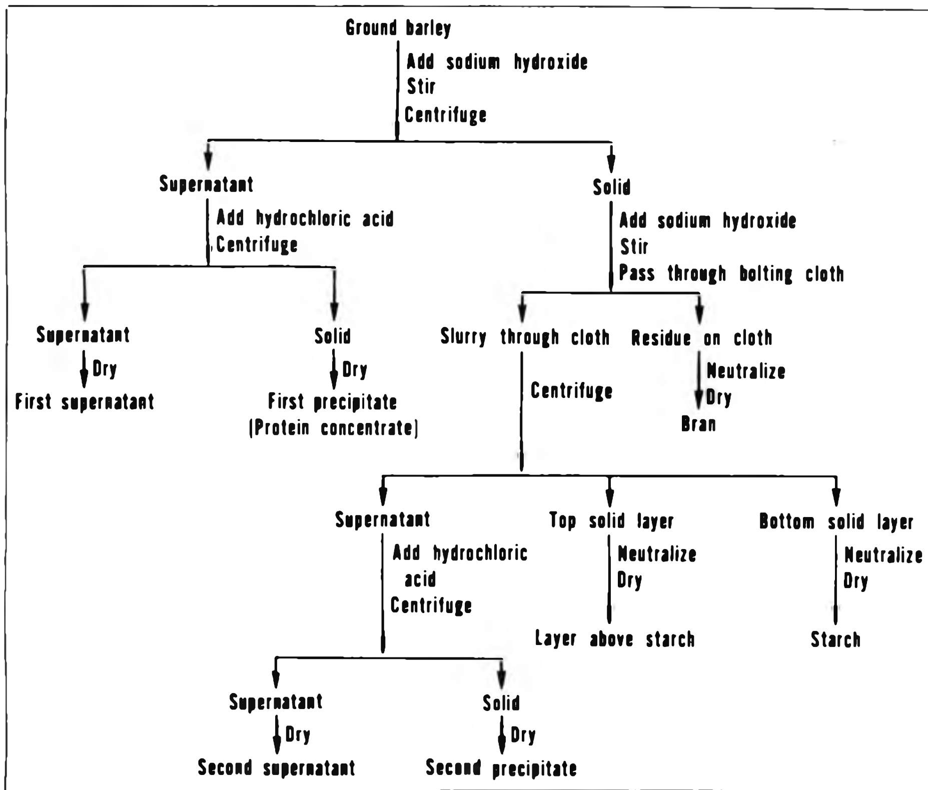

Fig. 1—Schematic diagram for preparing protein concentrate and by-products from ground barley.

Hiproly barley at a solid-to-solvent ratio of 1:6 (Table 1). Water extracted 19% of protein, and hydrochloric acid and acetic acid at different concentrations did not increase the percentage of protein extracted. As the molarity of alkali increased (slurry pH 9.1 to 11.2), the percentage of protein dissolved increased from 34 to 59%. In general, the protein content of the dried extracts (the supernatant from centrifugation, which was then freeze-dried) increased with the percentage of protein extracted. The highest percentage of protein dissolved was 59% at pH 11.2, and this pH value was used subsequently for making protein concentrate.

Optimum solid-to-solvent ratio

Ground Hiproly barley was extracted with sodium hydroxide solutions at various solid-to-solvent ratios from 1:4 to 1:18 (Table 2). Since the percentage of protein dissolved by sodium hydroxide solutions depends on pH of the slurry (Table 1), normality of the sodium hydroxide was adjusted to give the same approximate pH. As the solid-to-solvent ratio increased from 1:6 to 1:18, the percentage of the protein dissolved increased from 46 to 84% at pH 10.6 and from 59 to 83% at pH 11.2. A solid-to-solvent ratio of 1:10 at pH 11.2 seems a good compromise between the highest percentage of protein dissolved and minimum amount of extractant needed (Table 2), and that ratio and pH were used for the first alkaline extraction to make protein concentrate. The optimum concentration range for sodium hydroxide was determined by the desired pH.

Optimum precipitation pH

The effect of precipitation pH on alkaline extract of Hiproly barley was determined at eight pH values between 3.5–7.1 (Table 3). The amount of protein precipitated

Table 1—Extraction of Hiproly barley protein with various solvents[a]

Solvent	pH of slurry	Total protein extracted, %	Protein content of dried extracts, %
0.02N HCl	3.3	16	25
1N HOAc	3.3	16	28
0.2N HOAc	3.6	17	28
0.015N HCl	4.2	18	26
Water	5.0	19	24
0.05N NaOH	9.1	34	45
0.075N NaOH	10.3	45	53
0.09N NaOH	11.0	51	54
0.10N NaOH	11.2	59	48

[a] Solid-to-solvent ratio 1:6. Results expressed on dry basis.

Table 3—Effect of precipitation pH on alkaline extract of Hiproly barley

pH	% N precipitated
7.1	41
6.6	53
6.1	73
5.5	81
5.1	80
4.5	81
4.0	77
3.5	76

Table 2—Influence of solid-to-solvent ratio on extraction of Hiproly barley protein

Solid:solvent ratio	Solvent (sodium hydroxide) normality	Slurry pH	Total protein extracted (%)
1:4	0.1125	10.6	33
1:6	0.075	10.6	46
1:12	0.0375	10.5	65
1:18	0.025	10.5	84
1:6	0.10	11.2	59
1:8	0.075	11.2	73
1:10	0.06	11.2	76
1:12	0.05	11.1	77
1:14	0.043	10.9	76
1:16	0.0375	11.1	82
1:18	0.033	11.0	83

ranged from 41–81%. The maximum amount of protein precipitated was at pH 5.5 and 4.5, and a precipitation pH between 5.3–5.4 was chosen for recovering protein from alkaline extract.

Products from barley

The yield of protein concentrate in Table 4 ranged from 10% for Risϕ 1508 to 16% for CI 4362. The total protein accounted for by the concentrate ranged from 51–72%. The higher yields of concentrate for CI 4362 and Hiproly are due to higher initial protein content of those barleys as well as to higher percentage of total protein as concentrate compared with Risϕ 1508. The yield of first supernatant was comparable to that of concentrate, and as such accounted for 11–22% of total protein. Bran and starch were the two largest fractions by weight, and they ranged from 25–35% and 20–32%, respectively. The yields of first supernatant, second precipitate, second supernatant and layer above starch were similar for the three barleys.

Composition

The protein content of CI 4362, Hiproly, and Risϕ 1508 is 18.3, 21.4, and 14.5%, respectively. Since the protein content of barley will vary with location and year for the same variety, a comparison of protein content of the barley here with earlier reported value is not made. The protein, fat, ash and starch contents of ground barley, protein concentrate and other fractions appear in Table 5. The protein concentrates had 72–84% protein and 7–23% fat compared with 15–21% protein and 2–4% fat for the starting grain. The second precipitates had somewhat lower protein but higher fat contents compared with protein concentrate. The first and second supernatants had 16–26% protein, low fat and high ash. These supernatants contained albumin, globulin, salt, sugar, mineral and other water-soluble materials; their high ash content is partly due to sodium chloride formed by neutralization. The bran fraction was generally similar to the grain in ash and starch contents but had lower protein and fat. The layer above starch had considerably higher starch content than the bran or grain. The starch fraction had little or no protein or fat. In general, fractions high in protein were also high in fat, and the higher fat content of Risϕ 1508 barley compared with the other two varieties resulted in higher fat content for Risϕ fractions.

The essential amino acid compositions of barley, protein concentrate and other fractions from the three barleys were corrected to 100% N recovery and expressed in grams amino acid per 16g nitrogen (Table 6). CI 4362 barley with a normal lysine content of 2.9g per 16g nitrogen is deficient compared with that of the amino acid pattern for human requirement (FAO/WHO, 1973). Hiproly barley had 3.8g lysine per 16g nitrogen, and it represents a significant improvement over normal barley but is still deficient. Risϕ 1508 barley, with a lysine content of 5.4, meets the FAO/WHO pattern.

Amino acid composition of the protein concentrate in general does not differ greatly from that of the barley from which it originates (Table 6). CI 4362 concentrate had higher isoleucine and leucine but lower methionine + cys-

Table 4–Products from CI 4362 (C), Hiproly (H) and Risϕ 1508 (R) barleys[a]

Product	Yield,[b] %			Total protein, %		
	C	H	R	C	H	R
Protein concentrate (first precipitate)	16	15	10	72	59	51
First supernatant	13	15	13	11	16	22
Second precipitate	1	2	2	5	7	6
Second supernatant	2	3	2	2	3	3
Bran	25	32	35	6	10	12
Layer above starch	10	12	10	1	2	3
Starch	32	20	27	0	0	0
Total	99	99	99	97	97	97

[a] Solid-to-solvent ratio was 1:10 for the first extraction and 1:6 for the second extraction. Result expressed on dry basis.
[b] Yield was based on starting weight of barley.

Table 5–Composition of protein concentrate and by-products from barley (% dry basis)[a]

Material	Protein (nitrogen X 6.25)			Fat			Ash			Starch		
	C	H	R	C	H	R	C	H	R	C	H	R
Ground barley	18.3	21.4	14.5	2.1	2.7	4.2	2.2	2.4	3.0	43.2	39.7	54.2
Protein concentrate (first precipitate)	83.6	84.3	72.1	7.3	8.1	22.5	1.9	1.8	2.0			
First supernatant	15.9	23.7	25.6	0.2	0.1	0.1	32.3	17.3	18.2			
Second precipitate	71.5	66.7	41.9	18.7	21.4	49.1	3.4	2.1	1.2			
Second supernatant	15.7	23.4	21.5	0.6	0.5	0.7	33.0	22.4	35.2			
Bran	4.2	6.6	5.1	0.9	1.3	1.8	3.5	2.6	1.0	51.0	48.4	43.6
Layer above starch	2.0	3.8	4.7	0.6	0.6	2.1	2.8	3.0	4.9	79.6	70.4	68.6
Starch	0.0	0.0	0.0	0.1	0.1	0.4	1.7	2.3	1.4	102.0	98.4	105.6

[a] CI 4362 (C) and Hiproly (H) are high-protein barleys, while Hiproly and Risϕ 1508 (R) are high-lysine ones.

Table 6–Essential amino acid composition of protein concentrate and by-products from barley (g/16g nitrogen recovered)[a]

Amino acid	Ground barley			Protein concentrate (first precipitate)			First supernatant			Second precipitate			FAO/WHO (1973)
	C	H	R	C	H	R	C	H	R	C	H	R	
Isoleucine	3.4	4.8	3.6	4.2	4.4	4.4	3.6	3.4	3.1	4.2	4.4	3.8	4.0
Leucine	6.9	8.6	6.8	7.7	7.9	8.1	5.9	5.0	5.0	8.0	8.3	7.3	7.0
Lysine	2.9	3.8	5.4	2.9	4.2	5.0	4.6	5.2	5.6	5.0	3.6	4.7	5.5
Methionine + cystine	3.0	3.0	3.3	2.1	3.0	4.2	4.8	3.8	4.1	3.8	3.0	2.7	3.5
Phenylalanine + tyrosine	10.5	11.0	7.7	11.0	11.2	9.3	6.0	5.0	5.9	9.5	11.7	6.9	6.0
Threonine	3.3	4.0	3.7	3.1	3.3	3.9	4.0	3.5	3.2	3.9	3.6	3.7	4.0
Valine	5.0	6.2	5.3	4.5	5.4	5.9	5.4	5.5	4.5	6.0	5.9	5.1	5.0

[a] CI 4362 (C) and Hiproly (H) are high-protein barleys, while Hiproly and Risϕ 1508 (R) are high-lysine ones.

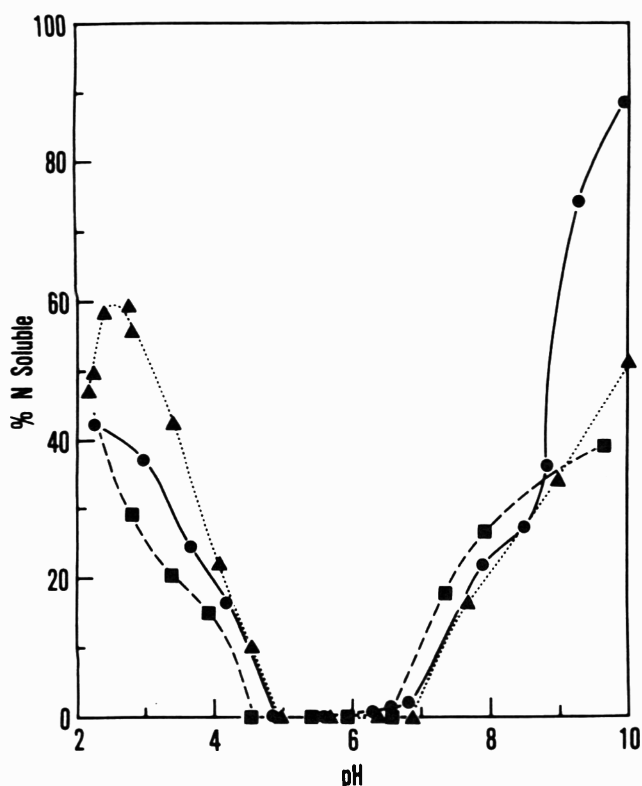

Fig. 2—Nitrogen solubility of barley protein concentrate at various pH values. Protein concentrate (0.1g) was stirred with 10 ml of water to which hydrochloric acid (below pH 5.0) or sodium hydroxide (above pH 5.9) solution was added to arrive at desired pH. ▲ CI 4362, dotted curve; ● Hiproly, solid curve; ■ Risø 1508, dashed curve.

tine, Hiproly concentrate had lower threonine and valine and Risø 1508 concentrate had higher isoleucine, leucine, methionine + cystine and phenylalanine + tyrosine than the respective barley. Risø 1508 concentrate essentially meets or exceeds the human requirement; Hiproly concentrate is somewhat low in lysine, methionine + cystine and threonine and CI 4362 concentrate is deficient in lysine, methionine + cystine and threonine. Each first supernatant has lower leucine and phenylalanine + tyrosine but higher lysine and methionine + cystine compared with the grain. The second precipitate has similar amino acid composition in general compared with protein concentrate.

Nitrogen solubility of protein concentrate

The percentages of nitrogen soluble at pH 2.2–10 for CI 4362, Hiproly and Risø 1508 protein concentrates were plotted in Figure 2. All concentrates were insoluble in aqueous solution between pH 5.0 and 6.5 except Hiproly began to show some solubility at pH 6.3. CI 4362 concentrate was more soluble below pH 5 than the other two concentrates; it was 60% soluble at pH 2.5, but solubility decreased to 47% at pH 2.2. Hiproly concentrate was considerably more soluble above pH 8.8 than the other two concentrates, and it was 89% soluble at pH 9.9.

Hydration capacity, emulsifying activity and emulsion stability

Some functional properties of the three barley protein concentrates were compared with soy isolate in Table 7.

Table 7—Some functional properties of barley protein concentrate compared with soy isolate

Protein concentrate	Hydration capacity	Emulsifying activity, %	Emulsion stability, %
CI 4362	2.7	2	1
Hiproly	3.4	11	6
Risø 1508	3.7	4	4
Soy isolate		45	42

Hydration capacity (weight of sediment per weight of sample) of the protein concentrates ranged from 2.7–3.7. The emulsifying activity and emulsion stability of each protein concentrate were poor compared with the corresponding values of 42 and 45% for soy isolate.

Potential uses of protein concentrate and by-products

Barley protein concentrate may find application in foods as a protein ingredient. The satisfactory hydration capacity suggests possible use as a water-absorbing agent in food. The alkaline solids after the initial protein extraction probably can be neutralized and extruded into breakfast food or convenience foods or used as a starch source for fermentation. Starch fraction can also be produced as shown in Figure 1 with a continuous centrifuge.

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Ms received 3/16/79; revised 6/17/79; accepted 7/1/79.

Presented at the 39th Annual Meeting of the Institute of Food Technologists, St. Louis, MO, June 10—13, 1979.

We thank Dr. E.A. Hockett, Plant and Soil Science Dept., Montana State University, Bozeman, for CI 4362 and Hiproly barleys, Dr. D.M. Wesenberg, Agricultural Research, Science and Education Administration, U.S. Department of Agriculture, Aberdeen, ID, for Risø 1508 barley and N.E. Harrison for technical assistance.

The mention of firm names or trade products does not imply that they are endorsed or recommended by the U.S. Department of Agriculture over other firms or similar products not mentioned.

APPLICATION OF IMMOBILIZED ALKALINE PROTEASE TO CHEESE-MAKING

KUNIO OHMIYA, SHUYA TANIMURA, TAKESHI KOBAYASHI and SHOICHI SHIMIZU

ABSTRACT

An alkaline protease immobilized on Dowex MWA-1 by glutaraldehyde was applied to a step of cheese-making process. By reversing the order of the lactic acid fermentation process and the "rennetting" (hydrolysis) process in the traditional cheese-making method, and by raising pH of milk to 7.0, coagulation of milk in the alkaline protease column was avoided. As a result of these modifications, it became possible to operate a stable hydrolysis process of milk proteins at 50°C. Average degree of hydrolysis was about 3%. Curd was prepared *from* this milk after lowering pH to 6.2 by lactic acid fermentation and was processed to make Cheddar-type cheese. After ripening this cheese for 6 months at 10°C, its ripening ratio was similar to that of cheese prepared by the traditional method. It is concluded that the immobilized alkaline protease could be used for cheese-making.

INTRODUCTION

THE TECHNOLOGY and use of immobilized enzymes have expanded rapidly during the last decade. Since the immobilized enzyme would not remain in the product, it may be possible to substitute a less expensive but more readily available enzyme such as crude microbial protease instead of the traditional milk-clotting enzyme, rennet. Ferrier et al. (1972), Cheryan et al. (1976) and Taylor et al. (1977) tried the possible use of pepsin covalently bound to porous glass or inorganic supports for the milk-clotting. They operated the immobilized pepsin column by passing skim milk acidified with phosphoric acid to pH 5.6–5.9 at low temperature (15°C) to avoid coagulation of milk in the column. Accumulation of white materials on the pepsin-glass, however, reduced flow rate during continuous supply of skim milk. They also reported the rapid inactivation of the immobilized pepsin at the normal pH (6.5) of milk. These disadvantages had to be overcome in attempting to employ immobilized enzyme for cheese-making.

In a previous report (Ohmiya et al., 1978), an alkaline protease with higher activity at more alkaline pH compared with rennet was immobilized on an anion exchange resin by glutaraldehyde. In the present work, the feasibility of using immobilized alkaline protease for cheese-making was examined by modifying the traditional cheese-making method.

MATERIALS & METHODS

Materials

Alkaline protease from *Bacillus subtilis* was kindly donated by Amano Pharmaceutical Co. Rennet was the product of Hansens' Laboratorium. *Streptococcus cremoris* H-61 was given by the National Institute of Animal Industry, Chiba, Japan. Glutaraldehyde

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0022-1147/79/0006-1584$02.25/0
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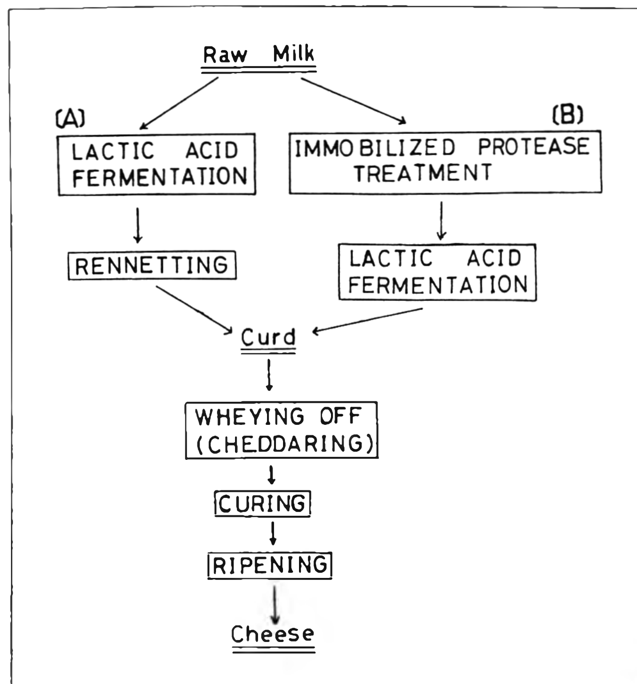

Fig. 1—Traditional (A) and modified (B) processes for making Cheddar cheese.

(25% aqueous solution) was obtained from Ohken Shoji Co. Dowex MWA-1 (20–50 mesh), an anion exchange resin of Dow Chemical Co. Ltd., was used as a carrier. Raw fresh milk was transported from the Nagoya University Farm within a few hours after suction. Hammersten casein was purchased from Merck Co. All the other reagents used in this experiment were of analytical grade.

Immobilization procedure

Immobilizations of the alkaline protease and rennet were carried out according to the method described in a previous report (Ohmiya et al., 1978).

Continuous operation of immobilized enzyme reactor

Immobilized protease (1g) was packed in a jacketed column (1 × 10 cm). Casein solution (0.5% or 3% at pH 7.0) or raw milk (adjusted pH to 7.0 with NaOH) was pumped peristaltically from a flask in a constant temperature water bath at various flow rates upward (fluidized-bed) or downward (packed-bed) through the column. The temperature of the column was kept constant by circulating water through the column jacket from the water bath. To determine stability of the immobilized enzyme during column operation, change of proteolytic activity was measured intermittently at a flow rate of 45 ml/hr (space velocity: 0.29/hr) and various temperatures. Half-lives of the immobilized enzymes were evaluated. For detection of the soluble enzyme bleeding from the reactor, both casein solution and milk passed through the columns were incubated at 37°C for 1 hr. Thereafter, proteolytic activity was determined.

Preparation of cheese

Immobilized alkaline protease cheese (AP-cheese). A schematic diagram to prepare AP-cheese is shown in Figure 1 (Scheme B). For the first step, the hydrolysis procedure was carried out as follows: fresh milk (5L) was pasteurized at 63°C for 30 min and the pH raised to 7.0 with concentrated NaOH solution. The milk was supplied at 50°C and constant flow rate (2 L/hr) upward through the

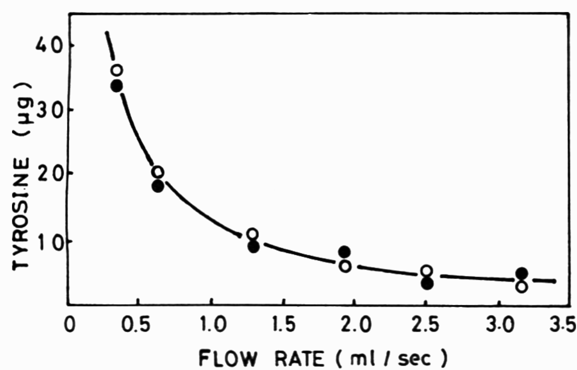

Fig. 2—Effect of flow rate on the proteolytic activity of immobilized alkaline protease using a packed-bed (•) and fluidized-bed (○) column. Casein solution (0.5%, pH 7.0) was used as the substrate at 50°C.

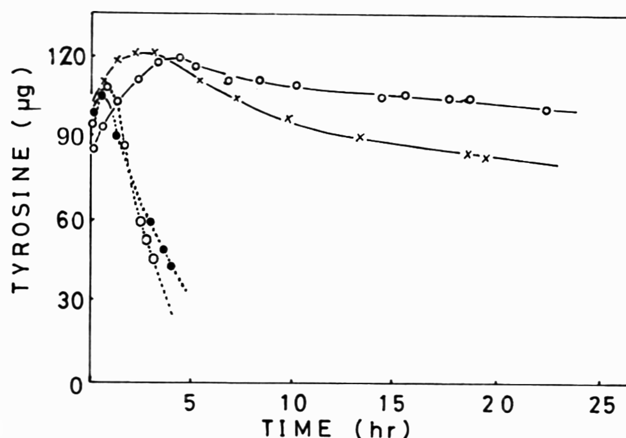

Fig. 3—Changes of proteolytic activity during continuous flow of 0.5% casein solution through an immobilized protease column: alkaline protease at 45°C (—○—) and 60°C (—x—); rennet at 37°C (---•---) and 45°C (---○---).

column (2.4 × 35 cm) in which the immobilized alkaline protease (40g wet weight) was packed. The volume of the fluidized-bed was twice that of the packed-bed at this flow rate. The milk effluent from the column was pooled in a beaker and $CaCl_2$ (final concentration: 0.02%) was added to it. *Streptococcus cremoris*, precultured in skim milk (200 ml), was inoculated in the treated milk as the starter and cultivated at 30°C. After about 2 hr cultivation, milk began to coagulate. Thereafter, cutting, wheying off (Cheddaring), curing and pressing procedures were employed according to the traditional method (Editorial Committee of Dairy Technology Series, 1968a) for making Cheddar cheese. Then the cheese was covered with paraffin and ripened at 10°C for 6 months.

Immobilized rennet cheese (R-cheese). To prepare R-cheese, the same procedure as described above (Fig. 1, Scheme B) was employed by using the immobilized rennet.

Normal Cheddar cheese (N-cheese). To compare the properties of AP- and R-cheeses, N-cheese was prepared according to a traditional method (Fig. 1, Scheme A).

Analytical methods

Determination of milk-clotting activity. Milk-clotting activity of protease was measured as the time (min) at which fine coagulants were recognized in the coagulation test milk (Iwasaki et al., 1967) during the incubation of the milk (50 ml) at 35°C with 5 ml of enzyme solution. The milk used was 10% aqueous solution of dried skim milk at pH 6.2. Clotting activity of the milk passed through the enzyme column was tested after lowering the pH to 6.2 with phosphoric acid.

Determination of proteolytic acitivity. Soon after coagulants of the milk were recognized, an equal volume of 10% trichloroacetic acid (TCA) solution was added to the milk for removal of proteins by filtration. When casein was used as a substrate in the immobilized enzyme column operation, an equal volume of the effluent was added to 10% TCA solution. This mixture was also filtrated. The amounts of tyrosine in the filtrate were determined by the Folin method (Hagiwara et al., 1956).

Determination of sialic acid. Sialic acid released from milk protein was quantitatively determined by the method of Kim et al. (1965).

Cheese analysis. Protein, fat, and moisture contents in the cheese at the initiation of ripening, and the number of viable cells, acidity, and amounts of water-soluble nitrogen in the cheese during ripening were determined according to standard methods (Editorial Committee of Dairy Technology Series, 1968b). Bitter taste of the cheese was determined organoleptically.

Agar gel electrophoresis of cheese proteins was done as follows: purified agar (0.5g) was dissolved in M/15 phosphate buffer (pH 8.5, 15 ml) with polyvinylpyrrolidone (0.25g) and urea (5g) by warming in a boiling water bath for 30 min. Thereafter, 25 ml of the hot agar solution were rapidly spread on the prewarmed glass (10 × 25 cm). After the agar was completely gelatinized, the samples adsorbed to the filter paper (0.3 × 1.5 cm) were placed on the gel. Electrophoresis was carried out for 24 hr by loading 10 mA/10 cm width of agar gel at pH 8.5 (buffer in the electrode chamber was M/15 phosphate buffer, pH 7.0). For the protein staining, the gel was immersed in Amido Black 10B solution (0.1%) for 5 min and then washed in 7% acetic acid solution. The samples for gel electrophoresis were prepared by dissolving 1g of cheese before and after ripening for 5 months in 4 ml of 8M urea solution at pH 8 (Ohmiya and Sato, 1969). Native whole casein, α_s-casein and β-casein prepared by the methods of Hipp et al. (1952) and of Tsugo and Yamauchi (1960) were also dissolved in the urea solution. These casein solutions were applied for the electrophoresis to get standard patterns.

RESULTS

Continuous hydrolysis of casein by immobilized enzyme

Effect of reactor type on the hydrolytic rate. The proteolytic activities of the immobilized alkaline protease were determined in the packed-bed and fluidized-bed reactors at various flow rates of 0.5% casein solution (Fig. 2). As the values obtained from both reactors were very close to each other, a fluidized-bed reactor was mainly used, which is known to be advantageous for keeping excellent heat- and mass-transfer characteristics and for avoiding a pressure drop in the column by plugging. The minimum flow rate in this reactor was 0.3 ml/sec (space velocity: 0.86/hr) when enzyme beads began to fluidize in the column.

Stability of immobilized enzyme during continuous operation. Continuous hydrolysis of casein by immobilized alkaline protease or by immobilized rennet was carried out at a given temperature (Fig. 3). In every trial, the proteolytic activity evidently increased at the beginning of the operation, and thereafter gradually decreased. From these data, half-life values of both immobilized enzymes were estimated on the basis of their maximum activities. Half-life values of the immobilized alkaline protease were 7.8 days at 45°C and 1.6 days at 60°C, whereas those of the immobilized rennet were only 0.13 days at 37°C and 0.10 days at 45°C; half-life of the former was 78 times as long as that of the rennet at 45°C. This is reasonable because the optimum temperature of alkaline protease in the soluble state was higher than that of rennet (Ohmiya et al., 1978). In the effluents from the columns, any significant activity of the protease was not detected.

Effect of whey on the proteolytic activity. The proteolytic activity in the alkaline protease column was monitored at various time intervals by supplying raw milk (pH 7.0) or 3% casein solution (Fig. 4). The values of maximum proteolytic activity and half-life at 60°C in the case of the former were about 60%, and 30% (6 hr) of those in the case of the latter, respectively. These results suggest that something in the milk inhibited the activity and accelerated the inactivation. Therefore, the proteolytic activity of the alkaline pro-

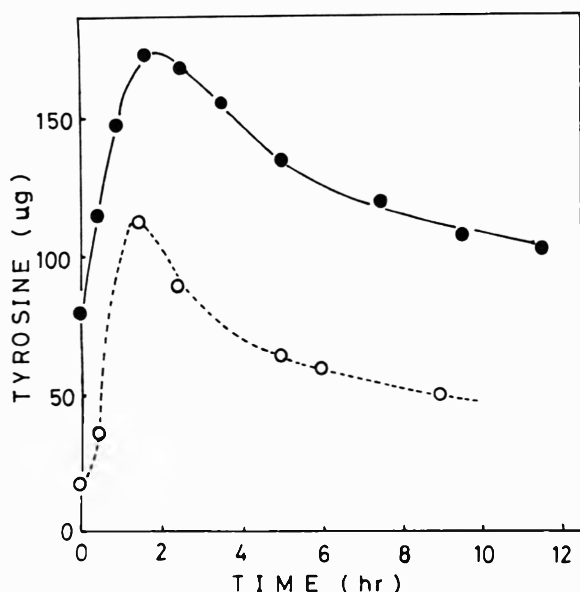

Fig. 4—Activity changes during continuous flow of raw milk (---○---) and 3% casein solution (—●—) through a column packed with immobilized alkaline protease at 60°C, pH 7.0.

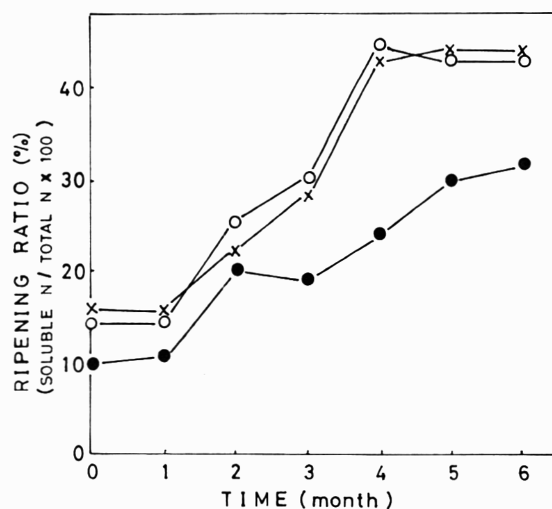

Fig. 5—Change of ripening ratio in N-cheese (○), R-cheese (●) or AP-cheese (x) during its ripening at 10°C for a given period.

Table 1—Composition (%) of cheese prepared by using immobilized protease

Component	Expt no.	N-cheese	R-cheese	AP-cheese
Moisture	I	43	44	50
	II	45	45	48
Protein	I	22	22	19
	II	22	24	20
Fat	I	19	18	16
	II	18	17	17

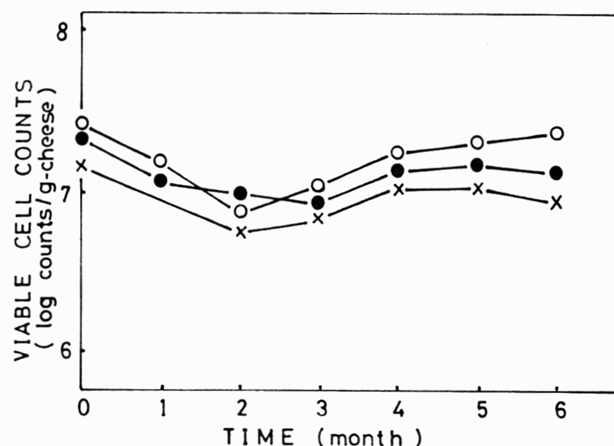

Fig. 6—Change of viable cell number in N-cheese (○), R-cheese(●) or AP-cheese (x) during its ripening at 10°C for a given period.

tease in the soluble state was determined in milk and found to be about one-third of the value in the case of casein solution. When the mixture of equal volumes of whey dialyzate and 1% casein solution was used as a substrate solution, the activity was less than half of the control (0.5% casein solution, without whey dialyzate). This indicates that whey components in the dialysis bag inhibited the proteolytic action of the alkaline protease.

Preparation of cheeses and their ripening

When milk clotted as a result of proteolytic action of the alkaline protease in soluble state (2 mg/ml), the amounts of tyrosine and sialic acid released from milk protein were about 3% and 40%, respectively. In the case of rennet, the value of the former was 1.7% and the latter 90%. According to these data, the flow rate of milk passed through each immobilized enzyme column was controlled so as to be the same hydrolysis degree as in the soluble state. The milk began to coagulate when lactic acid fermentation proceeded enough to lower the milk pH to 6.2. It took about 2 hr or more to form hard curd enough for the cutting procedure. The curd made by the immobilized alkaline protease was slightly softer than that obtained by using soluble and immobilized rennet. From these curds, AP-, R- and N-cheeses were prepared according to the same procedure (Fig. 1).

The appearance and hardness of AP-cheese were comparable to those of R- and N-cheeses. Moisture in AP-cheese was slightly higher than those in R- and N-cheeses (Table 1). Consequently, protein and fat contents in the former were somewhat lower than those in the latter. The difference of curd preparation method did not significantly affect these compositions.

Ripening ratios (Fig. 5) of AP- and N-cheeses increased gradually during ripening for 4 months and were higher than that of R-cheese. Acidity increase in AP- and N-cheeses was revealed after their ripening for 3 months but not in the case of R-cheese. Viable cell counts (Fig. 6) in these three cheeses declined in the first 2 months and thereafter increased slightly to the order of 10^7 at the end of ripening. The taste of AP-cheese was not inferior to that of the other two. No bitter flavor was detected in any of the three cheeses through the ripening period. In agar gel electrophoretic patterns of native whole casein [Fig. 7 (A) W and (B) W], two main bands from the starting point corresponded to β- and α_s-caseins in this order: the α_s-casein band from the AP-cheese [Fig. 7 (A) AP] was fainter than that from whole casein [Fig. 7 (A) W]. This change in the α_s-casein band was also recognized in the case of N-cheese [Fig. 7 (A) N] but not in the case of R-cheese [Fig. 7 (A) R]. After ripening for 5 months, α_s-casein bands from these

three cheeses remarkably disappeared [Fig. 7 (B)]. In the case of AP-cheese, a broad band trailed from the cathode at the initiation of ripening [Fig. 7 (A) AP] and was denser than that of the β-casein band after ripening for 5 months.

DISCUSSION

Continuous operation of the immobilized enzyme column

Proteolytic activities in the packed-bed and fluidized-bed reactors were very close to each other at the same flow rate (Fig. 2). In the present experiments, degree of hydrolysis was restricted to within 5% because excess hydrolysis of milk protein causes the formation of soft curd after lactic acid fermentation. Effect of back-mixing on degree of conversion was negligibly small when degree of conversion was less than 5% (Kobayashi and Moo-Young, 1971). Therefore, it is reasonable that activities in both reactors were almost the same.

Proteolytic activity increased at the beginning of the continuous operation when casein solution was supplied to the column (Fig. 3), whereas it did not increase when the immobilized enzyme ground in the mortar on ice was packed in the column. The operational time, in which proteolytic activity became the maximum, was shorter as the reaction temperature increased (Fig. 3), and it was also shorter at the same reaction temperature when benzoylarginine ethylester was supplied to the column instead of casein. When a casein concentration higher than 0.5% was used as a substrate, the effectiveness factor of the immobilized alkaline protease was 0.5 (Ohmiya et al., 1978). These results suggest that the initial increase of proteolytic activity might be caused by the diffusional limitation of substrate within the enzyme carrier. Simulations for the change of proteolytic activity were carried out by combining the rate of substrate diffusion within the carrier under unsteady-state and the rate of thermal inactivation, and the above results were satisfactorily simulated.

Preparation of cheese using immobilized proteases

The application of an immobilized enzyme to continuous coagulation of raw milk depends upon several critical points. These points are: (1) separation of the hydrolytic and clotting phases to prevent milk-clotting in the enzyme column; (2) long stability of enzyme activity under operating condition; (3) freedom of microbial infection; and (4) quality resembling the traditional cheese.

In the present study, separation of the hydrolytic and clotting phases at curd formation was accomplished by reversing the order of the lactic acid fermentation process and the protease treatment process of milk, i.e., rennetting (Fig. 1). In the traditional procedure for cheese-making [Fig. 1(A)], the first step is fermentation by lactic acid bacteria. This process increases the acidity of milk to about 0.2%. Thereafter rennet in the soluble state is added to release glycomacropeptide from κ-casein, i.e., the second step. Then milk begins to coagulate slowly. It is impossible to separate the hydrolytic and clotting phases from each other in this traditional procedure. Ferrier et al. (1972) employed this procedure to prepare curd continuously by using immobilized pepsin. In their experiment, separation of both phases was accomplished by proper control of milk temperature and the accumulation of white materials in the enzyme column was reduced by increasing the flow rates to make turbulent flow; however, the disadvantage of short retention of enzyme activity remained.

In the present study, protease treatment of milk was carried out as the first step for curd preparation by using the immobilized alkaline protease after raising the milk pH to 7.0 [Fig. 1 (B)]. Average hydrolytic extent of the milk during the first step [Fig. 1 (B)] was controlled to around

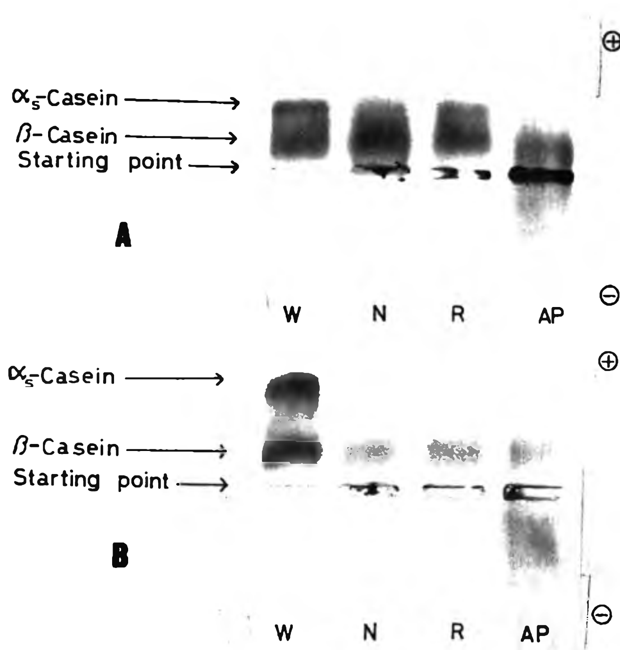

Fig. 7—Electrophoretic patterns of cheese solubilized in 8M urea at ripening initiation (A) and after ripening for 5 months (B). W: whole casein; N: N-cheese; R: R-cheese; AP: AP-cheese.

3% which was enough to trigger coagulation of milk acidified at the second step. By raising the pH of milk to 7.0 at the first step, coagulation of milk and accumulation of white materials around the enzyme carrier in the immobilized enzyme column were reduced significantly. Column operation at high temperature (50°C) was effective in preventing microbial infection in the enzyme column. This was allowed by using immobilized alkaline protease which showed longer retention of operational stability than rennet. After treatment of milk by the immobilized enzyme, proteolysis due to the milk-clotting enzyme did not proceed further because alkaline protease was not released in the effluent milk. As the second step, lactic acid fermentation in the milk was initiated by *S. cremoris;* within 2 hr milk began to coagulate.

The curds (AP- and R-cheeses) prepared by the present method did not include the clotting enzyme. This suggests that the ripening ratios of AP- and R-cheeses are lower than that of N-cheese which includes the soluble milk-clotting enzyme. In fact, this is revealed in Figure 5 which indicates that the ripening ratio of R-cheese was smaller than that of N-cheese, because R-cheese did not contain rennet but N-cheese did. As mentioned in a paper by Ohmiya and Sato (1972), coexistence of rennet and protease from lactic acid bacteria in the curd would enhance the hydrolysis of milk proteins more than only lactic acid bacterial protease.

In the case of AP-cheese, the ripening ratio was higher than that of R-cheese (Fig. 5), even though both cheeses did not contain any milk-clotting enzymes. The milk used for the preparation of AP-cheese, however, was hydrolyzed (hydrolysis degree: 3%) by alkaline protease more than the R-cheese (hydrolysis degree: 1.7%) was. This difference of proteolysis might affect the density of the α_s-casein bands in the electrophoretic patterns of AP- and R-cheeses [Fig. 7(A)]. Density of α_s-casein against β-casein in AP-cheese was fainter than that in R-cheese [Fig. 7(A)]. In both patterns, relative density of α_s-casein against β-casein was lower than that of native casein. These results suggest that casein in AP-cheese was in the more modified condition than that

in R-cheese. Consequently casein in AP-cheese may be more easily hydrolyzed by the protease of lactic acid bacteria than casein in R-cheese. This tendency was also shown in other papers (Sato and Ohmiya, 1966; Ohmiya and Sato, 1970). Thus, a slight excess of casein hydrolysis by alkaline protease compared with that by rennet accelerated the ripening ratio in AP-cheese.

In summary, alkaline protease could be used for cheese-making as a milk-clotting enzyme instead of calf rennet if its proteolytic activity to release sialic acid is properly controlled. Efficient utilization of the immobilized alkaline protease for cheese-making could be achieved by modifying the manufacturing procedure as described above.

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Ms received 3/21/79; revised 7/4/79; accepted 7/10/79.

The authors express their gratitude to Y. Sato for helpful discussion, and greatfully acknowledge Amano Pharmaceutical Co. for the kind donation of alkaline protease. This work was supported in part by a grant-in-aid for Co-operative Research No. 235056 and Scientific Research No. 256057 from the Ministry of Education, Japan.

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CHEMICAL, NUTRITIONAL AND MICROBIOLOGICAL QUALITY OF A PROTEIN CONCENTRATE FROM CULLED DRY BEANS

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ABSTRACT

A study was undertaken to develop a bean protein concentrate (BPC) from a mixture of five varieties of cull beans. The chemical composition, microbiological quality, and the nutritive value of the BPC were determined. The BPC was isolated from the bean flour by extraction with a dilute salt solution followed by acid precipitation. Protein recovery was 56% as BPC by extracting the cull bean flour with a 0.2% sodium chloride solution followed by precipitation at 90°C, pH 4.0. No detectable amount of toxic substance was found in BPC as assayed for heavy metals and mycotoxins. The microbiological and protein nutritional quality (PER) of the final products were determined. The PER data indicated that the BPC obtained by acid precipitation at room temperature had a higher nutritional quality than did BPC acid precipitated at 90°C.

INTRODUCTION

LARGE AMOUNTS of cull beans are produced each year as a result of adverse weather conditions at or near the time of harvest, mechanical damage during processing, mold growth, and insect infestation during storage. The world production of dry beans in 1974 was about 1.15×10^7 metric tons (FAO, 1975). At a cull rate of 5%, approximately 1.32×10^5 metric tons of bean protein would be potentially available for human consumption.

The production of protein concentrates from dry edible beans has been reported by many workers (Murphy et al., 1964; Pant and Tulsiani, 1969; Herrera, 1974; Molina and Bressani, 1975; Satterlee et al., 1975; Luh et al., 1975; Polit and Sgarbieri, 1976). Work done by Pant and Tulsiani (1969) showed that bean protein isolate, when supplemented with the respective limiting sulfur amino acids, was an efficient protein source.

Herrera (1974) isolated protein concentrate from Great Northern, pink and pinto beans, and determined the nutritive value of the bean protein concentrates (BPC). The best yield of BPC from dry beans was obtained by using a 0.5% NaCl solution extraction followed by acid precipitation at pH 3.5.

A protein-starch extraction process, which did not lower the nutritive value of the Jack bean protein, has been described by Molina and Bressani (1975). It is considered to be more economical than protein extraction alone, since it yields two final products, starch and protein.

Satterlee et al. (1975) prepared a protein isolate from Great Northern beans which contained 65% globulins and 35% albumins. Functional properties of the Great Northern bean isolate were also determined.

Castor bean (*Ricinus communs*) protein was isolated by filtration of the alkali-extracted bean flour after 5 min heating at 100°C (Polit and Sgarbieri, 1976). This heat treatment completely eliminated all toxic and allergenic materials present in the raw bean. The authors found the protein concentrate to be limiting in lysine and the sulfur amino acids.

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0022-1147/79/0006-1589$02.25/0
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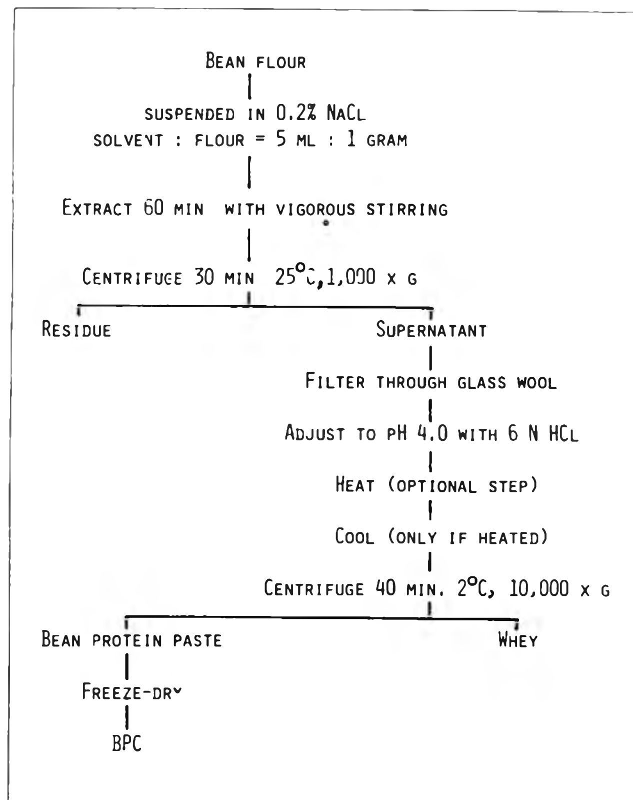

Fig. 1—Isolation Of Bean Protein Concentrate.

The objectives of this study were to:

1. develop a food ingredient from culled dry beans which are presently used solely as an animal feed;
2. develop a protein concentrate from the culled fraction of the five most prominent varieties of dry beans raised in the U.S.;
3. determine the chemical composition of the bean protein concenterate (BPC);
4. evaluate the protein nutritional quality of all BPCs via the PER assay; and
5. test the microbiological quality and mycotoxin content of all BPCs obtained.

EXPERIMENTAL

THE CULL BEANS used in this study were from the top five varieties produced in the U.S. They were navy, pinto, Great Northern, red kidney (*Phaseolus vulgaris*) and lima beans (*Phaseolus lunatus*). Navy and red kidney beans were obtained from Michigan, Great Northern beans from Nebraska, and pinto and lima beans from California.

Isolation of bean protein concentrates

The cull beans were finely ground by using a laboratory pulverizing mill purchased from Weber Brothers and White Metal Works,

Inc., Illinois. Duplicate 100-g samples of the bean flour from each variety were used to isolate bean protein concentrate according to the procedure outlined in Figure 1.

A study on the effect of heating on the isolation of the bean protein concentrate was also conducted. Duplicate 100-g samples of the mixed cull bean flour, which consisted of 41.4% navy, 29.5% pinto, 12.7% Great Northern, 8.9% kidney and 7.5% lima beans, were also processed according to the procedure illustrated in Figure 1. This mixture represents the relative amounts of these five bean varieties as they occur in the U.S. and, therefore, is representative of the composition of the culls available in the U.S. Heat was employed immediately after acid (pH 4.0) precipitation, followed by immediate cooling to 35°C after reaching the desired heating temperature (60, 70, 80, 90, 100, and 110°C). A room temperature (25°C) isolation was also conducted.

BPCs were also isolated from 3–4 kg of mixed cull bean flour, using a 0.2 or 0.5% NaCl solution for extraction. This was followed by heat + acid or just acid precipitation, in order to study the large-scale production of BPC. The BPCs obtained from this study were used in the following chemical, microbiological, and nutritional studies. BPC obtained by acid precipitation was labeled BPC (25) referring to acid precipitation at 25°C; whereas acid precipitation followed by heating to 90°C produced a BPC labeled as BPC (90).

Proximate analyses

Proximate analyses of the bean protein concentrates were performed according to AOAC (1975) procedures for moisture content (AOAC, 14.002-14.003), crude fat content (AOAC, 7.044-7.045), mineral content (AOAC, 14.006), and protein content (AOAC, 2.047-2.049 using a nitrogen-to-protein conversion factor of 6.25). The amount of carbohydrate was determined by difference.

Heavy metal analyses

Samples analyzed for heavy metals were BPC (25), BPC (90), and the mixed cull bean flour. The heavy metal content of the cull bean flour was determined to see if there was a concentration or dilution effect derived from the isolation procedure. Cd, Pb, Cu, Cr, Ag, and Mn were determined according to AOAC (1975) atomic absorption spectrophotometry on a Perkin Elmer 303 Spectrophotometer (Cd: AOAC, 25.026-25.030; Pb: AOAC, 25.060-25.064; Cu, Cr and Ag: AOAC, 25.041-25.045, and 33.089-33.094). As, Hg, and Se were determined according to AOAC (1975) (As: 25.006-25.011; Hg: 25.108-25.115; Se: 25.117-25.119) by the Dept. of Biochemistry at South Dakota State Univ., Brookings, S.D.

Mineral analyses

Samples used for mineral analyses were BPC (25), BPC (90), and the mixed cull bean flour. Ca, Fe, Mg, and Zn were determined according to AOAC (1975) atomic absorption spectrophotometry (AOAC, 2.096-2.097 and 25.143-25.147).

Na and K were also determined by atomic absorption spectrophotometry (Perkin Elmer, 1973). Samples were moistened with dilute H_2SO_4 in a quartz crucible, dried in an oven (AOAC, 3.024), and then dry ashed (AOAC, 7.080a). Subsequent dilutions were made with a 1,000 ppm LiCl solution to bring sample solutions into the analytical measurement range. Na and K were then determined by atomic absorption at 589.0 and 766.5 nm, respectively.

Phosphorus was determined by a colorimetric method (AOAC, 1975, 3.062-3.064).

Vitamin content

The vitamin assays performed included the quantification of vitamin B_1, vitamin B_2, niacin, and vitamin C. Procedures used were according to AOAC (1975) for vitamin B_1 (AOAC, 43.031-43.034), vitamin B_2 (AOAC, 43.039), niacin (AOAC, 43.044), and vitamin C (AOAC, 43.051).

Microbiological quality and mycotoxin analyses

Bean protein concentrates BPC (25) and BPC (90) were prepared under strict sanitary conditions to avoid product contamination during processing. The BPCs were then aseptically packaged and immediately analyzed for microbiological quality.

Microbiological quality examination consisted of the following determinations: total plate count, coliforms, *Salmonella*, *Staphylococcus*, yeast, and mold count. The mycotoxins examined were the aflatoxins B_1, B_2, G_1, G_2, patulin, ochratoxin, and sterigmatocystin. Microbiological examinations and mycotoxin analyses were performed according to AOAC (1975).

Table 1—Effects of temperature on yield of BPC protein

Heat temp (°C)	% of the total protein recovered
25	48.23
60	46.28
70	50.06
80	53.16
90	57.28
100	53.80
110	50.06

Table 2—Yields of BPC (25) and BPC (90) protein from each cull bean variety

	% of total protein recovered as BPC	
Variety	BPC (25)	BPC (90)
Navy	41.95	52.41
Pinto	53.23	57.18
Great northern	46.96	55.14
Kidney	43.05	46.80
Lima	18.98	50.99

Protein quality evaluation

Amino acid analysis. A 6N HCl hydrolysis for 24 hr at 110°C under vacuum was used to prepare a protein hydrolyzate for subsequent determination of all amino acid except tryptophan and sulfur containing amino acids. A separate protein hydrolysis was carried out in alkaline $Ba(OH)_2$ to prevent tryptophan destruction (Pataki, 1968). The procedure described by Moore (1963) was used to prepare a protein hydrolyzate for the accurate determination of the sulfur containing amino acids. All amino acids were determined using a Beckman 120C amino acid analyzer.

Protein efficiency ratio (PER). The PER of BPC was measured by the procedure described in AOAC (1975), with the single modification in that a Williams and Briggs modified salt mix (Teklad Co.) was used to replace the USP salt mix.

Preparation of protein samples for the PER study. Powdered samples of BPC (25) and BPC (90) were autoclaved in pans at a thickness not exceeding 1 cm, at 121°C for 15 min. This procedure was referred to as dry autoclaving. Samples of BPC (25) and BPC (90) were made into a slurry by adding distilled water before being autoclaved at 121°C for 15 min. These BPC samples were then freeze-dried after autoclaving. This procedure was referred to as wet-autoclaving.

Preparation of diets. BPC (25) and (90) based diets were prepared by the 1975 AOAC procedure with all diets containing 10% protein. A second set of BPC (25) and (90) diets, with their respective ANRC casein reference diet, was formulated to contain 14% protein. A third set of BPC (25) and (90) diets was supplemented with 0.3% lysine, 0.5% phenylalanine, 0.3% valine and 0.5% methionine. All diets contained the same proportion of fat, vitamin, mineral, and salt mixture. Corn starch and sucrose were used in equal portions as the carbohydrate source in each diet.

Assay procedure. Male weanling Holtzman rats (22 days old) were randomly divided into groups according to AOAC 43.185. Each rat was kept in an individual cage and provided with appropriate assay diet and H_2O ad libitum. Generally, ten rats were used per study group. However, six rats were used in the dry autoclaved BPC (25) and BPC (90) studies. At the end of the study, the PER for each group was calculated by dividing the average gram weight gain by protein intake for 28 days. The PER reported for all samples is based upon the corrected PER of the casein control being 2.5.

Apparent protein digestibility. Apparent digestibility of the test samples and all casein diets were determined during the PER assay. Feces were collected from day 18 to day 26. Food intake in this period was also recorded. The apparent digestibility was calculated for each group of rats by the formula:

$$\text{Apparent digestibility} = \frac{\text{N intake} - \text{N fecal}}{\text{N intake}} \times 100$$

Table 3—Heavy metal content of the BPCs and the mixed cull bean flour[a]

Sample	Cd	Pb	Mn	Cu	Cr	Ag	As	Hg	Se
BPC (25)	0.09	2.50	13.00	21.3	9.60	0	0.22	<0.05	0.33
BPC (90)	0.18	2.50	18.75	17.50	8.65	0	0.54	<0.05	0.34
Bean flour	0.89	2.70	24.75	10.75	6.40	<0.02	–	–	–

[a] All values are reported as ppm.

Table 4—Mineral content of the BPCs and the mixed cull bean flour[a]

Sample	Ca	Mg	Zn	Fe	Na	K	P
BPC (25)	42.0	100.0	2.5	35.0	1400.0	600.0	850.0
BPC (90)	39.0	112.0	3.6	29.0	840.0	972.0	993.0
Bean flour	143.7	175.0	3.1	36.0	16.0	1550.0	416.0

[a] All values are reported as mg mineral per 100g sample.

Table 5—Concentration of thiamin, riboflavin, niacin and ascorbic acid in the BPCs[a]

Sample	Thiamin	Riboflavin	Niacin	Ascorbic acid
BPC (25)	0.52	0.30	2.20	0
BPC (90)	0.40	0.22	1.30	0

Values are reported as mg vitamin per 100g sample.

RESULTS & DISCUSSION

THE EFFECTS of temperature on yield of BPC protein, as percent of total mixed cull bean flour protein, is shown in Table 1. The protein content of the mixed cull bean flour on the fresh weight basis is 23.55%. There was no significant increase in protein yield as the temperature increased from 25°C to 60°C; however, there was a 10% increase at 80°C and a 20% increase at 90°C. As the temperature was elevated from 90 to 100 and 110°C, there was a decrease in protein yield. All further studies utilized BPCs produced either via acid precipitation at room temperature (BPC 25) or at 90°C (BPC 90).

Table 2 shows the yield of BPC (25) and BPC (90) protein from each of the five cull bean varieties. The amount of BPC protein obtained with the 90°C heat step was significantly higher than that obtained with the 25°C (unheated) acid precipitation, especially from lima beans where the amount of BPC (90) was more than twice the amount of BPC (25) obtained. The increased yield obtained upon heating was due to the heat denaturation step aiding the initial acid denaturation step. Some bean proteins are believed to be resistant to acid precipitation, but are denatured and precipitated by heat; thus, when both acid and heating precipitations were used, BPC yield was increased.

Proximate analysis

Proximate analyses were carried out on the BPC samples which were used for the subsequent PER studies. All BPC samples produced in this study contained from 72–81% protein, 9–17% carbohydrate, 4–5% ash, 1–3% crude fat, and 2–6% moisture.

Heavy metals

The results of the heavy metal analysis are summarized in Table 3. The concentration of each heavy metal in the three samples lies in the normal ranges of common foods (Underwood, 1973), with the exception of lead and chromium. All samples contained about 2.5 ppm Pb, which is slightly higher than the <1 ppm level found in most foods. However, the 2.5 ppm level is still far below the maximum allowance level of 7 ppm set by the U.S. Food and Drug Administration (Underwood, 1973). The mixed cull bean flour, BPC (25) and BPC (90), contained 6.4, 9.6, and 8.6 ppm of Cr, respectively. This Cr level is higher than what is found in most common plant-based food ingredients, which contain Cr ranging between 0.1–0.5 ppm. The chromium toxicity has been summarized by Underwood (1977). Cats tolerate 1,000 mg/day and rats showed no adverse effects from 100 mg/kg diet. Life time exposure to 5 mg/liter of trivalent chromium in the drinking water induced no toxic effects in rats and mice, and exposure of mice for three generations to chromium oxide at levels up to 20 ppm of the diet had no measurable effect on mortality, morbidity, growth, or fertility. Chromium is known to play an important role in glucose, lipid and protein metabolism.

Minerals

Table 4 shows the mineral content of the BPC samples and the mixed cull bean flour. The mineral content of the mixed cull bean flour, with the exception of iron, is similar to the results reported by Ritchey et al. (1973) and USDA (1975). The mixed cull bean flour, as well as the BPC samples, are rich in iron since they contain from 29–36 mg Fe/100g sample. The amount of calcium in the BPC samples (about 40 mg Ca/100g sample) was about one-third of that found in the mixed cull bean flour (144 mg Ca/100g sample).

A drastic increase in Na content of the BPC samples was noted and apparently due to the use of NaCl in the extraction solvent. Potassium concentration was diluted by the isolation procedure. The phosphorus content of the BPC samples was about twice that in the mixed cull bean flour. It is believed that phytate is associated with bean proteins, therefore, any process that utilizes solution and isoelectric precipitation would concentrate the phytate into the resulting product.

Vitamins

The amount of thiamin, riboflavin, niacin, and ascorbic acid in the BPC samples are presented in Table 5. The BPC (25) contains amounts of these vitamins resembling the amount found in intact beans as reported by the USDA (1975). A significant decrease in thiamin, riboflavin, and niacin was observed in the BPC (90), presumably due to the degradation of these vitamins by heat.

Mycotoxins

No detectable level of any of the mycotoxins was found in either BPC (25) or BPC (90). This implies that the molds found on the cull beans either are not toxin producing molds or are unable to produce the mycotoxins in the environment of the dry bean. It also is possible that mycotox-

Table 6—Microbiological profile of BPCs

Sample	Total plate count	Coliforms	*Samonella*	*Staphylococci*	Yeast	Mold
BPC (25)	8,100/g	<10/g	Negative in 30g	<10/g	<10/g	10/g
BPC (90)	3,500/g	<10/g	Negative in 30g	<10/g	<10/g	20/g

ins are removed during the production of the BPCs.

Microbiological quality

The results describing total plate counts, coliforms, *Salmonella, Staphylococci*, yeast, and mold counts on the BPC samples are given in Table 6. Total plate count for the BPC (25) (8,100/g) was twice that of BPC (90) (3,500/g). More than one-half of the microorganisms present were destroyed by the heat treatment. However, the bacterial counts of both BPC samples were still well below the microbiological standard for dried egg, dried milk, and many other foods as described by Frazier (1967). The coliforms, *Salmonella*. *Staphylococci*, yeast, and mold counts of the BPC samples were also very low. The microbiological results indicate that the BPC samples are well under the limits required for similar food ingredients.

Protein quality

Amino acid analysis. The amino acid compositions of the nonautoclaved BPC (25), dry autoclaved BPC (25), nonautoclaved BPC (90), and dry autoclaved BPC (90) are shown in Table 7. The BPC samples had good amino acid profiles. In all cases, the sulfur containing amino acids, threonine, valine, and tryptophan are the limiting amino acids, as compared to the provisional pattern set by FAO/WHO (FAO/WHO, 1973). Dry autoclaving BPC (25) destroyed some lysine, and dry autoclaving BPC (90) destroyed a portion of the lysine and methionine. Nonautoclaved BPC (25) had a higher lysine content (6.59 g/100g protein) than did the nonautoclaved BPC (90) (5.86 g/100g protein) revealing that the heating step in the isolation process caused some destruction of lysine.

PER study. The PER values are listed in Table 8. Rats fed dry autoclaved BPC (25) averaged a 3-g increase in weight and yielded a PER value of 0.13. Rats fed dry autoclaved BPC (90) lost weight (11.4g) but did not die through the experimental period. Rats fed wet autoclaved BPC (25) gained an average of 13g and yielded a PER value of 0.52. Rats fed wet autoclaved BPC (90) lost weight, but the extent of weight loss was very small (2.8g). The above results indicate that wet autoclaving has a positive effect on the nutritive value of the BPC. Heating was employed to destroy the heat labile toxic factors such as protease inhibitors and lectins in the BPC; however, dry heat caused a detrimental effect on the nutritive value of the BPC. The color of the BPC turned dark brown during dry autoclaving, while the color of the BPC did not change through wet autoclaving. The color change can be attributed to the Maillard reaction between the reducing sugars and free amino groups of proteins. The unusually low PER values obtained for all BPC samples was attributed to (1) the low levels of feed consumption by the rats, a factor known to adversely affect PER; (2) the lack of essential amino acids; and (3) the browning reaction that caused a reduction in essential amino acid content.

The supplementation of the BPC with amino acids greatly improved the nutritive value of the BPC. PER values of 1.17 and 2.24 were obtained for the amino acid supplemented, wet autoclaved BPC (25), and BPC (90), respectively. The increase in bean protein level to 14% in the diet raised the PER values to 0.88 for the wet autoclaved BPC (25), and 0.65 for the wet autoclaved BPC (90). The above results imply that no significant amounts of toxic substances were present in either the BPC (90) or BPC (25) samples. Otherwise, neither amino acid supplementation nor diet protein level increase would have improved the PER values of the BPC.

Apparent protein digestibility. The results on the digestibility studies are also shown in Table 8. The apparent protein digestibility of the autoclaved BPC samples were fairly good ranging from 71% to 89%. This could be attributed to the heat destruction of the protease inhibitors present in the BPC samples. The amino acid supplementation did not affect the apparent protein digestibility of the wet autoclaved BPC samples as might be expected.

A discrepancy exists among the good amino acid composition, the good digestibility, and the poor PER values of

Table 7—Amino acid composition (g amino acid/100g protein) of the BPC samples

Amino acid	Non-autoclaved BPC (25)	Dry autoclaved BPC (25)	Non-autoclaved BPC (90)	Dry autoclaved BPC (90)
Aspartic acid	10.70	10.88	11.41	11.60
Threonine	3.28	3.22	3.72	3.85
Serine	4.88	4.97	5.59	5.67
Glutamic acid	15.85	14.91	14.90	15.13
Proline	3.75	3.29	3.64	3.67
Glycine	3.64	3.83	3.62	3.72
Alanine	3.80	4.02	3.83	3.91
Cystine	0.96	0.82	1.18	1.03
Valine	5.28	5.75	5.41	5.57
Methionine	2.20	2.24	2.03	1.19
Isoleucine	5.11	5.55	5.21	5.32
Leucine	8.65	9.30	8.69	8.87
Tyrosine	3.67	3.84	3.73	3.79
Phenyalanine	5.92	6.29	6.14	6.23
Lysine	6.59	5.98	5.86	5.15
Histidine	2.44	2.45	2.45	2.29
Ammonia	1.73	1.73	1.81	1.77
Arginine	5.82	5.24	4.98	5.40
Tryptophan	0.74	0.69	0.82	0.84

Table 8—PER and apparent protein digestibility of the BPC sample and casein reference diets

Protein source	Protein level in diet (%)	No. of rats	Sample treatment[a]	Adjusted PER	Apparent protein digestibility
BPC (25)	10	6	DA	0.13	84.68
BPC (90)	10	6	DA	—[c]	71.32
BPC (25)	10	10	WA	0.52	85.89
BPC (90)	10	10	WA	—[c]	84.92
BPC (25)[b]	10	10	WA	1.17	80.54
BPC (90)[b]	10	10	WA	2.24	84.27
BPC (25)	14	10	WA	0.88	89.43
BPC (90)	14	10	WA	0.65	88.65
Casein	10	10	—	2.50	92.68
Casein	14	10	—	2.50	93.40

[a] DA = Dry Autoclaved; WA = Wet Autoclaved.
[b] Diet with the supplementation of amino acids.
[c] Rats lost weight during study period.

the dry autoclaved BPC (25) and dry autoclaved BPC (90). One of the reasons for this difference is due to the low food intake by the rats throughout the experimental period. The low food intake could make part of the protein serve as an energy source and, therefore, unavailable for growth. Another factor responsible for the low PER values could be due to the fact that heat accelerates the Maillard reaction. Heat also causes specific interactions among the internal active groups on the bean protein and can form enzyme resistant crosslinkages which would lower bioavailability, but which can be released by acid hydrolysis and would not be revealed by the determination of amino acid composition.

CONCLUSIONS

THE FOLLOWING points were concluded from this study:

1. Temperature has a definite effect on the isolation of the bean protein concentrate. Fifty-six percent of the proteins from bean culls can be isolated by extracting the cull bean flour with 0.2% NaCl solution and with heating to 90°C following acidification to pH 4.0.

2. The bean protein concentrates isolated had good amino acid profiles. Nonautoclaved bean protein concentrate was abundant in lysine, but first limiting in methionine and cystine. Autoclaving caused a partial destruction of some essential amino acids.

A discrepancy exists between the amino acid composition of the BPCs and their nutritive values as measured by PER assay. The low PER values can be attributed to the low food intake of the rats, the limiting quantities of some of the essential amino acids, and the browning reactions which caused a loss of essential amino acids.

3. Wet autoclaving was less detrimental to the nutritive value of BPC than was dry autoclaving. The results of the PER study indicate that BPC (25) was better than BPC (90). However, when both diets were supplemented with specific essential amino acids, a higher PER value was obtained for amino acid supplemented BPC (90) than that of the amino acid supplemented BPC (25).

4. Under the described experimental conditions, no detectable levels of toxic substances were found in either BPC (25) or BPC (90) as assayed for heavy metals and mycotoxins. This suggests that the protein concentrate isolated from culled dry beans has the potential of being a food ingredient.

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Ms received 3/22/79; revised 7/4/79; accepted 7/20/79.

Published as Paper No. 5752, Journal Series, Nebraska Agricultural Experiment Station. Research reported was conducted in part by a grant from the Nebraska Department of Economic Development.

LEVELS OF CHLORINE AND CHLORINE DIOXIDE OF EQUIVALENT BACTERICIDAL EFFECT IN POULTRY PROCESSING WATER

H. S. LILLARD

ABSTRACT

Equivalent levels of chlorine and chlorine dioxide (generated on-site) were established on the basis of bactericidal action in bird chiller water. When introduced directly into the chiller water after processed birds had begun to enter the chiller, 5 ppm ClO_2 and 34 ppm Cl_2 were equally bactericidal. When introduced into the chiller with the fresh-water input, 20 ppm Cl_2 and 3 ppm ClO_2 were equally effective. The use of about 1/7 as much ClO_2 as Cl_2 would be less corrosive to processing equipment and provide the poultry industry with an alternative bactericide to chlorine.

INTRODUCTION

CURRENT REGULATIONS require the input of 0.5 gal (1.9L) fresh water per broiler carcass in continuous chillers (USDA, 1973). Wesley (1977) suggested that the intake requirement could be reduced to 0.25 gal (0.95L) of water per bird without affecting quality of the carcass or chiller water. A change in regulations permitting the reduction of fresh-water input in continuous chilling systems is being considered "provided that the fresh water intake, including that used to fill chillers but excluding ice, consists entirely of fresh water that contains 20 ppm available chlorine" (USDA, 1978). Wesley estimated that reducing the fresh water input by one-half could save the industry in the state of Virginia about $150,000 annually. Poultry processors in other areas may well realize greater monetary savings than would those in Virginia, judging from the current cost of water and of wastewater treatment.

Many in the industry have expressed concern about the effect on equipment of 20 ppm (20 mg/L) chlorine. Others have expressed concern that their potable water supply may not have the required pH to retain 20 ppm chlorine in solution. Some feel that they would not make use of this water conservation measure because of the chlorination requirement.

The use of chlorine in poultry processing water was suggested by Gorseline et al. (1951) and chlorine is now widely used to control spoilage bacteria and pathogens. The use of chlorine in chiller water has been widely investigated and found to significantly reduce bacterial populations in the water (Dawson et al., 1956; Mead and Thomas, 1973; Blood and Jarvis, 1974), and extend carcass shelf-life (Mallman et al., 1959; Nillson and Regner, 1963; Ranken et al., 1965; Patterson, 1968; May, 1974).

Chlorine dioxide (ClO_2) is not used as commonly as chlorine and had not been as widely investigated as a bactericide in poultry processing water. Benarde et al. (1965) showed that ClO_2 is a more effective bactericide than chlorine in sewage effluent at pH 8.5. Baran et al. (1973) studied the effect of ClO_2 in processing water at a turkey plant with an in-plant chlorination system. They reported microbial reductions with ClO_2 but had difficulty interpreting the results because both chlorine and ClO_2 were in use at the same time.

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Chlorine dioxide as an aqueous solution has been used for potable water and wastewater treatment, but its major use has been for bleaching pulp in the manufacture of paper (White, 1972). Chlorine dioxide is five times as soluble as chlorine in water, and has over two times the oxidizing capacity of HOCl. Its bactericidal efficiency is not impaired at high pH, and it does not react with ammonia or nitrogenous compounds, including the simple amino acids, as does chlorine (White, 1972). For all these reasons it seems likely that chlorine dioxide could provide bactericidal action equivalent to that of chlorine but at much lower levels.

Stabilized ClO_2 in chiller water (provided as a stock solution of 20,000 ppm) was reported ineffective as a bactericide against salmonellae on poultry carcasses (Dougherty, 1974). The bactericidal effect of ClO_2 generated on-site (Olin Water Services' system) has not been investigated in poultry processing plants.

This study was undertaken to determine the equivalent levels of chlorine and chlorine dioxide (generated on-site) as bactericides in the chiller water of a broiler processing plant.

MATERIALS & METHODS

Bactericidal treatment

Two approaches were used in determining the equivalent effective levels of chlorine and chlorine dioxide: (1) The two bactericides were metered into chiller water at a rate designed to result in about 0.5 ppm free residual bactericide. For safety reasons, both chlorine and chlorine dioxide were injected into the chiller water of a commercial processing plant at the bottom of the chill tanks, close to the circulating pump, after the first processed birds had been released into the full chillers; (2) Chlorine dioxide was injected into the potable water at the bottom of the chill tank, close to the circulating pump, as the chiller was being filled. For safety considerations the chlorine dioxide-generating equipment was not turned on until the injection point in the chiller was submerged in water, i.e. the chiller was 1/4–1/2 full. Chlorine gas (20 ppm) was injected into a 4-in. potable-water pipe by which the chillers were filled and into a 2-in. potable-water pipe that provided the fresh-water input (makeup). In order to provide and maintain 20 ppm available chlorine in the potable water supply, a rotameter setting of 50 was needed on the 4-in. line to the chiller and a separate rotameter setting of 20 on the 2-in. line for the fresh-water input during the 43 min required to fill the chiller. When the 4-in. line to the chiller was shut down, water pressure changed in the 2-in. line so that the rotameter setting on the makeup line had to be changed to 13 in order to maintain 20 ppm Cl_2 in the fresh water input for the rest of the 8-hr shift. Calculations are based on a 9,500-gal (35,960L) chiller and prechiller capacity, 43 min to fill the chiller and prechiller, 0.5 gal (1.9L) fresh-water input per broiler carcass, a line speed of 108 birds/min or fresh-water input at the rate of 54 gal/min (204L/min) and an 8-hr shift. Calculations for both Cl_2 and ClO_2 are expressed in one of two ways: (1) based on makeup volume (25,920 gal, 98,115L or 216,172 lb per 8-hr shift); (2) based on total water volume, consisting of chiller and prechiller capacity plus makeup water, (35,420 gal, 135,075L or 295,402 lb per 8-hr shift).

Chlorine gas was metered through an Advance cylinder-mounted gas chlorinator (Capital Controls Co., Inc.). Total chlorine and free residual chlorine were measured with a Fischer and Porter Model 1/T1010 amperometric titrator.

Chlorine dioxide was generated on-site by the reaction between a solution of sodium chlorite precursor (Olin 4107) and chlorine in an Olin Water Services system. Hydrochloric acid (J.T. Baker Chemical Co., Food Grade) or chlorine was used to adjust the pH of the

Table 1—Microbiological analyses of untreated chiller water

	Mean log_{10}/mL[a]						Salmonellae					
	Total aerobic count			Fecal coliforms			Incidence		No. samples <1 cell/mL		Range No. cells/mL	
Sample source	Overall mean[b]	a.m.[c]	p.m.[c]	Overall mean[b]	a.m.[c]	p.m.[c]	a.m.	p.m.	a.m.	p.m.	a.m.	p.m.
Chiller I	3.41d	3.40d	3.41d	2.37	2.34f	2.41f	13/30h	12/30h	10/30j	12/30j	<0.4– 6.6	<0.4–2.6
Chiller II	3.45d	3.41d	3.49d	2.47	2.41g	2.52g	15/30h	13/30h	11/30j	13/30j	<0.4–15.8	<0.4–4.4

[a] The same lower case letter (in columns and/or rows) follows values which do not differ significantly at the 5% level.
[b] Based on 60 samples taken 4 each on 15 sampling days
[c] Based on 30 samples taken 2 at each sampling time on 15 sampling days

Table 2—Chemical analyses of untreated chiller water

	Mean mg/L[a] Suspended solids			Mean mg/L[a] Dissolved matter			Mean mg/L[a] Grease		
Sample source	Overall mean[b]	a.m.[c]	p.m.[c]	Overall mean[b]	a.m.[c]	p.m.[c]	Overall mean[b]	a.m.[c]	p.m.[c]
Chiller I	80.23	71.57	88.90	288.61	260.87	317.31	124.93d	105.23e	144.63f
Chiller II	101.88	94.93	108.60	354.64	319.03	389.07	156.07d	112.14e	197.07f

[a] The same lower case letter (in columns) follows values which are not significantly different at the 5% level.
[b] Based on 60 samples taken 4 each on 16 sampling days
[c] Based on 30 samples taken 2 at each sampling time on 15 sampling days

reaction mixture to between 3–3.5, the optimum pH range for generating ClO_2 (Lillard, 1979a).

Chlorine dioxide was measured by a modified N,N-diethyl-p-phenylenediamine (DPD) method (APHA, 1971) using a Hach DR-EL/2 test kit with spectrophotometer. The modification of the DPD test consisted of adding five drops of Olin Water Services' Chlorine Dioxide Stabilizer Solution to the water sample prior to proceeding with the test. The stabilizer eliminated chlorine interference in the DPD procedure. Residual chlorine dioxide was read as Cl, and, therefore, the readings were multiplied by a factor of 1.9 to express the results as ClO_2.

Sampling

Prior to treatment, two water samples (500 ml) were collected from the midway point of the chill tank 2 hr and 7 hr after start-up time. After treatment with Cl_2 or ClO_2 had been initiated, two water samples (500 ml) were collected in the morning, 2 hr after start-up time, and in the afternoon, 7 hr after start-up time, from each of three sampling points: (1) the injection point of Cl_2 or ClO_2; (2) midway point of the chill tank; (3) the exit end of the chill tank. An appropriate amount of sodium thiosulfate (105 mg) was added to each container in which treated water was collected.

All samples were kept on ice and transported immediately to the laboratory, where microbiological tests were started within 30 min of sampling. The number of sampling days (4 samples of chiller water each day) for each treatment were as follows: untreated chiller water, 15 days; 34 ppm Cl_2, 11 days; 5 ppm ClO_2, 12 days; 20 ppm Cl_2, 13 days; 3 ppm ClO_2, 12 days.

Chemical analysis

Untreated water samples from two chillers were tested for suspended solids, dissolved matter and grease so that the loading of organic matter in the water could be established. Standard methods were used (APHA, 1976).

Microbiological analyses

Samples taken for total aerobic plate counts were decimally diluted in 1% peptone; the dilutions were pour-plated in duplicate in Difco Plate Count Agar and incubated at 20°C for 72 hr.

For enhanced recovery of damaged cells, fecal coliform densities were determined by the five-tube MPN procedure described in *Standard Methods for the Examination of Water and Wastewater* (APHA, 1976). Fermentation tubes with inoculated EC Medium were incubated in a Precision Scientific Coliform Incubator Bath at 44.5°C for 24 hr.

The incidence of salmonellae was estimated by enrichment methods, and levels were estimated by a five-tube MPN procedure (Lillard, 1977).

Statistical analyses

Data for total aerobic counts and fecal coliform levels were treated by standard analysis of variance. The Chi Square test was applied to data on salmonellae incidence. Significance is reported at the 5% level unless stated otherwise.

RESULTS & DISCUSSION

UNTREATED WATER from the two chillers in a commercial processing plant was tested to determine whether both chillers could be treated simultaneously, one with chlorine, the other with chlorine dioxide. Because any given treatment was to be applied for an 8-hr shift, water samples were taken in the morning (2 hr after start-up) and afternoon (7 hr after start-up) to determine whether the concentration of organic matter, including bacteria, differed throughout the day. Table 1 shows that there was no significant difference in total aerobic counts for the two chillers but that fecal coliform levels in chiller II were significantly higher than in chiller I. Fewer than 50% of the samples from either chiller were salmonellae positive; levels in most of the samples were very low (<0.4/mL). The levels of aerobic organisms and fecal coliforms, as well as salmonellae incidence, did not differ significantly for the two sampling times.

Table 2 shows that levels of suspended solids, dissolved matter, and grease all increased significantly in the afternoon samples from both chillers. Also, suspended solids and dissolved matter in samples from chiller II were significantly higher than in samples from chiller I. Since hypochlorous acid, the effective bactericide formed in water from chlorine, reacts with organic matter (ammonia and nitrogenous compounds) to form chloramines (White, 1972), it became apparent that for a fixed level of chlorine treatment the water should be tested at least twice a day. Furthermore, preliminary work showed that the differences between the chillers would confound the evaluation of results if different treatments were carried out in the two chillers. Therefore, all subsequent work was done with one chiller, and treatments were periodically alternated so that seasonal effects would be minimized.

The efficiency of chlorine is impaired at pH values greater than 5.5 because of a reduction in the effective

Table 3—Variation in Cl_2 and ClO_2 concentrations in various parts of the chill tank

Treatment	Conc (mg/L) of Cl_2 or ClO_2[a] based on Vol. of makeup water	Total vol. of water	Requirement of Cl_2 or ClO_2 lb/8-hr shift	Conc range (mg/L) of free residual Cl_2 or ClO_2 in chiller water[b] — 2 hr after start-up a.m. Injection point	Midway point	Exit end	7 hr after start-up p.m. Injection point	Midway point	Exit end
Cl_2[e]	46	34	10.00	0.5– 0.6	0.5–0.6	0.4–0.5	0.5– 0.7	0.4–0.5	0.3–0.4
ClO_2	7	5	1.51	0.6– 0.8	0.4–0.7	0.4–0.6	0.6– 0.9	0.4–0.7	0.4–0.6
Cl_2[f]	28	20	6.04	20 –21	0 –0.3	0	20 –21	0	0
ClO_2	4	3	0.88	0.3– 0.4	0.2–0.3	0.1–0.3	0.2– 0.4	0.2–0.3	0.2–0.3

[a] Rounded off to the nearest mg
[b] Rounded off to the nearest 1/10 unit
[c] Volume of makeup water, based on a line speed of 108 birds/min = 25,920 gal (98,115L) or 216,172 lb/8 hr.
[d] Total volume of water, based on prechiller and chiller (9,500 gal or 35,960L) plus makeup water (25,920 gal or 98,155L) = 35,420 gal (134,075L) or 295,402 lb/8 hr
[e] Rotameter setting of 30 lb/24 hr
[f] Rotatmeter settings were as follows: 50 lb/24 hr for 43 min on 4-in. quick-fill potable-water line to chiller; 20 lb/24 hr for 43 min on 2-in. potable-makeup water line; 13 lb/24 hr on 2-in. potable-makeup water line for 7 hr 17 min.

concentration of hypochlorous acid (White, 1972). The following pH values were noted at the processing plant: potable water, pH 6.8–7.1; chiller water during processing (no treatment), pH 6.1–6.5; chiller water chlorinated to 20 ppm (prior to processing), pH 5.4–6.3; chlorinated chiller water during processing, pH 5.6–6.8. Bactericidal efficiency of chlorine dioxide is not pH dependent, and because it does not react with ammonia or nitrogenous compounds, chlorine dioxide is always available (White, 1972).

A free residual of 0.5–1 ppm chlorine is an effective bactericide for *E. coli* and the various species of Salmonella and Shigella (Smith and Martin, 1948). Table 3 shows that 34 ppm total available chlorine from a gaseous source (concentration based on total water volume, rotameter setting of 30) was required to maintain this level of free residual. An equivalent level of free residual was obtained with 5 ppm chlorine dioxide (based on total volume). Adequate free residual levels were found throughout the chiller when these amounts of Cl_2 and ClO_2 were injected (Table 3). Table 4 shows that in reducing total aerobic counts, fecal coliform levels and salmonellae incidence, 34 ppm chlorine and 5 ppm chlorine dioxide in chiller water were not significantly different, but that aerobic counts and fecal coliform levels differed significantly between morning and afternoon samples from either treatment (probably due to the increase in organic matter in the afternoon). Reductions in aerobic counts from untreated chiller water approached 1 log with either treatment; reduction of fecal coliforms with either treatment was over 2 logs; both fecal coliforms and salmonellae were reduced to nondetectable levels. These data show that equivalent microbiological results can be obtained with both oxidizing agents as long as comparable levels of free residual are present in the processing water.

The next phase of this study was to determine the bactericidal characteristics of chlorinated chiller water when the input and makeup supply of potable water, but not ice, to the chiller was maintained at 20 ppm (as may be required by USDA, 1978), and to determine the concentration of ClO_2 that would have a comparable bactericidal effect.

When chillers were filled with potable water containing 20 ppm available chlorine, some free residual was occasionally detected in the morning at the midway point, but not at the exit end of the chiller after processing had started. During the afternoon no chlorine residual was detected at either the mideway point or the exit end of the chiller (Table 3). However, a free residual of 0.2–0.3 ppm was found throughout the chiller when it was filled with potable water containing 3 ppm ClO_2 (Table 3). Table 4 shows that in reducing total aerobic organisms, fecal coliform levels or salmonellae incidence 20 ppm Cl_2 was not significantly different from 3 ppm ClO_2 but that aerobic counts in the morning differed significantly from those in the afternoon.

Table 4 shows that the four treatments did not differ significantly in lowering aerobic counts, but that the treatments with the higher levels of chlorine and chlorine dioxide were significantly more effective than the other two

Table 4—Microbiological data for chiller water treated with various levels of Cl_2 and ClO_2 (calculated on the basis of total volume)

Type and level of water treatment	Mean log_{10}/ml[a] Total aerobic count — Overall mean	a.m.	p.m.	Fecal coliform a.m.	p.m.	Salmonellae[b] Incidence No. +/total a.m.	p.m.
Untreated chiller water	3.41	3.40p	3.41p	2.34g	2.41g	13/30r	12/30r
Cl_2–34 ppm	2.61fc	2.50g	2.72h	<1k	<1k	0/22j	0/22j
ClO_2–5 ppm	2.69fd	2.49g	2.89h	<1k	<1k	0/24j	0/24j
Cl_2–20 ppm	2.89fe	2.76g	3.02h	1.02n	1.19n	4/28m	5/28m
ClO_2–3 ppm	2.68fd	2.41g	2.95h	1.32n	1.41n	7/24m	5/24m

[a] The same lower case letter (in columns and/or rows) follows values which do not differ significantly at the 5% level.
[b] All positive samples were very low in numbers (estimated by MPN procedure at <0.4/ml).
[c] Based on 44 samples taken 2 at each sampling time on each of 11 sampling days
[d] Based on 48 samples taken 2 at each sampling time on each of 12 sampling days
[e] Based on 52 samples taken 2 at each sampling time on each of 13 sampling days

treatments in reducing fecal coliform levels and salmonellae incidence. These data confirm that both oxidizing agents are effective bactericides for potentially pathogenic organisms if present as free residual at 0.5–1 ppm. These data also show that in poultry processing water, chlorine dioxide is as effective a bactericide as chlorine at about 1/7 the concentration. Lillard (1979b) showed that all treatments significantly reduced bacterial counts of carcasses and exteuded shelf-life over those chilled in untreated water although there was no significant difference in carcass counts among different treatment groups.

Chlorine dioxide is more expensive than chlorine gas. The current price of a 150-lb cylinder of chlorine gas is $55.00 or 36.67 cents/lb. The cost to the processor for using 34 ppm Cl_2 would be $3.67/8-hr shift and cost of 5 ppm ClO_2 would be $9.36/8-hr shift, as estimated by the vendor. To supply 20 ppm available chlorine in the freshwater input to one chiller for 8 hr in the plant, 6.04 lb of Cl_2 gas was required at a cost of $2.21. Equivalent results were obtained with 3 ppm ClO_2, and the requirement was 0.88 lb of ClO_2 generated from a small amount of precursor reacted with Cl_2 at pH 3–3.5. The cost of ClO_2 to a processor is determined by the amount used. Therefore, it is difficult to make an exact comparison of cost. However, from the above conditions, the vendor estimated the cost of chlorine dioxide for one chiller at $5.45/8-hr shift. One of the benfits of using 1/7 as much ClO_2 as Cl_2 to provide the same bactericidal effect is that the smaller amounts of ClO_2 are apt to be less corrosive to equipment than the larger amounts of chlorine. Furthermore, because the bactericidal efficiency of chlorine declines with increasing pH, chiller systems operating with more alkaline water than that used in this study could require greater amounts of chlorine than I have reported. Chlorine dioxide, on the other hand, maintains bactericidal efficiency even with increasing pH.

According to a recent article (Anon, 1979), a report by the Food Safety and Quality Staff (FSQS) states that there are no data to demonstrate that a 20-ppm chlorine solution would effectively combat the increased bacterial loads that would result when the intake level in poultry chillers is reduced.

The FSQS report states that, as water intake is reduced, organic materials in the water increases, and the bactericidal effect of the 20-ppm chlorine solution is nullified by the organic material. Data presented in this study show that chlorine is tied up by increasing levels of organic matter in the water but chlorine dioxide is not (compare a.m. vs p.m., Tables 2 and 3). Therefore, a reduced water-intake program could be implemented on the basis of a chlorine dioxide requirement, rather than a chlorine requirement, without undue concern for the accumulation of organic matter in the chiller water. The savings in cost of water and discharge would probably exceed the cost of chlorine dioxide in most plants.

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Ms received 5/12/79; revised 7/4/79; accepted 7/10/79.

The author acknowledges R.L. Wilson for the statistical analyses and Olin Water Services for providing the chlorine dioxide generating equipment for this study.

Mention of firm names does not imply endorsement of the products by the U.S. Department of Agriculture. No discrimination or preference is intended. The use of firm names and products is for identification only.

VARIATION AND REPEATABILITY OF AN UNTRAINED BEEF SENSORY PANEL

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ABSTRACT

Estimates were made of panel variation and of repeatibility for an untrained sensory panel using beef loins as the test product. Prior to testing, the beef loins were placed in one of three groups (tender, intermediate, tough) based on Warner-Bratzler shear value. The sensory panel evaluated each steak for juiciness, tenderness, connective tissue amount, flavor desirability and overall desirability using an eight-point scale. The accuracy of the untrained panel was not different from the accuracy of trained sensory panels. A considerable amount of variation was observed for individual judges, but this did not affect the accuracy of the panel as a whole. The repeatability of the panel and of individual judges was used as a measure of precision. The repeatability of the whole panel was much higher than the repeatability of the individual judges. Tenderness was found to be the most repeatable palatability characteristic evaluated. The panel as a whole and each individual judge were more repeatable or precise in their evaluation of nine samples per session than three samples per session. This indicates that no panel fatigue occurred during this study. Repeatabilities for the panel and the individual judges were found to be higher for the tougher loins than for more tender loins.

INTRODUCTION

SENSORY TESTS are accepted as objective measures of product characteristics even though they are based on subjective judgments by panelists (Harries, 1953). Because of the numerous selection procedures and training techniques, development of analytical panels for in-house evaluation of new products has become a widely accepted practice (Amerine et al., 1965; Martin, 1971; Cross et al., 1978). It is generally assumed that if a trained analytical panel cannot detect a difference between two samples, an untrained (or consumer) panel will not be able to detect a difference either (Hirsh, 1975b). Ehrenberg and Shewan (1953) noted that an untrained panel will, in general, score a product the same way as a trained panel; but this does not negate the need for trained panelists in qualitatively assessing product characteristics (Harries, 1953). Trained panels function as a human analytical instrument—trained to give precise and consistent sensory judgments; untrained panels measure consumer response using subjects representative of a target population.

Research conducted on evaluation of products such as dried eggs, dried milk, fruit juices, and processed potato products has identified tests which are best suited to various test objectives and those statistics which will estimate validity of test results. However, because of the inherent variability in fresh meat, the types of tests which may be used in its evaluation are limited.

Scalar scoring is the most common method used in analytical evaluation of variable foods like fresh meat (Weir, 1953; Alsmeyer et al., 1957). Interpretation of results of such tests is often difficult because of lack of standardization in conducting the studies and in reporting the findings.

Scalar scoring has been done with varying numbers of panelists (five, Alsmeyer et al., 1957; six, Berry et al., 1974; Davis et al., 1977; eight, Weir, 1953; and sixteen, Rust et al., 1971) and varying numbers of sample characteristics evaluated (ranging from two, Rust et al., 1971, to nine, Alsmeyer et al., 1957). Number of samples per session, order of presentation of samples (Eindhoven et al., 1964; Sidel and Stone, 1976), and method-length of panel training are important, yet were not mentioned in the experimental procedures of Alsmeyer et al. (1957), Rust et al. (1971), Berry et al. (1974) or Weir (1953).

An effort to establish guidelines for experimental meat cookery and sensory evaluation was recently made by the American Meat Science Association (Cross, 1977). The development of recommended guidelines for sensory evaluation and the reporting of sufficient detail in procedures will make it possible to: (a) allow a researcher to understand a study, judge the appropriateness of the procedures and evaluate the probable reliability of the results; (b) allow the study to be repeated; and (c) allow comparison of results of different studies. Nevertheless, a change in test objectives may require modification of a method for sensory evaluation which would preclude establishment of standardized methods that would be unnecessarily restrictive. Cross et al. (1978) have reported methods for testing and training panelists for sensory evaluation of meat quality; however, little data are available on the ability of untrained sensory panelists to measure meat quality.

The present study examines the accuracy and precision of untrained sensory panelists in the evaluation of steaks of varying tenderness levels.

Table 1—Order of sample presentation[a]

	Day				
Wk	Monday	Tuesday	Wednesday	Thursday	Friday
1	Tender-A	Tough-W	Tender-B	Tough-X	Intermed.-M
7	Tender-C	Tough-Y	Tender-D	Tough-Z	Intermed.-N
2	Tender-A	Tender-B	Tough-W	Tough-X	Intermed.-M
8	Tender-C	Tender-D	Tough-Y	Tough-Z	Intermed.-N
3	Tough-W	Tough-X	Tender-A	Tender-B	Intermed.-M
9	Tough-Y	Tough-Z	Tender-C	Tender-D	Intermed.-N
4	Tough-W	Tender-A	Tough-X	Tender-B	Intermed.-M
10	Tough-Y	Tender-C	Tough-Z	Tender-D	Intermed.-N
5	Tough-X	Tough-W	Tender-B	Tender-A	Intermed.-M
11	Tough-Z	Tough-Y	Tender-D	Tender-C	Intermed.-N
6	Tender-B	Tender-A	Tough-X	Tough-W	Intermed.-M
12	Tender-D	Tender-C	Tough-Z	Tough-Y	Intermed.-N

[a] Weeks 1–6, 3 steaks per day; Weeks 7–12, 9 steaks per day; Groups A, B, C and D contained loins of mean shear value less than 49N. Groups M and N contained loins of mean shear value greater than 49, but less than 66N. Groups W, X, Y and Z contained loins of mean shear value greater than 66N.

MATERIALS & METHODS

BEEF LOINS from 60 animals of USDA quality grades Prime to

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Table 2—Means and standard deviations of tenderness scores and Warner-Bratzler shear values for three loin groups

Loin group[a]	Mean shear[b]	S.D.	Mean tend.	S.D.
Tender-A	39.13	3.40	6.01	0.61
Tender-B	47.17	3.12	6.02	0.46
Intermediate-M	51.68	4.06	5.76	0.13
Tough-W	104.24	15.23	3.30	0.38
Tough-X	89.31	6.33	3.81	0.54

[a] Groups A and B contained loins of mean shear value less than 49.0N. Group M contained loins of mean shear value greater than 49.0N and less than 66.0N. Groups W and X contained loins of mean shear value greater than 66N.
[b] Shear values expressed in Newtons.

Table 3—Means and standard deviations of tenderness scores and Warner-Bratzler shear values for 9 loin groups

Loin group[a]	Mean shear[b]	S.D.	Mean tend.	S.D.
Tender-C	40.29	4.00	6.12	0.33
Tender-D	36.66	6.39	6.19	0.38
Intermediate-N	50.75	2.32	5.63	0.35
Tough-Y	86.96	19.76	4.64	0.53
Tough-Z	78.48	21.27	5.03	0.71

[a] Groups C and D contained loins of mean shear value less than 49.0N. Group N contained loins of mean shear value greater than 49.0N and less than 66.0N. Groups Y and Z contained loins of mean shear value greater than 66.0N.
[b] Shear values expressed in Newtons.

Table 4—Overall repeatability estimates and repeatability estimates for individual judges

Trait	Overall R score[a]	Individual judge R score 1	2	3	4	5	6	7	8	9	10
Juiciness	0.51	0.26	0.05	0.04	0.07	0.14	0.16	0.28	0.20	0.12	0.06
Tenderness	0.56	0.58	0.62	0.52	0.54	0.55	0.43	0.54	0.46	0.57	0.44
Connective tissue amount	0.38	0.45	0.63	0.57	0.45	0.38	0.38	0.54	0.34	0.64	0.47
Flavor desirability	0.40	0.38	0.15	0.35	0.30	0.21	0.00	0.04	0.39	0.18	0.14
Overall desirability	0.36	0.46	0.48	0.54	0.53	0.42	0.15	0.38	0.46	0.55	0.39

[a] R = repeatability estimate; Overall R score is the repeatability estimate of all judges combined.

Canner were obtained from commercial sources. Each loin was cut into steaks 2.5–3.0 cm in thickness. Steaks were wrapped in polyethylene-coated freezer paper and held at 0°C prior to use.

Nine steaks were obtained from each loin and these were numbered in order from anterior to posterior. The loins were placed into one of three tenderness groups based on Warner-Bratzler shear value means of steaks 1, 5 and 9. The three groups consisted of a tender group containing 24 loins with mean shear force values less than 44N, an intermediate group of 12 loins with mean shear force values between 49 and 66N, and a tough group of 24 loins with mean shear values greater than 66N.

Six loins from the tender group, six loins from the tough group and three loins from the intermediate group were served during the first six weeks of the experiment. Eighteen loins from the tender group, 18 loins from the tough group and 9 loins from the intermediate group were served during the second 6-wk period of the experiment. No loins were common to both 6-wk periods. The serving order for both of the 6-wk periods is given in Table 1.

As nearly as possible, steaks for each session were from the same anatomical location to minimize within-loin variation.

Cooking and sampling procedures

All steaks were thawed in a 6°C cold room for 24 hr prior to cooking to facilitate the insertion of thermocouples. Each steak was broiled to a final internal temperature of 70°C on a preheated, "Farberware" electric grill at approximately 250°C. Following cooking, each steak was boned out. If the steak was to be used for Warner-Bratzler shear determination, the steak was allowed to cool to room temperature. Four 1.27 cm cores were obtained and each core was sheared twice. If the steak was presented to the sensory panel, ten 1.27 cubes were obtained. One cube was randomly selected and evaluated by each panelist. Panel members independently scored each sample according to 8-point rating scales (8=extremely juicy, extremely tender, no organoleptically-detectable connective tissue, extremely desirable flavor and extremely desirable overall palatability; 1=extremely dry, extremely tough, abundant organoleptically-detectable connective tissue, extremely undesirable flavor and extremely undesirable overall palatability).

Selection and orientation of panelists

The sensory panel consisted of 10 undergraduate Animal Science students. Age, sex or experience in sensory evaluation was not considered in selection of the panelists. These 10 students met for eight simulated panel sessions to allow them to become accustomed to the physical conditions and basic procedures involved in sensory evaluation. These sessions also allowed the panelists to become acquainted with the scoring system used and enabled them to understand the characteristics they were asked to evaluate. Open discussion of sample characteristics and the scoring method was allowed during these orientation sessions, but no samples were evaluated, thus no formal training occurred.

Statistical analysis

All data obtained in this study were analyzed through the facilities of the Data Processing Center of Texas A&M University. Means and standard deviations were computed. Analysis of variance was conducted on all loins, judges and groups of loins. Repeatability estimates were derived from analysis of variance tables using the procedure outlined by Sawyer et al. (1962).

The correlation of mean Warner-Bratzler shear values and mean tenderness values was computed.

RESULTS & DISCUSSION

THE INTERPRETATION of results from sensory panel evaluation has always presented the researcher with many difficulties. One must determine the precision and the accuracy of the results before they can be termed valid. A discussion of the precision and accuracy of the sensory measurement of tenderness in this study and the statistical methods which were used to establish them will be undertaken in this section. Because of the different methods involved, panel accuracy and panel precision will be discussed separately.

Accuracy

Accuracy is statistically defined as a measure of correctness. The most commonly used measure of sensory panel accuracy is the simple correlation coefficient between the sensory scores and some mechanical measurement of a specified trait, usually tenderness. Tables 2 and 3 show the mean sensory panel tenderness scores and the mean Warner-Bratzler shear values for the 3-loin and 9-loin groups used in this study. The correlation coefficient between sensory panel tenderness and Warner-Bratzler shear values for the 60 animals was -0.80 ($P < 0.01$), which indicates good agreement between the two measures. Earlier studies using

Table 5—Repeatability estimates when three samples per session were evaluated

Trait	Loin group[a]				
	A	B	M	W	X
Juiciness	0.18	0.06	0.39	0	0.004
Tenderness	0.50	0.64	0.04	0.51	0.71
Connective tissue	0.49	0.45	0.05	0.66	0.48
Flavor desirability	0.20	0.26	0.60	0.49	0
Overall desirability	0.47	0.46	0.07	0.35	0.58

[a] Groups A and B contained loins of mean shear value less than 49.0N. Group M contained loins of mean shear value greater than 49.0 and less than 66.0N. Groups W and X contained loins of mean shear value greater than 66.0N.

Table 6—Repeatability estimates when nine samples per session were evaluated

Trait	Loin group[a]				
	C	D	N	Y	Z
Juiciness	0.44	0.50	0.38	0.33	0.42
Tenderness	0.59	0.62	0.56	0.66	0.80
Connective tissue amount	0.64	0.65	0.40	0.67	0.80
Flavor desirability	0.54	0.18	0.37	0.41	0.59
Overall desirability	0.69	0.78	0.59	0.32	0.41

[a] Groups C and D contained loins of mean shear value less than 49.0N. Group N contained loins of mean shear value greater than 49.0N and less than 66.0N. Groups Y and Z contained loins of mean shear value greater than 66.0N.

trained sensory panels have shown correlations between Warner-Bratzler shear and sensory panel tenderness ranging from −0.60 (Sperring et al., 1959) to −0.88 (Hostetler and Dutson, 1978) for loin steaks broiled to the same final internal temperature. Ehrenberg and Shewan (1953) reported that untrained panelists, in general, scored the samples in the same way as a trained panel. The high correlation found between sensory panel tenderness and Warner-Bratzler shear values in the present study supports this theory.

Sather et al. (1963) suggested that a wide range in shear force values might be necessary for correlation coefficients between shear values and sensory panel scores to be high. Moreover, a wide range in tenderness may also be necessary to allow a sensory panel to more objectively assess the tenderness of any one sample. A wide tenderness range would present a more varied reference experience to the sensory judge. Increased objectivity would then be reflected as a wider range in sensory scores, which would also increase correlation coefficients. In the present study, both shear force values and sensory scores showed a wide range of values (Tables 2 and 3).

Precision

Precision may be defined as the consistency or exactness with which a specific sensitivity is repeatedly demonstrated (Hirsh, 1975a). One measure of precision which may be used in evaluating sensory panels is the repeatability estimate, R, which is widely used by animal breeders to predict performance ability. Repeatability is defined as the intraclass correlation of repeated measurements, or the measure of the constancy of repeated observations by a given judge (Sawyer et al., 1962). The estimation of repeatability used in this study is an adaptation of the formula given by Kempthorne (1957). R was estimated directly from the analysis of variance (ANOVA) for each loin, as follows:

Source of variation	d.f.	Mean square	Expectation of mean sq
Total	js-1		
Judge	j-1	M_j	$\sigma_e^2 + 6\sigma_j^2$
Steaks	s-1	M_s	$\sigma_e^2 + 10\sigma_s^2$
Residual	(j-1)(s-1)	M_e	σ_e^2

J = judges; s = steaks.

For each judge, R is estimated by:

Source of variation	d.f.	Mean square	Expectation of mean sq
Total	sℓ-1		
Loins	ℓ-1	M_ℓ	$\sigma_s^2(\ell) + 6\sigma_\ell^2$
Steaks within loins	ℓ(s-1)	$M_{s(\ell)}$	$\sigma_s^2(\ell)$

s = steaks; ℓ = loins.

and $R = \sigma_\ell^2 / [\sigma_\ell^2 + \sigma_s^2(\ell)$, where steaks within loins is the error component of variance.

An individual analysis of variance was done for each of the five palatability characteristics evaluated by the sensory panel for each of the 60 loins. In general, tenderness was the most repeatable trait (consistently high R values), while flavor desirability was the least repeatable. Connective tissue amount (CTA), juiciness and overall desirability showed wide ranges of values with no definite trends. Each R value was based on 60 scores (10 judges' scores on 6 steaks from each loin). The low repeatability for juiciness could have been due to variation among the steaks in each loin, cooking differences or lack of panelist precision.

The total repeatability for the five palatability traits (10 judges' scores on 6 steaks from each of the 60 loins) is shown in Table 4, along with the R values for each individual judge. The R values for tenderness are again the highest and have a smaller range of values than the other characteristics. CTA and overall desirability were relatively repeatable even though both show a wide range of values. It is interesting to note that although the total repeatabilities for juiciness and flavor desirability are higher than those for CTA and overall desirability, the R values for the individual judges are very low. This indicates that although no single individual scored the trait consistently, the panel as a whole, was consistent. The fact that no individual judge was consistent in scoring is possibly a reflection of inadequate replication and/or insufficient training for a particular palatability trait. Larger numbers of panelists would not necessarily reduce inconsistent scoring, but because the computations usually involve division by N (number of panelists), there is the impression that consistency is improved by increasing the number of panelists.

Tables 5 and 6 show the repeatability estimates for the loin groupings used in this study. As has been previously noted, tenderness was the most repeatable trait, followed by CTA and overall desirability. With three samples per session and nine samples per session, the tough loins (group W, X, Y and Z) had the highest repeatabilities while the intermediate tenderness groups (groups M and N) had the lowest. It is possible that panelists find it easier to identify and score samples that are undesirable.

In reviewing the repeatability estimates for each judge for each group of loins, it was observed that there was an increase in the R values when nine loins were served, with tenderness being the most repeatable trait (highest R value). This may indicate that the larger number of samples pre-

Table 7—Repeatability estimates for loins by class

	Class				
Trait	1[a]	2[b]	3[c]	4[d]	5[e]
Juiciness	0.37	0.38	0.66	0.36	0.18
Tenderness	0.50	0.77	0.79	0.61	0.64
Connective tissue amount	0.38	0.65	0.76	0.44	0.71
Flavor desirability	0.32	0.59	0.57	0.39	0.44
Overall desirability	0.39	0.72	0.79	0.45	0.50

[a] Class 1 contained 19 loins with mean shear values less than 44.0N.
[b] Class 2 contained 20 loins ranging in shear values from 44.0N to 66.0N.
[c] Class 3 contained 12 loins ranging in shear values from 66.0N to 89.0N.
[d] Class 4 contained five loins ranging in shear values from 89.0N to 111.0N.
[e] Class 5 contained four loins with mean shear values greater than 111.0N.

sented in each session enabled the judges to more consistently score each loin. The magnitute of the correlation coefficient is greatly influenced by the degree of difference between the products. Presentation of only three samples per session, coupled with a lack of experience, did not allow the judges to be as accurate as they were with an increased number of samples. The higher R values for the 9-loin groups (9 samples per session) also contradict the idea of sensory panel fatigue within a session. Daily observation of the panel gave no indication of panel fatigue.

To look more closely at the effect of tenderness on the repeatability of individual judges and the panel as a whole, the 60 loins were divided into five classes according to mean shear values. The results of these analyses are shown in Table 7. Class 1 was the least repeatable of the five. The highest R values were those for Class 3. These loins contained most of the loins in groups W, X, Y and Z, which also showed higher repeatabilities than the other six groups. It is interesting to note that the number of loins in each class seemed to have no effect on the R values.

CONCLUSIONS

THE VARIATION of scores on consecutive steaks from 60 loins was estimated for a 10-member, untrained sensory panel. Analysis of variance, mean separation and repeatability estimates were calculated for each loin, each judge and two groupings of loins.

The correlation between sensory panel tenderness scores and Warner-Bratzler shear was found to be −0.80. This is in the upper portion of the range of correlation coefficients reported for trained sensory panels.

Repeatability estimates showed that individual judges and the panel as a whole were more consistent in scoring the tough loins. Repeatability also increased when the number of samples per session was increased from three to nine. No evidence of panel fatigue was observed with the increased sample number.

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Ms received 6/10/78; revised 5/12/79; accepted 5/20/79.

Technical Article No. 14472 from the Texas Agricultural Experiment Station.

LOW-TEMPERATURE AIR OVEN VS A WATER BATH FOR THE PREPARATION OF RARE BEEF

E. M. BUCK, A. M. HICKEY and J. ROSENAU

ABSTRACT

Paired muscles were used to assess the merits of a water bath method for the preparation of rare beef as compared to a conventional oven using a long-time, low-temperature process. Samples prepared in a water bath were more uniformly rare in cross-section, had significantly greater cooked yields, and were significantly more tender than oven-cooked roasts. A sensory panel found water bath samples to be significantly more tender and preferred them overall to oven-cooked samples. In addition to the above advantages, the more rapid rate of heat penetration and the potential for reduced energy requirements suggest that the water bath procedure should be examined in greater depth with an eye towards more widespread use.

INTRODUCTION

LOW-TEMPERATURE COOKING of meat with its associated increases in tenderness and yield has been of great interest to researchers since Cover (1937) first reported that well-done roasts were more tender when cooked at 124°C than at 225°C. Many workers have since investigated the effects of low-temperature, and consequently longer time, cooking processes on the roasting of meat (Laakkonen et al., 1970; Funk et al., 1966; Bramblett and Vail, 1964; Toumy and Lechnir, 1964; Marshall et al., 1960). Machlik and Draudt (1963) reported a marked decrease in shear value of beef heated to a temperature of 56–59°C compared to beef heated to 66–70°C where hardening of the tissue became substantial and was not resolved until temperatures approached 80°C. This hardening effect, caused by denaturation of sarcoplasmic and myofibrillar proteins, undoubtedly accounts for the decreased tenderness associated with roasts and steaks cooked by dry heat methods beyond the rare or medium rare state.

There is ample evidence to suggest that quality and yield of roasted meats would be improved if cooking temperatures were lowered; however, there are practical limits below which one cannot go without seriously increasing microbiological risks and costs due to the increased heating time. It is well known that when water is used as the heating medium for cooking meat instead of air, the value of the heat transfer coefficient is on the order of 20 times greater. Use of water is required for the production of braised or stewed product; However, it is normally not suited for the production of a rare roast beef-type product. In recent years several purveyors to the hotel, restaurant, and institutional trade have developed a water bath method of producing rare beef by using very low bath temperatures. The meat is protected from actual contact with the water by placing it in a vacuumized bag. Oven-roasted color, flavor and appearance is produced by rubbing the roast with a blend of seasonings and spices prior to bagging.

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0022-1147/79/0006-1602$02.25/0
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A review of the scientific literature uncovered no information relative to the commercial processing of rare roast beef in a water bath. Several workers (Laakkonen et al., 1970; Howard and Judge, 1968; Marsh et al., 1966; Thomas et al., 1966) have heated meat samples in a water bath where the meat was protected from direct contact with the water by a plastic film or similar material; but none suggested a commercial application for the procedure.

Among the many potential advantages of a water bath method of cooking meat are: increased rate of heat penetration with resultant increases in microbiological safety and reduction in energy cost; increased tenderness and cooked yield; more precise control over degree of doneness; more uniform degree of doneness; and reduced cooking area ventilation requirements.

This study was undertaken to assess the merits of a water-bath method of beef cookery as compared to preparation in a conventional air oven using a long-time, low-temperature process, and to determine the suitability of such a procedure for use at point of consumption, i.e., in a restaurant or in a home. In addition, the information generated will prove valuable in future studies relative to microbiological safety, holding times after cooking, and overall development of the procedure.

EXPERIMENTAL

Source of experimental material

Nine trials were conducted with paired samples prepared from contralateral longissimus, semimembranosus, semitendinosis, biceps femoris, and rectus femoris muscles excised from both sides of three steer and one heifer carcass prepared in the University Meats Laboratory. Carcasses weighing 220–252 kg and judged to be equivalent to USDA Good grade in quality were aged 10–14 days at 2°C prior to removal of the paired muscles. A pair of samples for each trial was prepared from the same location on contralateral muscles removed from the right and left sides of a carcass. Although roasts used in the various trials varied in shape and wieght (1–4 kg), care was taken to ensure that the roasts within a pair represented the same location in contralateral muscles and were identical in terms of weight and geometry. Paired roasts were placed in polyethylene bags, vacuumized, frozen and stored at −20°C for 3–5 months until required for experimentation. Two days prior to the initiation of a trial, paired roasts were thawed in a 4°C refrigerator. The internal temperature of all roasts was at 4°C upon initiation of each trial.

Cooking procedures

Right and left muscles from each pair of samples were randomly assigned to each of the two treatments. Samples for oven roasting were placed on a wire rack in an open shallow roasting pan, and cooked in a conventional household-type air oven set at 94°C and cycling from 88–99°C. Samples from the logissimus dorsi were placed dorsal side up on the rack. Those from the semimembranosus were placed proximal side up, those from the semitendinosus and the biceps femoris were placed distal side up, while rectus femoris samples were placed anterior side up. Oven temperature and sample temperature were monitored with copper-constantan thermocouples, one located in the center of the oven, and the other located in the approximate geometric center of the sample. The sample was removed from the oven when its internal temperature reached 59.5°C. After removal, samples reached a final internal temperature of 60–60.5°C.

Samples to be cooked in the water bath were first browned on all sides by rotating the meat for a total of 1 min on the surface of a griddle maintained at 120°C. The samples thus browned on all

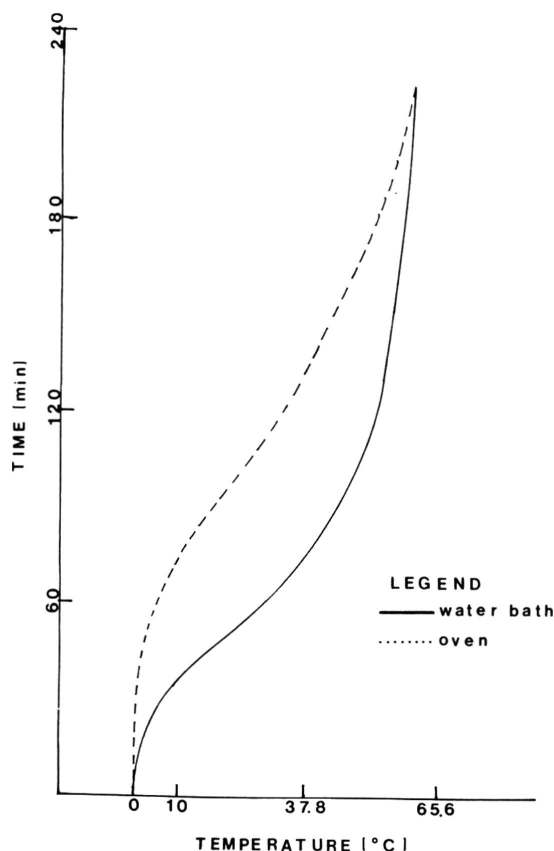

Fig. 1—Typical heating curves for low-temperature oven and water-bath cooked rare roast beef.

sides were immediately placed into heat stabilized nylon 66 bags, a thermocouple was placed in the approximate geometric center of the roast, and the bag was sealed under vacuum. The bagged meat was then completely submerged in a water bath operating at a temperature of 60–61°C. Water in the bath was constantly agitated by a mechanical stirrer to ensure uniform heat distribution, and water temperature was monitored with a themocouple. Bagged samples were removed when their internal temperatures reached 60°C.

The nylon bags used comply with applicable FDA regulations for food use and are extremely shrink resistant in hot water since they are commonly used for cooking at normal oven temperatures.

pH Determination

Each pair of samples was trimmed to a uniform size and weight just prior to cooking and three 10-g samples of trimmings were each blended with 100 ml of distilled water according to the method of Rogers et al., (1967). Triplicate pH readings were made on the slurries thus obtained. After cooking, a similar procedure was used on samples obtained from slices removed for that purpose.

Shear and press fluid values

Each roast was cut in half perpendicular to the long axis of its muscle fibers and a 2.3-cm slice was taken from each cut surface for pH determination. A 2-mm thick slice was then removed from each surface for press fluid determinations and a 4-cm slice was removed for shear measurement.

Three cores, 1.3 cm in diameter and parallel to the muscle fibers, were removed from each 4 cm thick slice. One core was taken directly under the dorsal, proximal, distal or anterior side of the roast depending upon the muscle used, one from the center of the slice, and one next to the outer surface opposite the first core. Each core was sheared in half perpendicular to its long axis; then each half thus produced was sheared again in half on a Warner-Bratzler shearing apparatus with a 25 kg dynamometer.

Similar sized cores for press fluid determinations were removed from the same three locations on the 2 mm thick slices. A Carver press exerting 900 kg per sq in. pressure was used to extract the fluid from samples weighing approximately 0.5g, according to the 1-min method of Sanderson and Vail (1963).

Sensory data

The sensory panel consisted of six volunteer graduate students who were semi-trained by their participation in several sessions during which they were familiarized with test procedures, sensory techniques, and terminology. Panelists were also given an opportunity to physically compare extremes of the attributes to be measured. Only individuals who did not find rare meat objectionable were used on the panel.

Panelists evaluated samples as to tenderness and juiciness using an 8-point descriptive scale where 8 was extremely tender or juicy and 1 was extremely tough or dry. There was no neutral category. Panelists were also asked to rate overall product acceptability by rating a sample on an 8-point Hedonic scale where 8 was like extremely and 1 was dislike extremely.

Seven trials for sensory evaluation were conducted using roasts prepared separately from those used in the first part of this study. Six slices 1.3 cm thick were removed from the center of each roast with a commercial slicer, perpendicular to the long axis of the muscle fibers. Two adjacent 1.3-cm cubes were then removed from each slice in three locations similar to those used for shear measurements. Each slice yielded two samples or cubes from each of the three locations for a total of six cubes. The six slices thus produced a total of 36 cubes per roast. Panelists made three judgments on each of two cubes, one from an oven cooked roast and the other from the identical location in the water bath prepared roast. This procedure was repeated two more times to account for the three locations sampled. The entire evaluation was then replicated. Warm samples were used and were presented on prewarmed white ceramic plates.

Two 1.3 cm thick slices were also removed for objective shear measurements which were done on two cores removed from the center of each slice.

Analysis of data

A factorial design was employed with analysis of variance used to determine the effects of cooking methods on tenderness, juiciness, yield, and overall acceptability. Variations in sample size and shape among trials was accounted for statistically in the analysis.

RESULTS & DISCUSSION

Cooking procedures

Oven-cooked roasts required, on the average 73.17 min per pound to reach an internal temperature of 60°C, whereas water-bath cooked roasts required 81.03 min per pound. The initial increased rate of heat penetration obtained with the water-bath method is clearly shown in Figure 1. When both roasts are started at the same time, the water-bath cooked roast typically shows a significant increase in center temperature approximately 45 min before the oven cooked roast. It would then heat at a much more rapid rate reaching 38°C approximately 45–50 min before the oven-cooked roast. Heating rate in the water-bath roast begins to slow as an internal temperature of 49°C is reached, however, and considerable time advantage is lost towards the end of the process when the water-bath cooked roast frequently requires 1 hr, or more, to achieve the last two degree rise in internal temperature to 60°C. This suggests, of course, that a higher bath temperature would be desirable; however, further work is needed to determine the optimal bath temperature which would decrease total cooking time without significantly decreasing yield or tenderness.

The microbiological safety of any low temperature cooking process must always be considered, especially with meat. The microbiological benefits of the water-bath process, if any, are presently under study in this laboratory; however, some preliminary conclusions may be drawn from a review of the current literature. In a recent study conducted by an independent research organization (Anon. 1977), beef rounds were inoculated with a mixture of seven Salmonella species, placed in vacuumized cooking bags, and cooked in a steam sparged water bath pre-calibrated to

Table 1—Average pH, cooking losses, shear values, and press fluid yields for paired muscles cooked by two methods

	pH		Cooking losses[a]			Shear values[c]				
	Before cook	After cook	Volatile losses[b]	Recovered drippings	Total cooking losses	Pt. 1	Pt. 2	Pt. 3	$\bar{X}$	Press fluid[d]
Oven roast	5.48	5.56	11.62	2.19	13.81	3.24	3.53	4.99	3.92	51.72***
Water bath	5.52	5.58	1.39**	9.32**	10.71**	2.54	3.05	2.92	2.84**	54.22

[a] Expressed as percent of uncooked roast
[b] Losses for water bath samples occurred during searing prior to cooking.
[c] Expressed as kg of force required to shear a sample 1.3 cm in diameter
[d] Expressed as percent of cooked roast weight (represents only 5 trials)
** Significantly different ($P < 0.01$)
*** Significantly different ($P < 0.10$)

Table 2—Average shear values and panel scores for tenderness and juiciness of rare beef roasts prepared in an oven or a water bath

	Tenderness[a]				Juiciness[b]				
	Pt.1	Pt.2	Pt.3	$\bar{X}$	Pt.1	Pt.2	Pt.3	$\bar{X}$	Shear values[c]
Oven	5.1	4.1	4.1	4.4	5.8	6.4	6.0	6.1	3.47
Water bath	6.0	6.1	6.3	6.1**	6.0	6.1	5.9	6.0	2.52*

[a] Eight-point descriptive scale (8 = extremely tender and 1 = extremely tough)
[b] Eight-point descriptive scale (8 = extremely juicy and 1 = extremely dry)
[c] Expressed as kg of force required to shear a sample core 1.3 cm in diameter
** Significantly different ($P < 0.01$)
* Significantly different ($P < 0.05$)

73.89°C. Rounds were removed from the bath when they reached various internal temperatures between 48.89–62.78°C and held, at similar temperatures, for varying periods of time up to 7 hr. It was found in this study that a final internal temperature of 51.67°C combined with 7 hr holding at that temperature was inadequate for the safe processing of beef since low levels of survivors could be detected under these conditions. A final internal temperature of 57.22°C was deemed safe if the product was held for at least 3 min at that temperature. Final internal temperatures over 57.22°C were considered safe even if 10 million Salmonella were present initially.

It would seem safe to conclude then that beef cooked in a water bath to an internal temperature of 60°C, as was done in our study, presents no problems with regard to Salmonella. One could not assume, however, that the oven-cooked roasts, assuming contamination, were also free of viable Salmonella since there is evidence to suggest that surface and near surface portions of dry-heat cooked roasts have lower water activity than interior portions, as a result of drying and crust formation during cooking, and that this contributes to the thermal tolerance of contaminants (Blankenship, 1978).

In recent years there has been considerable interest shown in *C. perfringens* as a primary agent in food borne illness. This organism is of special interest because it is frequently found as a contaminant in raw beef and because of its ability to grow rapidly at realtively high temperatures. In a recent study by Willardson et al. (1978), the growth and survival of *C. perfringens* was studied during constantly rising temperatures and it was found that temperatures above 51°C became increasingly inhibitory and that inactivation of *C. perfringens* began at approximately 55°C. As the temperature rose above 55°C, inactivation occurred at an increasing rate. The authors demonstrated that minimal exposure to temperatures near 60°C may not be sufficient to eliminate a vegetative cell population of *C. perfringens* and further stated that the potential exists for growth during certain low-temperature long-time cooking practices.

Holding for some period of time at 60°C would inactivate vegetative populations; however, the average cooking process contains too many variables, and there are insufficient data available at present to say with any degree of certainty, that use of a water bath cooking process as described in this paper will assure product free of *C. perfringens*, assuming a large initial population was present prior to cooking.

Examination of the data and heating curves do suggest, however, that the water-bath method presents some advantages over the low-temperature oven since water-bath cooked roasts have generally higher rates of heat penetration and spend less time in the temperature range 23.9–51.7°C, the zone of the most rapid growth. Also, the surfaces of water-bath cooked roasts remain moist contributing to increased heat sensitivity of the microorganisms which might be there.

After cooking, water-bath cooked roasts were uniformly rare in appearance when viewed in cross-section, as compared to the oven-cooked roasts which had rare centers but tended to become more well done towards the outer surfaces.

Total cooking losses expressed as a percent of the uncooked roasts are shown in Table 1. Total cooking losses ranged from 10.07% to 17.50% for oven-cooked samples, and from 7.61% to 14.19% for water-bath cooked samples. The percentage of cooking losses was consistently less for roasts prepared in the water bath, and these differences proved to be statistically significant at the 0.01 level.

Water-bath cooked roasts were browned before cooking to develop a characteristic roast appearance and to impart a more desirable roast flavor to the recovered drippings. This procedure resulted in an average loss of 1.39% of original weight due to evaporation of water and some slight rendering of external fats. All other cooking losses were recovered in the bag after cooking. Although recovered drippings are not considered part of the yield of a cooked roast, the water-bath method does offer the added advantage of making available for the preparation of gravy or for other uses, all of the drippings which are normally lost in a dry-heat cooking process. The percent recovered drippings for wa-

ter-bath samples was significantly greater ($P < 0.01$) than for the oven roasts.

The increased yield of cooked meat from water-bath prepared samples in this study is consistent with that found in commercial water-bath cooking operations (Anon., 1976).

pH, shear values and press fluid determinations

The pH of all roasts was monitored before cooking as a measure of post-slaughter tissue glycolysis with its resultant effects upon water-holding capacity. All samples were found to be within a normal range (Table 1). After cooking, pH values rose slightly with both treatments. This phenomenon has been reported by several workers (Vollmar et al., 1976; Laakkonen et al., 1970; Schock et al., 1970). Statistical analysis revealed no significant differences in pH values between treatments.

Tenderness, as measured by Warner-Bratzler shear values did not differ significantly between the two sample points within each roast; however, there was a highly significant difference between cooking methods and core location (Table 1). The greater overall toughness and wider spread in shear values from point-to-point found within over-cooked roasts is undoubtedly due to the variation in heat treatment. No part of a water-bath cooked roast was ever subjected to a temperature higher than 61.1°C while portions of oven-cooked roasts were heated above this level as a result of higher ambient cooking temperatures and differences in heat transfer rates between lean and fatty tissues. Several workers have demonstrated the higher shear values associated with increases in temperature (Machlik and Draudt, 1963; Tuomy and Lechnir, 1964; Bramblett et al., 1959; Bramblett and Vail, 1964). As measured objectively, water-bath cooked roasts presented a more uniform tenderness in cross-section.

Some of the increase in tenderness associated with water-bath cooked samples may have resulted from the fact that these roasts were held in a temperature range conducive to enzymatic tenderization for a longer period of time than oven-cooked samples. Further work is needed to determine if changes in tenderness are more closely related to decreased protein hardening or increased collagen solubilization.

Water-bath cooked roasts yielded significantly greater ($P < 0.10$) quantitites of press fluid than oven cooked roasts. This would be expected since water-bath cooked samples experienced significantly decreased cooking losses.

No attempt was made in this study to demonstrate the differences in shear values, or other factors, found among the different muscles used since these variations have been well documented in the literature. Treatment differences when applied to similar sample pairs was the primary purpose of the investigational design.

Sensory evaluation

Taste panel scores for tenderness differed significantly with cooking method ($P < 0.01$) (Table 2). Water-bath cooked roasts were significantly more tender and were also more uniformly tender as measured at the three sampling points. There were differences in tenderness within oven-cooked roasts with samples from point 1 proving to be significantly more tender than the other two points. There were no significant positional differences found in water-bath cooked roasts.

Warner Bratzler shear measurements taken at the centers of roasts used in sensory evaluation proved to be significantly different ($P < 0.05$) with water-bath roasts being more tender.

Panel scores for juiciness did not differ significantly between the two cooking methods used (Table 2). Since water-bath cooked roasts had significantly smaller cooking losses than oven-cooked roasts, it would normally be expected that a sensory panel would identify them as juicier. However, correlation coefficients for cooking losses vs juiciness scores are usually low. Two possible explanations for the panel's failure to detect a difference are offered. It is well known that there is an increasing shift from bound to free water as the internal temperature of meat is raised, resulting in greater moisture losses for those products cooked to higher tmeperatures (Ritchey, 1965). It seems possible that in the oven-cooked roasts there could have been a change in the physical properties of the remaining moisture which resulted in the panel scoring those samples as juicier. Another possibility is that the panel was permitting the sensation of juiciness to enhance its tenderness measurement thereby actually scoring some of the juiciness as tenderness.

When asked to compare water-bath and oven-cooked roasts for overall acceptability using an 8-point hedonic scale, the panel rated water-bath roasts significantly higher ($P < 0.01$) scoring them 5.9 on the average as compared to 4.7 for oven-cooked roasts.

The results of this study suggest that a water bath can be used effectively to prepare rare beef with the significant added advantages of greater cooked yield, tenderness, and overall acceptability. Increased rates of heat penetration permit use of lower bath temperatures and consequently production of a more uniformly rare product. Evidence in the literature suggests that the microbiological safety of the bath process in not impaired by use of lower temperatures since the more uniformly moist environment increases the heat sensitivity of microorganisms. Certainly more definitive information on the safety of the process is required before it can be recommended for widespread use. It does, however, offer some interesting potential.

Presently, a water-bath process is being used commercially to produce a ready-to-serve, rare beef for use by the hotel, restaurant, and institutional trade. The product is frequently delivered frozen in the same bag in which it was cooked. The simplicity of the procedure used in this study, suggests that it could be adapted for use by hotels, restaurants, and other food service organizations, for on-site preparation of rare beef and eventually many other meat and food products. Use of such a process could reduce equipment costs, energy requirements, and kitchen ventilation requirements.

Toumy et al. (1964) found that the color of cooked meat depended on the temperature rather than time of exposure and they reported that meat samples still appeared rare in color after 7 hr at 60°C. This suggests that use of a water-bath operating at the same temperature as the desired final degree of doneness would permit holding for up to 7 hr, or more, once cooking was complete. From a practical standpoint, this means that roasts could be prepared by inexperienced help many hours before they were actually needed for serving without becoming overdone, drying out, or losing the deisred color. With respect to vegetative cells, holding roasts in the bath at 60°C could even increase the microbiological safety of the process since Brown and Twedt (1972), working with various species of Salmonella, Staphylococcus, and *C. perfringens* demonstrated that populations of Salmonella and Staphylococcus decreased rapidly above 48.8°C and destruction of *C. perfringens* occurred above 51.1°C.

Although at the present time water-bath procedures for cooking rare meat should be restricted to those individuals with sufficient experience to guarantee the safety of the product, the method is certainly worthy of more investigation which would permit more widespread use.

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STORAGE STABILITY, PALATABILITY AND HISTOLOGICAL CHARACTERISTICS OF MECHANICALLY TENDERIZED BEEF STEAKS

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ABSTRACT

USDA Choice and Utility grade boneless beef loins were used to determine the palatability, bacteriological and histological characteristics of steaks mechanically tenderized. There was a significant improvement in taste panel ratings of tenderness for the Utility tenderized steaks, but only a small improvement ($P > 0.05$) for the Choice tenderized steaks. None of the total aerobic bacterial surface counts or the interior counts for total aerobic, psychrotrophic or anaerobic bacteria was significantly different between tenderized and nontenderized steaks compared at 1, 2, 5 and 10 days of storage at 5°C. Histological studies showed a definite pattern of blade penetrations in the tenderized samples at low magnifications. At very high magnifications, the muscle fibers were torn and fragmented by the blade rather than evenly cut.

INTRODUCTION

ONE OF THE NEWER and widely applied advances in tenderization of meat is the use of the blade-type mechanical tenderizer. Mechanical tenderization can improve tenderness of meat cuts that are traditionally too tough for acceptable consumer consumption.

Blade-type mechanical tenderization of beef, pork and lamb cuts has been shown to significantly increase their tenderness as measured by Warner-Bratzler shear (Goldner et al., 1974; Schwartz and Mandigo, 1974; Glover et al., 1975; Bowling et al., 1975, 1976). Glover et al. (1975), Davis et al. (1975) and Tatum et al. (1976) reported that blade-tenderized beef loin steaks and round roasts sustained greater cooking losses than their controls. Goldner and Mandigo (1974), Schwartz and Mandigo (1974) and Bowling et al. (1975, 1976), however, reported no significant difference in cooking loss attributable to mechanical tenderization of beef, pork and lamb cuts.

Very limited work (Boyd et al., 1978) has been reported concerning the effects of blade-type mechanical tenderization on the storage stability of the tenderized product, and no work has been reported on the histological characteristics of the tenderized muscle tissue.

This research was therefore conducted to determine the effects of blade-type mechanical tenderization on the storage stability, histological characteristics and palatability traits of beef steaks.

EXPERIMENTAL

Tenderizing

A commercial blade-type tenderizer, the Bettcher Tend-R-Rite Model TR2, was used at different conveyor speeds to control the tenderization level. For a tenderization level of 1X the belt advances 7.62 cm per plunge, for 2X it advances 5.08 cm per plunge, and for 3X it advances 2.54 cm per plunge. The tenderizing head consists of 609 spear-shaped blades arranged in an 8 × 30 cm configuration. With the belt adjusted so that it advances 2.54 cm per plunge (3X), an average penetration density of approximately eight penetrations per cm^2 is obtained. For a tenderization level of 3X, the conveyor belt speed was set at 2.54 cm per plunge, and the boneless beef top loin was placed fat side down and passed through the tenderizer. For a tenderization level of 6X, the conveyor belt speed was also set at 2.54 cm per plunge. The loin was placed fat side down and passed through the tenderizer. The loin was then turned over, placed lean side down and passed through the tenderizer again.

Bacteriological

Eight USDA Utility grade (small degree of marbling, D Maturity) boneless beef loins (NAMP 118) were selected from a local packer. These eight loins consisted of matching loins (right and left side) from four animals. At 3 days postmortem, one of each of the paired loins was mechanically tenderized at a tenderization level of 6X with a blade-type tenderizer. After tenderization, the loins were vacuum packed in barrier film (25–30 cc/m^2 for 24 hr at 23°C, "Freshstuff"® bag, American Can Co.) with a Model MG2 Multivac machine and a vacuum was drawn to 28–29 mm Hg. The steaks were placed in a cooler at 2 ± 2°C. At 6 days postmortem, the loins were removed from the cooler and cut into 3.8-cm steaks, wrapped with polyvinyl chloride (PVC) film, and placed in a commercial-type retail display case at 5°C.

Four tenderized and four nontenderized steaks were sampled each day at 0, 1, 2, 5 and 10 days of retail display storage, for a total of 40 steaks sampled. Surface and interior bacterial counts were made from each steak for total aerobic, psychrotrophic and anaerobic bacterial counts, according to the methods described by the American Public Health Association (APHA) in 1966 and 1972. Surface counts were performed by using a swab technique to obtain an estimate of contamination of outer portions of the meat. Internal counts were made on a gravimetric sampling basis for evaluating deeper contamination. The methods were not intended to be comparable, but did serve to compare meat treatment.

The medium used for all counts was Trypticase Soy Agar (TSA) medium. It was prepared according to the BBL *Manual of Products and Laboratory Procedures* (1973). The dilution blanks consisted of 0.1% Bacto-peptone dissolved in distilled water and autoclaved. The pour plate method was used for all counts.

A sterile cotton swab moistened in the dilution blank was used to swab the center of the exposed surface of the muscle, defined by a 10-cm^2 sterile aluminum template, for all surface counts. The cotton swab was then placed in a 99-ml dilution blank, and proper dilutions were made.

A 30-g sample was then obtained from the interior of the steak by careful dissection with sterilized forceps and scalpel. Alcoholic flaming was done periodically during the dissection to help reduce possible contamination of the sample being removed. The 30-g sample was then placed into an Osterizer jar containing 270 ml of sterile distilled water. This mixture was then blended on the Osterizer blender for 1 min at slow speed and 1 min at fast speed, with a 1-min rest period between the two blendings. The dilutions for all the interior counts were then made from this solution.

Plates for total aerobic counts (surface and interior) were incubated at 32 ± 1°C for 48 hr. Psychrotrophic counts (surface and interior) were incubated aerobically at 5 ± 1°C for 7–10 days. Anaerobic plates were placed in BBL Disposable Anaerobic Systems (GasPak 100) and incubated anaerobically at 32 ± 1°C for 48 hr.

Subjective sensory scores for odor were obtained by a three-member panel. Odor scores were based on subjectively smelling the samples and ranged from 1–5 with: 1, representing no odor; 2, slight odor; 3, slightly moderate off-odor; 4, moderate off-odor; and 5, extremely off-odor.

Histological

Samples for histological comparison were removed from the in-

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0022-1147/79/0006-1606 $02.25/0
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Table 1–Comparison of mean total aerobic surface bacteria, surface psychrotrophic and anaerobic bacterial counts[a] between tenderized and nontenderized fresh beef steaks during 5°C storage

Treatment	Initial sample	Days of storage at 5°C			
		1	2	5	10
Nontenderized					
Aerobic bacteria	2.37	2.62	3.34	5.25	7.97
Psychrotrophic bacteria	1.18	1.26	2.52b	5.13d	8.01
Anaerobic bacteria	1.53	1.57	3.15c	4.71	5.97
Tenderized					
Aerobic bacteria	2.65	3.07	4.69	6.61	7.91
Psychrotrophic bacteria	1.95	2.59	4.68b	6.60d	7.89
Anaerobic bacteria	2.26	2.52	4.54c	5.34	6.05

[a] Values expressed as log of number per sq cm; Comparative means between the tenderized and nontenderized steaks in the same column with the same letter are significant ($P < 0.05$).

terior of beef longissimus obtained from the shortloin portion of 20 Utility grade loins. Ten tenderized and 10 nontenderized samples were obtained and placed immediately into Helly's fixative and left there for 24 hr (Humason, 1972).

After fixing, the samples were washed for 24 hr in running tap water to remove any excess fixative. Iodine was then added to the water to help remove excessive mercuric chloride precipitate (5–8 hr). The samples were then washed in running tap water, again, and placed in a mixture of 50% dioxane and 50% distilled water for 2 hr. They were then placed in two changes of anhydrous dioxane for 2 hr each. The tissues were then transferred to a mixture of 50% dioxane and 50% paraffin (Tissuemat®) for a period of 2 hr. They were then placed in melted paraffin (Tissuemat®) overnight to complete infiltration and were then embedded in paraffin blocks (Tissuemat®) for sectioning.

The paraffin blocks were sectioned on an American Optical rotary microtome at a thickness of 12 microns. The sections were then placed in a Technicon water bath and mounted on glass slides coated with Mayers albumin. The slides were then allowed to dry on a warming plate and left to stand overnight before staining.

The slides were stained with Mallory's Triple Stain, as described by Humason (1972). Tissues stained with Mallory's Triple Stain show the following reactions: nuclei, red; muscle, red to orange; and collagen and other connective tissue, dark blue to blue. The stained muscle tissue sections were then observed under light microscopy. Proper shutter speeds and intensity of lighting were then determined according to the type of film and magnification level used (Hurtgen and Massios, 1975).

Sensory evaluation, Warner-Bratzler shear and cooking losses

Sixteen USDA Choice and 16 USDA Utility grade boneless beef top loins (NAMP 118) were used for this portion of the study. The loins were matching loins (right and left sides) in both the Choice and Utility grades.

One each of the paired loins was mechanically tenderized by a blade-type tenderizer. Choice loins were tenderized at a level of 3X and Utility loins at a level of 6X. Two 3.8-cm steaks were cut from the center of each loin for sensory taste panels. Sensory evaluations were made for flavor, juiciness, tenderness and overall acceptance (Dawson and Harris, 1951). A 10-member trained taste panel was used, and samples were rated on an 8-point hedonic scale (8 being the most desirable) according to methods reported by Parrish et al. (1969).

Thermometers were placed in the center of each steak, and the steaks were placed in a preheated oven for broiling. The oven temperature above the surface of the steaks was 241°C. When the internal temperature of the steak reached 35°C, the steak was turned over. At 65°C internal temperature the steak was removed from the oven. The steaks for the sensory taste panel were then cut into equal sized samples and distributed to the panel. Each member of the panel was given the same location of sample within the steak for all steaks sampled that day.

Two more 3.8-cm steaks were removed from the center portion of the loins and were used for Warner-Bratzler shear comparisons. The steaks for the Warner-Bratzler shear were cooked in the same manner and to the same internal temperature as just described. The steaks were then allowed to cool to room temperature before shearing. A mechanical boring device (Kastner and Henrickson, 1969) was used to remove five 1.27-cm cores from each steak. Cores were removed from the lateral, central and medial locations of the longissimus (Hedrick et al., 1968), plus two more cores were removed midway between the laterial and central cores and the central and medial cores. All five cores were then sheared once by the Warner-Bratzler shearing device (Bratzler, 1949).

The steaks used for the taste panel were weighed before cooking and after cooking to determine both the evaporative and drip loss during cooking. The broiling pans were weighed before and after cooking to determine the drip loss during cooking. Cooking time was recorded for each steak cooked for sensory evaluation.

Statistical analysis

The least squares analysis as applied to regression and analysis of variance was used to analyze differences in taste panel measurements, Warner-Bratzler shears, cooking losses, cooking time and microbiological counts. Simple and partial correlation coefficients were determined between variables, and least significant differences (LSD) were obtained. All statistical methods were carried out according to procedures by Snedecor and Cochran (1967) and Cox (1971). The probability levels used for the least significant differences (0.05 and 0.01) were divided by the number of comparisons made, before the tabled F-value was determined (Cooper, 1968).

RESULTS & DISCUSSION

Growth of microorganisms on fresh beef steaks

The total aerobic, psychrotrophic and anaerobic bacterial surface mean counts are given in Table 1. None of the total aerobic bacterial surface counts (Table 1) for tenderized or nontenderized beef steaks differed significantly ($P > 0.05$) on any of the five sampling days (0, 1, 2, 5 and 10 days of retail storage). Although total aerobic surface bacteria counts did not differ significantly between tenderized and nontenderized steaks, counts were generally higher for the tenderized samples.

Steak comparisons (tenderized vs nontenderized) for psychrotrophic (Table 1) bacterial surface counts did not differ significantly ($P > 0.05$) on 0, 1 and 10 days of retail storage display. On days 2 ($P < 0.01$) and 5 ($P < 0.05$) of retail display, however, the psychrotrophic surface counts were significantly higher on the tenderized steaks. Bacterial counts may have reached a peak before 10 days and then tended to become equal between tenderized and nontenderized steaks as spoilage occurred.

Anaerobic bacterial surface counts (Table 1) from tenderized and nontenderized beef steaks did not differ significantly ($P > 0.05$) on any of the sampling days, except for day 2 ($P < 0.01$) of retail display.

Off-odor was detected on the tenderized beef steaks at 5 days of retail storage when the surface count for total aerobes was 6.61 (log) organisms per cm^2 (Table 1). This is in close agreement with Ayres (1951; 1960a, b; 1965), who detected off-odor of fresh beef at bacterial counts with approximately 10^7 (log 7.00) organisms per cm^2. Slime was detected on the surface of both tenderized and nontenderized beef steaks by day 7 of retail storage.

Blade-type mechanical tenderization of beef may carry surface bacteria to the interior of the meat. Thus, interior bacterial counts were determined to find if this was occurring.

The bacterial interior mean counts for total aerobic, psychrotrophic and anaerobic counts, respectively, are reported in Table 2.

The interior total aerobic, psychrotrophic or anaerobic bacterial counts for tenderized vs nontenderized steaks were not significantly different ($P > 0.05$) on any of the five sampling days. The tenderized beef steaks, however,

Table 2—Comparison of mean[a] total aerobic, psychrotrophic and anaerobic bacteria counts[b] in the interior of tenderized and nontenderized beef steaks during 5°C storage

Treatment	Initial sample	Days of storage at 5°C			
		1	2	5	10
Nontenderized					
Aerobic bacteria	1.76	2.42	3.17	5.01	7.65
Psychrotrophic bacteria	1.70	2.30	2.84	4.86	7.57
Anaerobic bacteria	1.22	2.34	3.08	4.30	5.42
Tenderized					
Aerobic bacteria	2.74	2.83	4.06	5.65	7.05
Psychrotrophic bacteris	2.31	2.79	3.66	5.59	7.04
Anaerobic bacteria	2.01	2.32	3.76	4.86	5.96

[a] No significant ($P > 0.05$) difference between means
[b] Values expressed as log of number per gram

generally had higher interior aerobic, psychrotrophic and anaerobic counts (Table 2).

This study was not designed to test sampling methods; however, the method of sampling (surface or interior) had a significant ($P < 0.01$) effect on total aerobic counts, but not on psychrotrophic or anaerobic counts. The method × treatment interaction and method × day interaction had a small but significant ($P < 0.05$) effect on psychrotrophic counts, but not on total aerobic or anaerobic bacterial counts. All other variables and their interactions had no effect ($P > 0.05$) on bacterial counts (total aerobic, psychrotrophic or anaerobic).

The bacterial portion of the study would indicate that mechanical tenderization does cause some bacterial contamination and, thus, increased bacterial counts over the controls (nontenderized steaks). Boyd et al. (1978) reported similar trends on beef semimembranosus muscle.

Histological characteristics of beef steaks

Histological traits of the tenderized muscle are shown in Figures 1, 2 and 3.

Figure 1 represents a photomicrograph from raw longissimus muscle that has been tenderized by the blade-type mechanical tenderizer. There are four blade penetrations (shown by arrows) in this section, and a pattern of horizontal penetrations can be seen. There are two blade penetrations in the line above and two in the line below.

The lower left penetration (Fig. 1) lies in the line with the space between the two upper blade penetrations. This is caused from the "offset" position of the blades in the tenderizing head. A general disruption and severing of muscle fibers and a severing of connective tissue is seen in the area where the blade penetrated the muscle tissue. Except in the area of blade penetration, however, the muscle fibers and connective tissue are not disrupted and appear normal.

Figure 2 represents a photomicrograph with 75× magnificantion in the area of a blade penetration. A general severing and fragmentation of muscle fibers is quite apparent. This general disruption, breaking and fragmentation of muscle fibers was shown by Hoke (1958) and Wang et al. (1957) to occur in muscle tissue treated with an enzyme tenderizer. The observed muscle fiber disruption in their studies occurred throughout the muscle tissue; whereas, the disruption of muscle fibers in this study occurred only in the area of blade penetration.

A higher magnificantion (290×) photomicrograph of muscle fibers severed by blade penetration is shown in Figure 3. It seems that the muscle fibers have been torn by the blade when it penetrated the muscle tissue, rather than receiving a straight or even cut. The separation of the individual myofibrils can be seen at the ends of the severed muscle fibers. Striations in the muscle fibers can be seen upon close examination.

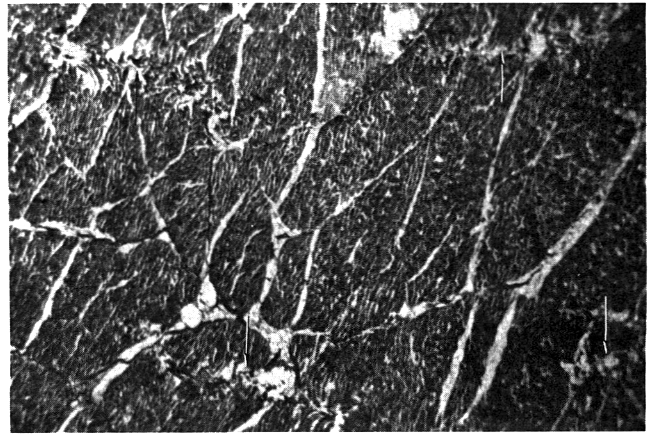

Fig. 1—Raw longissimus muscle that has been mechanically tenderized with a blade-type tenderizer (Magnification 5X). Arrows in upper and lower one-third of the figure designate blade penetrations.

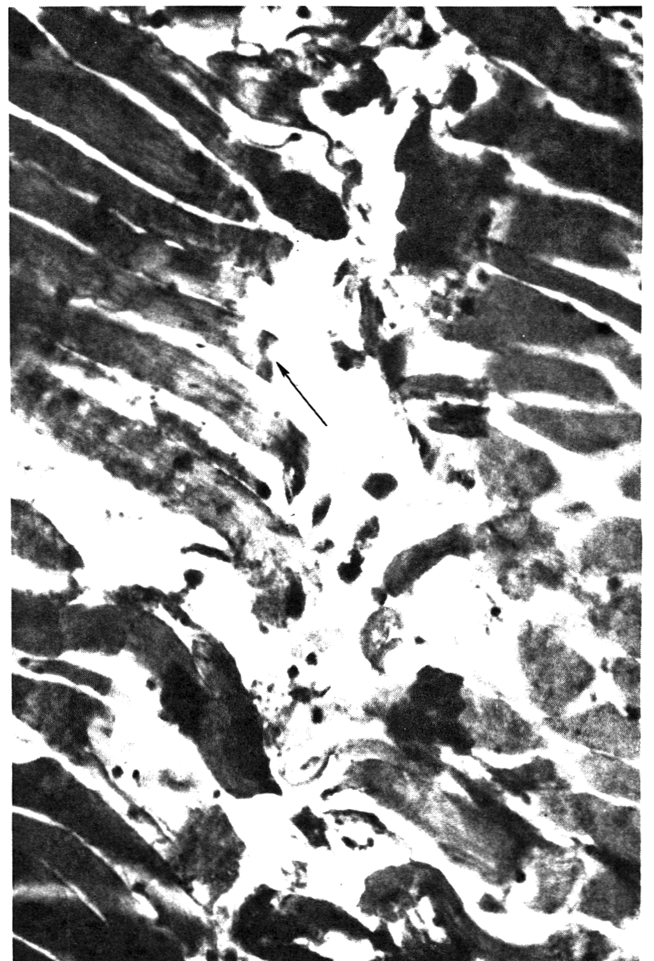

Fig. 2—Raw longissimus muscle that has been mechanically tenderized with a blade-type tenderizer (Magnification 75X). Arrow in center of figure designates severed muscle fiber and space left after blade penetration.

Since the disruption and severing of muscle fibers and connective tissue occurs only in the area of the blade penetration, the blade-type mechanical tenderizer's method of tenderization is considerably different from that of enzyme tenderizers. Enzyme tenderizers disrupt and fragment the

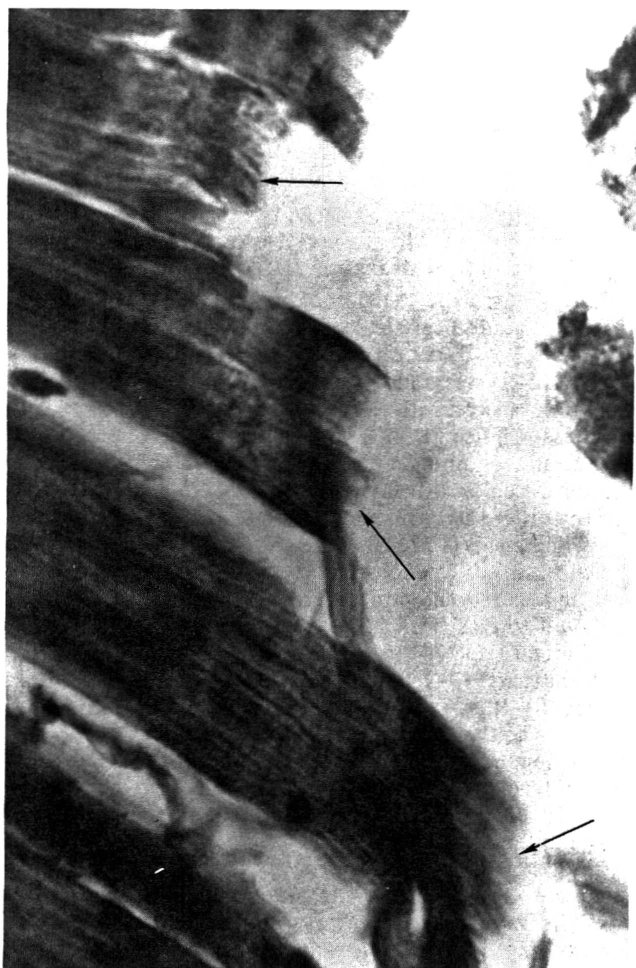

Fig. 3—Raw longissimus muscle that has been mechanically tenderized with a blade-type tenderizer (Magnification 290X). Arrows designate fragmented muscle fibers.

Table 3—Comparison of mean taste panel scores for flavor, tenderness, juiciness, and overall acceptance between choice and utility grade beef steaks (tenderized and nontenderized)[a]

Grade and treatment	Flavor	Tenderness[b]	Juiciness	Overall acceptance[c,d]
Utility nontenderized	5.23a	3.66a	5.63a	4.30a
Utility tenderized	5.37a	5.84b	5.34a	5.31b
Choice nontenderized	5.99b	6.07b	5.66a	5.84b
Choice tenderized	5.94b	6.47b	5.10a	5.83b

[a] Means within the same column with different letters differ significantly at $P < 0.01$.
[b,d] Utility tenderized and Choice tenderized steaks differ significantly at $P < 0.05$.
[c] Utility tenderized and Choice nontenderized steaks differ significantly at $P < 0.05$.

Table 4—Comparison of mean Warner-Bratzler shear values, mean drip percentages, mean evaporation percentages, and mean cooking times for choice and utility grade beef steaks (tenderized and nontenderized)[a]

		Cooking losses		
Grade and treatment	W.B. shear[b] (Kg)	Drip[c] (%)	Evaporation (%)	Cooking time[c,d] (min)
Utility nontenderized	5.18a	7.58a	11.28a	15.98a
Utility tenderized	2.99b	8.54a	10.93a	14.88a
Choice nontenderized	3.04b	10.72b	11.36a	17.31a
Choice tenderized	2.26c	11.51b	12.16a	17.86a

[a] Means within the same column with different letters differ significantly at $P < 0.01$.
[b,d] Utility tenderized and Choice tenderized steaks differ significantly at $P < 0.05$.
[c] Utility tenderized and Choice nontenderized steaks differ significantly at $P < 0.05$.

muscle fibers and connective tissue throughout the muscle tissue (Wang et al., 1975). Enzyme tenderizers, however, do not penetrate the muscle evenly or to any depth, and, thus, tenderization is often very uneven. With blade-type mechanical tenderization, the muscle is tenderized much more evenly because the blades pass completely through the muscle during tenderization.

Palatability characteristics of beef steaks

Mean taste panel flavor scores are given in Table 3. The Choice and Utility nontenderized and tenderized beef steaks did not differ significantly ($P > 0.05$) in taste panel flavor scores. The Utility grade beef steaks, both tenderized and nontenderized, had significantly ($P < 0.01$) lower taste panel flavor scores than the Choice grade beef steaks.

The mean taste panel tenderness scores are given in Table 3. A highly significant increase in taste panel tenderness scores was observed for Utility tenderized steaks over Utility nontenderized steaks. No significant increase ($P > 0.05$) in taste panel tenderness scores due to mechanical tenderization, however, resulted within the Choice grade beef steaks. Also, no significant difference ($P > 0.05$) in tenderness scores existed between Utility tenderized and Choice nontenderized steaks. Thus, blade-type mechanical tenderization upgraded the tenderness scores of Utility grade beef steaks to almost that of Choice grade nontenderized steaks.

The significant ($P < 0.01$) grade × treatment interaction is evident in Table 3, by the fact that mechanical tenderization did not increase taste panel tenderness scores as much within the Choice grade as it did within the Utility grade. The Choice grade steaks were significantly more tender ($P < 0.01$) to start with (before tenderization) than the Utility grade steaks, which accounts for the greater effect of mechanical tenderization on Utility grade beef.

This nonsignificant ($P > 0.05$) increase in taste panel tenderness score with mechanical tenderization in higher grade beef (Choice) is in agreement with work by Davis et al. (1975). These workers stated that the initial quality of the steaks used in their study was such that the effects of mechanical tenderization were masked by inherent tenderness. The results of this study would tend to agree with this conclusion when looking at the Choice beef steaks.

The mean taste panel juiciness scores are listed in Table 3. A general trend of decreased juiciness from mechanical tenderization can be seen in both grades (Choice and Utility), but none of the means differed significantly when compared within grade (Choice or Utility) or between grades (Choice vs Utility).

Bowling et al. (1975, 1976), Davis et al. (1975) and Glover et al. (1975) all found that blade-type mechanical tenderization had no significant effect ($P > 0.05$) on the taste panel juiciness ratings of meat (beef, lamb and goat). Davis et al. (1975) reported a general trend (nonsignificant) of decreased juiciness scores with mechanical tenderization, which agrees with results of this study.

A significant difference ($P < 0.05$) in the drip loss (5) during cooking was found as a result of the grade (Choice and Utility) of the beef steaks (Table 4). Also, blade-type mechanical tenderization had a significant effect ($P < 0.05$)

on the drip loss (%) of beef steaks. There was, however, no grade × treatment interaction ($P > 0.05$).

There was no significant effect ($P > 0.05$) due to either grade (Choice or Utility) or treatment (tenderized or nontenderized) on the evaporation loss (%) of beef steaks during cooking (Table 4). Also, there was no significant ($P > 0.05$) grade × treatment interaction for evaporation loss (%).

A trend toward increased drip loss (%) with mechanical tenderization in both grades (Choice and Utility) was found. This increase was not significant ($P > 0.05$), however, for the Choice or Utility grade beef steaks. This is in agreement with work by Tatum et al. (1976). Glover et al. (1978), however, found drip loss was significantly greater ($P < 0.05$) for tenderized round roasts when compared with the controls.

There was no significant ($P > 0.05$) effect of either blade-type mechanical tenderization or grade (Choice or Utility) on the evaporation loss (%) of beef steaks during cooking. There seems to be no trend for evaporation losses (%) due to tenderization, even though there was with juiciness scores and drip losses (%).

From these results, it seems that blade-type mechanical tenderization does not significantly affect ($P > 0.05$) the cooking losses (drip and evaporation) of Choice and Utility grade beef steaks, even though it does slightly increase the drip loss (%). This is in agreement with work by Goldner et al. (1974), Schwartz and Mandigo (1974) and Bowling et al. (1976). Davis et al. (1975), however, reported a significant increase in cooking losses of loin steaks due to blade-type mechanical tenderization.

Blade-type mechanical tenderization had a significant effect ($P < 0.01$) on the overall acceptance of beef steaks by the taste panel. Also, as would be expected, the grade (Choice or Utility) of the beef steaks had a significant effect ($P < 0.01$) on the overall acceptance. A significant ($P < 0.01$) grade × treatment interaction was also found. Thus, both grade and treatment play an important role in determining the overall acceptance of beef steaks by the taste panel.

The mean overall acceptance scores of Choice and Utility grade beef steaks are listed in Table 3. Mechanical tenderization has significantly increased ($P < 0.01$) the overall acceptance of Utility grade beef steaks, but did not affect ($P > 0.05$) the Choice grade steaks. There is a significant difference ($P < 0.05$) between the tenderized Utility and nontenderized Choice grade steaks in overall acceptance. This difference may be due mainly to flavor differences of the two grades since it has already been shown that mechanical tenderization increased Utility grade steaks to a tenderness level (taste panel) almost equivalent to the nontenderized Choice steaks.

Nontenderized Utility grade beef steaks had significantly ($P < 0.01$) lower overall acceptance scores when compared with the other treatment groups.

Warner-Bratzler shear of beef steaks

As with taste panel tenderness scores, mechanical tenderization had a significant effect ($P < 0.01$) on tenderness, as measured by the Warner-Bratzler shear, of Choice and Utility beef steaks. Also, the grade of the steak and the grade × treatment interaction had a significant effect ($P < 0.01$) on Warner-Bratzler shear values and, thus, tenderness.

Table 4 shows the mean Warner-Bratzler shear values for Choice and Utility grade beef steaks (tenderized and nontenderized). There is a much greater decrease in Warner-Bratzler shear value due to blade-type mechanical tenderization within the Utility grade steaks than within the Choice grade steaks. A main point is that the tenderized Utility steaks are not significantly ($P > 0.05$) different in Warner-Bratzler shear values from the nontenderized Choice steaks. Indeed, the tenderized Utility steaks have a slightly lower Warner-Bratzler shear value than the nontenderized Choice steaks.

This significant decrease in Warner-Bratzler shear of beef loin steaks, due to mechanical tenderization, also was found by Glover et al. (1975) and Tatum et al. (1978). However, Davis et al. (1975) did not find a significant decrease ($P > 0.05$) in Warner-Bratzler shear for tenderized loin steaks (Choice and Good grade).

Cooking time of beef steaks

Table 4 gives the mean cooking time (min) of Choice and Utility grade beef steaks. Mechanical tenderization had no effect ($P > 0.05$) on the cooking time for either the Choice or Utility grade steaks. Tenderized Utility grade steaks took significantly less time to cook than either nontenderized Choice ($P < 0.05$) or tenderized Choice ($P < 0.01$) steaks. Nontenderized Utility grade steaks, however, did not differ significantly ($P > 0.05$) in cooking times from the other three treatment groups.

Bowling et al. (1976) and Tatum et al. (1978) reported no significant difference ($P > 0.05$) in cooking time between tenderized and nontenderized samples. Cooking time was found to be significantly less for tenderized samples by Goldner et al. (1974) and Glover et al. (1975). Schwartz and Mandigo (1974) found tenderized Choice steaks took significantly ($P < 0.05$) less time to cook than nontenderized steaks of the Good grade. Tatum et al. (1976) reported that blade-tenderized steaks requied more cooking time (nonsignificant) than control steaks. Thus, it is clear from this study and past studies that no apparent trend for cooking time of tenderized steaks has been established.

From the results of this study, the following summary statements may be made: (1) tenderness of Utility grade steaks, as measured by taste panel and Warner-Bratzler shear, is significantly improved by blade-type mechanical tenderization; (2) tenderness of Choice grade steaks is significantly improved by mechanical tenderization according to Warner-Bratzler shear measurements, but only slightly improved as measured by taste panel; (3) the effects of mechanical tenderization on tenderness tends to be more pronounced within the Utilitiy grade, probably because initial tenderness of Choice steaks is much greater than the initial tenderness of Utility steaks; (4) blade-type mechanical tenderization is an effective and reasonable method for upgrading the tenderness of lower grades of beef to within a close proximity to that of higher grades; (5) mechanical tenderization has no significant effect on taste panel ratings of flavor or juiciness, cooking losses (drip and evaporation) and cooking time of Choice and Utility grade beef steaks; (6) the storage life of beef steaks is not significantly affected by mechanical tenderization; the steaks may have a tendency to discolor faster, but this does not occur until 5 days of retail storage; (7) tenderized beef steaks had higher initial (day 0) bacterial counts than nontenderized steaks, although not significantly higher; this seems to be caused by bacterial contamination of the loins by the blades and conveyor belt of the mechanical tenderizer; (8) blade-type mechanical tenderization seems a more even method of muscle fiber and connective tissue tenderization, and, thus, the steaks should be more evenly tender than when other methods of tenderization are used.

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Journal Paper No. J-9418 of the Iowa Agriculture and Home Economics Experiment Station, Ames. Project No. 2175. Appreciation is expressed to Bettcher Inc. for providing the tenderizer used for the study.

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This work was supported in part by the Massachusetts Agricultural Experiment Station.

PREPARATION AND PROPERTIES OF DEHYDRATED CLAM FLAVOR FROM CLAM PROCESSING WASH WATER

Y. JOH and L. F. HOOD

ABSTRACT

Surf clam wash water was concentrated and dehydrated in order to convert it to a marketable clam flavor ingredient (CFI). Concentration by boiling to 2% solids and freeze drying with 4.6% dextrin yielded the best product. Ultrafiltration, and spray- and drum drying were also evaluated. CFI had acceptable dispersibility, solubility, and color. Quality did not deteriorate during 90 days storage. Addition of 0.5% CFI to seafood chowder improved the overall acceptability of the chowder. Chowder containing 1% CFI and no clam meat was equivalent in aroma and clam flavor intensity to clam chowder made with 3.5% clam meat.

INTRODUCTION

WASH WATER from the mincing operation in surf clam processing plants contains soluble solids and pieces of clam tissue. Traditionally, it has been discarded. This has resulted in a waste disposal problem and the underutilization of a valuable resource. Recently, a method has been described for converting wash water into a marketable clam juice (Hood et al., 1976). The procedure entails boiling the wash water in order to concentrate the liquid and to preserve the clam flavor.

We were interested in finding other uses for the wash water. The development of a dehydrated clam flavor ingredient (CFI) seemed logical since the increasing demand for and the price of fresh clams makes them a very expensive flavor ingredient. The manufacturing process for, and the properties of CFI are described here.

EXPERIMENTAL

Sampling and preparation of raw materials

Clams were washed three times during processing (Fig. 1). The wash water collected from the third washing, following the mincing operation, contained substantially more clam solids than the wash water from the two preceding washings (Hood et al., 1976). Thus only the third wash water was collected and utilized.

After mincing 500 kg of shucked clams, which took 2–3 hr, 40 gal of the wash water in the collection tank was transferred to a steam-jacket kettle and boiled immediately (Fig. 1). Total solids in the unboiled and boiled wash waters, as determined with a moisture balance (Central Scientific Co., Chicago, IL), were 1.3–1.6% and 1.9–2.1%, respectively. The prompt boiling of clam wash water prevented development of an undesirable fishy flavor in the wash water (Hood et al., 1976). Boiling was mild for the first 20–30 min because the liquid foamed easily near the boiling temperature and tended to overflow the kettle. When the foam subsided, the wash water was boiled vigorously at 95–100°C for a total boiling time of 45–50 min, until the total solids were approximately 2.0%. The boiled-concentrated wash water was held undisturbed, without further heating, until the insolubles that were formed during boiling precipitated. The concentrated clam wash water (CCWW) was decanted, canned in 50-oz cans and retorted at 122°C, 15 psi, for 45 min in order to preserve it for dehydration experiments. The canning step may not be necessary when the CFI manufacturing process is commercialized, particularly if concentration and dehydration can be accomplished at the same location. In our work, it was necessary to preserve the CCWW by canning so that it could be transported to the research laboratory (7 hr) for further processing and analysis. Interestingly, previous studies have shown that retorting enhances clam flavor (Hood et al., 1976).

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0022-1147/79/0006-1612$02.25/0
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Dehydration of CCWW

CCWW was further concentrated by boiling or ultrafiltration (Amicon UM05, Lexington, MA). Dehydration methods evaluated were drum-, spray-, and freeze drying. Various conditions were evaluated. Only the optimum conditions for each method are described here.

Drum drying. CCWW was blended with 16% dextrin (w/w), DE 10, (Maltrin 10, Grain Processing Co., Muscatine, IA) in a Waring Blendor at low speed for 2 min. The blended mixture was dried on a double drum dryer (Model ALC-4, Blaw-Knox Co., Bufflao, NY) at 20–25 psi steam pressure, 1–1.5 rpm and 0.01 mm nip distance.

Spray drying. CCWW was blended with 6.7% dextrin (DE 10) in a Waring Blendor at low speed for 2 min and dried in a conical-type laboratory spray dryer (Boen Engineering, Inc., North Branch, NJ, Serial No. BE 278, spray nozzle type NS2). The nozzle pressure was 42–45 psi, intake air temperature was 170–205°C, and the temperature at the collecting pan was 110–135°C.

Freeze drying. CCWW was blended with 4.6% dextrin (DE 10) in a Waring Blendor for 1 min at low speed. The blended mixture was

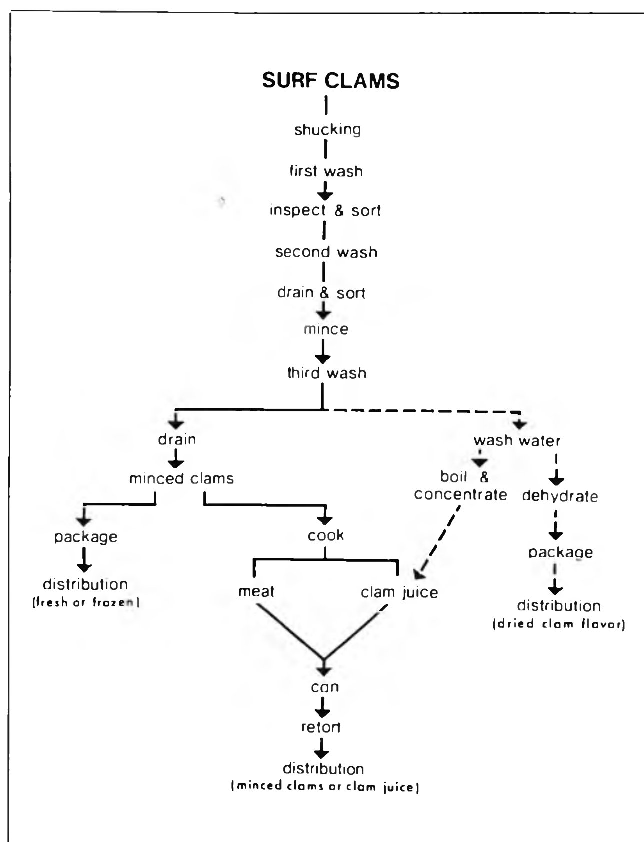

Fig. 1—Flow diagram of surf clam processing.

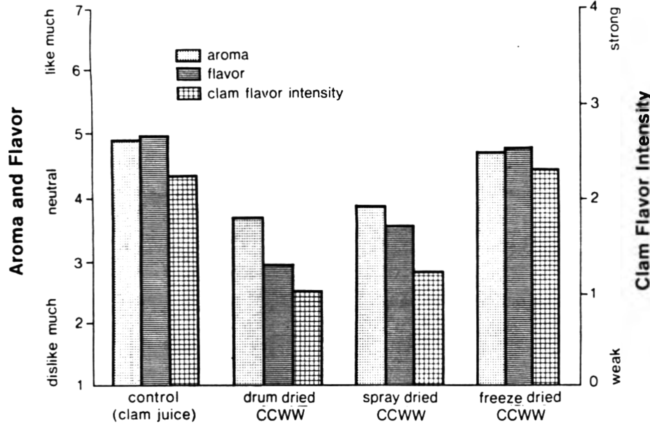

Fig. 2—Effect of dehydration methods on the organoleptic quality of rehydrated products.

frozen to −50°C and dried to 7% moisture under 0.05–0.1 mm of mercury (FFD-15 WS, Virtis Co., Gardner NY). No shelf heat was applied for the first 24 hr. During the next 24 hr, 10°C was used. Thereafter, 24°C was applied until the temperature of the product reached 24°C.

All dried products were ground in a Thomas-Wiley Mill (Model ED5, A.H. Thomas Co., Philadelphia, PA) with a 2 mm sieve.

Sensory evaluation

Eleven people were selected from the Department of Food Science staff and graduate students. They were trained to be familiar with aroma and flavor of clam juice, clam flavor attributes, and the procedures of testing and scoring. In the training sessions, a freshly dehydrated sample (reference) was compared with an off-flavored sample which had been opened and stored at 3°C for 2–3 wk. Clam juice with different degrees of off-flavor were made by mixing the fresh reference sample and the off-flavored sample in different ratios. Triangle tests were used to train the taste panelists to distinguish the reference sample from other samples. After the panelists were trained, a seven-point hedonic scale (dislike much:1, like much:7) and a five-point clam flavor intensity scale (weak:0, strong:4) were used to evaluate the dehydrated products.

Dried CCWW was rehydrated with distilled-deionized water at 22–24°C to 2.0% solids. Commercial clam juice without added salt, (2% solids Shelter Island Oyster Co., Greenport, NY) was included in each set of samples. Rehydrated CCWW and clam juice were each mixed well before pouring into 2 fl oz white plastic cups. Approximately 5 ml of sample were sufficient for each panelist.

Composition

Total solids and ash content were determined by drying sample aliquots in tared crucibles in a forced air oven overnight at 100°C. The crucibles were cooled in a desiccator and weighed. After the dry weights of samples were determined, the samples were ashed in a muffle furnace for 5–7 hr at 550°C. The ashed samples were cooled in a desiccator and weighed.

Nonprotein nitrogen was determined, after precipitating the protein with 24% trichloroacetic acid, by a modified Kjeldahl method (30 ml of 98% H_2SO_4 for digestion, 110 ml of 50% NaOH and 10% potassium sulfide for neutralization). Total nitrogen content of the products were also determined by the modified Kjeldahl method. Protein (%) content was calculated by: (% total nitrogen − % total nonprotein nitrogen) × 6.25.

NaCl concentration was measured by the AOAC method using 0.1N $AgNO_3$ and 0.1% 2'7'dichlorofluorescein in ethanol (AOAC, 1970).

Properties

Bulk density, foam expansion and foam stability of the products were measured by the methods of Wang and Kinsella (1976a, b).

Dispersibility and percent solubles were determined as follows: 1-g aliquots of dehydrated CCWW were weighed into 30 ml polypropylene centrifuge tubes and 10 ml of 24°C or 100°C distilled-deionized water added. Tubes were shaken for 3 min in a shaker (Magic Whirl Shaker, Blue M Co., Blue Island, IL) at 24°C, which traveled horizontally 2.5 cm, 120 times per min. Required time for the samples to be completely dispersed in the water was measured and is reported as dispersibility. Percent solubles were determined on the completely dispersed samples by centrifuging at 12,000 × G for 10 min. Supernatants were carefully siphoned off and the remaining precipitate dried in a forced air oven for 24 hr at 100°C. Percent solubles in the product were calculated as:

$$\frac{\text{wt of dehydrated CCWW (dry wt basis)} - \text{wt of dried ppt}}{\text{wt of dehydrated CCWW (dry wt basis)}} \times 100$$

Product colors were evaluated subjectively by the trained panel and objectively with a color-difference meter (Hunter Lab, Model D25, Hunter Assoc. Lab., Fairfax, VA) using a white standard plate (L:93.1, a:−0.8, b:−0.6). A seven-point hedonic scale (too brown:1, just right:4, too light:7) was used for panel evaluation.

Storage stability

Storage stability tests were designed to check the effects of different storage temperatures, packaging conditions and time on product quality. Dried products were vacuum packed in 2.5 × 3.5 in. cans (about 35 g/can) at 50 mm mercury using a vacuum can sealer (Continental D106622, Continental Can Co., Chicago, IL). The lids of the cans were sealed 30 sec after the 50 mm mercury pressure was attained. Products were also packaged in screw-cap jars. Cans

Table 1—Effect of concentration methods on the organoleptic quality of CCWW[a]

Sample	Aroma	Flavor	Clam flavor intensity	Flavor criticisms
1[b]	4.8 ± 1.2	4.9 ± 1.0	2.2 ± 0.9	
2[c]	4.7 ± 1.3	5.1 ± 1.0	2.1 ± 1.2	
3[d]	4.5 ± 0.8	3.8 ± 0.7	2.1 ± 1.3	Burnt, musty
4[e]	4.0 ± 1.3	3.0 ± 1.7	1.9 ± 1.0	Musty, caramel, sl. fishy
5[f]	3.0 ± 1.4	2.1 ± 1.1	0.2 ± 0.3	No flavor or aroma

[a] Mean ± SD, n = 11
[b] Reference (canned commercial clam juice without NaCl added)
[c] Concentrate to 2% solids by boiling
[d] Concentrate to 4% solids by boiling
[e] Retentate (2.4% solids) by ultrafiltration
[f] Permeate (0.7% solids) by ultrafiltration

Table 2—Composition of clam flavor ingredient (CFI)

	%
Total solids	92.9
Ash	4.7
Total nitrogen	2.57
Nonprotein nitrogen	2.04
Protein	3.31
NaCl	3.5

Table 3—Functional properties of CCWW freeze dried without and with 4.6% dextrin[a]

	Without dextrin	With dextrin[b]
Bulk density (g/ml)	0.25 ± 0.01	0.11 ± 0.01
Dispersibility (sec)		
25°C	12 ± 1.0	65 ± 1.5
100°C	12 ± 1.0	85 ± 0.6
Solubles (%)	100 ± 0.6	100 ± 0.3
Foam expansion (ml)	28.5 ± 0.4	24.0 ± 0.4
Foam stability (min)	10.0 ± 0.5	8.0 ± 0.3

[a] Means ± SD, n = 3
[b] CFI

and jars were stored at 4, 24 and 40°C, and evaluated by the taste panel after 30, 60 and 90 days storage.

Product applications

Dried CCWW with dextrin (CFI) was added to the Cornell Seafood Chowder fromulation (Baker et al., 1976), made with 3.5% clam meat and without clam meat, at 1 and 2% (w/w). The chowder was diluted with whole milk (1/1 v/v) and heated to 100°C. Aroma, flavor, clam flavor intensity, and overall acceptability of the chowders were evaluated by the trained panel.

RESULTS & DISCUSSION

METHODS for concentrating the wash water prior to dehydration were evaluated. CCWW concentrated by ultrafiltration did not have a good sweet clam flavor. This was probably because low molecular weight compounds that contribute to clam flavor were removed. In addition to the loss of clam flavor, other undesirable flavors such as musty, burnt, caramel-like, and fishy were more pronounced in the concentrate (Table 1).

CCWW concentrated to greater than 2% solids by boiling also had a burnt and musty flavor (Table 1). Considering the undesirable flavors formed during the extended boiling and the high energy costs involved, further concentration beyond the 2.0% solids level by boiling was not practical.

The CCWW (2% solids) was dehydrated by three conventional methods: drum-, spray- and freeze drying. All of the dehydrated products were hygroscopic. Therefore, a low DE dextrin was blended with the CCWW before drying. All products codried with the dextrin were nonhygroscopic and free-flowing.

Freeze drying was the only dehydration technique that yielded a satisfactory product under the conditions evaluated (Fig. 2). Drum drying produced a powder that had a burned, caramelized flavor. When rehydrated, it scored significantly lower than commercial clam juice. The drum-dried powder also had poor solubility and dispersibility in water. Spray drying yielded a more soluble and dispersible powder with slightly better flavor than the drum-dried powder. Spray drying and drum drying required larger concentrations of dextrin than freeze drying in order to produce a powder with acceptable color and physical properties. The larger additions of dextrin diluted the clam flavor and a grain-like flavor resulted. Overall, the products made by spray- and drum drying had unacceptable organoleptic qualities.

CCWW freeze dried with 4.6% dextrin is subsequently referred to as clam flavor ingredient (CFI). Freeze drying 14 lb of CCWW (2% solids) with 4.6% dextrin yielded about 1 lb of CFI (92.9% solids).

Table 4—Color of spray- and freeze dried CCWW and CFI

	CCWW		CFI
	Spray dried[a]	Freeze dried	
Panel score[b]	3.4 ± 0.8	1.9 ± 0.9	4.4 ± 1.0
L value[c]	84.2 ± 0.3	78.3 ± 0.3	85.3 ± 0.2
a value[c]	1.8 ± 0.1	−2.0 ± 0.2	0.8 ± 0.1
b value[c]	12.9 ± 0.1	13.8 ± 0.3	10.5 ± 0.4

[a] With 6.7% dextrin
[b] Mean ± SD, n = 10
[c] Mean ± SD, n = 3

Table 5—Organoleptic quality of CFI after 90 days storage[a]

	Aroma	Flavor	Clam flavor intensity
Control[b]	4.6 ± 0.5	5.2 ± 0.8	2.3 ± 0.7
Vacuum			
4°C	4.6 ± 0.7	4.8 ± 1.4	2.5 ± 0.9
24°C	4.3 ± 0.8	4.8 ± 1.3	2.2 ± 0.7
40°C	4.8 ± 0.8	5.2 ± 1.6	2.8 ± 0.7
Jar			
4°C	4.4 ± 0.9	5.1 ± 1.3	2.4 ± 0.9
24°C	4.3 ± 0.8	4.7 ± 1.0	2.3 ± 0.8
40°C	4.4 ± 0.6	4.8 ± 1.4	2.3 ± 0.9

[a] Mean ± SD, n = 10
[b] 0 day

Table 6—Organoleptic quality of seafood chowder made with CFI[a]

Chowder	Aroma	Flavor	Clam flavor intensity	Overall acceptability
A[b]	5.0 ± 0.7	5.6 ± 0.7	2.5 ± 0.8	5.2 ± 1.3
A[c]	5.4 ± 1.0	5.8 ± 1.0	2.5 ± 0.9	5.4 ± 1.5
B[d]	5.0 ± 1.1	5.0 ± 1.0	2.2 ± 1.0	4.4 ± 1.5
B[e]	4.9 ± 0.8	5.2 ± 1.2	2.4 ± 0.9	4.6 ± 1.2
B[f]	5.6 ± 0.7	5.7 ± 0.6	2.8 ± 0.4	5.6 ± 1.3

[a] Mean ± SD, n = 10
[b] Cornell Seafood Chowder with 3.5% clam meat
[c] Cornell Seafood Chowder with 3.5% clam meat + 0.5% CFI
[d] Cornell Seafood Chowder without clam meat
[e] Cornell Seafood Chowder without clam meat + 0.5% CFI
[f] Cornell Seafood Chowder without clam meat + 1.0% CFI

Properties of CFI

The total solids content of CFI was 92.9% (Table 2). Approximately 80% of the total nitrogen was nonprotein nitrogen. Various amine compounds (e.g. trimethylamine, cadaverine) and protein degradation products such as small peptides, amino acids, urea and ammonia probably were responsible for the nonprotein nitrogen.

The microbiological quality of CFI was good. Standard plate counts were 900 per gram. No yeast and mold were detectable. These low counts were probably due to the fact that CFI was prepared from retorted concentrated clam wash water.

CFI had good dispersibility and solubility (Table 3). When it was mixed with water, some solids floated before going into solution. A little shaking noticeably increased the dispersion rate. Floating was probably due to the low bulk density (Table 3). The dispersibility of CFI might be improved (to eliminate the floating solids) by wetting the powder to 8–10% moisture and redrying. The addition of dextrin as a codrying agent did not appear to enhance the functional properties, except for the bulk density. Nevertheless, it was essential to include it to reduce the hygroscopicity of the dried product.

Dextrin also influenced the color of the dehydrated product (Table 4). The whiteness of products was directly proportional to the dextrin concentration in the freeze-dried products. Powders codried with dextrin were preferred by the panel. CFI was preferred to the spray-dried powder with dextrin, even though the latter contained more dextrin.

CFI was stable when stored under various conditions for 90 days (Table 5). Apparently the product can be packaged either in vacuum or air-tight containers. Vacuum packaging may insure better quality if the product is to be stored longer than 90 days. Package selection should take into consideration the fact that CFI becomes sticky at relative humidities above 70%.

Taste panel results indicated that the addition of 0.5% CFI to seafood chowders slightly improved the aroma, flavor, clam flavor intensity, and overall acceptability of the chowder (Table 6). When CFI was added at the 1.0% level, organoleptic quality of the chowders was better than the

PROCESSING AND INGREDIENT INFLUENCES ON TEXTURE OF COOKED COMMINUTED FISH MUSCLE

C. M. LEE and R. T. TOLEDO

ABSTRACT

The study was conducted to determine process requirements and formulations necessary to prepare a coarse textured smoked fish sausage. Sausages prepared from Spanish mackerel, using two different comminution processes and different levels of shortening, soy protein fiber (SPF) and added ice, were evaluated for texture using both instrumental methods and taste panels. Previous work has shown that fish muscle chopped with NaCl and polyphosphate in a silent cutter had a texture similar to a hard gelatin gel when steam cooked and a mushy texture when cooked in a smokehouse. The cooked fish sausages had a sponge-like texture after freezing and thawing. These unacceptable textural characteristics were overcome by the incorporation of at least 12g shortening/100g fish muscle, and SPF at 15:85 SPF/fish muscle using a two-stage comminution process. Shear and compressive strength were markedly diminished when ice was added in excess of 15%. The addition of shortening at the level of 12 g/100g fish muscle and SPF significantly improved taste panel ratings on texture relating to the structure of material and increased juiciness. The mechanical properties of the sausages were significantly modified by addition of SPF in a ratio 30g SPF/70g of fish muscle. A comminution process for the formulation containing 25g shortening/100g fish muscle resulted in significantly reduced strength and water-holding capacity and increased brittleness of the cooked product. There were no significant differences in texture and general acceptability between products prepared from the mechanically deboned as compared to the filleted fish if the moisture content and bone residue in the raw material were carefully controlled.

INTRODUCTION

IN RECENT YEARS much effort has been made in developing a new minced fish product based on deboned fish muscle (Rekhnina et al., 1973; Morehead, 1974; King et al., 1974). Texture and flavor were the primary considerations for quality assessment in this type of product. In a previous study (Lee and Toledo, 1976), the textural characteristics of comminuted fish muscle was found to be very strongly affected by addition of NaCl and polyphosphate and by the time of comminution, moisture content and cooking temperature. One-stage comminution of fish muscle with 2.5% salt in a silent cutter developed a gel-like texture after steam-cooking, a mushy texture after smoke-cooking and a sponge-like texture when the products were frozen and thawed. Taste panels indicated that such textural characteristics were not acceptable. Such unacceptable texture appeared to be favorably modified by the incorporation of shortening and soy protein fiber using a two-stage comminution process. The present study was conducted to determine the potential of soy protein fiber (SPF) in modifying texture of sausages from fish muscle. The effects of the level of SPF added, type and level of fat, the level of added ice, and manner of comminution on texture of fish sausage were investigated.

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0022-1147/79/0006-1615$02.25/0
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EXPERIMENTAL

Source and preparation of materials

Spanish mackerel (*Scomberomorous maculatus*) were purchased from a retailer in Athens, GA, and filleted. Fillets were skinned, packaged in polyethylene bags, immediately frozen in a blast freezer (−32°C) overnight, and kept in a freezer (−13°C) until used. Frames were passed through a deboner (small model, Yanagiya Machinery Works, Inc., Japan) equipped with a drum having 3.5 mm diam holes. The deboner was installed in a room at 3°C and the deboner drum was precooled with dry ice prior to each operation. Deboning was carried out with the belt tension set at medium resulting in an 80% yield. The mechanically deboned fish flesh was frozen and stored in the same manner as the fillet.

Formulation and processing

Unless otherwise indicated, the formulation used (in percent) was: fish flesh, 56.9; SPF, 14.1; corn syrup, 3.6; shortening, 8.6; ice, 15.0; and salt, 1.8 (total 100%). SPF was 20% of the combined weight of SPF and fish flesh. In studies on effect of SPF, combined SPF and fish flesh were held at 71% and SPF was added at ratios of 0:100; 10:90; 20:80; and 30:70 SPF/fish flesh by weight. Fat was added in the form of shortening (Crisco, Proctor and Gamble). The effect of levels of water was evaluated by taking the base formulation above without the ice and adding ice at levels of 0, 15, 20, or 25%.

The influence of six different comminution procedures was evaluated. Figure 1 summarizes process I, a two-stage comminution process. Soy protein fiber (SFP 200, Ralston Purina Co., St. Louis, MO) was chopped for 2 min in a silent cutter (Hobart, Model 8181D) with 0.1 ml 400 ppm Na_2CO_3 solution per g SPF added to improve functionality. An equal weight of fish flesh was added to SPF, and chopping was continued for 3 min adding salt (2.5% of the combined weight of SPF and fish) and the half of the total ice used in the formulation. Chopping was continued for 3 min at which time the remaining ingredients were added in the following order: corn syrup (80 DE, Clinton Corn Processing Co., Inc., Clinton, IA), ice, and vegetable shortening. The comminuted batter was ground with the remaining fish on a small laboratory meat grinder (Enterprise, Philadelphia, PA) fitted with a plate having 4.5 mm diam holes. The grinder was pre-chilled with crushed ice before introduc-

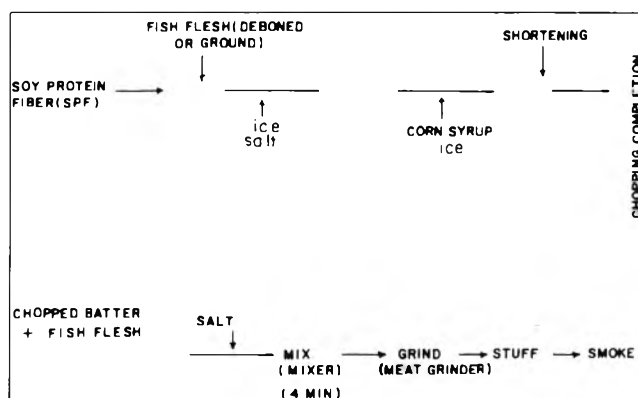

Fig. 1—Schematic flow diagram of the two-stage comminution procedure for comminuted fish muscle.

tion of the fish mixture. After grinding, the batter was mixed mechanically using a paddle mixer for 4 min, at which time the remaining salt was added.

A variation of this two-stage process is a one-stage process II. After the initial 3 min chopping of a 50:50 mixture of all the SPF and fish flesh, all the ingredients were added and chopping was continued for a total of 7 min.

The other one-stage processes include: Process III–Chop SPF with the Na_2CO_3 solution on a silent cutter for 2 min, mix all the ingredients by hand, and grind on a meat grinder (4.5 mm diam openings on plate); Process IV–Chop SPF with Na_2CO_3 solution for 2 min. Add the rest of the ingredients and continue chopping for 1 min; Process V–Similar to IV except that chopping was for 2 min; Process VI–Similar to IV except that chopping was for 4 min.

The batters were stuffed into 1.5 cm diam cellulose casing (Precision Nojax Casing, Union Carbide Corp., Chicago) and linked in 7 cm length. The links were kept under ice and transferred into a cold room (3.8°C) until smoke-cooked to minimize thermally induced syneresis of solubilized protein. Smoke-cooking was done within a few hours in a one-cage air-conditioned smoke house (Alka, Lodi, WI) employing the following schedule: Cold smoke at 80% R.H. with low fire for 1 hr during which the smoke house temperature increased to 50°C (dry bulb); hot smoke at 80–85% R.H. and 72–77°C for 30 min and at 60% R.H. and 72–77°C for another 30 min. Finally, with the dry bulb temperature still at 77°C, smoking is continued for another 10 min with the steam off, followed by a cold shower for 2 min. This schedule was chosen from among several schedules tested since it was found to provide the most desirable textural characteristics and maximum smoke penetration.

Determination of water and fat holding capacity

The raw batter was tested for thermal stability, i.e. the ability to hold fat and water in cooking. A 20-g plug of batter from each batch was transferred into a 125 ml Erlenmeyer flask. After the flask was sealed with parafilm, it was immersed in a water bath at 70°C for 1 hr. The amount of fat (in the form of oil) and water released per 100g of batter (% ml/g) was used as an index of thermal stability.

Mechanical properties

Products were evaluated for texture using instrumental methods and sensory panels. An Instron Universal Testing Machine (Type 1130, Instron Eng. Corp., Canton, MA) was used to evaluate mechanical properties related to texture. These mechanical properties evaluated were: shear strength (SS) and compressive strength (CS). Specimen loading, test conditions and specimen preparation were done according to the procedure used by Lee and Toledo (1976) with appropriate modifications. Specimens that had been frozen or refrigerated were either warmed to room temperature or were broiled before testing in order to test the effect of specimen temperature on mechanical properties. The specimens were 10 mm in diam for shear and 10 mm diam × 10 mm long for compression. Ten replicate measurements were made on products from each treatment.

The amount of fluid expressed upon compression with a hydraulic press was used as an index of juiciness. The procedure (Lee and Toledo, 1976) was based on the method of Grau and Hamm (1957).

Textural characteristics were also evaluated subjectively by panels consisting of 3 females and 7 males from the Department of Food Science, University of Georgia (Athens, GA). Products were broiled before presentation to the panels. All samples that were compared with each other were presented to the panel at the same time. Each panelist was asked to make 3 evaluations each on different days. Terms proposed by Jowitt (1974) were adopted for use in scoring by the panel.

RESULTS & DISCUSSION

FIGURE 2 shows the effect of ice addition on both the cook stability of the batter and the mechanical properties of the cooked product. Both SS and CS gradually diminished with increasing level of ice up to 15% and dropped markedly with ice additions beyond this level. The point where the marked decrease in SS and CS appeared to correspond with the point where the amount of water released from the batter in cooking, also started on a sharp increase, indicating some relationship between the cook stability of the batter and the mechanical properties of the cooked

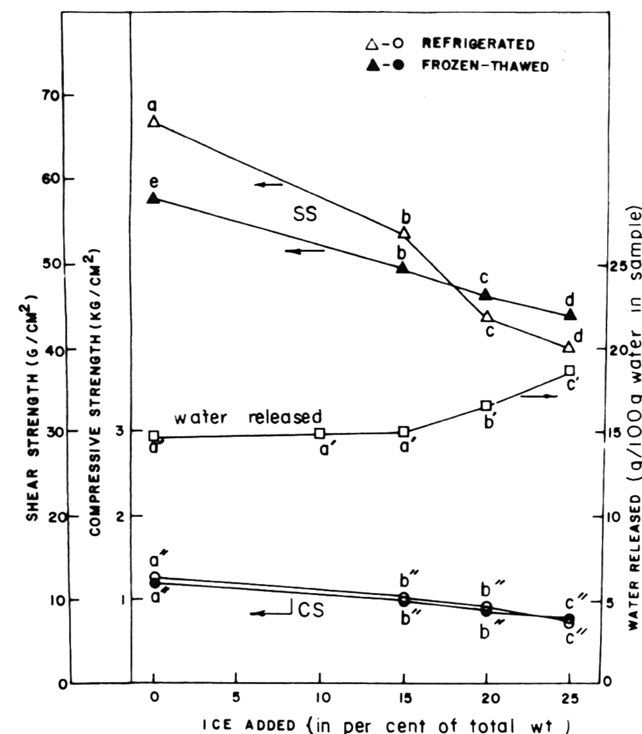

Fig. 2—Effect of the added ice on the stability of raw batter and textural strength of cooked product. Points represented by the same letters are not significantly different at p < 0.01 (n=10). CS=compressive strength and SS=shear strength.

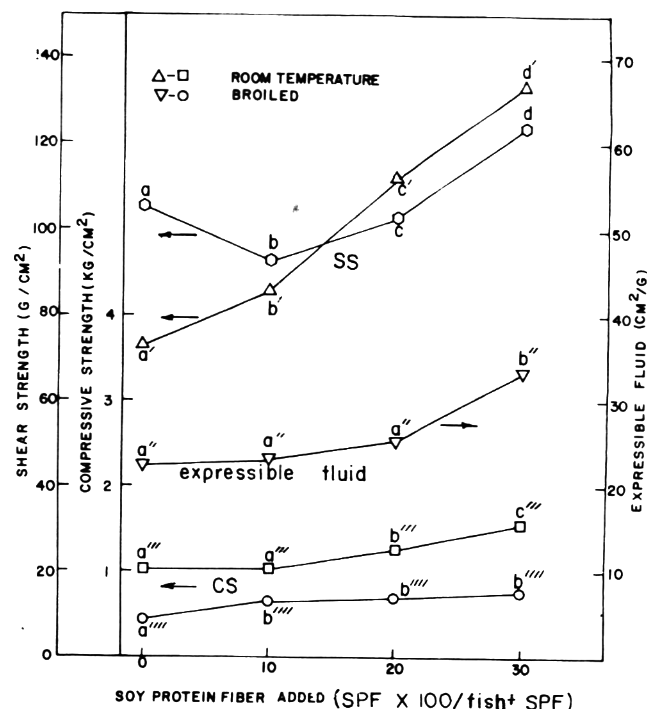

Fig. 3—Effect of soy protein fiber on the textural strength and the amount of expressible fluid of cooked product. Points represented by the same letters are not significantly different at p < 0.01 (n=10). CS=compressive strength and SS=shear strength.

Table 1—Effect of comminution procedure on WHC and mechanical properties of cooked comminuted fish sausage containing SPF and shortening[a]

Comminution process	Expressible fluid (cm²/g)	Compressive strength kg/cm²		Shear strength g/cm²	
		Room temp	Broiled	Room temp	Broiled
Process I	65.13 ± 8.88a	0.988a ±0.19	0.447a ±0.163	114.38a ± 11.94	43.6 ± 3.0a
Process II	51.52 ± 8.00b	1.256b ±0.12	0.703b ±0.16	112.06a ± 4.65	50.76 ± 3.68

[a] Values followed by the same letter in a column are not significantly different at $p < 0.05$.

product. In a previous study (Lee and Toledo, 1976), it was shown that when fish muscle was chopped with NaCl and polyphosphate, a considerable reduction in CS and a marked increase in SS occurred after the material was frozen and thawed compared to a fresh material. The extent of the change that accompanied freezing and thawing became more pronounced with increasing moisture content. A sponge-like texture was also manifested by the product. The results in the present study shown in Figure 2 show no significant difference ($p < 0.01$) in the CS and SS for the frozen and thawed product compared to the unfrozen product. The addition of SPF and shortening, therefore, improved the freeze-thaw stability of cooked comminuted fish muscle.

Figure 3 shows the effect of SPF addition on the expressible fluid and the textural strength of the cooked comminuted fish muscle. The shear strength exhibited a sharp increase with increasing SPF addition when the cooked product was tested at room temperature. However, when broiled, the influence of SPF addition did not become pronounced until the added SPF exceeded 20:80 SPF/fish flesh.

When tested at room temperature, the product gave increasing CS with increasing SPF additions over 10:90 SPF/fish flesh. On the other hand, the CS of the broiled product only increased significantly up to 10:90 SPF/fish flesh ratio and further increase in SPF did not give a significant effect. Overall, the extent of increase in SS was greater than that in CS, indicating that the fibrous structure of SPF contributed to the increase in SS. The quantity of expressible fluid increased gradually with increasing SPF indicating a decrease in the water holding capacity (WHC). Such diminution of WHC could be attributed to the low WHC of SPF.

The influence of the comminution process on mechanical properties of the cooked product are shown in Tables 1 and 2. Products of the one-stage process (II) showed a significantly lower CS and SS than those from the two-stage process (I) (Table 1). Such diminution in CS was observed in samples tested both at room temperature and after broiling, whereas differences in SS can only be observed in the broiled samples. The WHC of the one-stage comminution process (II) was significantly lower than that of the two-stage process (I). This could explain the preference of test panelists for the products of process I (Table 3). Test panelists indicated that products showing high quantities of expressible fluid were less juicy. In a previous study (Lee and Toledo, 1976) it was shown that the magnitude of the textural strength of a cooked comminuted fish product depended upon the integrity of the product matrix and the degree of immobilization of water in the matrix. The low textural strength and high expressible fluid in products of a one-stage comminution process can be attributed to the diminished continuity of the protein matrix as solubilized protein was surrounded by fat during extensive chopping. However, in the two-stage comminution process such discontinuity of protein matrix was minimized by minimal chopping which minimized loss of protein functional properties resulting in the formation of a stable matrix. The mixing that follows the short chopping period in the two-stage comminution process also reduced the gel-like texture that could result when most of the protein is solubilized and fine muscle particles result from a thorough chopping in the one-stage comminution process.

Table 2—Comparison of mechanical properties of cooked fish sausage prepared using different comminution techniques[a]

Comminution process	Mechanical properties at room temp	
	Compressive strength kg/cm²	Shear strength g/cm²
Process II (no chopping)	1.18 ± 0.19b	119 ± 24c
Process IV (single stage 1 min comminution)	0.97 ± 0.20a	108 ± 15bc
Process V (single stage 2 min comminution)	1.15 ± 0.22b	95.7 ± 9.8b
Process VI (single stage 4 min comminution)	1.18 ± 0.19b	78.6 ± 2.7a
Process I (2 stage comminution)	1.26 ± 0.20c	127 ± 17c

[a] Values followed by the same letter in a column are not significantly different at $p < 0.05$.

The results in Table 2 show that the mechanical properties of products from the one-stage comminution processes III, IV, V, and VI are significantly different from that of the two-stage process I. The products showing the properties in Table 2 were all prepared from the same batch of unfrozen fish flesh. The properties of the product from fresh fish using comminution process I are similar to those of products from frozen fish flesh shown in Table 1. Comminution time in a one-stage process up to 4 min showed a gradual increase in CS and a gradual decrease in SS. The effect of chopping in reducing particle size and giving a more uniform texture is shown by a reduction in the standard deviations of SS with prolonged chopping. The results in Table 2 follow a trend previously shown by Lee and Toledo (1976) on fish flesh without SPF, where prolonged comminution resulted in an increase in the CS. The presence of SPF modified the trend on change in SS showing a decrease in SS for the first 4 minutes of chopping for the fish flesh SPF mixture, as opposed to a gradual increase in SS with fish flesh alone. Values of the SS with SPF at 4 min of comminution are almost double those for fish flesh alone, as reported by Lee and Toledo (1976).

The results in Table 2 show that comminution reduces the resistance of SPF fibers to shear. These results also show that the improved textural attributes of the two-stage comminution process is not merely due to the reduced chopping time for the fish muscle in process I, but rather to

Table 3—Sensory evaluation scores on textural attributes of cooked comminuted fish muscle[a]

Attributes	Sensory evaluation scores[b] A[c]	B	C	D	E	F
Firmness	3.4a	2.7a	3.5a	3.6a	4.6b	4.0
Brittleness	–	1.2a	1.4a	1.5a	2.2b	2.3*
Chewiness	2.4a	2.4a	2.7a	3.0a	3.4b	3.2
Rubberiness	–	1.8a	1.9a	1.9a	3.1b	2.0
Elasticity	2.2a	2.1a	2.2a	2.4a	2.6a	2.0
Mushiness	2.1a	1.5b	1.1c	1.2c	1.2c	1.4
Smoothness	–	3.8a	3.4a	2.8b	2.2b	2.1
Fineness	3.2a	3.4a	3.2a	3.0a	2.0b	1.9
Lumpiness	2.4a	1.5b	1.3b	2.3a	2.3a	2.3
Mealiness	2.6a	1.6b	1.8b	1.6b	2.1b	2.7
Fibrousness	–	1.0a	1.1a	1.4a	1.9b	2.0
Moistness	–	4.1a	3.6a	2.6b	2.3b	1.9
Juiciness	–	3.8a	3.4a	2.4b	2.0b	1.9
Oiliness	–	1.9	1.6	1.7	1.6	1.6
Overall Texture	2.5a	3.9b	4.3b	2.7b	3.1a	2.3

[a] Values followed by the same letters are not significantly different at $P < 0.05$. F was compared only with D and (*) indicates a significant difference ($P < 0.05$) from the corresponding values under D.

[b] Sensory evaluation scores from 1 to 5 with 1 representing the least and 5 the most that the panelist could perceive for the indicated attribute. Fineness was scored with 1 as fine and 5 as coarse. Overall texture was scored with 1 as poor and 5 as good.

[c] Treatment designations: A—no shortening, no SPF, Process I; B—12g shortening/100g fish muscle, no SPF, Process I; C—12g shortening/100g fish muscle and SPF, 10:90 SPF/fish muscle; Process I; D—12g shortening/100g fish muscle and SPF, 20:80 SPF/fish muscle, Process I; E—12g shortening/100g fish muscle and SPF, 30:70 SPF/fish muscle, Process I; F—12g shortening/100g fish muscle and SPF, 20:80 SPF/fish muscle, Process II.

the proper blend of particle size reduction of the SPF and the development of the appropriate bonding strength of the fish muscle itself through protein solubilization during comminution. The two-stage comminution process I appears to be the optimum in providing the most desirable texture.

Table 3 shows the result of the panel evaluation on the textural properties of products prepared using a one-stage or a two-stage comminution process with or without shortening at different levels of SPF. The behavior of the test material under stress and strain (firmness, brittleness, chewiness, rubberiness and elasticity) was significantly modified when the level of SPF in the batter exceeded the 30:70 SPF/fish flesh ratio. Brittleness was significantly increased in products prepared by the one-stage comminution. The addition of SPF at 10:90 SPF/fish flesh shortening at 10 g/100g fish flesh and SPF significantly reduced mushiness.

The textural characteristics related to the structure of the test material (smoothness, fineness, lumpiness, mealiness and fibrousness) were improved by the addition of shortening and SPF. In general, shortening appeared to play a major role in textural modification.

SPF additions in excess of 20:80 SPF/fish flesh significantly reduced smoothness ($p < 0.05$). The fineness score was poor at a level of 30:70. Scores for lumpiness increased at 20:80 and fibrousness at 30:70. Chopping in one-stage comminution resulted in a poor fineness score and increased mealiness ($p < 0.05$) with SPF additions at the 20:80 level. Moisture and juiciness were significantly diminished at 20:80 SPF/fish flesh ratio. Addition of SPF at 10:90 SPF/fish flesh did not alter moistness and juiciness that developed with the addition of shortening. Comminution in a silent cutter in one-stage process significantly reduced the WHC resulting in poor moistness and juiciness responses.

In the development of a coarse textured sausage, overall texture as judged by panels was improved by the addition of SPF and shorteining. The overall texture score was significantly impaired when using a one-stage comminution process.

Table 4 shows the panel response to the product formulated with different levels of deboned fish flesh. There was no significant difference in texture and general acceptability between products prepared from the mechanically deboned fish flesh and those from the ground fillet when moisture content and bone residues were carefully controlled.

Table 4—Panel response to the product formulated with different levels of mechanically deboned fish flesh and processed using the two-stage Process I

Formula	Score: Texture[a]	Acceptability[b]
100% ground fillet	4.0 ± 1.07[c]	4.25 ± 0.88
50% deboned flesh + 50% ground fillet	3.8 ± 1.23	4.12 ± 0.83
100% deboned flesh	3.7 ± 0.95	3.87 ± 0.99

[a] Texture: 1 (poor) – 5 (good)
[b] Acceptability: 1 (poor) – 4 (good) – 5 (very good)
[c] n = 30 responses
* Products were formulated with SPF and shortening at the respective levels of 20% and 12% of the combined weight of fish and SPF.

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Ms received 5/22/78; revised 5/29/79; accepted 6/5/79.

Support by the National Sea Grant Program under Grant No. 04-7-158-44126 and the University of Georgia Agricultural Experiment Station is gratefully acknowledged.

CHANGES IN LIPID COMPOSITION OF COOKED MINCED CARP (Cyprinus carpio) DURING FROZEN STORAGE

J. MAI and J. E. KINSELLA

ABSTRACT

Minced carp tissue was cooked by baking and deep-fat frying and stored at −18°C for periods up to 8 wk. Phospholipid (PL) levels decreased whereas free fatty acids (FFA) increased during frozen storage of all samples. Samples treated with antioxidants gave significantly ($P < 0.05$) higher values for FFA compared to the controls Thiobarbituric acid (TBA) values were higher in the cooked samples compared to the raw samples. Samples without antioxidants had considerably higher TBA values than those containing antioxidants. The carbonyl content of the samples fluctuated during storage. There was no significant change in the composition of the fatty acids during storage.

INTRODUCTION

COMMINUTED minced fish obtained from whole fish or filleted fish residue represents a novel source of food protein (Keay and Hardy, 1974; Crawford et al., 1972). Problems related to the lipids may arise in these products because mechanical destruction of tissue exposes lipids to accelerated oxidative and enzymatic reactions. Research is warranted to develop the information required to maintain the quality of these new fish products during storage.

In a related study, we reported the effects of cooking and antioxidants on lipids of minced carp (Mai and Kinsella, 1979). In the present study we determined changes in content and composition of total lipids, phospholipids, free fatty acids, TBA-reactive materials and carbonyls in cooked minced carp tissue.

MATERIALS & METHODS

Preparation of antioxidant and fish samples

The antioxidant solutions (BHA and TBHQ) were prepared according to the procedure given in publication No. ZG-109B, Eastman Kodak Company, Kingsport, TN (1976). The antioxidant solutions were applied to the raw fish at 0.01% (w/w) based on the lipid content of the minced fish.

The minced carp were cooked by baking and deep-frying (Mai and Kinsella, 1979). The cooking conditions were described previously. After cooking, the cooked samples were individually wrapped in polyethylene bags and stored at −18°C. Samples were withdrawn at bi-weekly intervals for analyses. All samples were prepared and analyzed in triplicate.

Lipid extraction and analyses

The lipid content was determined by the method described by Kinsella et al. (1977). The colorimetric method of Lowry and Tinsley (1976) was used to quantify FFA. The palmitic acid standard was dissolved in chloroform to make standards ranging from 0.25–4.0 mg. The amounts of FFA were reported as equivalents of palmitic acid.

Phospholipids were determined by the method of Raheja et al. (1973).

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0022-1147/79/0006-1619$02.25/0
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Thiobarbituric acid (TBA) test

The TBA values were determined by the method of Lemon (1975) which has a sensitivity range from zero to 0.05 μmoles malonaldehyde. The TBA values were calculated from a standard curve prepared using 1,1,3,3-tetraethoxypropane (TEP) as a standard.

Total carbonyls

Total carbonyls were measured by the procedure of Lawrence (1965) using the molar extinction coefficient reported by Schwartz et al. (1963).

Gas liquid chromatography (GLC)

The boron trifluoride-methanol method was used to prepare the fatty acid methyl esters (FAME) (Metcalfe et al., 1966). The FAME's were analyzed by a computerized automated Hewlett Packard 5830A GC. The parameters and column conditions have been described previously (Kinsella et al., 1977). The fatty acid composition was reported as mg fatty acid per 100g wet tissue.

RESULTS & DISCUSSION

Lipids and phospholipids

There was no detectable change in total lipids of samples during storage (Table 1). The phospholipid (PL) decreased in all the samples during frozen storage (Table 2). The decreases were very consistent in the case of the raw samples. The PL dropped from 530 to 371 mg/100g wet weight for the control, from 515 to 385 mg/100g wet sample for the BHA treated samples, and from 450 to 337 mg/100g wet weight for the TBHQ treated samples. The antioxidants apparently did not provide any protection against PL degradation. The loss of PL may be caused by both enzymatic and oxidative reactions (Olley and Lovern, 1960; Quaglia et al., 1974; Lee and Dawson, 1976). In the case of frozen fish, enzymatic reactions (e.g. phospholipases) are of more significance (Braddock and Dugan, 1973; Audley et al., 1978). The PL of the cooked samples remained unchanged during the first 6 wk. After the sixth week, a drop of PL was observed in the cooked samples. Whether this decrease of PL was due to the action of the residual phospholipases or to the nonenzymatic oxidation is a question of interest. Olley and Lovern (1960) reported that nonenzymatic hydrolysis did not occur in cooked frozen cod stored at −14°C. They also found that cooking of cod filet for 30 min at 100°C did not result in any appreciable loss of phospholipase activity. The minced carp in this study was cooked at a higher temperature (195°C) for less than 6 min corresponding to an internal temperature of approximately 80°C. With the data available it is not possible to ascertain whether enzymatic or oxidative reactions were responsible for the decrease of phospholipids.

Free fatty acid

The free fatty acid (FFA) of the raw frozen fish increased during the storage indicating that the lipases and phospholipases were active at −18°C (Table 3). The samples with antioxidants showed a higher production of FFA compared to the control and statistical analyses indicated that the effect of antioxidants on the accumulation of FFA was highly significant ($P < 0.01$) (Steel and Torrie, 1960). In a study of oxidation of fish fat, Ushkalova et al. (1974) found that in the presence of phenolic compounds, FFA accumulated more quickly due to some catalytic action of the phenols on fat hydrolysis. In the present study the FFA

Table 1—Total lipid content of raw and cooked minced carp (Cyprinus carpio) stored for 8 wk at −18°C*

Duration of storage (wk)	Total lipids (g/100g sample)								
	Raw			Baked			Deep-fried		
	Control	BHA	TBHQ	Control	BHA	TBHQ	Control	BHA	TBHQ
0	5.44[a] ± 0.16	5.93 ± 0.29	5.44 ± 0.22	5.85 ± 0.32	6.03 ± 0.37	6.28 ± 0.27	11.31 ± 1.38	12.50 ± 1.48	13.83 ± 1.72
	24.54[b] ± 0.74	27.11 ± 1.32	24.61 ± 1.01	23.64 ± 1.31	22.42 ± 1.42	22.24 ± 0.96	44.41 ± 5.41	37.47 ± 4.43	41.33 ± 5.14
2	5.23 ± 0.21	5.36 ± 0.18	5.02 ± 0.14	5.78 ± 0.17	6.33 ± 0.24	5.86 ± 0.21	9.75 ± 1.23	12.07 ± 1.54	13.60 ± 1.68
	24.20 ± 0.97	24.49 ± 0.83	23.20 ± 0.65	24.00 ± 0.71	24.50 ± 1.12	23.19 ± 0.83	37.62 ± 4.75	44.65 ± 5.70	45.09 ± 5.57
4	5.04 ± 0.13	5.03 ± 0.19	5.11 ± 0.20	5.84 ± 0.19	6.48 ± 0.28	5.80 ± 0.31	11.23 ± 1.37	9.33 ± 1.21	9.76 ± 1.26
	22.09 ± 0.57	20.99 ± 0.79	22.99 ± 0.91	23.67 ± 0.77	24.22 ± 1.05	23.44 ± 1.25	34.17 ± 4.17	34.96 ± 4.39	36.13 ± 3.85
6	5.28 ± 0.32	5.18 ± 0.25	4.96 ± 0.17	6.50 ± 0.31	6.67 ± 0.27	6.19 ± 0.25	12.40 ± 1.37	9.30 ± 1.42	10.53 ± 1.39
	24.57 ± 1.48	22.61 ± 1.09	22.34 ± 0.77	26.91 ± 1.28	25.24 ± 1.04	22.78 ± 0.92	35.13 ± 3.88	35.71 ± 5.45	38.75 ± 5.12
8	5.44 ± 0.25	5.47 ± 0.23	5.17 ± 0.16	6.18 ± 0.29	6.41 ± 0.24	6.14 ± 0.27	11.16 ± 1.43	11.30 ± 1.49	9.67 ± 1.40
	23.83 ± 1.10	24.25 ± 1.03	23.08 ± 0.71	22.42 ± 1.05	24.51 ± 0.92	22.51 ± 0.99	39.35 ± 5.04	41.98 ± 5.53	36.38 ± 5.27

[a] g/100g wet sample
[b] g/100g dry sample
* Mean of triplicate samples ± S.D.

Table 2—Phospholipid content of raw and cooked minced carp stored at −18°C with and without antioxidants for 8 wk*

Duration of storage (wk)	Phospholipids (mg/100g wet sample)								
	Raw			Baked			Deep-fried		
	Control	BHA	TBHQ	Control	BHA	TBHQ	Control	BHA	TBHQ
0	530 ± 23	515 ± 18	450 ± 24	510 ± 25	515 ± 14	585 ± 24	510 ± 25	530 ± 27	570 ± 38
2	430 ± 22	410 ± 31	430 ± 27	531 ± 27	580 ± 30	515 ± 19	555 ± 30	605 ± 40	560 ± 34
4	397 ± 9	398 ± 17	420 ± 16	501 ± 18	593 ± 32	545 ± 31	557 ± 25	500 ± 17	565 ± 23
6	379 ± 12	394 ± 23	390 ± 17	511 ± 32	592 ± 29	532 ± 27	537 ± 18	506 ± 23	500 ± 21
8	371[a] ± 11	358[a] ± 9	337[a] ± 16	459[a,b] ± 21	471[a,b] ± 16	482[a,b] ± 18	501[b] ± 14	504[b] ± 14	479[a,b] ± 17

[a] $P < 0.01$ vs samples at 0 wk
[b] $P < 0.01$ vs raw samples
* Mean of triplicate samples ± S.D.

Table 3—Fatty acid content of minced carp stored at −18°C with and without antioxidants for 8 wk*

Duration of storage (wk)	Free fatty acid (mg/100g wet sample)								
	Raw			Baked			Deep-fried		
	Control	BHA	TBHQ	Control	BHA	TBHQ	Control	BHA	TBHQ
0	229 ± 11	246 ± 12	248 ± 9	224 ± 14	234 ± 16	215 ± 8	207 ± 9	221 ± 7	238 ± 11
2	259 ± 7	283 ± 18	259 ± 8	233 ± 18	242 ± 22	246 ± 14	216 ± 12	229 ± 12	236 ± 14
4	258 ± 22	271 ± 21	275 ± 16	213 ± 7	227 ± 13	250 ± 13	213 ± 11	236 ± 17	219 ± 9
6	281 ± 13	312 ± 27	294 ± 18	252 ± 12	252 ± 21	277 ± 21	232 ± 13	239 ± 20	243 ± 12
8	336[a] ± 24	339[a] ± 17	338[a] ± 26	241[b,c] ± 12	240[c] ± 17	251[b,c] ± 17	215[c] ± 10	234[c] ± 11	227[c] ± 10

[a] $P < 0.01$ vs samples at 0 wk
[b] $P < 0.05$ vs samples at 0 wk
[c] $P < 0.01$ vs raw samples
* Mean of triplicate samples ± S.D.

Table 4—Thiobarbituric acid (TBA) values of minced carp samples stored at −18°C for 8 wk*

Duration of storage (wk)	TBA number (μmole malonaldehyde/100g wet sample)								
	Raw			Baked			Deep-fried		
	Control	BHA	TBHQ	Control	BHA	TBHQ	Control	BHA	TBHQ
0	0.44 ± 0.08	0.33 ± 0.03	0.38 ± 0.06	2.11 ± 0.23	1.16 ± 0.10	0.37 ± 0.04	0.94 ± 0.11	0.63 ± 0.08	0.70 ± 0.12
2	0.41 ± 0.03	0.33 ± 0.02	0.34 ± 0.08	0.96 ± 0.10	0.44[c] ± 0.08	0.36[c] ± 0.07	0.83 ± 0.04	0.84 ± 0.11	0.68 ± 0.08
4	0.42 ± 0.06	0.40 ± 0.05	0.30 ± 0.04	0.93 ± 0.12	0.44[c] ± 0.07	0.42[c] ± 0.08	0.67 ± 0.03	0.56 ± 0.06	0.47 ± 0.03
6	0.49 ± 0.04	0.33 ± 0.03	0.32 ± 0.09	0.96 ± 0.14	0.44[c] ± 0.06	0.31[c] ± 0.03	0.84 ± 0.06	0.65 ± 0.09	0.52 ± 0.04
8	0.44 ± 0.09	0.51[a] ± 0.16	0.42 ± 0.12	1.11[a] ± 0.16	0.76[a] ± 0.11	0.47[b] ± 0.05	1.06[b] ± 0.17	0.94[a] ± 0.13	0.82[a] ± 0.11

[a] $P < 0.01$ vs 0 wk
[b] Not significantly different from the raw samples
[c] $P < 0.01$ vs deep-fried samples
* Mean of triplicate samples ± S.D.

concentration reached the same level for all raw samples by the eighth week of storage.

For the cooked samples, especially the deep-fried samples, the production of FFA was reduced (Table 3). This suggested that heat treatment inactivated the lipolytic enzymes in these samples. In all the cooked samples, the FFA increased slightly after the fourth week. The increase corresponded to the increase of total carbonyls. However, there was no correlation between these two indices. The samples containing antioxidants gave significantly ($P < 0.05$) higher values of FFA. Whether the stimulation of FFA release by the antioxidants is caused by the enzymatic or nonenzymatic hydrolysis should be further tested.

TBA values

The TBA values of those samples without antioxidants were considerably higher than those containing antioxidants (Table 4). The cooked carp showed higher TBA values than the raw carp during frozen storage. Oxidative rancidity usually occurs more rapidly in cooked meat than in raw meat (Yamauchi, 1973). Lee and Toledo (1977) also found that cooking significantly raised the TBA values of minced mullet (Mugil spp.) during refrigerated storage. Younathan and Watts (1959) proposed that the iron in the heme protein changes from the ferrous to the ferric state upon denaturation and this enhanced catalysis of lipid oxidation. Both BHA and TBHQ proved to be effective in

Table 5—Carbonyl values of minced carp samples stored at −18°C for 8 wk*

Duration of storage (wk)	Carbonyl number (μmoles/100g wet sample)								
	Raw			Baked			Deep fried		
	Control	BHA	TBHQ	Control	BHA	TBHQ	Control	BHA	TBHQ
0	18.92 ±0.27	18.77 ±1.14	19.40 ±1.43	17.54 ±1.11	21.37 ±1.10	19.86 ±0.98	22.99 ±2.29	23.15 ±1.10	25.65 ±1.98
2	47.50 ±1.38	36.12 ±0.95	47.00[c] ±2.12	24.76 ±1.25	16.66[d] ±1.37	31.75[c,d] ±2.22	8.48[e] ±1.12	19.45[e] ±1.02	28.55[e] ±2.05
4	38.14 ±0.70	21.57 ±1.65	22.02 ±1.09	16.67 ±1.40	12.97 ±1.17	24.92[c] ±1.49	30.76 ±3.10	20.59 ±2.01	19.57 ±1.12
6	61.60 ±0.80	52.04 ±3.48	53.35 ±3.14	50.72 ±0.82	50.32 ±4.12	53.88 ±3.38	59.95 ±3.38	50.39 ±3.18	62.87[c,d] ±4.16
8	40.74[a] ± 4.32	36.37[a] ±2.10	44.55[a,c] ±3.53	32.73[a] ±2.15	32.97[b] ±1.17	40.93[b,c] ±2.12	40.22 ±2.52	51.80[a] ±4.01	43.08[c] ±2.32

[a] $P < 0.01$ vs 0 wk
[b] $P < 0.05$ vs raw samples
[c] $P < 0.05$ vs BHA treated samples
[d] $P < 0.01$ vs control
[e] $P < 0.001$ vs raw control
* Mean of triplicate samples ± S.D.

Table 6—Fatty acid composition of raw frozen minced carp stored at −18°C for 8 wk*

Fatty acid (mg/100g wet fish)	Time of storage (wk)								
	0			4			8		
	A[a]	B	C	A	B	C	A	B	C
C14:0	176	169	161	160	157	156	159	159	150
C15:0	48	51	48	55	46	48	49	49	47
C16:0	695	776	710	623	627	644	695	705	664
C17:0	49	54	43	44	45	48	55	53	50
C18:0	128	224	163	185	184	184	200	190	186
εSaturates	1096	1274	1125	1067	1059	1080	1158	1156	1097
C14:1	77	82	78	76	76	77	80	80	76
C15:1	20	23	21	19	20	20	25	25	24
C16:1ω7	948	1005	940	871	871	897	922	930	879
C17:1ω8	138	148	136	128	127	133	139	136	131
C18:1ω9	966	1073	963	821	833	827	939	929	873
C20:1ω9	59	64	59	54	56	55	75	75	69
εMonoenes	2208	2395	2197	1969	1983	2009	2180	2175	2052
C18:2ω6	290	311	315	235	238	238	266	250	249
C18:3ω6	11	11	13	10	9	10	8	9	7
C18:3ω3	387	404	383	357	357	359	355	355	341
C18:4ω3	76	75	73	77	76	75	74	75	71
C20:2ω6	10	11	9	10	10	10	10	10	10
C20:3ω9	7	8	5	9	10	9	9	8	9
C20:3ω6	11	12	8	10	8	11	10	11	10
C20:4ω6	141	153	139	133	136	136	142	143	139
C20:4ω3	44	49	43	53	56	52	48	50	49
C20:5ω3	353	404	371	374	379	370	377	378	363
C22:4ω6	32	36	38	36	37	39	38	41	37
C22:5ω6	2	8	6	11	10	13	12	13	12
C22:5ω3	87	102	86	78	81	84	98	101	95
C22:6ω3	118	133	118	117	118	128	134	132	126
εPolyenes	1569	1717	1607	1510	1525	1534	1581	1576	1518
Total	4930	5390	4930	4546	4567	4623	4919	4907	4667

[a] A: Control; B: BHA treated; C: TBHQ treated
* Mean of triplicate samples

preventing the formation of the TBA-reactive materials in the cooked fish (Table 4). Tertiary butylhydroquinone (TBHQ) seemed to be slightly more effective than BHA in this regard. It was also noted that the antioxidants were more effective in the baked samples than in the deep-fried samples during storage. During deep-frying, volatilization and leaching may have reduced the concentration of antioxidants in the cooked samples.

The TBA values of the raw fish remained rather constant during the storage period though the antioxidant treated samples in general gave lower TBA values. For the baked samples, the TBA values of both the control and BHA treated samples decreased to about half of their original values during the first 2 wk of storage but then remained steady until the sixth week when the values started to rise slightly. For the deep-fried samples, the TBA values remained relatively constant until the sixth week and then increased as in the baked samples.

Carbonyls

Initial carbonyl values of samples were close (approximately 20 μmoles carbonyls/100g wet sample) except the deep-fried samples showed higher values (Table 5). During storage, the levels of carbonyls varied and no specific patterns were observed. The highest level of carbonyls was observed in all samples at the sixth week. Interaction between protein and carbonyls may have made the carbonyls unavailable or nonreactive during the test (Shin et al., 1972). This might account for the drop of carbonyl values at the eighth week. In the baked samples, the carbonyl contents of the control were significantly lower ($P < 0.01$) than the TBHQ treated samples (Table 5). In the raw samples, the carbonyl content of the BHA treated sample was significantly ($P < 0.05$) lower than in the controls (Table 5). TBHQ did not seem to be effective on the raw samples.

From these results, it seems that the effectiveness of antioxidants on the control of carbonyl production depends on the sample and the status of the sample, i.e. whether it is cooked and the particular method of cooking.

Fatty acid composition

There was no significant change in fatty acid composition of samples during storage (Tables 6, 7, 8). Antioxidant treatment did not give any superior retention of the fatty acids. A typical GLC chromatogram of carp fatty acids is shown in Figure 1.

General discussion

During frozen storage phospholipids (PL) decreased and free fatty acids (FFA) increased in all samples. The degradation of PL and the concomitant accumulation of FFA indicated that lipolytic enzymes remained active in the uncooked samples at $-18°C$. A correlation coefficient of 0.6 was found between the decrease of PL and accumulation of FFA in the raw samples. The changes of PL and FFA of the cooked samples were apparently caused by chemical hydrolysis or by the action of residual lipolytic enzymes. The degradation of PL was completely independent of the antioxidants present in the samples, while the production of FFA was significantly correlated with the antioxidants applied. It is conceivable that the electron or proton donating property of phenols facilitated the release of FFA. Free fatty acid release was enhanced in both the raw and cooked samples by the antioxidants indicating that this stimulation might be independent of lipolytic enzymes.

Text continued on page 1624

Table 7—Fatty acid composition of baked minced carp stored at $-18°C$ for 8 wk*

Fatty acid (mg/100g wet fish)	Time of storage (wk)								
	0			4			8		
	A[a]	B	C	A	B	C	A	B	C
C14:0	155	173	172	176	196	177	180	186	179
C15:0	47	52	54	52	57	52	56	64	56
C16:0	771	796	826	725	799	716	791	825	790
C17:0	39	56	59	37	48	36	57	67	59
C18:0	215	213	227	207	228	209	226	164	225
ΣSaturates	1227	1290	1338	1197	1328	1190	1310	1306	1309
C14:1	74	83	84	85	93	84	90	96	92
C15:1	21	23	25	24	25	21	29	33	29
C16:1ω7	932	1026	1043	998	1109	992	1048	1087	1053
C17:1ω8	128	148	150	144	164	144	157	164	158
C18:1ω9	1111	1063	1131	970	1167	951	1068	1127	1040
C20:1ω9	60	53	68	64	64	64	84	87	82
ΣMonoenes	2326	2396	2501	2285	2622	2256	2476	2594	2454
C18:2ω6	406	304	349	280	317	308	310	343	300
C18:3ω6	12	13	13	10	12	11	9	13	9
C18:3ω3	349	427	429	410	452	436	405	425	411
C18:4ω3	70	78	80	86	92	85	85	88	87
C20:2ω6	11	12	12	12	14	12	11	12	12
C20:3ω9	2	7	7	11	12	12	10	11	10
C20:3ω6	6	13	13	12	14	10	12	12	12
C20:4ω6	144	157	162	162	183	161	162	166	167
C20:4ω3	43	49	51	72	92	68	57	57	58
C20:5ω3	380	409	423	456	517	418	429	442	437
C22:4ω6	32	37	38	40	48	38	46	45	44
C22:5ω6	6	7	9	7	4	21	18	15	13
C22:5ω3	94	99	106	92	99	90	114	110	112
C22:6ω3	132	140	149	138	150	141	164	151	152
ΣPolyenes	1732	1752	1841	1788	2006	1811	1832	1890	1824
Total	5285	5438	5680	5270	5956	5257	5618	5790	5587

[a] A: Control; B: BHA treated; C: TBHQ treated
* Mean of triplicate samples

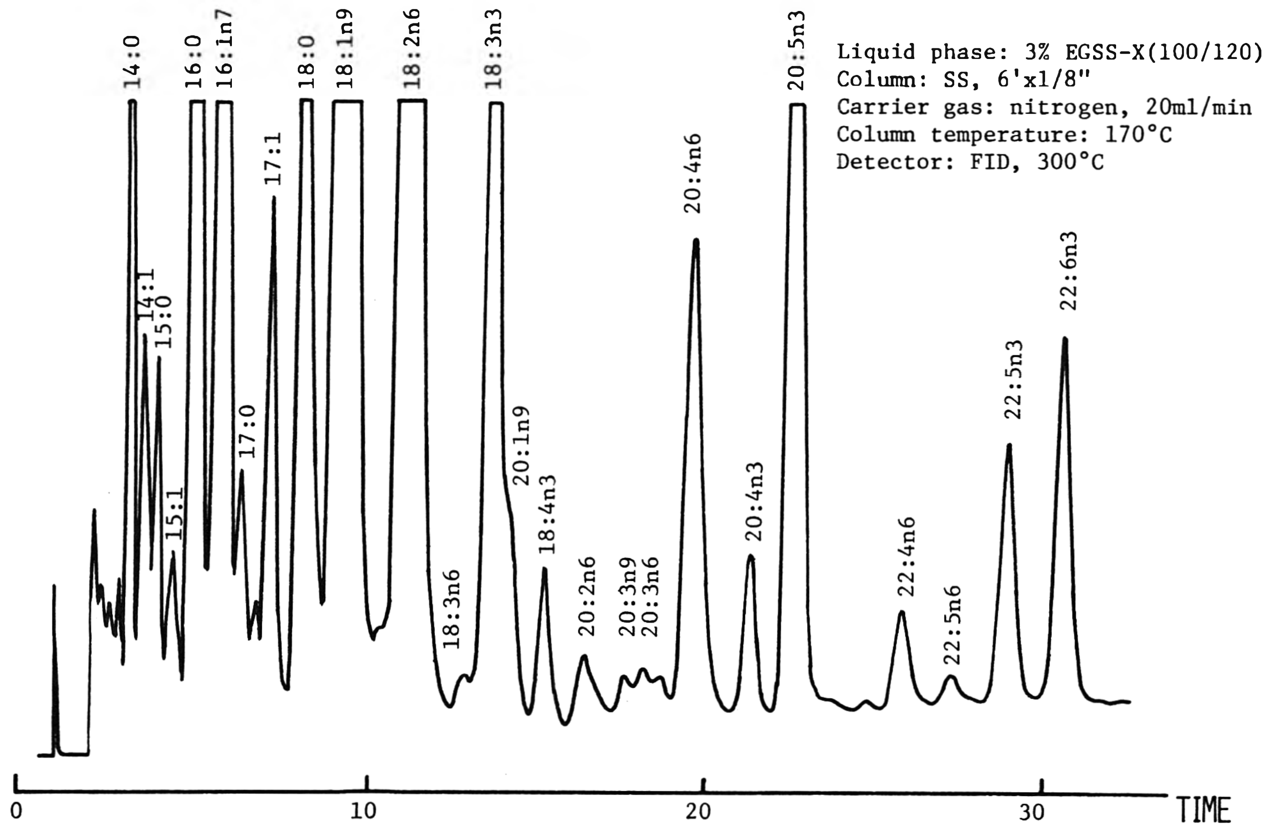

Fig. 1—A typical GLC chromatogram of fatty acids from raw minced carp separated on EGSS-X liquid phase.

Table 8—Fatty acid composition[a] of deep-fried minced carp stored at −18°C for 8 wk

Fatty acid (mg/100g wet fish)	Time of storage (wk) 0 A	0 B	0 C	4 A	4 B	4 C	8 A	8 B	8 C
C14:0	156	175	174	173	175	158	205	189	173
C15:0	47	54	54	51	51	47	61	56	54
C16:0	1265	1385	1497	1201	1042	1081	1301	1223	1110
C17:0	59	65	63	40	40	39	74	60	59
C18:0	425	467	522	405	336	362	422	406	345
ϵSaturates	1952	2146	2310	1870	1626	1687	2063	1934	1741
C14:1	72	81	79	79	80	74	98	92	86
C15:1	22	22	23	24	23	23	31	29	28
C16:1ω7	945	1048	1037	958	987	900	1158	1062	1017
C17:1ω8	134	150	148	136	141	128	173	158	148
C18:1ω9	3001	3303	3754	2829	2173	2410	2799	2727	2257
C20:1ω9	76	85	89	79	68	72	85	77	82
ϵMonoenes	4250	4685	5130	4105	3472	3607	4344	4145	3618
C18:2ω6	2658	2968	3518	2624	1773	2126	2390	2409	1796
C18:3ω6	18	22	22	15	18	13	14	10	11
C18:3ω3	612	687	741	641	553	574	680	648	563
C18:4ω3	72	81	82	75	81	73	89	82	79
C20:2ω6	8	9	9	14	12	13	12	17	12
C20:3ω9	12	2	3	11	7	10	11	20	10
C20:3ω6	9	2	11	11	12	10	11	10	5
C20:4ω6	134	146	152	158	156	143	174	161	164
C20:4ω3	41	45	45	76	74	51	57	53	55
C20:5ω3	372	416	420	421	443	385	474	426	427
C22:4ω6	28	31	32	39	50	32	38	41	50
C22:5ω6	2	2	3	9	15	8	17	19	22
C22:5ω3	94	103	107	88	87	91	125	130	123
C22:6ω3	130	144	151	138	139	135	172	182	174
ϵPolyenes	4190	4658	5296	4320	3420	3664	4264	4208	3491
Total	10392	11489	18012	10295	8518	8958	10671	10287	8850

[a] A: Control; B: BHA treated; C: TBHQ treated
* Mean of triplicate samples

Both TBA and carbonyl values are general indices for oxidative rancidity. Several TBA-sensitive substances other than malonaldehyde do not give an absorption maximum at around 534 nm (Marcuse and Johansson, 1973; Asakawa et al., 1975). The 2,4-dinitrophenylhydrazine procedure for determination of carbonyls is not sensitive enough to detect the early stages of off-flavor development (Erickson and Bowers, 1976). The direct quantification of peroxide decomposition products using gas liquid chromatography may provide a better method for more accurately determining the oxidative changes in foods (Erickson and Bowers, 1976; Marcuse and Johansson, 1973).

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Ms received 1/19/79; revised 6/13/79; accepted 7/1/79.

Supported in part by New York State Sea Grant program. We thank Mr. George Houghton for the interpretation of statistical results.

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chowder containing 0.5% CFI. CFI improved the organoleptic qualities of the chowders made with and without clam meat. Chowder containing 1% CFI and no clam meat was slightly better than the chowder made with 3.5% clam meat. Thus, it is apparent that CFI could be used as either a replacement for clam meat or a flavor enhancer. The effective level of CFI would vary with food products.

CFI has several advantages over clam juice which is used widely as a flavoring base for clam-flavored food products. An initial investment is required for a surf clam processor to produce CFI. However, economies will be realized from transportation, storage, and packaging when compared with heavier and bulkier clam juice. Higher profits may be realized from CFI than from clam juice because (a) as a dried flavor ingredient, CFI belongs to a higher value commodity group than clam juice, (b) the versatility of CFI in product application may result in a higher demand for CFI than clam juice, and (c) the increasing consumption of shellfish products in the U.S. coupled with the declining supply will make shellfish flavors an attractive economic substitute for shellfish in formulated foods.

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Ms received 3/21/79; revised 7/4/79; accepted 7/10/79.

The helpful advice of Dr. R.R. Zall and Mr. John Plock is appreciated. This research was sponsored by the New York Sea Grant Institute under a grant from the Office of Sea Grant, National Oceanic and Atmospheric Administration (NOAA), U.S. Department of Commerce.

STABILITY OF ADENOSINE DEAMINASE AND ADENOSINE MONOPHOSPHATE DEAMINASE DURING ICE STORAGE OF PINK AND BROWN SHRIMP FROM THE GULF OF MEXICO

WAI LUN CHEUK, GUNNAR FINNE and RANZELL NICKELSON II

ABSTRACT

The stability of adenosine deaminase and AMP deaminase from pink and brown shrimp muscle extracts was assayed during postmortem ice storage. AMP deaminase activity was lost rapidly during the early stage of ice storage, and no activity could be detected after 10 days for pink and 16 days for brown shrimp. Even though there was a gradual loss in activity, adenosine deaminase could be detected in both species through the entire storage period of 21 days. The stability of these two enzymes was also correlated with the traditional spoilage indicators: total volatile nitrogen (TVN), total plate count (TPC) and organoleptic evaluations. During the lag-phase of bacterial growth, AMP deaminase lost 50% of its original activity while adenosine deaminase remained active. During the log phase, bacterial counts and TVN values gradually increased to 10^8/g and 30 mg TVN-N/100g, respectively, while AMP deaminase lost its activity. Finally, during the stationary phase, adenosine deaminase (with low activity) was the only ammonia producing tissue enzyme that could be detected. The activity of adenosine deaminase and AMP deaminase could potentially be used as quality indices for fresh shrimp held on ice.

INTRODUCTION

QUALITY CHANGES that occur in iced shrimp during storage are considered to be the result of combined action of tissue enzymes and microbial activity (Nair and Bose, 1964; Cobb and Vanderzant, 1971; Flick and Lovell, 1972). Changes in pH (Bethea and Ambrose, 1962; Bailey et al., 1956), microbial numbers (Campbell and Williams, 1952), trimethylamine (Iyengar et al., 1960; Fieger and Friloux, 1954), total volatile nitrogen (TVN), nonprotein nitrogen (NPN), free amino acids (Velanker and Govindan, 1957, 1958; Gagnon and Fellers, 1957; Cobb et al., 1974), volatile acids (Fieger and Friloux, 1954), indole (Duggan and Strasburger, 1946) and carotenoid levels (Collins and Kelley, 1969) have been either used or proposed as quality indices of shrimp held on ice. The importance of ammonia formation during spoilage is evident since many of the above quality indices are indicators of either microbial or tissue enzyme ammonia production.

The effect of ammonia producing tissue enzymes on shrimp quality during postmortem ice storage was shown by Yeh et al. (1978). Among a large number of potential ammonia producing enzymes tested, they found in their study that only adenosine deaminase and AMP deaminase could be detected in significant levels in white shrimp (*Penaeus setiferus*) tails. Furthermore, at every temperature tested, enzymatic ammonia production from shrimp tissue during initial postmortem storage accounted for more than half of the total ammonia production indicating the importance of these two enzymes.

The nucleotide degradation pathway in shrimp and stability of the enzymes catalyzing the reactions involved have not been clearly established. Arai (1966) observed two pathways for the breakdown of adenine nucleotides in Japanese prawn (*Pandalus hypsinotus*). One involves the direct deamination of adenosine monophosphate (AMP) to inosine monophosphate (IMP), while the second involves dephosphorylation of AMP to adenosine, which is followed by deamination to inosine. Both these pathways will subsequently lead to hypoxanthine. Stone (1971) working on Alaskan shrimp (*Pandalus borealis, Pandalus platyceros* and *Pandalopsis dispar*) held on ice, showed the major pathway for adenine nucleotide degredation to result in accumulation of IMP rather than inosine. Flick and Lovell (1972) showed the opposite to be true for shrimp from the Gulf of Mexico (*Penaeus setiferus*) where inosine instead of IMP was first detected after death.

The purpose of this study was to investigate the stability of AMP deaminase and adenosine deaminase during iced storage of pink (*Penaeus duorarum*) and brown shrimp (*Penaeus aztecus*) caught in the Gulf of Mexico. Concurrently the shrimp were also analyzed for total plate count (TPC) and total volatile nitrogen (TVN) and were also subjected to sensory evaluation. When the relative activity of the two deamination enzymes are known, one can also determine the predominant nucleotide degradation pathway in Gulf shrimp.

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0022-1147/79/0006-1625$02.25/0
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MATERIALS & METHODS

Shrimp

Pink shrimp (*Penaeus duorarum*) and brown shrimp (*Penaeus aztecus*) were obtained directly from fishing boats in Aransas Pass and Corpus Christi and immediately packed in ice and shipped to the laboratory. The shrimp were deheaded, thoroughly washed, and immediately stored in ice chests well mixed with twice their weight of ice. The ice chests were held at room temperature, and the shrimp were sampled, drained, and re-iced on a daily basis for a period of 21 days.

Chemical and microbiological analysis

Protein determination was done according to Kjeldahl nitrogen content × 6.25 (AOAC, 1975) or the Biuret procedure (AOAC, 1965). Total volatile nitrogen was determined by the microdiffusion method using $Na_3PO_4 \cdot KOH$ as the releasing agent as described by Cobb et al. (1973). Ammonia was determined by the colorimetric method described by McCullough (1967). Total plate counts were estimated by the agar pour plate method (APHA, 1970).

Enzyme assay

Extraction. Extracts for enzyme assay were prepared from each species by homogenizing 15–21g of muscle tissue in a Waring Blendor with 7 parts of ice cold extraction buffer: 0.3M KCl, 0.09M KH_2PO_4 and 0.06M K_2HPO_4 adjusted to pH 6.5 (Lee, 1957). After 1 min of homogenizing, the mixture was transferred to a 500 ml beaker and stirred slowly for 1 hr at 3°C, forming a firmly packed precipitate which was shown to contain no deaminase activity. Insoluble particles were removed from the supernatant liquid by filtration through two layers of cheese cloth. Adenosine deaminase and AMP deaminase activities were measured by a modification of the method of Stone (1970), where the decrease in optical density (OD) of the substrate solution was monitored at 265 nm (Kalckar, 1947). The standard assay was conducted at 25°C using a 1.0 cm path length quartz curvette containing 3.0 ml substrate in a Perkin-Elmer, Model 124, double-beam grating spectrophotometer with an automatic recorder. The reaction was initiated by the addition of 0.4 ml

enzyme extract to the substrate, whereupon the reaction was followed for 10–20 min. Initial velocity was determined from the first part of the reaction curve by drawing a tangent to the curve at zero time. The activity of the enzymes was calculated according to Stone (1970) using differences in extinction coefficients between substrates and products of 7900 for adenosine and inosine (Hoagland and Fisher, 1967) and 8860 for AMP and IMP (Smiley et al., 1967).

Adenosine deaminase. The substrate for adenosine deaminase assay consisted of 12 mg/L adenosine in 0.05M phosphate buffer, pH 7.5.

AMP deaminase. The substrate for AMP deaminase assay consisted of 15 mg/L AMP in 0.1M Na_2-succinate buffer, pH 6.5.

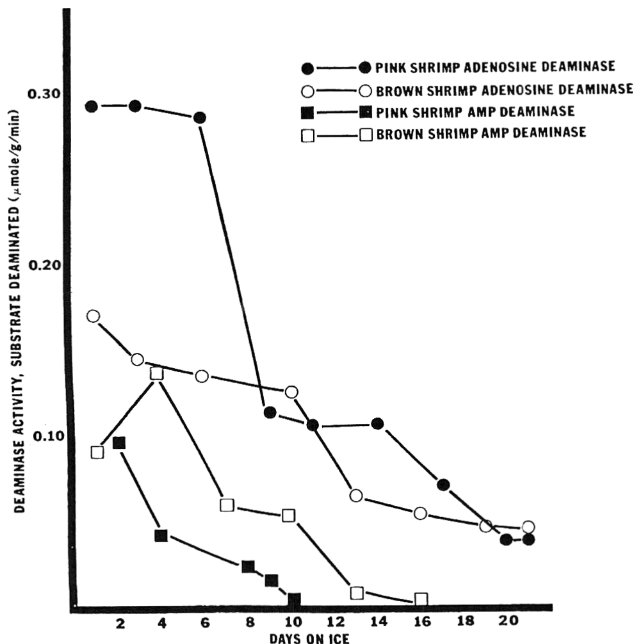

Fig. 1—Stability of adenosine deaminase and AMP deaminase activity in pink and brown shrimp held in ice.

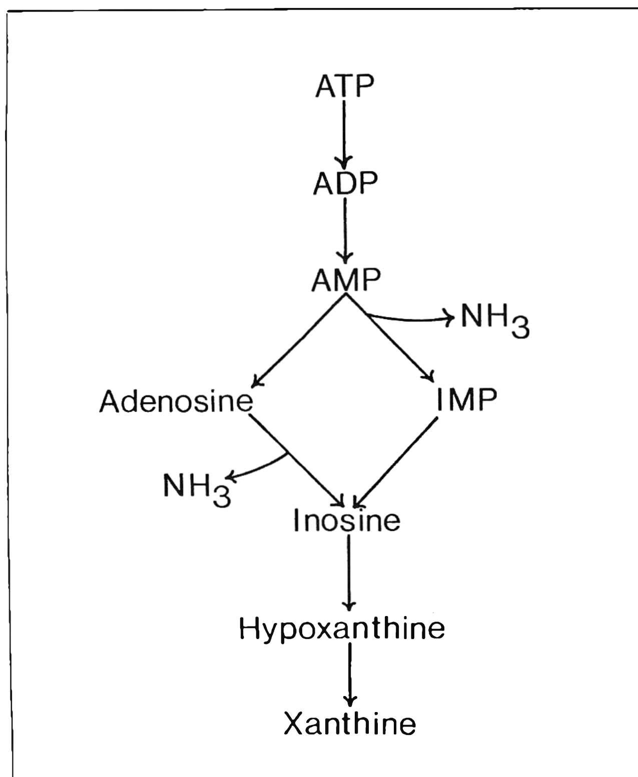

Fig. 2—Degradation pathway of ATP in shrimp.

Organoleptic evaluation

For organoleptic evaluation, shrimp (shell on) were heated in closed plastic bags in a boiling water bath for 3 min. By using this "boil in bag" method, volatiles released during heating are retained within the bag and onset of off-odors and flavors are more easily detected. The samples were served in coded bags to panelists seated in individual booths. The 12 panelists, consisting of graduate students and staff at the Seafood Laboratory, experienced in evaluating quality of seafoods, were asked to open the bags and evaluate the shrimp for odor, flavor, texture, juiciness and desirability. An intensity scale ranging from a high of 9 ("highest value") to a low of 1 ("lowest value") was used.

RESULTS & DISCUSSION

Activity of adenosine deaminase and AMP deaminase in shrimp stored on ice

The activity of adenosine deaminase and AMP deaminase in muscle extracts from pink and brown shrimp are shown in Figure 1. The figure indicates there was a gradual loss in activity as storage progressed in all four cases tested.

Adenosine deaminases from pink and brown shrimp not only showed a higher activity than the corresponding AMP deaminases but these two enzymes also retained close to optimum activity during the early part of the storage period. In pink shrimp, adenosine deaminase showed an initial activity of 0.29 μmole/g/min, 95% of which was retained after six days of iced storage. From the sixth day on, there was a rapid decline in activity to a value of 35% of original activity on the ninth day of storage after which the rate of loss in activity leveled off. Adenosine deaminase activity from brown shrimp, although somewhat less active, exhibited the same phenomenon as the adenosine deaminase of pink shrimp. The decline in activity of this enzyme in muscle of pink and brown shrimp stored on ice was approximately 50% of original activity after 9 and 12 days, respectively. However, both enzymes retained activity throughout the entire storage period of 21 days.

After 10 days of iced storage, no AMP deaminase activity could be detected in muscle extracts from pink shrimp. Even after only 4 days on ice, pink shrimp lost 50% of original AMP deaminase activity. Apart from an increase during the first few days, AMP deaminase from brown shrimp showed a similar stability pattern as the corresponding enzyme from pink shrimp. After 16 days on ice no AMP deaminase activity could be detected in brown shrimp muscle extracts. The instability of AMP deaminase is in agreement with earlier work done on crustaceans (Dingle and Hines, 1967; Flick and Lovell, 1972). Loss of enzyme activity during ice storage can be due to a number of factors. The enzymes may be washed out by melting ice, degraded by a constantly increasing bacterial population or may eventually run out of substrate. Accumulation of inhibitors may be another reason for activity loss. Since the enzymes were assayed in crude extract preparations, the activity may be inhibited by the presence of heavy metal ions, such as mercury or others. Feedback inhibition by accumulation of reaction products may still be another reason for enzyme activity loss. AMP deaminase has been shown to be inhibited by inorganic phosphate (Nikiforuk and Colowick, 1955) seemingly due to competition between free phosphate and the phosphate group on AMP for the binding site on AMP deaminase. This feedback inhibition may be a contributing factor to the rapid loss of AMP deaminase activity in ice stored shrimp.

Nucleotide breakdown sequence in Gulf shrimp

The postmortem degradation of adenine nucleotides in-

volves a sequence of continuous reactions. The rate at which the products of deamination accumulate in the muscle tissues depends both on the activity of the enzymes catalyzing the formation of these products and the activity of the enzymes which further change these products to other products. A simplified reaction sequence for the degradation of nucleotides in shrimp is shown in Figure 2 (Flick and Lovell, 1970). Based on the relative enzyme activity shown in this study, we suggest that the principal nucleotide degradation pathway for postmortem pink and brown shrimp held on ice is through adenosine rather than inosine monophosphate (IMP). The reason for this is the much higher initial activity observed for adenosine deaminase as compared to AMP deaminase and the rapid loss in AMP deaminase activity during the storage period. Both adenosine and AMP deaminases are principal ammonia producing enzymes during early stages of ice storage, while adenosine deaminase is the sole ammonia producer during the later stages.

Total volatile nitrogen and total plate count

Total volatile nitrogen (TVN) production is the combined result of tissue enzymes and microbial activities (Cobb and Vaderzant, 1971). Figure 3 shows the TVN analyses of shrimp extracts together with total aerobic bacterial count from both pink and brown shrimp over a period of 20 days ice storage. Total volatile nitrogen values showed similar patterns for both pink and brown shrimp. Apart from some high values for brown shrimp during the first few days, the TVN values remained constant for approximately 11 days for brown shrimp and 15 days for pink shrimp after which there was a rapid increase in both cases. Cobb et al. (1973) reported that the TVN value for spoiled Gulf shrimp usually exceeded 30 mg TVN-N/100g. In this study brown shrimp reached this value after 16 days on ice and pink shrimp after 19 days.

The microbial population on shrimp stored on ice was shown to increase from approximately 10^2/g to around 10^9/g over the 20-day time period. The low initial numbers, specifically for pink shrimp, signified high quality. As indicated in Figure 3 there was a good correlation between total counts and TVN production. Brown shrimp with highest microbial count also showed higher TVN values reaching 30 mg TVN-N/100g 3 days before pink shrimp.

Organoleptic evaluation

The designations of organoleptic quality given in Figure 4 were based on the "boil in bag" method. Each point on the graphs represent the average score for: odor, texture, juiciness, flavor and desirability, each of which was determined as the average of 12 panel members. Highest affirmative value was 9 and lowest 1. The shrimp, according to the taste panel, retained its prime quality up to 10 days after which there was a loss of the characteristic sweet flavor often associated with fresh crustaceans. From the 10th to approximately the 16th day, the shrimp were still of acceptable commercial quality, bland in flavor but without pronounced off-odor or off-flavor. From the 16th day on, a pronounced amine odor and off-flavor developed and the shrimp became unacceptable to the panelists.

Ammonia producing enzyme activity as potential quality index for fresh shrimp

From the relationship between TVN values, organoleptic evaluation and deaminase enzyme activity, there is a strong indication that the instability of AMP deaminase can be used as a quality parameter for shrimp held on ice. This study showed that AMP deaminase activity could not be detected in pink shrimp after 10 days of ice storage which coincided with the time that the taste panel determined the shrimp to no longer be of prime quality. It also corresponds to the time when TVN values started to increase. Brown shrimp lost AMP deaminase activity on the 16th day when the shrimp were still judged to be of acceptable commercial quality. If a rapid and technically simple test for AMP

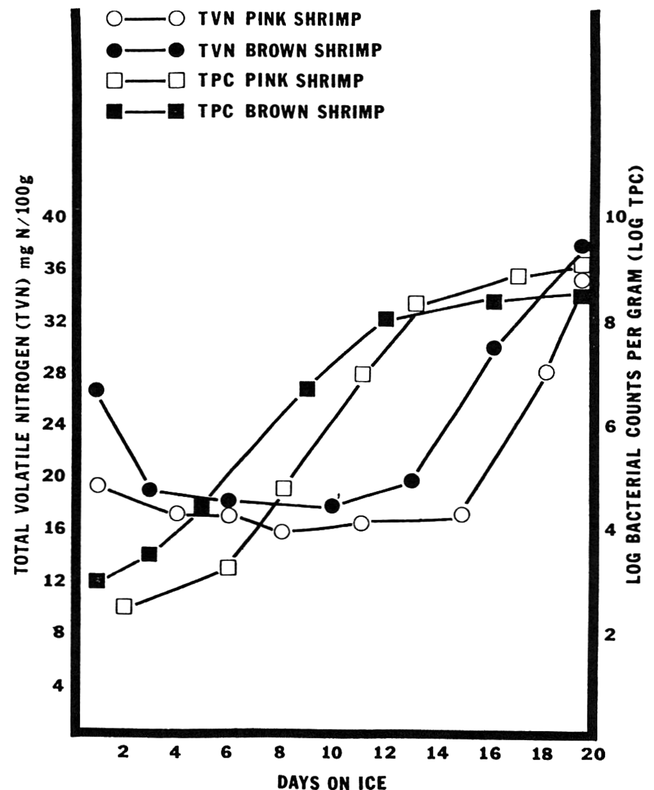

Fig. 3–Effects of storage on the agar plate counts, and total volatile nitrogen on pink and brown shrimp stored in ice.

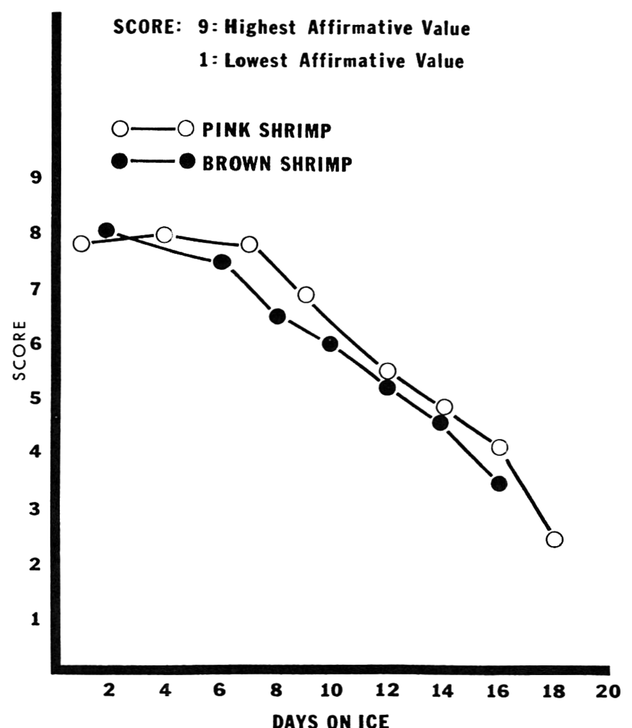

Fig. 4–Effect of storage on the organoleptic evaluation of pink and brown shrimp stored in ice.

deaminase activity can be developed, detection of activity can give a good indication of post-harvest storage time. Since both AMP deaminase and adenosine deaminase gradually lose activity during the storage period, the rate of these enzymatic reactions can also give an indication of ice storage time.

In order to test the possibility of using the activity of these two enzymes as quality indices for fresh shrimp, we sampled the catch from 21 commercial Texas shrimp trawlers. Approximately 1 lb of iced shrimp were sampled from the hull of each vessel and immediately repacked in ice and shipped to the laboratory. The trawlers that were sampled were selected on the basis of age of catch (days at sea). The shrimp samples were analyzed for AMP deaminase and adenosine deaminase activity and organoleptically evaluated by the Seafood Laboratory personnel. The enzyme activity data showed a close relationship to the days after harvest and to the organoleptic evaluations. Since the enzyme assays require well trained personnel and sophisticated instrumentation, the method described here cannot be expected to be part of the daily quality control in seafood processing operations. However, by simplifying the method into a colorimetric test, this method could hopefully find its way into the shirmp industry in the form of a test strip similar to that developed by Jahns et al. (1976) for hypoxanthine.

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Ms received 4/14/79; revised 6/13/79; accepted 7/1/79.

Technical Article 15108 from the Texas Agricultural Experiment Station. This work was supported through Institutional Grant 09-7-158-44105 to Texas A&M University by the National Oceanic and Atmospheric Administration's Office of Sea Grants, Department of Commerce.

TEXTURAL CHARACTERIZATION OF SQUID (Loligo pealei LESUER): SCANNING ELECTRON MICROSCOPY OF COOKED MANTLE

W. STEVEN OTWELL and DONALD D. HAMANN

ABSTRACT

SEM was used to reveal progressive structural alterations in the tissue components of cooked squid mantle. Progressive changes were observed at various cook temperatures, 50, 60, 70, 80 and 100°C and after different periods of cooking, 1, 2, 4, 8, 16, 32 and 64 min at 100°C. Thermal alterations of connective tissues first appeared as interfibril 'melting,' then total fiber gelatinization at higher temperatures. The onset temperatures of 'melting' and gelatinization differed depending on the anatomical location of the connective tissue tunics. Thermal alterations of muscle fibers appeared as a loss of myofibril distinction first evident at 50°C. Increasing temperature of muscle fibers caused, in order, coagulation of sarcoplasmic proteins, disintegration of the sarcoplasm, and continuous fiber shrinkage and dehydration. These changes in tissue structure were partially explained by simultaneous changes in gross body dimensions, and mantle pH, moisture, and protein content.

INTRODUCTION

THE MAIN EDIBLE PORTION of a squid is the mantle. It is the cone-shaped trunk of the body which provides locomotion, contains the viscera, and accounts for 45% of the total body weight (Otwell, 1978). Mantle can be fried, smoked, roasted or boiled. The method of preparation and duration of cook will affect texture. Mantle texture is a dominant and unique characteristic which can determine marketability of squid.

American consumers are hesitant to accept squid as a new food item because it has a tough, rubbery texture (Kalikstein, 1974). Most consumption of squid in the United States centers around certain ethnic groups, but even in Japan where squid is eaten more as a staple item, complaints of improper squid texture are not uncommon (Takahashi and Takei, 1955; Takahashi, 1965). In Europe, a recognized difference in texture between species *Loligo pealei* and *Illex illecebrosus*, influences market acceptance (Galus, 1975). *I. illecebrosus*, the deepwater or short-fin squid harvested along the eastern coast of the United States, is considered more leathery.

This study was initiated to illustrate the structural features of the mantle which are involved in the textural character of cooked squid. Scanning electron microscopy (SEM) has been used effectively to demonstrate structural alterations in cooked beef (Alexander and Fox, 1975; Cheng and Parrish, 1976; Jones et al., 1977), poultry, and fish (Schaller and Powrie, 1972). Stanley and Geissinger (1972) tried to relate sarcomere lengths of raw bovine muscle, as meassured with SEM, with toughness ratings and taste panel scores. No work was found which correlates SEM illustrations of the structural attributes of cooked meat with tests of physical strength and subjective responses, i.e. rheology and human panels, respectively.

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0022-1147/79/0006-1629$02.25/0
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This report uses SEM results to illustrate the progressive structural alterations which occurred during cooking of squid mantle. A second report, Otwell and Hamann (1979), demonstrates the progressive changes by physical tests and texture panel evaluations of the cooked mantle texture. A comparison of the results from these two studies gives an in-depth understanding of squid texture, and demonstrates the utility of SEM in combination with rheology and sensory perception to give a total textural characterization of food.

MATERIALS & METHODS

SQUID, *Loligo pealei*, harvested by commercial vessels fishing along the North Carolina coast, were held on ice for at most 72 hr after harvest. Rigor mortis occurred shortly after the squid were caught. To assure homogenity of samples only squid mantles 10–20 cm in length, which showed no observable signs of spoilage, were used. Pigment 'staining' of the outer surface, the initial and most obvious sign of spoilage (Takahashi, 1965), was never observed on any mantles used in this study. All squid were cleaned (skin, head and viscera removed), packed in 10 x 20 cm Whirl-pak bags, then slowly frozen (–29°C). Frozen storage was a convenience which allowed continuous work throughout the year irrespective of harvest seasonality, and suited the experimental design.

Prior to cooking, frozen mantles were thawed at 4°C ambient, then directly submerged in a pot of distilled water at room temperature (25°C). To assure homogenity of cook treatments all squid were cooked in the same type aluminum pot, filled with a 3:1 ratio of water to squid (w/w). Total water volume was 500–800 ml. Samples were heated at a rate of 5°C/min to specific cook temperatures, 25 (raw), 50, 60, 70, or 80°C and then immediately removed from the hot water. Other samples were heated to 100°C and continually boiled for specified cook times, 1, 2, 4, 8, 16, 32 and 64 min before removal. Thermometers and a stopwatch were used to monitor cook temperatures and times. The internal temperature of the thin mantle was assumed to be the same as the cook medium.

Analytical procedures were used to determine changes in gross weight, dimensions and basic mantle chemistry. Changes in gross weight and dimensions of whole mantle were calculated from changes in measurements before and after cook treatments. Thickness of the mantles was measured to the nearest 1/100 cm with a conductive micrometer stage (Otwell and Hamann, 1979). Protein, moisture, and fat content were determined by standard methodology (AOAC, 1975). Hydrogen ion activity (pH) of mantles at each test treatment was determined at the specific cook temperatures, and at room temperature subsequent to cooling from the specific cook temperature. A Fisher Model 420 digital pH meter was calibrated with standard buffers (Fisher Scientific Co.) warmed to specific temperatures. The pH was determined by direct contact of a Fisher standard combination probe in a slurry (3:1 ratio) of distilled water and mantle. Amount of protein leached during cook was determined by biuret analysis (Gornall et al., 1949) of filtered (No. 4 Whatman paper) volumes of cooled cook water. The final total volume of cook water was measured to determine water loss during cook.

Raw and cooked mantles, tempered to room temperature by air cooling, were prepared for SEM by the method of Otwell (1978). Glutaraldehyde was used for primary fixation. The fixed samples were freeze fractured in liquid nitrogen and critical point dried. Both fracture and nonfractured surfaces were examined with SEM. Photomicrographs shown in this report are representative of numerous views of at least three samples from each cook treatment. Each cook treatment was replicated in triplicate.

Gelatinization and melting are terms used interchangeably to denote the thermal phase transition of collagen from the highly ordered molecular state to a random state, gelatin. Terms such as

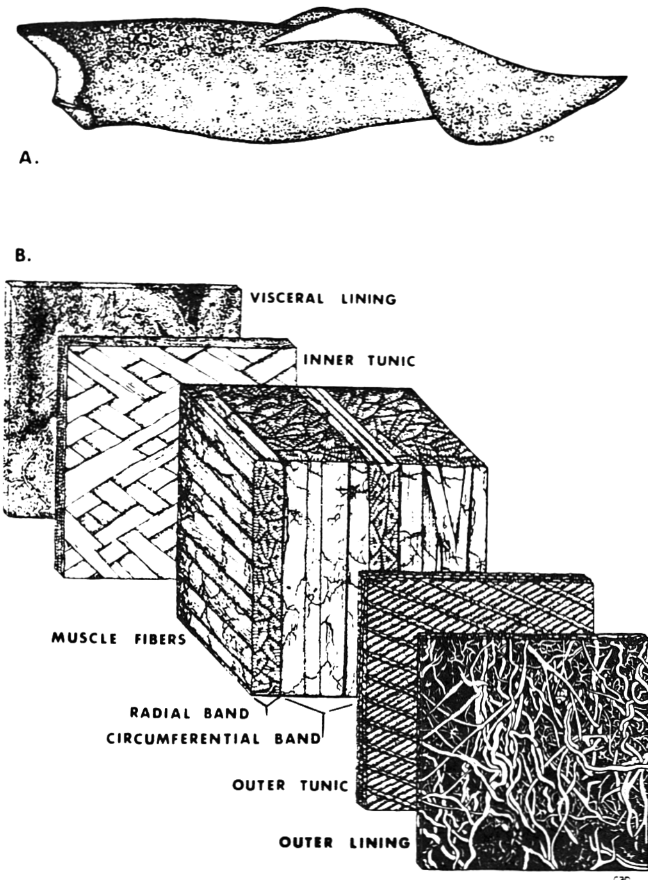

Fig. 1—Artist's rendition of squid mantle. (A) Whole mantle structure with skin attached, and head, tentacles, and viscera removed. Black dots on the surface are chromatophores. (B) Expanded cube cut from the entire mantle thickness. The drawing is not to scale to emphasize specific structural features. Skin has been omitted to reveal muscle fiber structure.

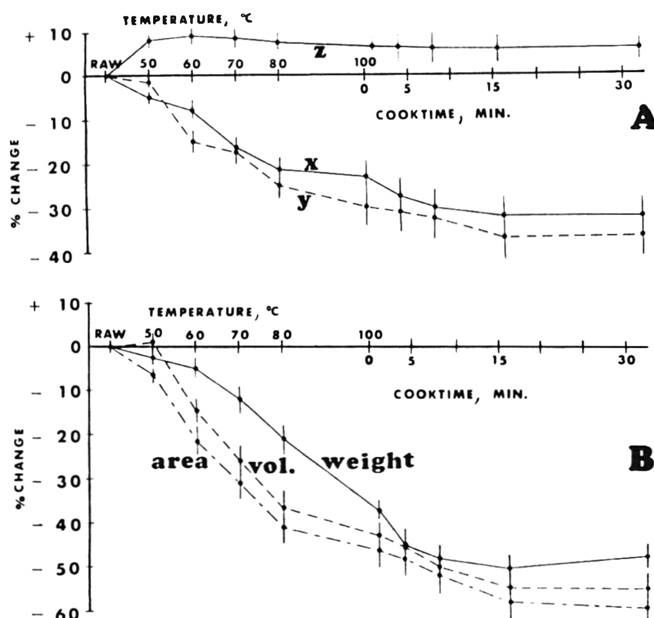

Fig. 2—Effect of cook temperature and cook time on mean % change in mantle physical measurements. (A) z is change in thickness, y is change in longitudinal axis dimension and x is change in lateral axis dimension. (B) Area, volume and weight changes.

protein denaturation, conformation change and coagulation, imply thermal alterations of proteins from their native structure.

RESULTS

Raw mantle

The mantle with skin on and head and tentacles removed appears like a cone with fins attached to the dorsal surface (Fig. 1A). The fins were not considered in this study, but their structure is identical with that of the mantle (Tanaka, 1958). An expanded cube cut from the entire thickness of the mantle (Fig. 1B) illustrates specific structural attributes of the various component tissues. This expanded drawing was made with reference to numerous photomicrographs taken during a SEM study by Otwell (1978) in which a complete structural analysis of these raw tissues is given.

Briefly, the mantle is composed of muscle tissue sandwiched between two tunics of connective tissue. Muscle fibers are grouped in bands which are arranged orthogonally. Circumferential muscle fibers run within the mantle thickness circling the entire circumference of the cone. Radial fiber direction is perpendicular to both tunics of connective tissue. All muscle fibers are small ($\bar{x} \cong 3.5 \pm 2.5$ μ diam) elongated cells of myofibrils surrounding a central core which houses sarcoplasmic proteins, mitochondria, and at least one nucleus. Squid muscle fibers are obliquely striated and covered with a thin sarcolemma. Descriptive terminology used in this report is adapted from Ward and Wainwright (1972) and Moon and Hulbert (1975). Fibers of connective tissue are arranged in a specific pattern in the outer tunic, but appear less ordered in the inner tunic. All connective tissue fibers are composed of aggregates of smaller fibrils ($\bar{x} \cong 0.1$ μ diam), but fiber size and shape differs in each tunic. The inner and outer tunics are covered by a nonfibrous visceral lining, and an outer lining of randomly oriented fibers, respectively.

Cooked mantle

When the mantle is cooked it retains its original pure white color, emits a unique strong odor, and curls. The curling is an instaneous phenomena which occurs just as the mantle is heated to 60°C. A flat strip cut from the cone-shaped mantle, will curl head-to-tail toward the outside surface. Cut sample dimensions do not affect the curling response.

Cooking mantle swells in thickness (Z) and simultaneously shrinks along the longitudinal (y) and transverse (x) axes (Fig. 2A). The mean maximum swelling (+ 9.0%) occurs at 60°C, and the swollen thickness is partially retained through the entire cook time. Shrinkage is not significantly different in the different directions (x and y). Primary shrinkage (> 25%) occurs as the meat is heated up to 100°C. Most shrinkage during cook at 100°C occurs within the initial 5 min of cook time, and is essentially complete at 100°C 15 min.

The mean volume of edible mantle tissue decreases to less than 60% of its original size when heated to 100°C 1 min, and approached 50% mean volume loss after 100°C 5 min (Fig. 2B). Since the decrease in mantle weight during cook does not parallel the graphed decrease in volume (Fig. 2B), the density of the mantle increases during cook. Most of the decrease in mantle weight during cook at 100°C occurs by 100° 5 min. Maximum reduction in original mantle volume, and weight occurred at 100°C 15 min.

Connective tissue

The visceral lining is the most heat labile tissue in the mantle. In raw tissue (Fig. 3A) the lining appears as a nonfibrous sheet tightly attached to the fibers of the inner tunic. When heated, obvious thermal degradation, evident at 50°C, slowly progresses to total degradation at 100°C 1 min. (Fig. 3B). After prolonged cooking at 100°C only coagulated remnants of the visceral lining remain meshed

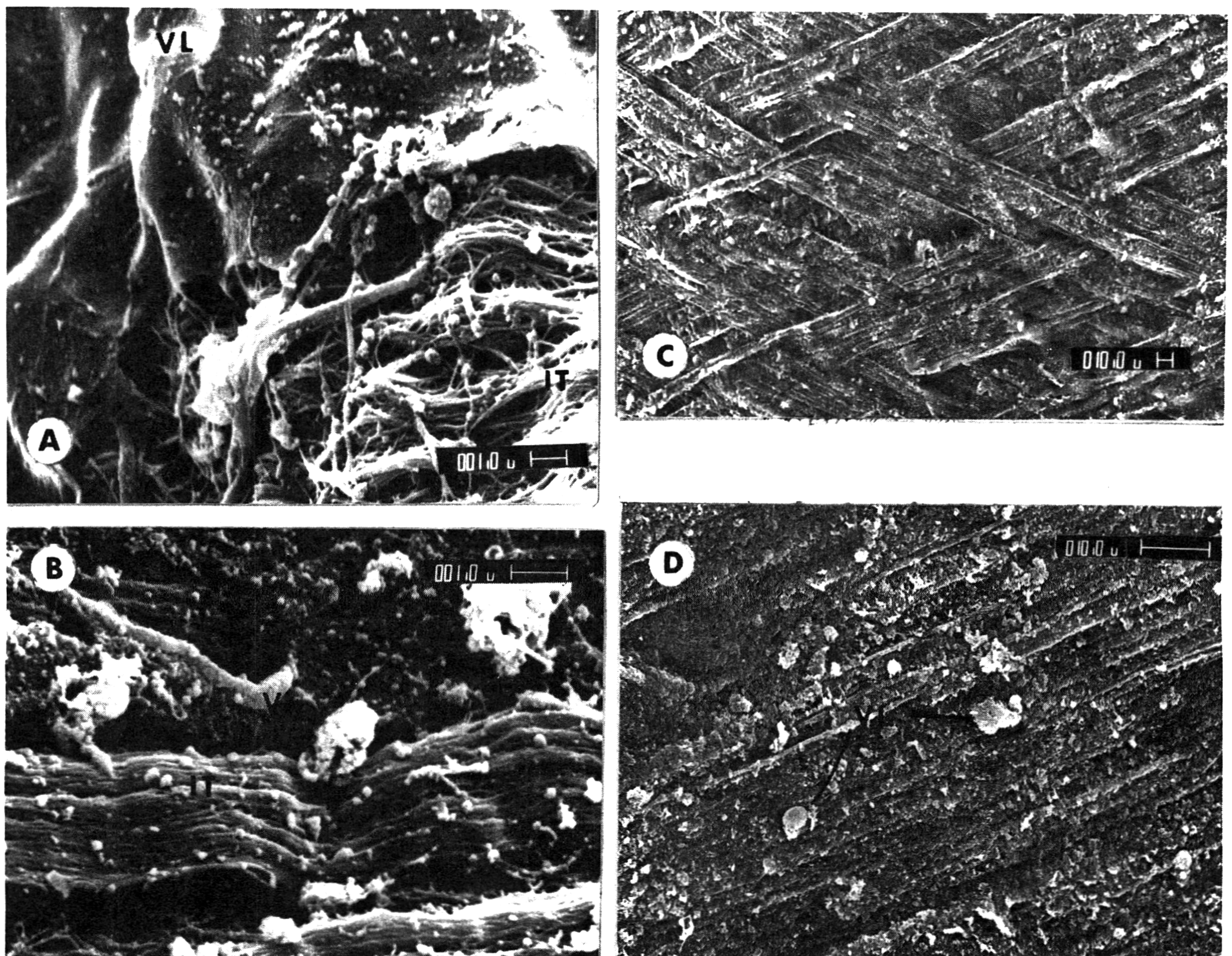

Fig. 3–Effect of cook temperature and cook time on visceral lining (VL), and the inner tunic (IT) of connective tissue. (A) Raw mantle. (B) Mantle cooked to 100°C 1 min. (C) Mantle cooked to 100°C 16 min, low magnificantion. (D) Mantle cooked to 100°C 16 min, high magnification.

among gelatinized fibers of the inner tunic (Fig. 3D).

Gelatinization or 'melting' of the inner tunic is first noted at 100°C 1 min (Fig. 3B). Fibril integrity was visible at all lower temperatures, but at 100°C signs of interfibril 'melting' are obvious. At longer cook times at 100°C, the fibril aggregates in the inner tunic have melted into a solid sheet (Fig. 3C and D). the crossed pattern in the heat denatured tunic is the only evidence of fiber order in the inner tunic, because in raw tissue the visceral lining conceals any discernible pattern (compare Fig. 3A and C).

Fiber and fibril structure in the outer tunic of connective tissue is more visibly ordered (Fig. 4A and B). Thermal destruction of this order initially appears as interfibril 'melting' at 70°C (Fig. 4C). Interfibril gelatinization progresses at 80°C (Fig. 4D) and 100°C 1 min, but individual fibers appear intact at 100°C 1 min (Fig. 5A and B). Figure 5 (A and B) is a cut, rather than fractured surface which shows individual fibers, but does not give a clear indication of the interfiber 'melting' which becomes visible at 100°C 1 min. During continued cooking at 100°C the fibers gradually gelatinize into a solid sheet (Fig. 5C and D). Note in the views of gelatinized outer tunic that a coagulated mass of the outer lining remains attached to the surface of the tunic.

Muscle tissue

At 50°C the muscle fibers still retain much of their original form (Fig. 6A); the 'sarcolemma' (Moon and Hulbert, 1975) is intact, and the protein material in the sarcoplasmic cores does not appear coagulated. There is a slight loss of myofibril distinction. Degradation of myofibril units is more obvious at 60°C, and the sarcoplasmic proteins appear coagulated in the central cores (Fig. 6B). The 'sarcolemma' is completely disintegrated at 70°C, the central cores have begun to shrink and dehydration of myofibrils is evident along the outside surface (Fig. 6C). Prolonged heating of the mantle at 100°C continually dehydrates the muscle tissue such that the fibers appear as densely packed, hardened units of myofibrillar proteins (Fig. 6D). Although excessive hardening continues, muscle fiber integrity remains even after cooking for 64 min at 100°C.

Mantle chemistry

The moisture content of the mantle decreased continuously through all cook temperatures, and cook times (Fig. 7). An abrupt moisture loss (2.0%) occurs within the first 5 min of cook time at 100°C. The moisture content in the mantle was still greater than 70% even after 100°C 64 min.

Naturally, the protein content in the cooked mantle increased as moisture was lost (Fig. 7), but approximately 25% of the original protein content (wet weight basis) is lost by the end of the first minute of cook after reaching 100°C (Fig. 8). The protein content in the cook water did not account for all protein lost. Filtration of cook water removed proteineous particulate matter, and the biuret reagents may not have been reactive with all the heat denatured proteins in the water. Protein continually leached from cooking mantle during all cook time.

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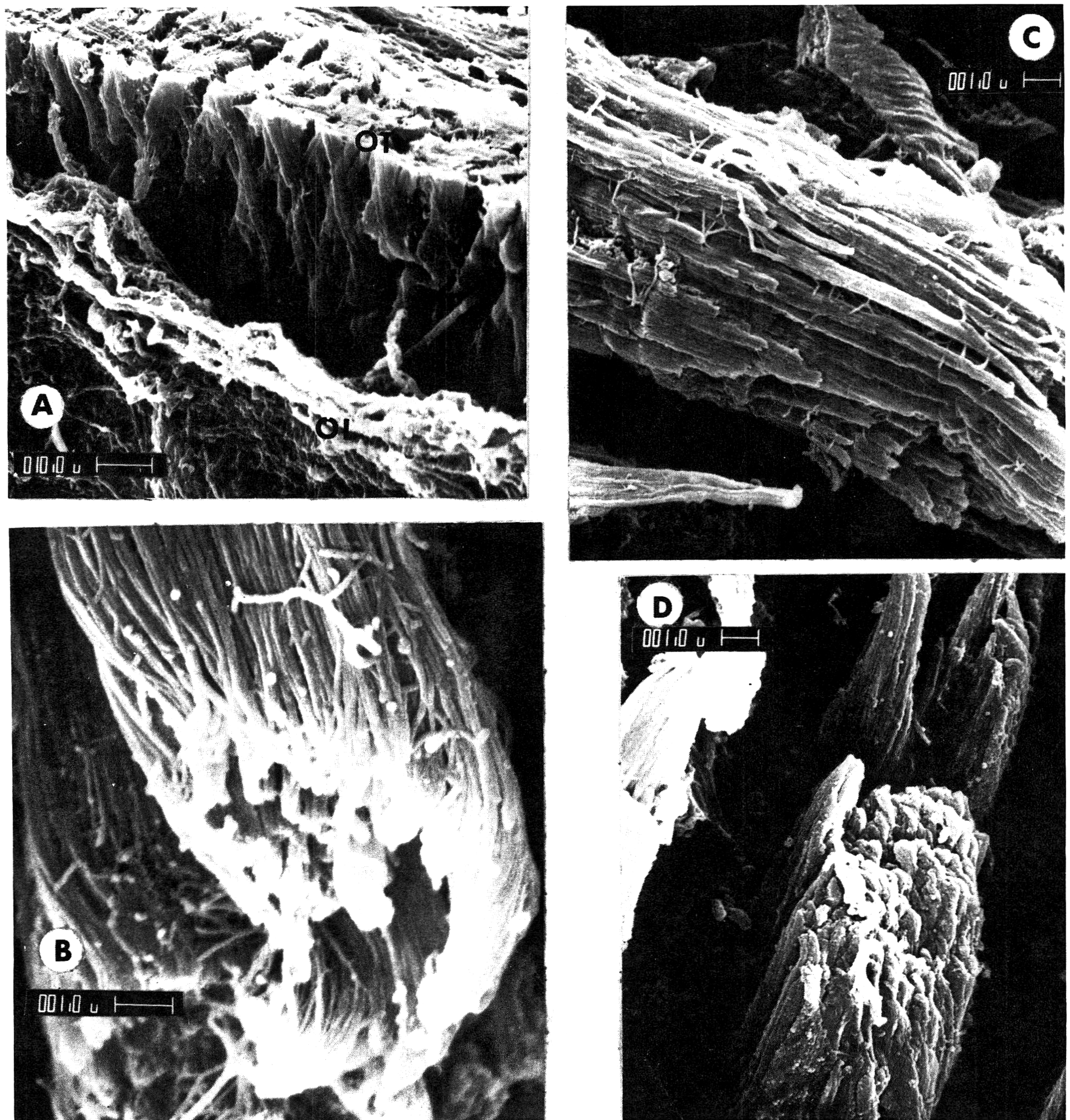

Fig. 4—Effect of cook temperature on the outer tunic. (A) Raw outer lining, OL, covering the ordered pattern of connective tissue in the outer tunic, OT. (B) Individual raw connective tissue fiber from the outer tunic; note the aggregation of fibrils. (C) Individual fiber cooked to 70°C showing interfibril melting. (D) Individual fiber cooked to 80°C showing advanced interfibril melting.

The pH of the mantle tissue increased during heating to 100°C (Fig. 9). The methods of pH determination gave conflicting results as to the initial temperature for rapid pH change. When the mantle pH was recorded at a specific cook temperature the results indicate an abrupt rise in pH at 70°C, which leveled at 6.9 during the remaining cook time. Recordings taken after the cooked mantle had cooled to room temperature indicated an increase in pH at the same temperature, 70°C, but the abrupt change occurred at 80°C and peaked at pH ≅ 7.25 throughout the remaining cook time.

DISCUSSION

SQUID MANTLE TISSUES are structurally unique and different from those described in more common edible meats like beef, poultry and fish. The most obvious difference is the relatively small size of squid muscle fibers ($\bar{x} \cong 3.5\mu$ diam), as compared to mammalian muscle fibers ($50-100\mu$, diam). Most of the sarcoplasmic proteins, commonly associated with mature, healthy mammalian muscle systems, are also found in mature squid muscle (Horie et al., 1975; Storey and Hochachk, 1975a–f), but they are located in a central sarcoplasmic core. This core is surrounded by distinct myofibril units which contain myofibrillar proteins that form an obliquely striated pattern (Hanson and Lowry 1957; Kawaguchi and Ikemoto 1958; Ward and Wainwright, 1972; Moon and Hulbert, 1975). Most common meats are typically cross striated. Mantle connective tissue structure

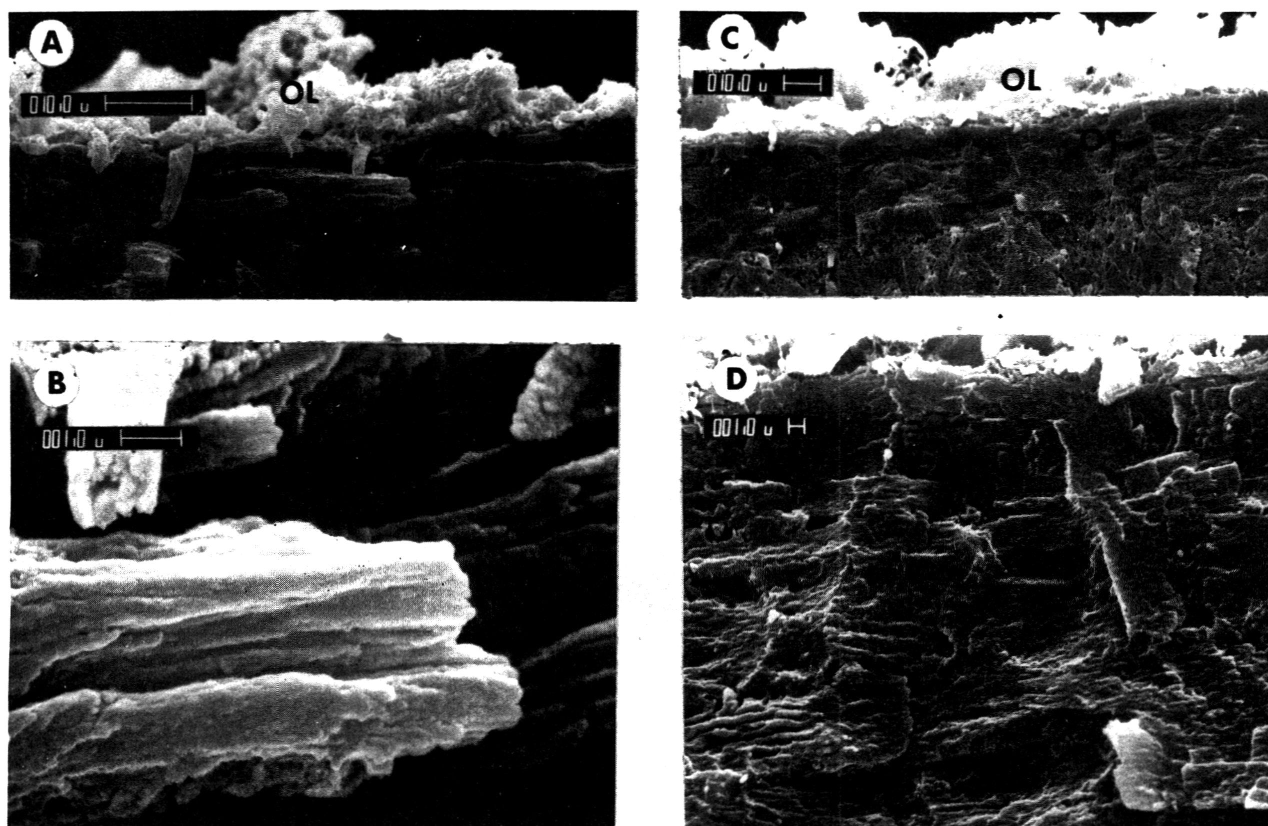

Fig. 5–Outer tunic of connective tissue covered by coagulated outer lining, OL, cooked to 100°C 1 min (A and B) and cooked to 100°C 15 min (C and D). Fibers have melted into a solid sheet at 100°C 15 min.

at the fibril level is similar to most meats, but the fiber pattern and overall location relative to the muscle tissue is unique in squid. Two structurally different tunics of connective tissue sandwich the mantle muscle tissue, and serve as flexible supports to aid in locomotion (Ward and Wainwright, 1972).

Despite these obvious structural differences, the thermal properties of the mantle tissues appear similar to those reported for more common meats. The connective tissue in the squid tunics is reported to be collagen based on its physical attributes (Ward and Wainwright, 1972), hydroxyproline content (Otwell, 1978) and the amino acid composition (Hunt et al., 1970). The fibrous structure in the tunics shrinks when heated, and is thermally denatured into a gelatinized mass. The onset of shrinkage, denoted by curling toward the dominant tunic surface, begins just as the tissue has been heated to 60°C. This shrinkage temperature is similar to that reported for collagens in beef (Machlik and Draudt, 1963; Field et al., 1970; Snowden et al., 1977) and chicken (Schaller and Powrie, 1972). The onset of gelatinization, 'melting' as viewed by SEM, was different in the separate tunics. Interfibril 'melting' was observed at a lower temperature (70°C) in the outer tunic than in the inner tunic (100°C 1 min). A difference in thermal denaturation temperatures of beef collagen from different anatomical locations had been reported by Field et al. (1970), Schmidt and Parrish (1971) and Pfeiffer et al. (1972). Possible explanations for this difference in squid collagen could be the difference in hydroxyproline content (Otwell, 1978), type and amount of carbohydrates associated with the collagen molecules, and/or the observed difference in interfibril spacing.

Seifter and Gallop (1966) contend that the differences in the temperatures of helical-coil transition in isolated collagen molecules depend on proline and hydroxyproline content, but Pfeiffer et al. (1972) could not explain the difference in thermal stability of beef collagen based on a difference in amino acid content. It may be that a difference in amino acid sequence within the primary structure, rather than content, affects the steric entropic factors such that there would be a difference in collagen molecular stability. The carbohydrate content of squid collagen molecules has been identified and quantified (Hunt et al., 1970), but there is no indication of a difference in distribution of these saccharides per tunic. 'Insulating' properties of carbohydrates warrant further investigation. Interfibril spacing was more pronounced in the inner tunic (compare Fig. 3A and 4B), and the gelatinized mass of inner tunic was less dense than that observed in the outer tunic (compare Fig. 3D and 5D). Thus, it appears that the observed difference in interfibril spacing has equal speculative value in explaining the obvious difference in thermal properties of the separate tunics of connective tissue.

Ultrastructure of the muscle fibers in cooked squid is grossly distorted in a manner similar to that reported for other meats. Thermal coagulation of myofibrillar and sarcoplasmic proteins and excessive fiber dehydration cause the muscle fibers to become hardened and densely packed. Thermal denaturation of myofibrillar proteins in squid was noted as a loss of SEM resolution of myofibrils at 50°C. Jacobson and Henderson (1973) report the 'melting' temperature, T_m, of myosin and actomyosin for rabbit muscle was 43 ± 2°C. Alterations of bovine myofibrils at 40–60°C have been determined by changes in myofibrillar protein solubility (Hamm, 1966; Paul et al., 1966), two-dimensional structure (Hostetler and Landman, 1968; Paul et al., 1970; Schmidt and Parrish, 1971), and three-dimensional structure (Schaller and Powrie, 1972; Cheng and Parrish, 1976; Jones et al., 1977).

The heat coagulation of squid sarcoplasmic proteins at

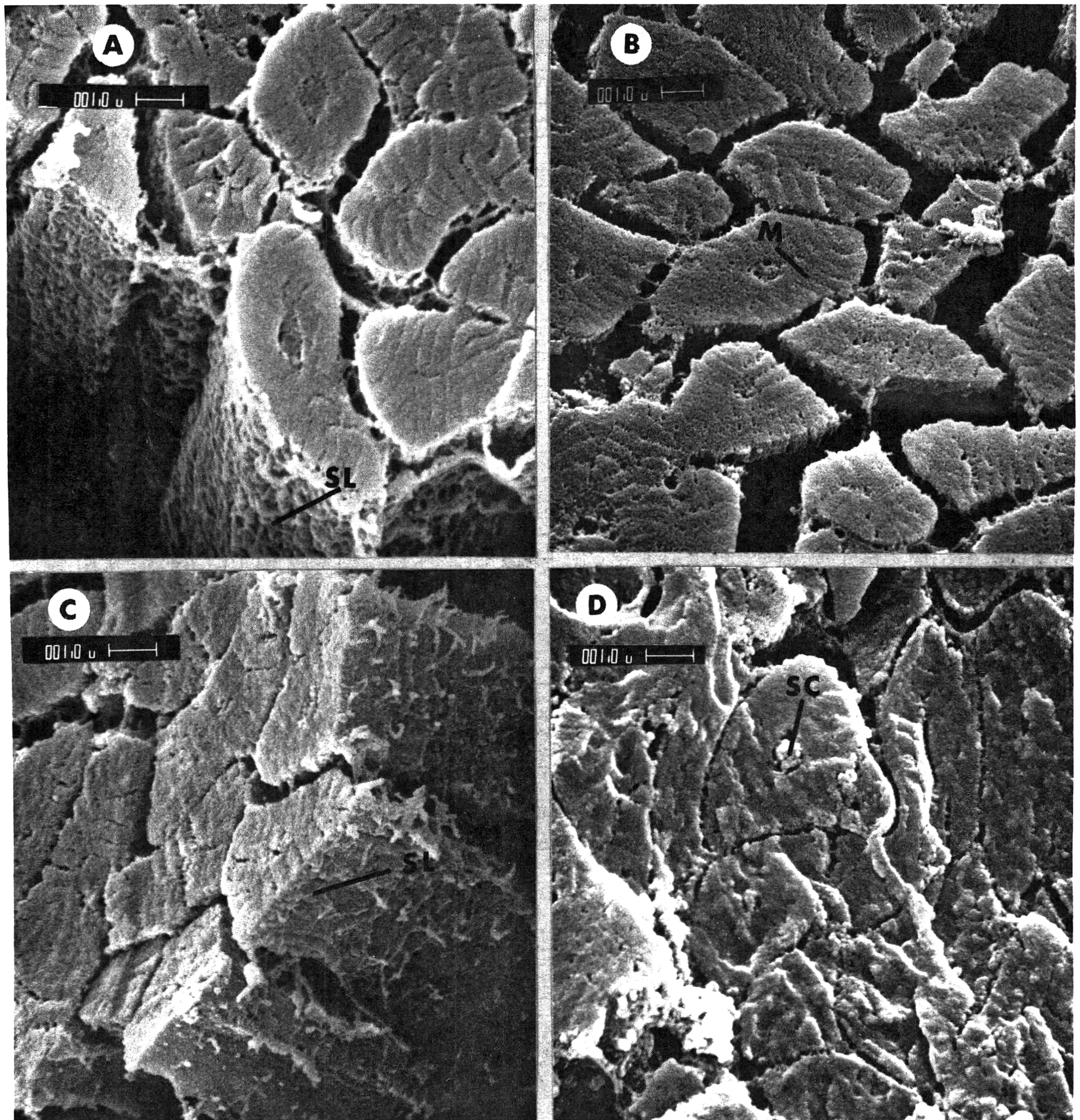

Fig. 6—Mantle muscle fibers cooked to 50°C (A), 60°C (B), 70°C (C), and 100°C 8 min (D). Note changes in the sarcoplasmic core (SC), the 'sarcolemma' (SL), and the myofibrils (M).

60°C is similar to the results observed with SEM by Schaller and Powrie (1972), Cheng and Parrish (1976) and Jones et al. (1977). These same authors were interested in thermal degradation of the Z-lines and I-bands and shrinkage of sarcomeres. Small fiber diameter and oblique fiber striations outlined by less distinct Z-bodies may have been reasons for the failure to detect such changes in squid.

Loss of moisture from muscle tissue is thought to be a result of spatial and conformational changes in myofibrillar proteins (Hamm and Deatherage, 1960; Buttkus, 1974). This seems to be true in cooked squid mantle. As the muscle fiber structure continued to distort at higher temperatures, the moisture content of the mantle continually decreased. When gross fiber distortion occurred, 100°C for first 15 min (Fig. 6A–D), moisture loss increased rapidly (Fig. 7). As the mantle tissues began to shrink, the whole mantle weight decreased. Weight loss was attributable to leaching proteins, as well as moisture loss. The loss of mantle volume caused an increase in mantle density as noted by the decrease in interfiber spacing, especially absent in Figure 6D.

The pH of the raw mantle tissue is higher than that commonly associated with raw beef, but the pH increases in both meats when cooked (Hamm, 1966). Since no glycogen reserves have been detected in the squid musculature (Moon and Hulbert, 1975), the lactic acid production typical of postmorten glycolysis in mammalian tissue is apparently of minimal consequence in squid. Similarly, the lack of energy reserves in squid (Moon and Hulbert, 1975) may account for the rapid onset of rigor immediately following harvest (Otwell, 1978).

Changes in mantle pH during cook result from a decrease

in hydrogen ion activity most likely due to conformation changes in mantle proteins. It is common knowledge that alterations in secondary and tertiary structure of proteins

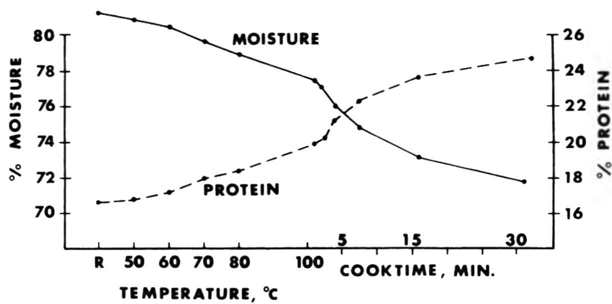

Fig. 7—Average percent protein and moisture in whole squid mantle at each cook treatment. Each treatment was replicated three times, and each analysis twice. Average standard deviation was ± 0.3% moisture, and ± 0.5% protein. Average fat content in raw mantles was 1.65 ± 0.2%.

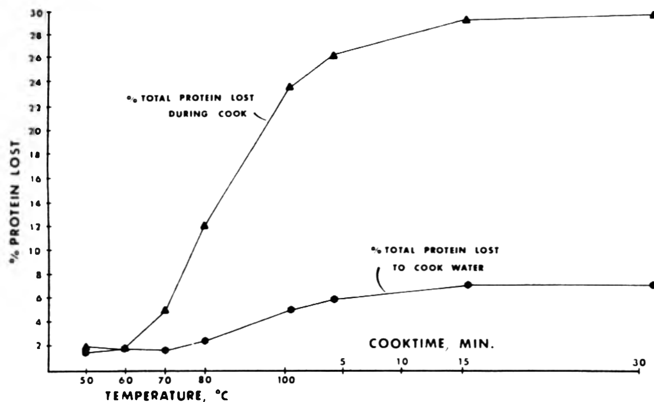

Fig. 8—Average percent of protein loss from the squid mantle during cook treatments. Each treatment was replicated three times, and analysis twice. Average standard deviation was ± 0.6% protein lost, and ± 1.5% protein lost to cook water.

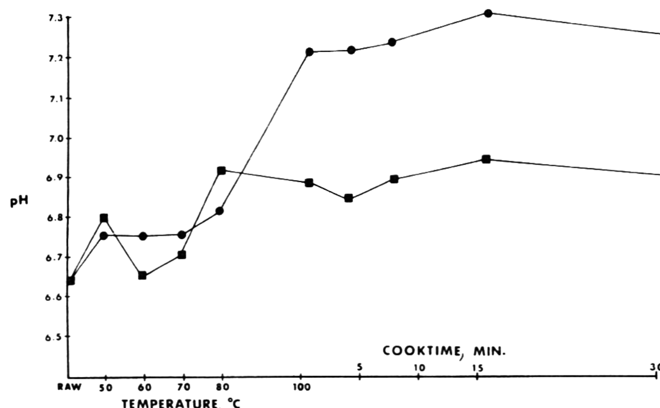

Fig. 9—Average pH of the squid mantle at each cook treatment. Each treatment was replicated twice, and each pH reading duplicated. Average standard deviation in pH readings was ± 0.1 pH unit. ■: pH determined at the specific treatment; ●: pH determined at room temperature (25°C).

involve hydrogen bonding and ionization of prototropic groups on amino acid residues in the peptide chains. The degree of involvement of individual mantle proteins cannot be determined from the simple pH pattern recorded, but the simultaneous rise in pH and interfibril 'melting' in the outer tunic at 70°C is cause to speculate that heat alteration in collagen is a major contributor to the decrease in hydrogen ion activity in cooked squid. Jackson et al. (1974) have shown that covalent linkages in guinea pig collagen are not broken until the tissue is heated above 71°C. Heat denaturation below this temperature was primarily a result of change in hydrogen bonding and electrostatic interactions. Further research is necessary to determine if similar heat denaturation forces occur in squid collagen and, if so, how do they influence the pH of the whole cooked mantle slurry. Also, care should always be used in selection of a temperature for pH determination in cooked meat. Apparently, an irreversible change in conformation occurred in squid proteins cooked beyond 80°C which affected the pH determinations at room temperature. The pH values recorded at room temperature for squid cooked beyond 80°C were significantly higher than determinations made at the specific cook temperature and time.

In summary, SEM has been effective in revealing progressive alterations of tissue components in cooked squid mantle. The changes in tissue structure are partially explained by simultaneous changes in gross body dimensions and mantle pH, moisture and protein content. The temperatures of specific thermal denaturation in connective tissue and muscle fibers are similar to those reported for other meats. These results can be compared with physical tests of tissue strength and texture panel evaluations of mouth feel to complete the textural characterization of the squid mantle.

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TEXTURAL CHARACTERIZATION OF SQUID (Loligo pealei L.): INSTRUMENTAL AND PANEL EVALUATIONS

W. STEVEN OTWELL and DONALD D. HAMANN

ABSTRACT

Instrumental tests and profile panel evaluations were used to characterize the textural attributes of squid mantles cooked in water to 50, 60, 70, or 80°C, or cooked for 1, 4, 8, 16, or 32 min after reaching 100°C. A comparison of results from each specific cook treatment was used to interpret textural changes caused by heat and duration of heating at 100°C. Shear type tests, a single blade, and a punch and die, demonstrated that the amount of force and energy required to cut cooked mantles was dependent upon the orientation of the shearing apparatus relative to the direction of muscle fibers and on the side of the mantle initially cut. These anisotropic and nonhomogeneous aspects of the cooked mantle are due to thermal hardening of muscle fibers and to the distribution and heat gelatinization of connective tissues. A unique isometric tension test demonstrated that hydrothermal shrinkage of connective tissues also influenced squid texture. Panel evaluations indicated that two groupings of textural character notes best described texture of cooked squid. Character notes assessing mantle strength correlated with the results of rheological tests. Character notes denoting mantle moisture were not explained by instrumental tests, but were an essential supplement describing the mouthfeel of cooked squid.

INTRODUCTION

Recent advocates (Rathjen, 1974; Anonymous, 1975, 1977; Learson and Ampola, 1977) for domestic utilization of the abundant and nutritionally valuable squid, *Loligo pealei*, have been discouraged by limited marketability (Kalikstein, 1974; Galus, 1975). A common consumer complaint is tough meat texture. "Connoisseurs" (Berk, 1974; Ampola, 1974; Bloom, 1976) argue that the texture depends on the recipe and method of cook. Takahashi and Takei (1955) tried to limit adverse squid texture by varying the composition of the cook media. Takahashi (1965) advised that methods for cooking squid meat would have to suit regional habits. To date, results are lacking that demonstrate the effects of cooking on squid texture.

The intention of this study was to characterize the textural attributes of cooked squid mantle. The mantle is the main edible portion from squid. Textural attributes of cooked mantle are the result of its inherent structure which resists deformation and breakdown during mastication. The physical strength of cooked mantle was determined by instrumental methods. Sensory perception of cooked mantle resistance to mastication was determined by trained texture profile panelists. Results from the instrumental tests and panel evaluations are compared with a structural analysis of cooked mantle tissues, (Otwell and Hamann, 1979) demonstrating the utility of an interrelated approach to understanding the link of food structure to texture, and suggesting methods for providing a more tender squid.

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0022-1147/79/0006-1636$02.25/0
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MATERIALS & METHODS

SQUID, *Loligo pealei*, used in this study were harvested, packaged, and stored frozen as outlined by Otwell and Hamann (1979). Only the main edible portion, the mantle, was used. Mantle sizes ranged from 10–20 cm in length. The cone-shaped mantles were cut open along the dorsal surface to yield a flat triangular shaped 'fillet' of squid meat. Mantles were cooked in water with a controlled temperature rise of 5°C/min up to specific cook temperatures: 25 (raw), 50, 60, 70, 80, and after reaching 100°C, for specified cook times: 1, 4, 8, 16, or 32 min (Otwell and Hamann, 1979). The cook temperatures and cook times at 100°C accounted for ten overall cook treatments. Henceforth, cook times at 100°C will be referred to as 100°C 1 min, 100°C 4 min, etc. After each cook treatment the mantles were cooled to 25°C by exposure to room air temperature before preparing samples for instrumental and texture panel evaluations.

Mantle thickness was considered as a test variable. A conductive micrometer stage was designed to accurately (nearest 1/100 cm) measure mantle thickness without compressing the soft tissues. Recorded mantle thickness was the micrometer reading when a needle deflection on an ohmmeter indicated contact of the micrometer with moist squid had completed the circuit with the metal stage support. The micrometer was secured and initially zeroed by direct contact with the stage.

Instrumental evaluations

Single blade. A single blade shear cell with a flat cutting end (Food Technology Corp.) was used to study the anisotropic nature of the squid mantle. Blade width was 0.30 cm, and it moved through a 0.32 cm wide cutting slot. The force and displacement required to cut rectangular test samples across the sample width were supplied and recorded by an Instron Universal testing machine (Model 1130) operating at 8.33 mm/sec crosshead speed and 8.33 mm/sec chart speed. Maximum force, displacement, and energy to failure were recorded. Failure was considered to be the point of peak force required to cut the samples. Chart patterns were near right triangles, thus, energy was half the product of peak force and displacement to peak force. Each dependent variable value was divided by the sample thickness to decrease variation due to intrinsic differences in cooked mantle thickness.

Special note needs to be made at this point of an instrumental deficiency that may be reflected in some of the absolute values of force, displacement, and energy. After the study was completed, it

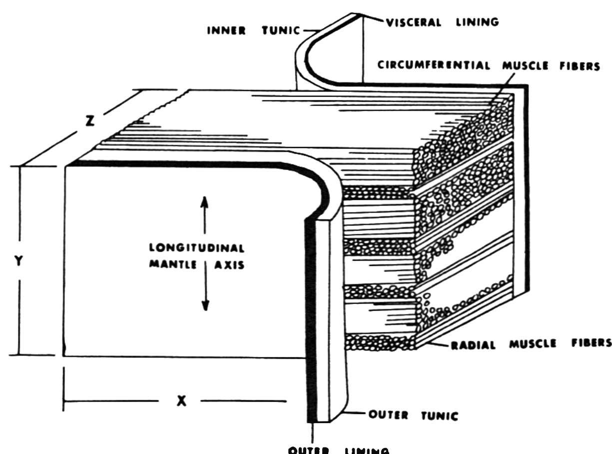


Fig. 1—Illustration of a transverse sample cut from the mantle. Note the dominant muscle fiber direction is circumferential.

was pointed out by one of the reviewers that the Instron Model 1130 recorder response was not fast enough to give full value forces at the crosshead speeds used for the single blade shear. Subsequently, this was checked by testing cooked squid and recording simultaneously on an external recorder (0.025 sec response time). Results showed that the force record could be attenuated by as much as 20% at the pen amplitudes used. This does not change the general observations and conclusions given but does mean the numerical values for force and energy may be somewhat greater than shown in the graphs and tables. Displacement could be slightly less than that shown.

Rectangular shaped test samples were cut from the cooked mantles so they would have prescribed fiber orientations. Samples cut such that the long axis of the rectangle was parallel to the dominant fiber direction will henceforth be referred to as transverse samples. Samples cut with the long axis perpendicular to the dominant fiber direction will be referred to as longitudinal. Figure 1 illustrates a typical transverse sample cut from the entire thickness (Z) of the mantle. Note the dominant muscle fiber direction is circumferential. One rectangular test sample cut from squid cooked to a particular cook temperature was defined as one replication of squid subjected to that particular cook temperature. Numerous rectangular samples were cut, in reference to muscle fiber orientation, to allow for replication of test factors.

A factorial analysis of variance was used to determine the effects of cook treatment, shear direction, and shear side, on the shear force, energy, and displacement required to cut through the squid samples. Shear directions were parallel and perpendicular and shear sides were inside and outside. When the shear blade cut through longitudinal samples, the blade orientation was parallel to the dominant fiber direction. Transverse samples were cut with the blade oriented perpendicular to the fiber direction. Inside 'shear' implied initial blade contact on the inside mantle surface. Outside 'shear' was initial blade contact on the outside surface. The effect of shear direction and shear side were tested at all of the ten cook treatments previously discussed. Each test combination of shear direction, shear side, and cook treatment was replicated four times. This experimental design allowed examination of main effects (shear side, shear direction, and cook treatment) and interactions between factors. Treatment effects were subjected to analysis of variance and significant mean differences in main effects and interactions determined at the 5% T-distribution. The entire experimental design was run on two separate series of sample sizes, 3.0 and 1.0 cm wide rectangular samples.

Punch and die. A second shear type test was to use a cylindrical probe to puncture cooked squid mantles. Shearing action of the puncture differed with respect to the single blade in that the circular cutting edge of the probe could be applied to mantle samples irrespective of the dominant muscle fiber direction.

A 0.635 cm diameter flat-faced probe, mounted on the Instron machine operating at 3.33 mm/sec crosshead speed and 8.33 mm/sec chart speed, was used to puncture whole mantle samples placed over a 0.655 cm diameter die (probe-die clearance was 0.01 cm). Each whole squid mantle 'fillet' was punctured in nine preselected locations. Mantle thickness at each location was measured prior to puncture. Use of a circular probe eliminated the preparation time required to cut sample widths as used in the single blade shear tests. Reducing the crosshead speed greatly reduced attenuation of the force indication due to pen response.

Two probe experiments were conducted on whole cooked mantles. Design of the initial experiment was a factorial analysis of three factors; cook treatment, squid 'fillet,' and location of puncture. Three separate squid 'fillets' from each cook treatment were each punctured in three locations, upper (open end of the mantle), middle, and lower third (apex end) of the whole mantle 'fillet.' All punctures were initiated on the outside surface of the mantles. Each location within one squid 'fillet' was punctured three times, thus allowing three observations of each factorial combination of cook treatment, squid 'fillet' and puncture location. Design for the second probe experiment was a factorial analysis of the three factors; cook treatment, squid 'fillet' and mantle side initially punctured. Three separate squid 'fillets' from each cook treatment were each punctured from the outside and inside mantle surface. Punctures in the second experiment were all located in the upper third of the mantle 'fillet.' Each squid 'fillet' was punctured three times from each side, thus allowing three observations for each factorial combination.

Variables recorded for both probe experiments were maximum force, displacement, and energy to failure. Each variable was divided by mantle thickness. Methodology used to determine significant differences and level of significance was the same as outlined for the single blade shear tests. F-values calculated with the mean square error term to account for 'nesting' of puncture location within squid 'fillets' were lower but gave the same significant differences as indicated by use of the overall mean square error.

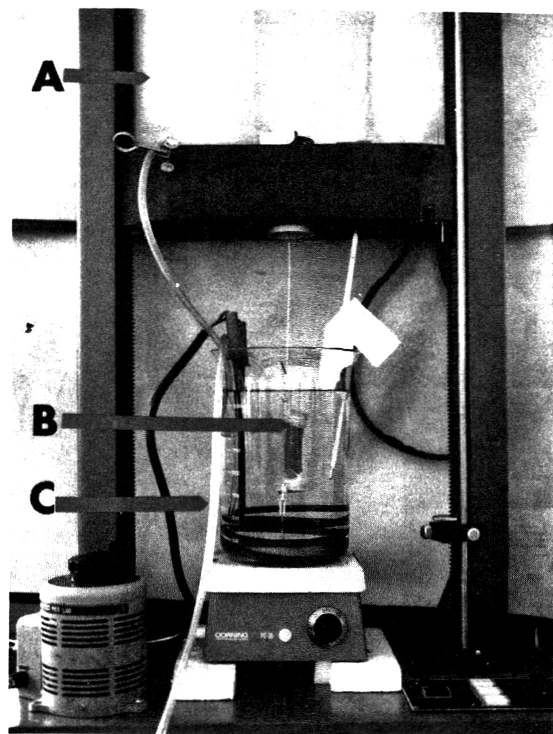


Fig. 2—Instron universal testing machine equipped for studying isometric tension developed by a cooking sample while submerged in the cook medium: (A) water supply; (B) simulated sample suspended between the anchor and tension load cell; (C) syphon drain.

Hydrothermal shrinkage. A third instrument test was designed to measure the hydrothermal shrinkage force developed while a strip of mantle was suspended in isometric tension within heating cook medium. Cook methodology employed during these tests was different from the cook treatment previously described for the shear type tests. Common laboratory apparatus was arranged on the Instron universal testing machine which could record tension (Fig. 2). A strip of mantle was suspended between an anchor placed in the center of a 4.0 liter beaker, and a 500 gram tension load cell. The mantle was attached by string tied about a pre-wrap of cheesecloth which prevented slippage, and sample tear. The beaker was filled with 3.5 liters of distilled water. A 10-g initial test load was placed on the suspended sample to remove any slack from the system. A submerged copper heating coil regulated by a rheostat and a hot plate with thermostat were used to heat the water. Heating rate from room temperature to boil was 5°C/min. All test samples were continually heated from room temperature to boiling. Bubble interference during boil was minimized by heating only with a copper coil, so as to keep bubbles on the periphery of the beaker. Temperature of the water was continuously monitored. The entire test was considered static, or isometric because the distance of load cell movement required to make a force registration was negligible relative to the sample length.

Experiments were designed to test the tension developed by 2.0 × 6.0 cm strips cut parallel and perpendicular to the dominant muscle fiber direction. Sample length was the distance between string ties. Parallel strips were cut from the upper, middle, and lower third of the mantle 'fillets.' Perpendicular strips were cut from the upper and lower half of the mantle 'fillet.' At least six replications of tension measurements were recorded for each combination of strip direction and location. A replication implies one strip cut from one squid mantle cooked in one beaker of medium. Thickness of

test strips was 0.51 ± 0.11 cm. Control tests run without sample present, indicated tension developed by the string was negligible.

Texture panel evaluations

A trained (Civille and Liska, 1975) texture profile panel was used to evaluate the texture of squid which had been cooked at 100°C for 1, 2, 4, 8, 16, or 32 min. At least six members of the ten member panel were present at all panel sessions. Initially, the panel conducted sessions to become familiar with the product, and define basic terminology needed to describe its unique texture. Then test samples of unidentified cook time were presented in a randomized order for textural evaluation. Samples were evaluated in three separate sessions. Sample variation was the difference in consensus ratings per cook time per session.

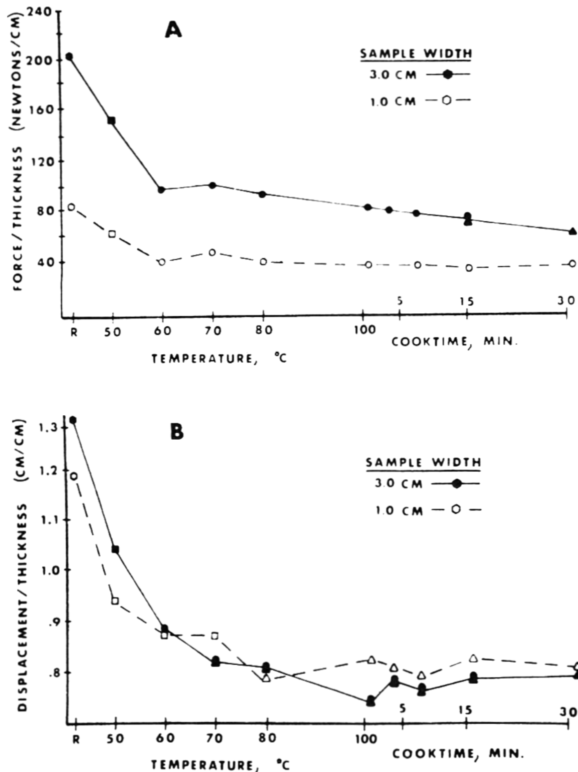


Fig. 3—Effect of cook treatment on force/thickness (A) and displacement/thickness (B) required to shear wide and narrow width squid mantle samples with a single blade. Any two means denoted by the same symbol are not significantly different as determined by the least significant difference. Two symbols per data point are necessary to show this in some cases.

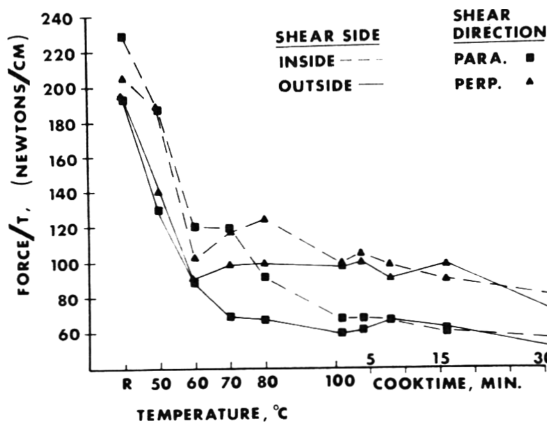


Fig. 4—Effect of shear side and shear direction on the force/thickness required to cut wide (3.0 cm) squid samples with a single blade shear at each specific cook treatment. Samples cut with the blade oriented perpendicular to the dominant muscle fiber direction are labelled perp.; para. denotes blade orientation parallel to muscle fiber direction. The least significant mean difference calculated at the 5% t-distribution was 17 Newtons/cm.

RESULTS

Single blade shear

Cook treatments affected all test variables. There was a slight tendency for mantle thickness to increase at higher cook temperatures, a swelling phenomenon noted by Otwell and Hamann (1979), but there was no significant difference in thickness across other factors or interactions. The use of thickness as a correction factor for intrinsic variation did decrease the coefficient of variation for corrected values, but did not affect the significant patterns as detected by uncorrected variables. The correlations between force/thickness (F/T) and energy/thickness (E/T), calculated across all main treatments, were high (average 0.956 ± 0.03 std dev), thus F/T and displacement/thickness (D/T) alone describe the effect of cook treatments (Fig. 3). Force, energy, and displacement required to shear the total thickness of cooked squid mantle decreased as cook temperature increased to 60°C, but at cook treatments in excess of 60°C there was no significant difference in force or energy required to shear the cooked samples. More force was necessary to shear the wider mantle samples (Fig. 3A). Larger samples were three times wider, but required 2.25 ± 0.13 times more force to peak failure. The displacement to failure decreased across all cook treatments in a similar pattern regardless of sample width (Fig. 3B). The greater displacement (D/T > 1) for the raw samples indicates the shear blade forced the flexible, raw tissue fibers further into the blade/slot gap prior to severing the tissue.

Shear side and shear direction as main effects significantly influenced the F/T and E/T required to cut through the squid samples. More force and energy were necessary to shear samples initially cut from the inside surface (Table 1). Significantly more force and energy were required to shear narrow samples with the blade oriented perpendicular to the dominant fiber direction (Table 1). This difference due to shear direction was significant at the 7.25% level for larger squid samples. There was no significant difference in D/T due to shear side or direction for either size squid samples cut with the single blade.

An illustration (Fig. 4) of the different second order interactions for large width squid samples indicates that patterns of significant differences in mean F/T required to

Table 1—Mean force/thickness (F/T), energy/thickness (E/T), and displacement/thickness (D/T) required to shear squid samples under the main effects of shear side and shear direction

	Large width			Small width		
	F/T[a]	E/T[b]	D/T	F/T[a]	E/T[b]	D/T
Shear side						
inside	137.3	0.375	0.97*	62.8	0.160	0.92*
outside	109.4	0.286	0.94*	44.5	0.107	0.88*
Shear Direction						
parallel	114.9*	0.312*	0.95*	46.6	0.114	0.89*
perpendicular	126.6*	0.347*	0.96*	60.8	0.152	0.91*

[a] Force/thickness is expressed as Newtons/centimeter.
[b] Energy/thickness is expressed as Joules/centimeter.
* Values not significantly different at 5% level of significance in the F-distribution.

Table 2—Mean force thickness (F/T), energy/thickness (E/T), and displacement/thickness (D/T) required to puncture squid 'fillets' under the main effects of location of puncture and side punctured in the respective experiments one and two

Experiment one				Experiment two			
Location of puncture	F/T[a]	E/T[b]	D/T	Side punctured	F/T[a]	E/D[b]	D/T
Upper 1/3	49.4*	0.080*	0.67*	Inside	43.6	0.070	0.64*
Middle 1/3	44.6*	0.009*	0.70*	Outside	52.3	0.086	0.66*
Lower 1/3	57.3	0.090*	0.66*				

[a] Force/thickness is expressed as Newtons/centimeter.
[b] Energy/thickness is expressed as Joules/centimeter.
* Values not significantly different at 5% level of significance in F-distribution.

Table 3—Hydroxyproline content[a] in separate zones of the squid mantle 'fillet'

Zones	% Protein	% Hydroxyproline
Upper 1/3	16.37 ± 0.40	0.058 ± 0.02
Middle 1/3	16.47 ± 0.32	0.065 ± 0.02
Lower 1/3	16.07 ± 0.25	0.091 ± 0.03

[a] Method of Woessner (1961); Each analysis was performed in triplicate on each of three samples from each zone.

shear mantle samples at each cook treatment were dependent on side of initial blade contact, and direction of the shear blade relative to the dominant muscle fiber direction. A similar pattern of F/T required to shear narrow samples was evident. At cook temperatures less than 80°C generally more mean F/T was reuqired to shear samples from the inside surface, but at all cook times at 100°C there was no significant difference in mean F/T required to shear samples from either side per similar shear direction. At cook temperatures less than 70°C, there was no pattern of significant differences in mean F/T required to shear samples in either direction, but at all cook times at 100°C significantly more F/T was required to shear perpendicular to muscle fibers. In summary, the second order interactions reveal the pattern of mean differences at lower cook temperatures was stratified by the influence of shear side, but at boiling the mean differences are stratified by the influence of shear direction. The transition in pattern occurred between 70°C and 80°C. There was no significant difference in D/T at each cook treatment due to side or direction of shear.

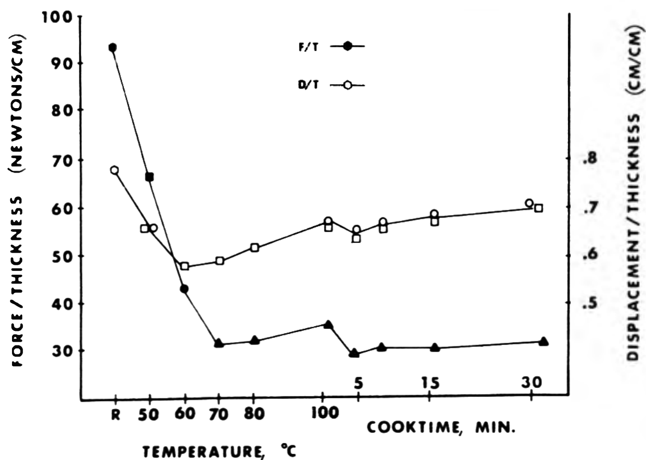


Fig. 5—Effect of cook treatment in the first probe experiment on the mean force/thickness (F/T) and displacement/thickness (D/T) required to puncture squid mantles with a 0.635 diameter probe. Any two means denoted by the same symbol are not significantly different as determined by least significant difference. Two symbols per data point are necessary to show this in some cases.

Punch and die shear

Results from use of the probe to puncture squid mantles were generally in agreement with the single blade results. Again, mantle thickness increased slightly at higher cook temperatures, but the difference was not significant in either probe experiment. Despite a low mean correlation coefficient (0.705 ± 0.05) between F/T and E/T, the patterns of force and energy required to puncture the mantle thickness were essentially the same across all cook treatments per experiment. Cook treatments in both probe experiments significantly influenced the amount of force and displacement required to puncture the squid samples (Fig. 5). In both experiments as cook temperatures initially increased, F/T and E/T decreased significantly. As cook temperatures increased beyond 60°C, there was no significant difference in F/T. The D/T before failure decreased as mantles were heated to 60°C, but after 100°C 1 min the D/T began to increase across cook time at 100°C.

There were no significant differences in mantle thickness, F/T, E/T, or, D/T in either probe experiment due to squid 'fillet' as main effect. There were significant differences in dependent variables due to the location and side initially punctured (Table 2). In experiment one, significantly more F/T was required to puncture the lower (apex end) zone of the squid 'fillet.' In the second experiment, significantly more F/T and E/T were required to puncture squid 'fillets' from the outside surface.

Figure 6 illustrates the influence of the first order interaction, location of puncture times cook treatment, on the sample thickness, F/T, and D/T. The middle zone of the mantle 'fillet' was significantly thicker at all cook treatments, but the F/T was significantly larger for the thinner, lower zone samples up to 70°C. At cook treatments in excess of 70°C, there was no significant difference in F/T due to location of puncture. A partial explanation of the greater amount of force required to puncture the thinner, lower zone samples may be due to the variation in collagen content per zone (Table 3). Hydroxyproline, as an indication of collagen content, was higher in the lower zone, as opposed to the upper two-thirds. The E/T did not differ significantly by location of puncture at any specific cook treatment, but punctures in the thicker, middle zone developed significantly more D/T before failure. This pattern was consistent at each cook treatment up until 100°C 16 min, after which there was no significant difference in D/T due to zone. The D/T tended to increase after 60°C for all locations. This trend is confusing in comparison with shear displacement data generated by single blade shear tests (compare Fig. 3B with 6). This difference could be due, in part, to the slower shear rate of the probe allowing more time for stress relaxation.

Deformation patterns resulting from punctures in thin upper zone samples were more linear than those in the thicker, middle zone samples. The more linear deformations indicated more structural resistance at equivalent displacement. More displacement was required in thicker samples

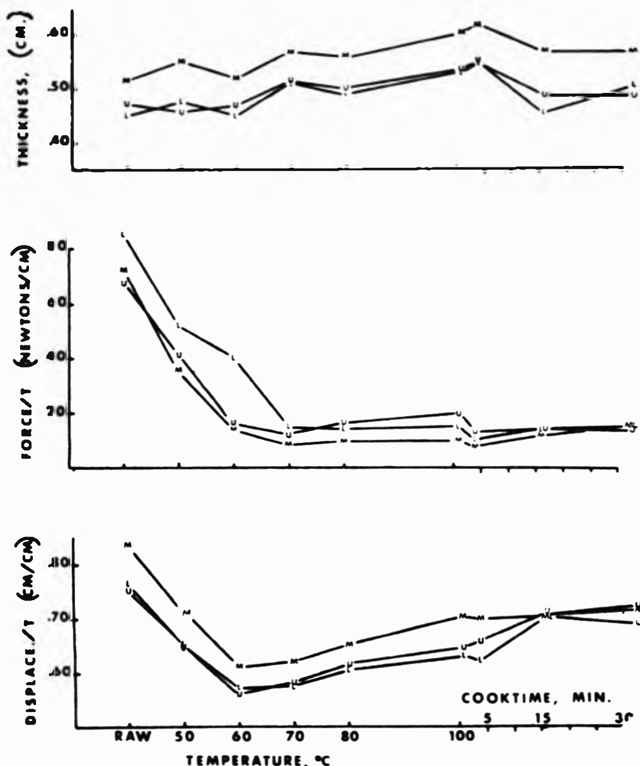
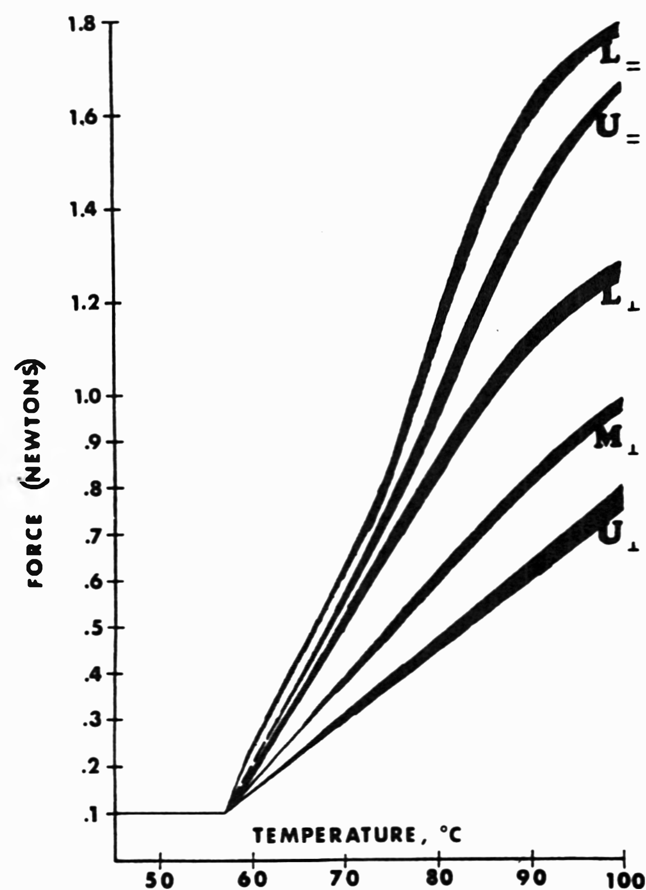


Fig. 6—Effect of the location of puncture on thickness, F/T and D/T at each cook treatment. Locations on the mantle 'fillet' are: U—upper 1/3; M—middle 1/3; and L—lower 1/3.



than in thin samples to reach a given force/displacement (F/D) ratio. Apparently, as the probe surface approaches the cutting edge of the die, F/D increases. The most linear response detected was in thin, lower zone samples heated up to 70°C (Fig. 6). These samples contained the most hydroxyproline (Table 3) and temperatures below 70°C were too low to gelatinize collagen (Otwell and Hamann, 1979). This evidence suggests that the tunics of connective tissue were most likely a dominant structural feature affecting F/D.

Results in the second experiment provide further evidence of the influence of connective tissue tunics on the rheological features of squid mantle as detected with a punch and die. For all interactions of cook treatment and side punctured, more force was required to puncture the total thickness of the samples initially probed from the outside surface. This differences was only significant at the 6% level at each cook treatment, but the consistency of the mean patterns suggests an influence of connective tissue tunics. The tunics on each surface of the mantle have been shown to differ in thickness and fiber organization (Otwell and Hamann, 1979).

The above differences due to side sheared were opposite from those detected with single blade shear. The difference in instrumental crosshead speed may be a factor. Further research would have to be conducted to fully explain the effect of tunics on the failure response of the squid mantle, but the differences due to side in both shear tests simply suggest that the tunics of connective tissue are involved in the texture of cooked squid mantle.

Iosmetric tension

The most obvious feature of all the isometric tension tests was the consistent sensing of shrinkage force at 57.7°C (Fig. 7). Variance from this initiation temperature did not exceed ± 0.2°C regardless of the location, direction, or replication of the sample strip from squid mantles. Strips cut perpendicular to the dominant fiber direction generated more force when heated, than did strips cut parallel to the same. The perpendicular strips cut from the lower half of the mantle 'fillet' generated the greatest amount of shrinkage force. Less force was generated from strips cut from higher zones of the 'fillet' regardless of cut direction. The force pattern at 100°C remained constant across all cook times up to 16 min. Samples held in tension to 32 min tore apart.

Texture panel

The terminology used to describe cooked squid texture was divided into two main categories of character notes, mantle strength and mantle moisture (Table 4). A similar categorization of panel ratings was noted for beef texture by Harris et al. (1972). The researchers and panelists were not aware of this categorization during their analysis, but the similarity in definitions (Otwell, 1978), and ratings of the textural characters indicated such grouping would better explain the overall subjective response. Mantle strength was a group of textural features (hardness, cohesion, snappiness, and springiness) which would be adverse factors if rated high. Hardness was an evaluation of failure strength. Springiness was defined as the degree to which a sample returns to shape after partial compression by the teeth. However, springiness correlates almost perfectly with the other strength characteristics and it was an adverse feature

Fig. 7—Force patterns generated by strips (2 X 6 cm) of squid mantle in the isometric tension. Specimen locations are U—upper, M—middle and L—lower. Longitudinal direction of the rectangular samples relative to the direction of the dominant muscle fibers are ⊥ (perpendicular) and = (parallel). Shaded area includes the complete range from six or more replications.

Table 4—Textural character notes deemed pertinent for describing the texture of cooked squid mantle as rated by the texture profile panel. Cook treatment was time of cook after reaching 100°C

Cooktime (min)	Mantle strength character notes[a]				Mantle moisture character notes[a]		
	Springiness	Cohesion[b]	Hardness	Snappiness	Smoothness	Moisture release	Slipperiness
1	11.0	10.9	11.5	10.7	12.0	11.5	11.0
2	11.0	10.3	11.5	10.7	11.0	10.7	11.0
4	10.2	10.2	11.0	11.3	9.7	9.3	9.3
8	10.2	10.0	11.0	10.0	8.3	8.3	6.0
16	9.0	9.1	9.7	9.0	6.7	6.7	6.0
32	8.4	8.0	8.3	8.3	6.7	6.3	4.7

[a] The terms "strength" and "moisture" as used here are very loose descriptive terms to encompass a group of quite precisely defined "notes." Quantitatively, for each note the range is 1 (not detectable) to 14 (very intense).
[b] This cohesion note is defined as relative deformation of the food before it ruptures.

in squid texture. Snappiness was the detection of a 'snap' at failure during primary mastication. Mantle moisture was a group of desirable features (smoothness, moisture release and slipperness) which depend on the 'wet' feeling of the sample in the mouth. Rating was done on a 14-point scale. Variation in ratings of individual character notes across three replicate sessions per cook time never exceeded ± 0.3 points and most ratings varied less than ± 0.1. The larger variations between replicates occurred for squid cooked to 100°C 16 min and 100°C 32 min for all textural character notes.

The ratings for strength characters decreased gradually across all cook times. The ratings for mantle moisture characters decreased more rapidly during initial cook at 100°C. No attempt was made to correlate these ratings with the physical results of the instrumental tests, but Figure 8 demonstrates the typical agreements. The gradual decrease in mantle strength characters is similar to the decrease in F/T for the single blade shear and probe, but the moisture characters deviated from the rheological pattern after 100°C 4 min. Original mantle moisture content (≥ 80%) decreased gradually to 75% at 5 min boil, and to 71% at 30 min boil (Otwell and Hamann, 1979).

The instrument tests appear to show general agreement with the panel characteristics which describe mantle strength, but differences in panel characteristics which describe mantle moisture characteristics are important textural attributes in squid, as evident from the panel's awareness of them.

DISCUSSION

EACH RHEOLOGICAL TEST and each texture panel evaluation describe somewhat different textural attributes of cooked squid. No one test was as informative as the comparison of the results for all tests which indicates the textural character of squid is influenced by two dominant structural features, muscle fibers and connective tissues. Most of the conclusions drawn from these tests can be verified by chemical and visual evidence from earlier work (Otwell and Hamann, 1979).

Muscle fibers

The most striking difference in instrument results caused by thermal change was the rapid decrease with increased temperature up to 60°C in force, energy, and displacement required to shear or puncture squid mantle. Structural changes during cook to 60°C, noted in accompanying work (Otwell and Hamann, 1979) were gradual heat destruction of the inner outer linings, sarcoplasmic protein coagulation, and denaturation of myofibrils. Jacobson and Henderson (1973) reported the 'melting' temperature of myosin and actomyosin as 43 ± 2°C, and numerous researchers have demonstrated thermal coagulation of sarcoplasm within the temperature range, 40–60°C (Hamm, 1966; Schaller and Powrie, 1972; Davey and Gilbert, 1969). Thermal destruction of the thin inner and outer linings is unique to squid and not considered to be of any major consequence. Initial heat denaturation of the myofibrillar and sarcoplasmic proteins, and the loss in mantle moisture (Otwell and Hamann, 1979) are likely causes for the decrease in rheological variables.

In the raw state, the squid mantle has been described as a highly elastic 'balloon' with structural features adapted for jet propelled swimming (Ward, 1972). Apparently, the raw mantle structure is an unusually tough meat, but the decrease in shear forces required to cut the cooked squid meat is a similar response to that reported for beef (Paul et al., 1973) and poultry (Pool, 1967). In contrast, Bouton et al. (1975a, b) and numerous researchers cited in his articles contend that shear force required to cut beef increases as the meat is cooked. Differences between the present results using squid and those reported for beef are most likely due to the cook temperature prior to instrumental tests, and the unique structural attributes of the muscle studied. As demonstrated with the single blade shear, at cook temperatures greater than 60°C more force was required to shear the cooked mantle when the single blade was oriented perpendicular to the dominant fiber direction. The persistence of this difference during all cook times at 100°C demonstrates the influence of thermally hardened muscle fibers on squid texture. Denatured myofibrillar and sarcoplasmic proteins lose their water-holding capacity (Hamm and Deatherage, 1960; Bendall and Wismer-Pederson, 1962), thus, at cook temperatures greater than 60°C the muscle fibers con-

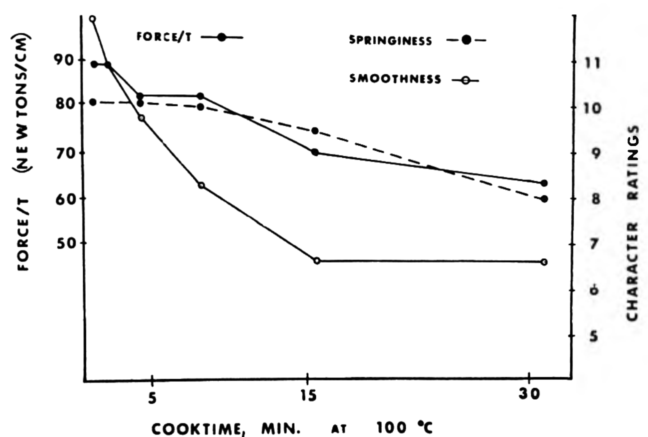


Fig. 8—Comparison of force/thickness required to shear cooked squid with a single blade with textural character notes rated by a texture profile panel. Springiness is a typical rating of mantle strength. Smoothness is a typical rating of mantle moisture.

tinually dehydrated and became a dense mass of hardened myofibrillar proteins (Otwell and Hamann, 1979).

Connective tissue (tunics)

Each side of the squid mantle is covered by a tunic of connective tissue fibers. These tunics differ in thickness, fiber construction, hydroxyproline content (Otwell, 1978) and thermal stability (Otwell and Hamann, 1979). Results from the two shear tests (single blade or punch) were opposite in differences due to sample side sheared and neither test could distinguish the specific structural influence of an individual tunic. The differences in stress pattern, cutting edge sharpness, crosshead speed and sample orientation are all possibly involved.

The increase in force and energy required to puncture mantle 'fillets' in the lower zones which contain a higher hydroxyproline content (as a measure of connective tissue) suggests a structural influence of tunics on the probe shear. Consistently more force and energy at all cook treatments was necessary to puncture the squid from the outside surface. These results were opposite for nonboiled mantles cut with a single blade, but at all cook times at 100°C there was no significant difference in shear force and energy due to side cut with the blade. This lack of difference due to side sheared with the blade is most likely a response to thermal changes in the tunics. At 100°C 1 min the outer and inner tunics begin to gelatinize (Otwell and Hamann, 1979). Penfield and Meyer (1975) demonstrated that thermal alterations in connective tissue occurred simultaneously with a decrease in beef muscle resistance to shear. Thus, the deformation patterns as affected by the side sheared with the single blade indicate that the tunics were involved in squid texture.

The influence of connective tissue tunics on squid texture was not entirely a direct effect. Hydrothermal shrinkage of the connective tissues would tend to increase squid toughness by increasing the muscle fiber density per cross-sectional area and squeezing 'loose' water from the mantle. The increase in patterns of shrinkage force in the isometric tension tests are thought to occur as a result of hydrothermal shrinkage in squid collagen. The average temperature of initial force readings (57.7 ± 0.2°C) is similar to those reported for beef (Field et al., 1970) and rat (Lawson et al., 1966) collagens. More pronounced shrinkage force patterns due to strips cut from the lower zones of the mantle 'fillet' which contains more hydroxyproline, suggest the shrinkage force was generated by thermal changes in the tunics. This hydrothermal shrinkage is most likely the cause of the curling phenonmena of mantels cooked to 60°C (Otwell and Hamann, 1979). Fibers of connective tissue in the outer tunic are arranged in a crossed pattern with each layer of fibers oriented about 25° from the longitudinal axis of the mantle, giving a 50° angle between consecutive layers, (Otwell, 1978). This fiberal arrangement is partial explanation for the greater amount of force generated by longitudinal strips of mantle. Thus, it seems that connective tissue tunics are involved in squid texture directly as a structure resistant to shear, and indirectly as a force to increase the density of shear resistant muscle fibers.

In conclusion, squid is not unlike most common meats in that its texture is influenced by two dominant structural features, muscle fibers and connective tissue. Thermal effects on these structures were determined by instrument tests in comparison with simultaneous changes in chemical composition, and ultrastructure as viewed with SEM in the companion paper (Otwell and Hamann, 1979). Instrument results demonstrated the influence of the cook treatments on the mantle resistance to shear and development of shrinkage force. The analysis of chemical composition and ultrastructure defined which structural features altered by the cook treatments caused changes in mantle strength. For example, the single blade shear tests demonstrated the influence of muscle fiber direction on mantle resistance, the SEM photomicrographs revealed the muscle fibers were becoming hardened, and the basic chemical analysis indicated the reason for hardening was fiber dehydration. This interrelated approach gives a better understanding of the relation of food structure to texture, but does not give a complete textural characterization of the food.

Evaluations by trained panelists were required to emphasize the importance of the loss in mantle moisture. The gradual decrease in shear force and texture panel ratings of mantle strength indicate prolonged cooking could be used to tenderize the mantle, but the much larger decrease in ratings of mantle moisture at cook times in excess of 5 min would limit the advantages of tenderization of squid by prolonged (> 5 min) cooking. High moisture content in cooked squid acts as a 'lubricant' to decrease structural resistance during mastication. To assure tender cooked squid, the mantle should be cut into longitudinal strips which have less muscle fiber resistance, and boiled for less than 5 min to avoid excessive mantle dehydration. Further research can determine which cook medium, i.e., pH, ionic strength, specific ion composition, etc., is best suited to cook tender squid. This medium should rapidly gelatinize the tunics of connective tissue and prevent excessive moisture loss from the muscle fibers.

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Ms received 2/8/79; revised 7/4/79; accepted 7/10/79.

The authors express their appreciation to the North Carolina State University, Food Science Department's texture profile panel members and to Ken Diehl and Win Duersch for their assistance in preparing samples and rheological tests, and to F.G. Giesbrecht of the North Carolina State University Statistics Department for statistical advice.

Paper no. 5877 in the Journal Series of the North Carolina Agricultural Research Service.

The use of trade names in this publication does not imply endorsement by the North Carolina Agricultural Research Service, nor criticism of similar ones not mentioned.

This research was sponsored, in part, by NOAA Office of Sea Grant, Department of Commerce under Grant No. 04-6-158-44-54 and the North Carolina Department of Administration. The U.S. Government is authorized to produce and distribute reprints for governmental purposes notwithstanding any copyright that may appear hereon.

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Ms received 2/8/79; revised 7/4/79; accepted 7/10/79.

The authors express their appreciation to Carol Daniels for her excellent drawings of the squid tissues, and to the NCSU SEM Center for assistance in learning operational use of the electron microscope.

Paper no. 5876 in the Journal Series of the North Carolina Agricultural Research Service.

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This work is a result of research sponsored, in part, by NOAA Office of Sea Grant, Department of Commerce under Grant No. 04-6-158-44054 and the North Carolina Department of Administration. The U.S. Government is authorized to produce and distribute reprints for governmental purposes notwithstanding any copyright that may appear hereon.

SEASONAL VARIATION AND FROZEN STORAGE STABILITY OF BLUE MUSSELS (Mytilus edulis)

JUDITH KRZYNOWEK and KATE WIGGIN

ABSTRACT

Proximate composition of blue mussels *Mytilus edulis* collected in Duxbury Bay, Duxbury, MA, was determined monthly on fresh mussels and after frozen storage. There was seasonal variation in proximate composition. This seasonal variation in the blue mussel affects the freezer life of the cooked product. Mussels harvested in the spring retain their sensory acceptability for longer than fall or winter mussels. Frozen storage does not affect the composition of the initial product, fat, moisture, ash and extractable protein remaining constant over frozen storage.

INTRODUCTION

WHILE THE BLUE MUSSEL enjoys great popularity in European countries, consumer acceptance has been minimal in the United States. This is unfortunate, because it is an excellent source of inexpensive protein. Because mussels feed on plankton, they contain vitamins A, B, C, D, and E, and are low in lipid content. They grow abundantly in the wild in the coastal waters of the northeastern United States. The meat may be processed in a myriad of ways: as fresh mussels for the gourmet, as canned mussels for retail sales, as protein concentrate (Joyner and Spinelli, 1969) for mass distribution to under-developed countries, and as meal for animal rations (Grce, 1975). A treatise on their mariculture and harvest has been presented by Hurlburt and Hurlburt (1975).

The lack of enthusiasm for the blue mussel in the U.S. may be due to the competition from the more "popular" and at one time more plentiful clams and oysters. The risk of paralytic shellfish poisoning is real but is monitored in shell-fishing areas by regulatory agencies. The vagaries of seasonal variation in the composition and sensory characteristics of blue mussels (Dare and Edwards, 1975; de Zwaan and Zandee, 1972; Drzycimsky, 1961; Slabyj et al., 1978) are a deterrent to potential processors and harvesters. This obstacle could be overcome if harvesting were done only when seasonal compositional data indicated that the mussels were nutritionally and organoleptically at their peak. Mussels could then be canned or frozen for later distribution.

The object of this investigation was to determine the effects of seasonal variation and long term frozen storage on the composition and sensory characteristics of raw and cooked blue mussels.

MATERIALS & METHODS

THE BLUE MUSSELS used in this study were obtained from natural beds in Duxbury Bay, Duxbury, MA They were harvested monthly over a 2-yr period starting May 1977 and ending May 1979 by a commercial source using a hand-raking technique. Sampling was random with no size or sex selection over an area of five acres. Spawning occurred in September 1977 and 1978.

The mussel shell stock was received in the laboratory 1–2 days after harvesting. They were hand scrubbed and shucked after cooking in boiling water for 2 min. The shucked mussels were packed in pint polyethylene tubs (at least 24 mussels per tub), covered with broth or distilled water, and quick frozen at –30°C for 24 hr. They were subsequently removed to –20°C for long term storage. Samples were thawed at ambient temperature for analysis on a monthly basis.

Taste tests were conducted monthly to assess changes with storage in appearance (A), odor (O), flavor (F), and texture (T). The mussels were steamed for taste testing in their own glaze, for 1 min. A reference sample of freshly harvested mussels was used as a control against which to grade the stored mussels. Both a known (labeled fresh control) and an unknown control were always served to the panelists as an internal check on sensory reliability. Twelve judges familiar with seafood comprised a panel. A numerical value of 9 was assigned to a rating of excellent, decreasing to a value of 1 for inedible. The sensory data were analyzed by the analysis of variance. Dunnett's test (Dunnett, 1955) was applied to those tests having a significant F ratio in order to determine which test sample(s) varied from the control. A sample was dropped from study if the average of all the A, O, F, T scores (overall score) decreased to a value of 5 (borderline acceptance) or less for two consecutive months.

The sample tubs used for chemical analysis contained a known initial weight of mussel meat (usually 200–400g meat). The sample tubs were placed in water at ambient temperature until the sample thawed. Drip was determined by evenly distributing the thawed mussels onto a Number 8 sieve inclined about 20°. After 2 min, the mussels were weighed, and this weight compared against their initial weight.

The entire drained sample was blended in a VirTis homogenizer until homogeneous for use in chemical analyses. Moisture content was determined by drying approximately 10g of sample to constant weight at 100°C. Total ash was determined by heating dried samples to constant weight at 525°C (AOAC, 1975). Lipid content was determined on two 25-g samples according to Bligh and Dyer (1959). Total protein for fresh mussels was determined on three 400-mg samples by a micro-Kjeldahl method described by the American Instrument Company (1959).

Extractable protein (XP) was determined by blending three 4-g samples in a VirTis homogenizer with 50 ml of 0.5 ionic strength buffered KCl for 2 min. The extractant was made according to Connell (1958). The samples were centrifuged for 30 min at 1800 × G at 0°C. Three ml of supernatant were used in the micro-Kjeldahl determination for protein content. Sample variation was tested as being significant at $p \leq 0.01$, and points of difference were detected by Duncan's (1955) multiple range test.

RESULTS & DISCUSSION

THERE WAS seasonal variation (Table 1) in proximate composition for the raw mussels. Moisture content varied from significantly low values (76%) in the spring months to a significantly high value (86%) for post spawning mussels in October. Slabyj et al. (1978) and Dare and Edwards (1975) noted a similar fluctuation. Fat content was significantly high (~2.5%) in May, June, and November. Fat content for the other months averaged about 2%. Ash content did not change significantly from May through December, averaging about 1.6%.

Proximate analyses were done monthly on cooked mussels (Table 1), the trend being similar to the raw mussels. Moisture content was low in the spring months with an increase in the fall and winter months. Fat content went from a high of 2.8 in the spring to a low value of 1.6 in the winter. Extractable protein as a percent of the total protein reaches low values in the fall months. Fat, moisture, ash,

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Table 1—Proximate composition of blue mussels

Sample	% Moisture Raw	% Moisture Cooked	% Fat Raw	% Fat Cooked	% Ash Raw	% Ash Cooked	% Total Protein Raw	% Total Protein Cooked	% Extractable protein Cooked	% XP of total Cooked
March	78.7	76.8	1.7	2.8	2.1	1.7	12.4	15.0	3.1	20.7
April	76.0	77.8	1.7	2.5	2.0	1.6	12.2	13.6	4.8	35.3
May	77.0	76.5	2.5	2.5	1.4	1.7	12.3	14.7	3.2	21.8
June	77.5	77.0	2.4	2.4	1.5	1.2		14.0		
July	81.5	79.1	2.0	2.3	1.7	1.0		15.2	3.1	20.4
Aug.	81.5	80.3	1.8	2.4	2.2	1.3		15.0	2.0	13.3
Sept.	82.7	78.9	1.8	2.4	1.7	1.5		14.6	1.9	13.0
Oct.	86.1	78.7	2.2	2.5	1.1	1.4		13.8	2.5	18.1
Nov.	81.2	81.6	2.6	1.8	1.7	2.2		13.8		
Dec.	79.8	80.0	1.8	2.2	1.6	1.4		13.1	1.5	11.4
Jan.		81.3		1.6		1.8		11.7	4.6	39.3

and extractable protein did not vary over storage. The effects of processing on proximate composition is well documented by Slabyj and Carpenter, 1977.

There was no seasonal variation in sensory scores for cooked fresh mussels (Fig. 1). There was, however, a marked difference in sensory scores during frozen storage. Meat from mussels low in initial moisture content (Table 1) such as the June sample (77% by raw weight) was not rated significantly lower than the control until the sixth month of storage and remained above borderline of acceptability in flavor for about 12 months in storage at $-20^\circ C$ (Fig. 1). The meats from mussels high in initial moisture content (Table 1) such as the September sample (83% by raw weight) were rated significantly lower in flavor than the control after 1 month frozen storage, and below borderline in acceptability after 2 months. This is in agreement with various authors (Anderson and Ravesi, 1969, 1970; Deng, 1978; Dyer, 1967; Sikorski et al., 1976) when it is noted that high quality products cooked live with minimum handling and quick frozen will obtain a longer shelf life.

Mussels harvested in late winter and early spring may be suffering from a shortage of food. Just prior to spawning in late summer, the females are grotesquely misshapen, and the appearance of the product suffers. Post spawning mussels have very little meat content (Slabyj et al., 1978) and a short shelf life in frozen storage as indicated by taste panels. It appears that mussels in the northeast United States should be harvested in late spring and early summer for best frozen storage results. Mussels may be harvested year round and served fresh with no difference in organoleptic appeal. Canning or freezing with flavor enhancers and protective additives might prolong the shelf life. Cultivated mussels might prove to be a better candidate for frozen storage (Slabyj et al., 1978; Hurlburt et al., 1975, 1979).

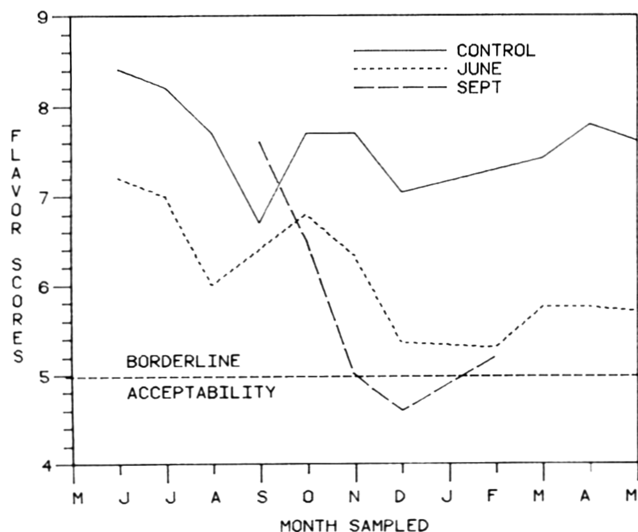

Fig. 1—Taste test flavor scores for June 1977 (cooked) mussels and September 1977 (raw and cooked) mussels in frozen storage at $-20^\circ C$.

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EFFECT OF ELECTRICAL STIMULATION ON ATP DEPLETION AND SARCOMERE LENGTH IN DELAY-CHILLED BOVINE MUSCLE

P. A. WILL, R. L. HENRICKSON, R. D. MORRISON, and G. V. ODELL

ABSTRACT

This study was undertaken to assess the effectiveness of electrical stimulation as a means of speeding postmortem metabolism as measured by ATP (adenosine triphosphate) depletion in delay-chilled bovine carcasses. Six animals of similar weight and age were used. Electrical stimulation was initiated 30 min postmortem. The stimulated side received a DC square-wave pulse of a magnitude (peak) of 300v at 400 cps (frequency), with a duration of 0.5 msec, and a current of 1.9 amps. ATP measurements were taken at 8 time periods postmortem. Results indicated muscles from the stimulated sides of beef exhibited significantly faster reductions of ATP than unstimulated controls. Electrical stimulation is an effective means of speeding postmortem glycolysis. Shear force data taken on the same muscles showed increased tenderness from electrically stimulated sides. However, measurement of sarcomere lengths from the same muscles revealed no significant differences between electrically stimulated and unstimulated sides. These data further suggested that some effect, in addition to the prevention of cold shortening, brought about the tenderization of the meat.

INTRODUCTION

ELECTRICAL STIMULATION of the meat carcass is not a new idea. Benjamin Franklin in 1749, in writings published by Lopez and Hurbert (1975), discovered that electrical stimulation of turkey carcasses made the resultant meat "uncommonly tender." Franklin's discovery was once again applied by Harsham and Deatherage (1951) in a patent which found that electrically stimulated meat promoted tenderization (U.S. Patent 2,544,681). The use of an electrical current for tenderization was confirmed by Carse (1973) in working with lamb carcasses. Since 1973, several research projects have been initiated studying different aspects of electrical stimulation as it affects the muscle systems of the carcass (Chrystall and Hagyard, 1975; Davey et al., 1976; Bendall et al., 1976; Gilbert et al., 1976; Smith et al., 1977; Shaw and Walker, 1977). The application of an electrical current to freshly slaughtered beef carcasses has been shown to increase the rate of glycolysis and reduce the time for onset of rigor mortis (Carse, 1973; Locker et al., 1975; Davey et al., 1976; McCollum and Henrickson, 1977; Shaw and Walker, 1977).

Working toward the goal of removal of muscle and muscle systems prior to chilling, the application of an electrical stimulus may have a great influence on the use of muscle for food. The rate and extent of postmortem decline in pH and ATP at the onset of rigor mortis is reflected in major variations in tenderness of the resulting meat. The objective of this study was to assess the effectiveness of electrical stimulation as a means of speeding postmortem metabolism as measured by ATP (adenosine triphosphate) depletion in delay-chilled bovine muscle. (Delay-chill processing denotes fabrication of the beef carcass prior to chilling. Hot muscle boning was initiated 2 hr postmortem followed by chilling at 1°C.) In addition, sarcomere lengths for electrically stimulated and unstimulated muscles were examined in relation to tenderness of the resultant meat.

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0022-1147/79/0006-1646$02.25/0
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Table 1—Effect of electrical stimulation on sarcomere length

Treatment	Muscle[a]	n[b]	DF[c]	Average	Mean square
Control	LD	60		1.70	
Stimulation	LD	60	5	1.65	0.07
Control	PM	60		3.22	
Stimulation	PM	60	5	3.44	1.75
Control	SM	60		1.76	
Stimulation	SM	60	5	1.65	0.08
Control	ST	60		1.90	
Stimulation	ST	60	5	2.03	0.15

[a] Longissimus dorsi (LD); Psoas major (PM); Semimembranosus (SM); Semitendinosus (ST).
[b] n = number of observations
[c] DF = Degrees Freedom

MATERIALS & METHODS

SIX HEREFORD STEERS of similar weight (363–407 kg) and age were slaughtered and the carcasses split in the conventional manner. Both sides were placed in a temperature control chamber cooled to 16°C with circulating air. At 30-min postmortem, the stimulated side received a DC square-wave pulse of a magnitude (peak) of 300v. The frequency of the stimulus was 400 cps with a duration of 0.5 msec and a current of 1.9 amps. This stimulus was applied to the side of beef for a period of 15 min. The control side received no electrical stimulation. Samples for ATP were taken from the longissimus dorsi (LD), semimembranosus (SM), and supraspinatus (SS) muscles using a 1.9 cm hand coring device. Each muscle was sampled at eight time periods (0.5, 1.0, 2.0, 4.0, 6.0, 8.0, 12.0, and 24.0 hr postmortem). The longissimus dorsi samples were taken from the 10th rib; semimembranosus samples were removed from an area 2 in. posterior to the border of the symphysis pubis. The supraspinatus samples came from the medial third of that muscle.

Both the control and stimulated sides of beef were fabricated into streamline hindquarters 2 hr postmortem. This fabrication consisted of chuck removal at the fifth thoracic vertebra with the flank and plate removed as in the commercial trade. Individual muscles were then removed from the hindquarters and placed in Cry-o-vac plastic wrap (no vacuum was drawn). These muscles were placed in a room 1°C for an additional 46 hr. All muscles studied were handled in this manner.

To eliminated ATP losses, samples were immediately stored in liquid nitrogen (LN_2) until extracted. ATP concentration was determined by homogenizing the 1.5-g frozen samples with a Kinematica Polytron. A double (2×) extraction of muscle samples using 0.4M perchloric acid ($HClO_4$) and centrifugation for 10 min at 15,000 × G was followed by neutralization using 5N KOH. The neutralized extract (pH range 6.8–7.2) was stored in an ice bath for 1 hr before being centrifuged at 15,000 × G for 10 min. The neutralized supernatant was frozen using a dry ice, acetone freezing bath. Samples were held at −20°C for 12 hr. Duplicate samples for all time periods

from a specific carcass and muscle were analyzed as an assay unit. The Firefly Lantern Extract (Sigma Chemical Company) and a Model 3000 Integrating Photometer (SAI Tech. Co., La Jolla, Calif.) were used to quantitate ATP concentrations (Marshall, 1977; Bowling et al., 1978).

Sarcomere length measurements were accomplished using samples taken from the LD, PM, SM and ST muscles in accordance with procedures developed by Falk et al (1975).

RESULTS & DISCUSSION

DATA RELATING to sarcomere length are shown in Table 1. Sarcomere lengths from all muscles studied (LD, PM, SM, and ST) indicated no significant differences between the control and stimulated samples. Shear force and taste panel data taken from the same muscles (published by Pierce, 1977) showed increased tenderness in muscles from electrically stimulated sides of beef. These conflicting results on the same muscles imply a simple prevention of cold shortening as expressed by Carse (1973); Locker (1976); Chrystall and Hagyard (1975, 1976); Davey et al. (1976); Chrystall (1976); and Shaw and Walker (1977) as the explanation for tenderization found in the electrically stimulated muscles should be seriously questioned (Smith et al., 1977; Bowling et al., 1978).

The postmortem depletion of ATP in stimulated and unstimulated muscles is presented in Figures 1, 2, and 3. The ATP content at the 0.5 hr showed no significant differences between stimulated and unstimulated semimembranosus (4.38 mg ATP/g tissue stimulated to 4.74 mg ATP/g tissue unstimulated), longissimus dorsi (3.65 to 3.68), and supraspinatus (3.78 to 3.83) muscles. The effect of electrical stimulation was to increase the subsequent decline of ATP in the semimembranosus (Fig. 1), longissimus dorsi (Fig. 2), and supraspinatus (Fig. 3) msucles. The ATP concentration was significantly lower ($P < 0.001$) in the stimulated SM, LD, and SS muscles at 1.0, 2.0, 4.0, 6.0, and 8.0 hr postmortem. At the 12.0-hr time period, significance ($P < 0.05$) was indicated in the SM and LD muscles with no difference being indicated in the SS. Nonsignificant differences in the SM, LD, and SS muscles were indicated at the 24-hr sampling period (Fig. 1, 2, and 3). The accelerated depletion of ATP in electrically stimulated muscles is in agreement with published work on rabbit and lamb, Bendall (1976); beef, Bendall et al. (1976); and lamb, Bowling et al. (1978)

Using a criteria set by Bendall et al. (1976) of ATP levels $\leqslant$ 50% of its base level as a safe limit below which rapid cooling can be initiated, a potential savings in time can be realized in stimulated beef muscles (Fig. 1, 2, and 3). The stimulated SM muscle has an immediate drop in ATP to below 50% by 2.75 hr postmortem, while the control reached this value by 6.75 hr. This represents a 59% savings of time (Fig. 1). A similar savings of 70% in the supraspinatus (Fig. 3) muscles favoring the stimulated treatment were realized.

These findings lend credence to the concept of electrical stimulation and fabrication of the beef carcass prior to chilling.

CONCLUSIONS

THIS RESEARCH clearly demonstrates that the application of an electrical stimulus to freshly slaughtered beef carcasses increases the postmortem metabolism of muscles of the carcass. The importance of cold shortening has been well documented in the production of less tender meat; however, the extent of its influence in electrically stimulated carcasses is in question.

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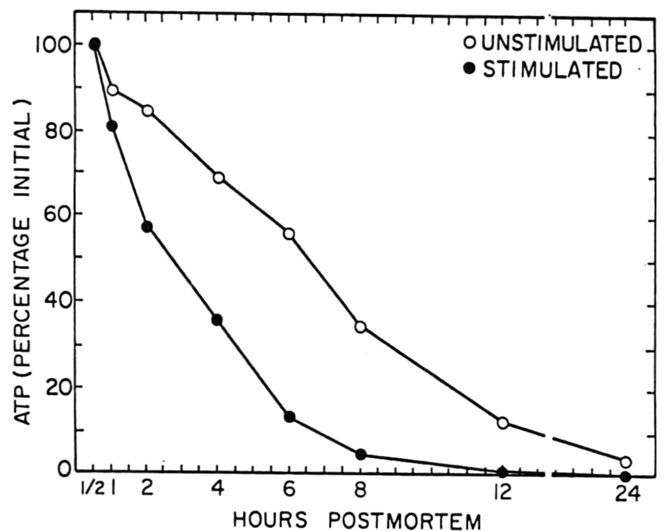

Fig. 1—Percentage of ATP depletion in stimulated and unstimulated semimembranosus muscle. Each point is an average of 12 observations.

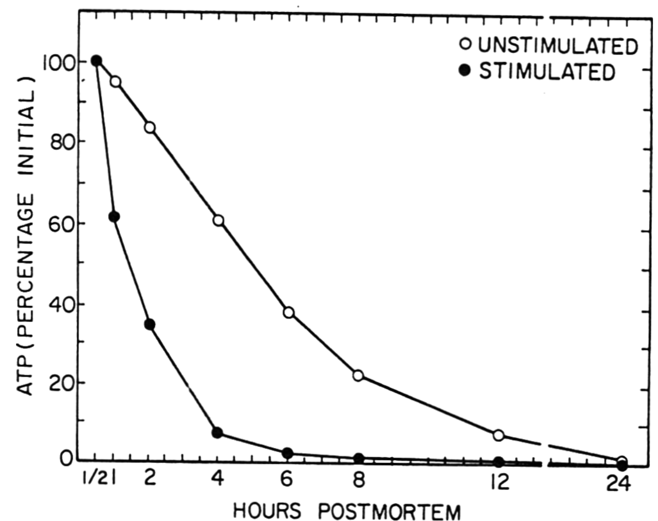

Fig. 2—Percentage of ATP depletion in stimulated and unstimulated longissimus dorsi muscle. Each point is an average of 12 observations.

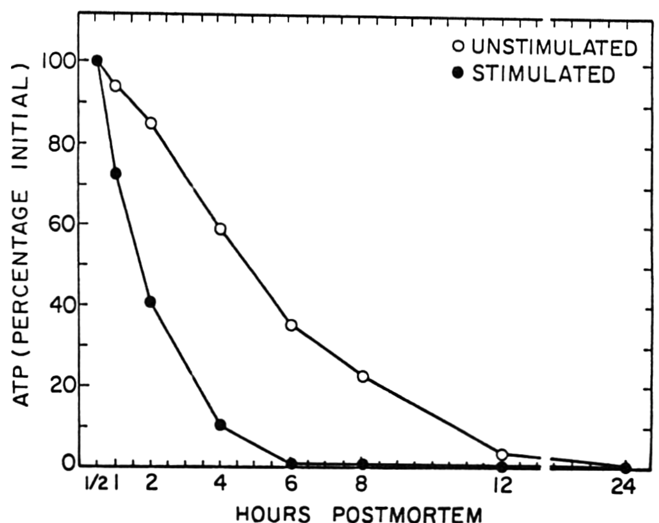

Fig. 3—Percentage of ATP depletion in stimulated and unstimulated supraspinatus muscle. Each point is an average of 12 observations.

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Journal Series Paper 3539 of the Oklahoma Agricultural Experiment Station. Financed in part by the Energy Research & Development Administration Contract No. E(40-1)5097. Appreciation is extended to B.N. Pierce, R.D. Noble, J.T. Thompson, C.M. Amundson, B.O. Brooks, and D. Doray for technical assistance.

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The authors thank Wilma Hill and Mary Haskins, National Marine Fisheries Service, Northeast Fisheries Center, Gloucester Laboratory, for technical assistance; Roger Cushing, independent shellfish dealer, Charles Mercer, Ipswich Shellfish Company, for supplying the samples; and C. Graham Hurlburt, President, Blue Gold Sea Farms, Portsmouth, RI, for technical advice.

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INHIBITION OF BACTERIA ISOLATED FROM GROUND MEAT BY STREPTOCOCCACEAE AND LACTOBACILLACEAE

GEORGES DUBOIS, HELENE BEAUMIER and RAYMOND CHARBONNEAU

ABSTRACT

Cultures of the genera *Pediococcus, Lactobacillus, Leuconostoc* and *Streptococcus* were tested for their ability to inhibit 103 bacterial isolates from ground beef by a cross-streaking technique. The bacterial isolates that were inhibited to the greatest extent were Gram-negative and were classified as species of *Serratia, Enterobacter* and *Pseudomonas* in decreasing order. Among lactic strains, the genus *Streptococcus* showed the strongest inhibitory power and the *Pediococcus* and the *Lactobacillus* the least strong. The *Streptococcus lactis* strains, in particular, were the most inhibitory, even though this power may vary from one strain to another in the same species. A nonspecific inhibitory substance seemed to be responsible for this phenomena.

INTRODUCTION

THE PRESENCE of psychrotrophic bacteria in ground beef shortens its shelf-life even if refrigerated (Jay, 1967; Pearson, 1968a, 1968b; Reddy et al., 1970, 1975; Stringer et al., 1969). Researchers have shown that 90% of psychrotrophic bacteria found in meat were Gram-negative, the maximum counts attained were, in decreasing order: *Pseudomonas* > *Achromobacter* > *Aeromonas* > *Enterobacter* > *Flavobacterium* (Daly et al., 1972; Gardner and Stewart, 1966; Gill and Newton, 1977; Kirsh et al., 1952; Stringer et al., 1969).

Reddy et al. (1970, 1975), Genske and Branen (1973), Haines (1972), Sorrels and Speck (1970), showed that lactic bacteria could lessen the growth of these Gram-negative bacteria. The production of microbial substances by the members of the *Lactobacillaceae* and *Streptococcaceae* was particularly studied by Cousin and Marth (1977a, b), Gilliland and Speck (1975) and Marth and Hussong (1963). The utilization of the lactic bacteria was mainly found in the milk and in the fermented meat industries. Reddy et al. (1970) proved that the inoculation of ground meat with cultures of *Streptococcus lactis* and *Leuconostoc citrovorum* delayed the growth of indesirable bacteria at 7°C. The same effect was also observed by Daly et al. (1972) in ground beef meat, in milk and in cheese, when they were inoculated by *Streptococcus diacetilactis*. Gilliland and Speck (1975) induced the same phenomena in ground meat using *Lactobacillus bulgaricus* and *Pediococcus cerevisiae*. In fact little is known on the use of the lactic bacteria as preserving agents in meat.

The objective of this work was to compare the inhibitory ability of different species in the following genera: *Lactobacillus, Streptococcus, Pediococcus* and *Leuconostoc*, and to select the most inhibitory ones, against bacteria isolated from ground meat. This information could be useful to improve meat shelf life.

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0022-1147/79/0006-1649$02.25/0
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MATERIAL & METHODS

Bacterial counts

Two samples of ground meat bought in April-May were divided in 50-g lots and refrigerated at 5°C. On the purchasing day, the second, the fifth and on the seventh day of storage, 50g were taken and crushed in phosphate buffer as recommended by Thatcher and Clark (1975). Proper dilutions were inoculated on Plate Count Agar (PCA) and incubated at 21°C for 2 days.

Isolation and characterization of bacteria present in meat

From a Petri dish containing between 30 and 300 colonies, different colonies were selected and purified by three reinoculations on

Table 1—Lactic bacteria

Lactic strains		Culture no.	Origin[a]
Streptococcus lactis		40	Rosell Inst.
Streptococcus lactis	NP07	42	A.C.
Streptococcus lactis	14871	43	A.T.C.C.
Streptococcus lactis	8007	153	N.R.C.C.
Streptococcus lactis	ML3	163	A.C.
Streptococcus cremoris	8029	154	N.R.C.C.
Streptococcus cremoris	AM3	162	A.C.
Streptococcus diacetilactis	18–16	164	A.C.
Streptococcus thermophilus-Z	(exp)	165	A.C.
Leuconostoc citrovorum		41	Rosell Inst.
Leuconostoc citrovorum	23006	44	A.T.C.C.
Leuconostoc citrovorum	91404	171	A.C.
Leuconostoc mesentero'ides	30001	161	N.R.C.C.
Pediococcus cerevisiae	NIRD#3	169	A.C.
Pediococcus sp.	217–35	172	A.C.
Lactobacillus plantarum	13004	155	N.R.C.C.
Lactobacillus casei	13005	156	N.R.C.C.
Lactobacillus casei	NCDO 242	168	A.C.
Lactobacillus acidophilus	13017	157	N.R.C.C.
Lactobacillus acidophilus	NCDO1375	167	A.C.
Lactobacillus bulgaricus-Z	(exp)	166	A.C.
Lactobacillus sp.	FRI #82	170	A.C.

[a] A.C.: Agriculture Canada; A.T.C.C.: American Type Culture Collection; N.R.C.C.: National Research Council Canada

Table 2—Bacteria isolated from ground meat stored at 5°C

Groups and species	No. of isolated strains	%	No. of isolation for various days of storage period. Both samples: 0	2	5	7
Gram-	6	5.8	2	3		1
Serratia sp.	33	32	13	3	5	12
Enterobacter sp.	7	6.8	2		1	4
Citrobacter freundii	1	0.9			1	
Achromobacter sp.	4	3.8	1	1	1	1
Pseudomonas putida	1	0.9	1			
Pseudomonas maltophilia	1	0.9		1		
Pseudomonas cepacia	5	4.8	2	1		2
Pseudomonas aeruginosa	9	8.7	2	1	1	5
Pseudomonas fluorescens	10	9.7	3		3	5
Pseudomonas sp.	25	24.2	3	4	9	13
Unclassified	1	0.9	1			
Total	103	100	30	10	21	42

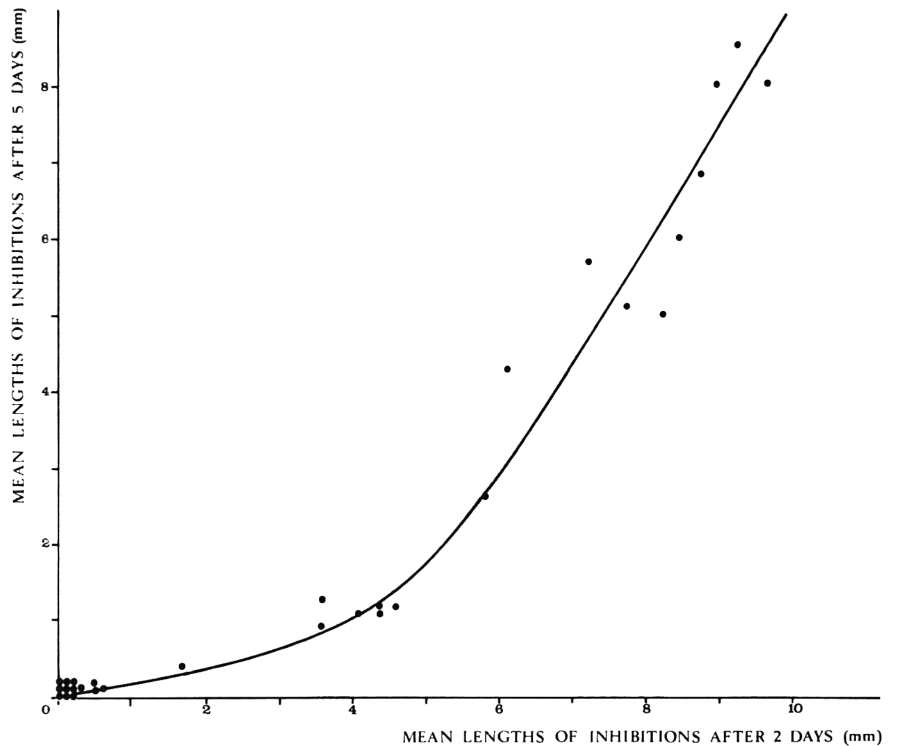

Fig. 1—Relation between the inhibitory power of the lactic strains after 2 and 5 days storage at 5° C.

PCA. Identification of these different strains of bacteria isolated from ground meat was made with the API system (Analytical Profile Index, Analytab Products, N.Y.) (Washington et al., 1971). This method used for identification of the enteric bacteria also permits the identification of other Gram-negative such as *Pseudomonas, Flavobacterium, Achromobacter,* etc . . .

Origin of the lactic bacteria

In this research, 22 strains of lactic acid bacteria were studied: 7 *Lactobacillus,* 2 *Pediococcus,* 4 *Leuconostoc* and 9 *Streptococcus.* Some of these bacteria were obtained from the Rosell Institute (Chambly, Canada), the National Research Council of Canada, Agriculture Canada and from American Type Culture Collection (USA) (Table 1).

Inhibition

It was decided to use the cross-streak technique (Daly et al., 1972). In order to obtain a satisfactory growth of the lactic bacteria, they were striated on PCA and incubated 24 hr at 37°C. After this incubation, the microorganisms isolated from meat were striated at right angle to the lactic acid bacteria. The Petri dishes were placed in a refrigerator at 5°C and maintained at this temperature during the storage period. Two and five days later, we measured the length in mm of the inhibitory zones and submitted the data for

Table 3—Percentage of the meat bacteria strains inhibited versus mean length of inhibitions after 2 days and 5 days storage at 5° C

Culture no.	Lactic strains		Mean lengths of inhibition (mm) 2 days	% Meat bacteria is inhibited	Mean lengths of inhibition (mm) 5 days	% Meat bacteria is inhibited
170	*Lactobacillus* sp.	FRI # 82	0.1	3.0	0.0	0.0
172	*Pediococcus* sp.	217–35	0.1	2.0	0.1	1.0
169	*Ped. cerevisiae*	NIRD#3	0.2	3.1	0.1	1.0
165	*Strep. thermophilus-Z*	(exp)	0.2	3.0	0.1	0.0
156	*Lactobacillus casei*	13005	0.3	7.7	0.1	2.0
166	*Lac. bulgaricus-Z*	(exp)	0.5	9.0	0.1	2.0
168	*Lactobacillus casei*	NCDO 242	0.5	9.2	0.2	4.0
155	*Lac. plantarum*	13004	0.6	15.5	0.1	2.0
154	*Strep. cremoris*	8029	3.6	84.0	1.2	48.0
42	*Streptococcus lactis*	NP07	3.6	100.0	1.3	45.1
162	*Strep. cremoris*	AM3	4.1	97.0	1.1	35.0
43	*Streptococcus lactis*	14871	4.4	100.0	1.2	47.5
41	*Leu. citrovorum*		4.4	97.7	1.1	38.0
44	*Leu. citrovorum*	23006	4.6	100.0	1.2	43.6
164	*Strep. diacetilactis*	18–16	5.8	100.0	2.6	73.0
163	*Streptococcus lactis*	ML3	6.1	100.0	4.3	100.0
161	*Leu. mesentero'ldes*	30001	7.2	100.0	5.7	99.0
40	*Streptococcus lactis*		8.4	100.0	6.0	98.0
153	*Streptococcus lactis*	8007	9.6	100.0	8.0	100.0
40 – 43			8.2	100.0	5.0	100.0
161 – 153 – 40			7.7	100.0	5.1	100.0
161 – 153			8.7	100.0	6.8	100.0
40 – 161			8.9	100.0	8.0	100.0
153 – 40			9.2	100.0	8.5	100.0

N.B. other lactic strains give negative results

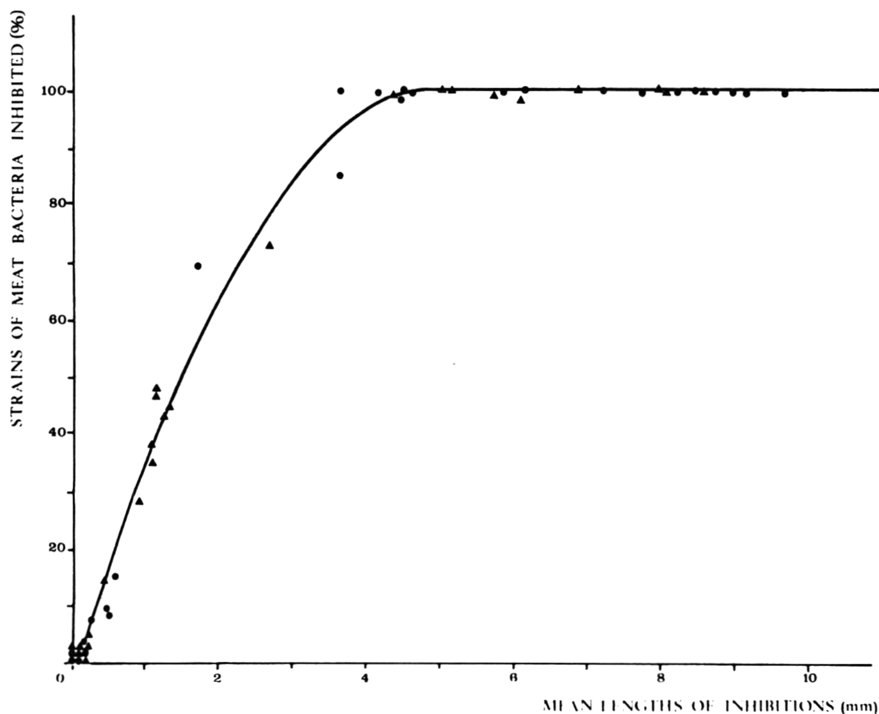

Fig. 2—Relation between the inhibitory power of the lactic strains and the percentage of the meat bacteria strains inhibited after 2 days ●—● and 5 days ▲—▲ storage at 5°C.

every lactic strain used to a statistical analysis to establish the means (P < 0.05).

RESULTS & DISCUSSION

THE PROPORTION among bacterial genera isolated (Table 2) did not show variations from one meat sample to another. We found genera usually present in meat (Gardner and Stewart, 1966; Genske and Branen, 1973; Gilliland and Speck, 1977; Kirsch et al., 1952; Stringer et al., 1969). *Pseudomonas* was the dominating genus at the end of our storage period, though this was not so obvious on the first day. The Gram-positive bacteria, while present during the first day, were eliminated during the storage period. These findings are in agreement with the works of Gill and Newton (1977), Jay (1967) and Stringer et al. (1969). The growth of some mesophilic bacteria occurred at a slow pace. This accounted for the absence of results for inhibition measurements in certain cases after two days of incubation.

The mean length of the inhibitory zone provoked by the lactic acid bacteria varied between 0 and 9.6 mm. Among strains showing good inhibition, we found strain 161 of the *Leuconostoc* and strain 40, 153, 163 and 164 of the *Streptococci.* Bacteria of the *Streptococcus lactis* specie gave the highest inhibitory effect. However, it is important to note that strains of this specie did not possess the same inhibitory power; for example, strain 43 gave an inhibitory zone with a mean length of 4.4 mm while it was 9.6 mm for strain 153.

The *Lactobacillus* and the *Pediococcus* had practically no inhibitory strength, while the *Leuconostoc* are average in this regard. The *Streptococcus,* as already mentioned, displayed the strongest effect amongst the lactic strains included in this study. This would account for the fact that Gilliland and Speck (1975) had to utilize large amounts of *Lactobacillus* and *Pediococcus* to obtain a marked inhibition (Table 3).

The results also show that for lactic strains displaying the strongest inhibitory power, this faculty was retained longer than for the weaker strains. In fact, the bacteria producing an inhibitory zone of 4 mm after 2 days were the ones losing about 75% of this power; those showing a 6 mm zone lost 50%, and finally the lactic acid bacteria giving a 9 mm inhibition zone kept 83% of their inhibitory power (Fig. 1).

The same trend was observed after a 5-day incubation period, although the *Lactobacillus* and the *Pediococcus* had very little effect on the growth of other bacteria. The *Leuconostoc* still exerted some effects and strains 153, 40 and 153 of the *Streptococcus* were those with the highest inhibitory action on the bacteria isolated from ground meat.

In studying the degree of inhibition (i.e. the percentage of strains isolated from meat inhibited) and the inhibitory power (i.e. mean length of the zone of inhibition), a relation was established between these two parameters. Lactic bacteria having high inhibitory power exerted their effect on almost all bacteria isolated from our meat samples, this was true for a 2-day and a 5-day period of conservation in the refrigerator (Fig. 2). The inhibitory substance secreted by the lactic acid bacteria seemed to be almost nonspecific: the spectral action varying with the quantity of the substance excreted (Fig. 2, Table 3).

The mean length of the inhibitory zones was also measured for each species of bacteria isolated from ground meat. Gram-positive bacteria were the most inhibited ones, followed by the enteric bacteria and the *Pseudomonas.* When the inhibitory substance was present, we observed the same phenomena whichever lactic strains were used (Table 4 and 5). Hurst (1973) in a review on the microbial antagonists, mentioned that *Streptococcus lactis* produce nisin, a substance inhibiting the growth of Gram-positive bacteria. On the other hand, we have observed also some inhibition on the Gram-negative bacteria more particularly with strains of *Leuconostoc* and *Streptococcus.* Thus we are inclined to think that another inhibiting substance is secreted by these bacteria. Moreover preliminary works carried out in vivo extracting the proteins secreted by these strains showed that this proteinaceous extract still had a power of inhibition on the growing capacity of the meat bacteria. We therefore assume that *Streptococcus lactis* produced a sub-

Table 4—Mean inhibition (mm) of the species isolated from meat by the most inhibitory lactic strains after 2 days storage at 5° C

Bacteria isolated from meat	Total no. isolated (103)	Lactic strains										
		154	42	162	43	41	44	164	163	161	40	153
Gram-	6	6.8	8.5	9.4	5.8	7.5	6.3	9.8	8.8	8	12.2	10.7
C. freundii	1	—[a]	—	—	—	—	—	—	—	—	—	—
P. putida	1	0[b]	11	4	8	7	7	4	4	6	15	8
Unclassified	1	—	5	—	4	5	8	—	—	5	9	10
P. maltophilia	1	5	5	6	7	—	4	7	9	10	11	11
Achromobacter sp.	4	1.5	4	2	3	4.2	3.5	6	5.5	5.3	8	8.7
P. cepacia	5	1	2.5	3	3	3.8	4.4	4.2	6.4	6	6.6	6.3
Enterobacter sp.	7	4.8	3.8	3.8	5.8	5.6	5.8	6.8	5.8	12	10.3	9.7
P. aeruginosa	9	1.6	2.2	3.4	3.1	3.4	3.7	5.0	6.2	5.6	7.7	9.1
P. fluorescens	10	2.2	2.6	3.4	3.4	3	3.8	3.7	4.8	6.3	7.4	9.3
Pseudomonas sp.	25	2.3	2.6	2.8	3.4	3.5	3.4	4.6	5.7	5.8	6.0	9.0
Serratia sp.	33	4.1	4.3	5.1	7.2	5.6	5.0	6.5	6.4	9.1	9.5	10.4
Total mean[c] (mm)		3.6	3.6	4.1	4.4	4.4	4.6	5.8	6.1	7.2	8.4	9.6

[a] No results after 2 days.
[b] No inhibition
[c] Total mean $\bar{X} = \frac{\Sigma \rho i \bar{X}i}{\Sigma \rho i}$ where $\bar{X}i$ represents the means and ρi the frequency for each species.

Table 5—Mean inhibition (mm) of the species isolated from meat by the most inhibitory lactic strains after 5 days storage at 5° C

Bacteria isolated from meat	Total no. isolated (103)	Lactic strains										
		154	42	162	43	41	44	164	163	161	40	153
Gram-	6	3.5	5.5	5.6	1.6	2.6	2	6.8	7.5	6	9.1	11.4
C. freundii	1	—[a]	—	7	—	—	—	6	7	0	—	—
P. putida	1	0[b]	8	0	5	5	5	1	2	4	12	6
Unclassified	1	5	1	8	0	1	0	11	9	5	4	10
P. maltophilia	1	2	0	2	4	—	0	5	8	8	9	9
Achromobacter sp.	4	0	1	0	1	1.2	1	2.2	3.2	4.5	6	5.7
P. cepacia	5	0	1	0	0.6	1.4	1.8	1.4	3.6	3.6	4.2	5.8
Enterobacter sp.	7	2.4	1.9	1.4	1.9	1.6	1.6	3.3	4.7	8	8.7	10
P. aeruginosa	9	0.1	0.3	0.4	1.3	1.1	1.4	2.3	3.8	3.9	4.8	5.8
P. fluorescens	10	0.5	0.4	0.3	1.4	0.3	1.1	1.9	3.5	4.7	4.8	6.9
Pseudomonas sp.	25	0.4	0.2	0.1	0.8	0.5	0.4	1.4	5.1	4.1	4.1	6.7
Serratia sp.	33	1.2	1.6	1.1	1.2	1.0	1.3	2.8	4.2	7.2	7.1	8
Total mean[c] (mm)		1.2	1.3	1.1	1.2	1.1	1.2	2.6	4.3	5.7	6	8

[a] No results after 5 days
[b] No inhibition
[c] Total mean $\bar{X} = \frac{\Sigma \rho i \bar{X}i}{\Sigma \rho i}$ where $\bar{X}i$ represents the means and ρi the frequency for each species.

stance that could be used to extend the shelf life of ground meat.

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Ms received 1/10/79; revised 5/18/79; accepted 5/27/79.

This research was supported by Agriculture Canada, contract no. 02SU.01531-7-0433.

The authors thank Dr. M. Gagnon, CRESALA®, for his assistance in preparing the manuscript.

THERMAL INACTIVATION OF CLOSTRIDIUM BOTULINUM TOXINS TYPES A AND B IN BUFFER, AND BEEF AND MUSHROOM PATTIES

J. G. BRADSHAW, J. T. PEELER and R. M. TWEDT

ABSTRACT

To determine the time-temperature relationships for the inactivation of botulinal toxins, crude toxins were prepared from three type A strains (62A, 73A, V141) and two type B strains (Beans-B and 999B). Toxins were diluted to 6,700–32,000 mouse LD_{50} units/0.5 ml in beef and mushroom patties and in 0.1M phosphate buffer, both at pH 6.05, and 0.1M acetate buffer, pH 5.0, and were heated at temperatures from 67.8–80.0°C. In the patties, inactivation times ranged from 53.15 min to 0.62 min and from 51.20 min to 1.08 min for the most thermostable type A (73A) and type B (Beans-B) toxins, respectively, whereas significantly less time was required in phosphate buffer.

INTRODUCTION

DATA DESCRIBING time-temperature relationships of botulinal toxin heat inactivation are somewhat limited. Shortly before the turn of the century, Van Ermengun (1897) reported that toxin was rendered almost inert when heated at 80°C for 30 min and was completely destroyed at 100°C. Dickson (1917), Landmann (1904) and Orr (1920) reported inactivation of toxins from various strains of *C. botulinum* at temperatures ranging from 65–80°C and times varying from 1 hr to 30 sec.

After *C. botulinum* toxin types were recognized, Sommer and Sommer (1932) investigated the heat inactivation of types A and C in buffers and natural juices. They reported that the rates of inactivation appeared to be curvilinear functions. Both toxins were most stable at or near pH 5.0. Inactivation rates at a given pH were greatly influenced by the addition of fruit or vegetable juices to the heating menstrua. Those experiments were marred, however, by the use of fourfold dilutions of heated toxin, a single mouse for each dilution, and irregularly spaced heating intervals. Beginning with a toxin type A concentration 500 times the mean lethal dose (LD_{50}) for mice, these investigators reported typical thermal inactivation times of 10 min at 75°C and <5 min at 80°C in pH 5.0 acetate buffer to reach 0.01 of the initial titer.

Two decades later, with an essentially identical experimental design, Scott and Stewart (1950) confirmed these observations in their studies of heat inactivation of toxin types A and B in canned vegetables. Scott (1950) identified some of the protective substances in vegetable juice as divalent cations and organic acid anions.

Cartwright and Lauffer (1958) described the curvilinear rates of inactivation that resulted when they heated botulinal toxins type A in 0.1M phosphate buffer (pH 6.9) at 50 and 60°C as biphasic. They interpreted their findings as the result of a dissociation of toxins into two entities with different thermal inactivation rates.

The heat inactivation of botulinal toxin type E was studied by Ohye and Scott (1957), who reported that the toxin was destroyed in 5 min in cooked-meat medium at 60°C at pH 7.5 and 3.5; at pH 4.6–4.9 the toxin was more stable. Abrahamson et al. (1966) found that botulinal toxin type E was more thermostable in meat broth and fish dialyzate than in 0.1M phosphate buffer (pH 7.2) or in buffer supplemented with 0.1% gelatin. Licciardello et al (1967a) demonstrated that, like botulinal toxin types A and B, type E toxin's heat inactivation could best be described as a curvilinear function. From the results of their studies (Licciardello et al., 1967b) of heat penetration and inactivation rates of botulinal toxin type E (5,000 mouse LD_{50} units/0.5 ml) in haddock fillets (pH 6.7), they calculated the theoretically required cooking times. They concluded that normal cooking procedures should suffice to inactivate toxin. They also confirmed that the pH of maximum heat stability of toxin was about 5.5.

Woolford et al. (1978) studied the heat inactivation of purified botulinal toxin type A in some convenience foods after frozen storage. They showed that toxicity of type A held in beef pie fillings or phosphate buffer (pH 5.9), mushroom soup (pH 6.2), or tomato soup (pH 4.1) remained the same throughout the frozen storage. Losikoff (1978) heated botulinal toxin type A in beef broth at different pH's and confirmed that pH 5.0 was optimum for heat stability. Woodburn et al. (1979) heated purified toxins types A, B, E and F in certain high- and low-acid canned foods and buffers. They reported that toxins were generally more stable in foods at pH 4.2–5.0 than at pH 6.2.

Our objective in the present study was to gather more data on the thermal inactivation of crude *C. botulinum* toxins types A and B in buffer and in beef and mushroom patties. The latter were selected to obtain data that are useful for evaluating the potential of these toxins to persist through the heat processing of convenience foods.

MATERIALS & METHODS

Cultures

Three strains of *C. botulinum* type A (62A, 73A, and V141) and one type B (999B) were obtained for this study from FDA stock cultures. The Beans-B strain was obtained from C. Hatheway, Center for Disease Control, Atlanta, GA. Stock cultures were grown on cooked meat medium (Difco) for 24 hr at 35°C and stored at −17°C.

Toxin production

For toxin production, type A strains were grown in beef heart casein broth (Stumbo, 1965) for 5 days at 30°C. Sac cultures of each type B strain were grown in corn steep liquor supplemented with 0.1% calcium chloride (Sterne and Wentzel, 1950) for 21 days at 35°C. Pooled cultures of each strain were centrifuged at 14,000 × G for 15 min, and the recovered supernatant fluid was titrated for toxicity in mice. Serial tenfold dilutions were prepared in gel phosphate buffer (pH 6.2) (Dowell and Hawkins, 1973). A 0.5-ml inoculation of each dilution was given intraperitoneally (i.p.) to each of four 15- to 20-g Swiss white mice.

The mice were observed for 4 days, and the deaths were recorded. The toxin content (mouse LD_{50} units/0.5 ml) of the culture fluid (crude toxin) was calculated by the Reed and Muench (1938) method. The sensitivity of a given lot of mice was determined by inoculating them with known dilutions of a purified *C. botulinum* type A toxin standard furnished by E. Schantz, Food Research Institute, Univ. of Wisconsin, Madison, WI.

Thermal inactivation studies

Crude toxin was diluted 100-fold to give 6,700–32,000 mouse LD_{50} units/0.5 ml in 0.1M acetate buffer (pH 5.0), in 0.1M phosphate buffer (pH 6.05), and in beef and mushroom patties (pH 6.05). The test concentration was selected to provide a tenfold multiple of the level that could be expected in contaminated convenience food. Botulinal type B toxin, ranging from 2–10,000 mouse

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Table 1—Time required at different heating temperatures to inactivate C. botulinum toxins from initial concentration to one mouse LD_{50} unit at pH 5.0[a]

Heating temp (°C)	F value (min)									
	Type A toxin						Type B toxin			
	62A		73A		V141		Beans-B		999B	
	C_0=3.2(10⁴)[b]	% CV[c]	C_0=2.9(10⁴)	% CV	C_0=2.9(10⁴)	% CV	C_0=3.0(10⁴)	% CV	C_0=3.0(10⁴)	% CV
70.0	–	–	–	–	–	–	–	–	31.60 31.00	1.3
71.1	143.30 143.50	0.1	–	–	65.20 65.60	0.4	–	–	–	–
72.2	–	–	–	–	–	–	39.80 41.60	3.1	5.21 5.50	4.0
73.3	29.60 30.90	3.0	38.80 37.90	1.7	19.80 20.70	3.1	–	–	–	–
74.4	–	–	–	–	–	–	11.40 9.50	12.8	2.40 2.40	0.0
75.6	9.70 9.90	1.4	11.00 12.00	6.1	6.70 6.40	3.2	–	–	–	–
76.7	–	–	–	–	–	–	1.10 1.70	30.3	0.18 0.17	4.0
77.8	0.67 0.58	10.2	3.00 3.00	0.0	2.40 2.60	5.7	–	–	–	–
78.9	–	–	–	–	–	–	0.96 0.64	28.3	–	–
80.0	–	–	0.45 0.45	0.0	–	–	–	–	–	–

[a] In 0.1M acetate buffer.
[b] Initial concentration in mouse LD_{50} units/0.5 ml.
[c] Percent coefficient of variation.

LD_{50} units/ml, was detected in 29 cans of commercially canned mushrooms during an examination of all warehouse stocks by the FDA (Read et al., 1974). It is believed that most ready-prepared food would, as a consequence of including such a contaminated constituent, have less than 10^3 mouse units/g.

A single lot of commercial beef and mushroom patties was purchased for these studies and stored at −17°C. The product contained 5.9% chopped, canned mushrooms and was flame-broiled to an internal temperature of approximately 65.5°C. For use in heat experiments, patties were minced and ground while still frozen in a stainless steel blender. Crude toxin in an amount to give the desired initial concentration was added to the contents and mixed. The mixture was dispensed by a modified steel grease gun.

Two-milliliter aliquots of buffer-diluted toxin, or approximately 2g of beef toxin mix, was dispensed into borosilicate glass tubes (13 × 100 mm). The tubes were heat-sealed by a technique that did not raise the temperature of the contents above 32°C in preliminary tests using thermocouples.

The sealed tubes were immersed and heated in a water bath at various temperatures between 67.8 and 80.0°C, and cooled immediately in ice water to 4.4°C. Quadruplicate tubes were heated at each temperature selected for approximately 12 time intervals. Duplicate studies were made at each temperature.

Heating and cooling curves for all three suspending substrates were prepared for each final heating temperature from data obtained with thermocouples and a temperature recorder. Using these curves, the lethality during heating and cooling was determined and used for calculations of the F values.

For determining thermal inactivation, 0.5-ml aliquots of heated toxin were inoculated into each of 10 mice.

Statistical methods

In preliminary experiments, the time of inactivation for serial dilutions of crude toxin was determined. A plot of the data revealed the inactivation rate to be nonlinear (that is, not expressable by traditional D values). Therefore, the present study was designed to determine F values, or the times required at a selected temperature to inactivate toxin from a known initial concentration (C_0) of mouse LD_{50} units/0.5 ml to one unit/0.5 ml. The statistical method of estimating LD_{50} values together with associated assumptions has been described by Finney (1964). The F values were corrected for lags in heating and cooling (Anellis et al., 1954) by the derivation of approximate D values from the slope of a two-point linear curve. The error in this approximation is not significant at low heating temperatures. A linear regression of $\log_{10}$ F values versus heating temperatures was computed (Ostle and Mensing, 1975) for each toxin-substrate-pH combination tested at each temperature to estimate the rate of decrease in toxin strength with temperature. The absolute value of the reciprocal of this rate is the z value or temperature increment (°C) needed to change the F values by 90%.

To test the hypothesis ($\alpha = 0.01$) that either substrate or pH had affected the rate of thermal inactivation of crude toxin, linear regression of $\log_{10}$ F values versus temperature were compared (Ostle and Mensing, 1975). Estimates of times at a selected temperature to achieve a reduction of C_0 were made and 99% confidence intervals computed according to Land (1972).

RESULTS

THE THERMAL CHARACTERISTICS of toxin from three type A and two type B strains of *C. botulinum* heated in 0.1M (pH 5.0) acetate buffer at temperatures between 70 and 80°C are shown as F values in Table 1. The z values were 2.94, 3.48, 4.69, 3.69 and 3.13°C, whereas the correlation coefficients were −0.982, −0.994, −0.999, −0.974,

Table 2—Time required at different heating temperatures to inactivate C. botulinum toxins from initial concentration to one mouse LD_{50} unit in different heating substrates

	F value (min)											
	Toxin 73A						Toxin Beans-B					
Heating temp (°C)	C_o=7.5(10^3)[a] in beef pH 6.05[b]	% CV[c]	C_o=6.7(10^3) in buffer pH 6.05[d]	% CV	C_o=7.8(10^3) in buffer pH 5.0[e]	% CV	C_o=7.6(10^3) in beef pH 6.05	% CV	C_o=7.8(10^3) in buffer pH 6.05	% CV	C_o=3.0(10^4) in buffer pH 5.0	%CV
67.8	–	–	–	–	–	–	–	–	106.50 102.40	2.8	–	–
68.3	–	–	107.50 121.50	8.6	–	–	–	–	–	–	–	–
70.0	–	–	–	–	–	–	–	–	37.50 33.40	8.2	–	–
71.1	52.50 53.80	1.7	36.90 35.50	2.7	210.60 218.50	2.6	–	–	–	–	–	–
72.2	–	–	–	–	–	–	52.50 49.90	3.6	11.90 10.50	8.8	39.80 41.60	3.1
73.9	10.40 11.60	7.7	9.00 8.70	2.4	53.10 58.10	6.4	–	–	–	–	–	–
74.4	–	–	–	–	–	–	9.40 9.30	0.8	2.80 2.90	2.5	11.40 9.50	12.8
76.7	2.80 2.80	0.0	1.60 1.30	14.6	8.20 8.10	0.9	3.10 2.90	4.7	–	–	1.10 1.70	30.3
78.9	–	–	–	–	–	–	1.19 0.97	14.4	–	–	0.96 0.64	28.3
79.4	0.67 0.57	11.4	–	–	2.20 2.00	6.7	–	–	–	–	–	–

[a] Initial concentration in mouse LD_{50} units/0.5 ml.
[b] In beef and mushroom patty.
[c] Percent coefficient of variation.
[d] In 0.1M phosphate buffer.
[e] In 0.1M acetate buffer.

and −0.981 for 62A, 73A, V141, Beans-B and 999B toxins, respectively.

A comparison of F values shows that although the initial concentrations of crude toxins, identically diluted from culture supernatants, were very similar, the rates of thermal inactivation among the three type A toxins and between the two type B toxins were dissimilar. The difference between the two type B toxins is more dramatic. For the remainder of the study, the type A (73A) and type B (Beans-B) toxins that exhibited the greatest thermal stability within each group were used.

The pH of the commercial beef and mushroom patties used in this study was 6.05. Therefore, thermal behavior of the two most heat stable toxins was studied in the beef and mushroom patties, in 0.1M phosphate buffer at the same pH (6.05), and in acetate buffer at pH 5.0. The F values obtained are shown in Table 2. The z values for 73A toxin were 4.34, 4.41, and 4.05°C in beef and mushroom patties, pH 6.05 buffer, and pH 5.0 buffer, respectively. Corresponding z values for Beans-B toxin were 4.02, 4.28, and 3.69°C. Correlation coefficients for 73A toxin were −0.999, −0.994, and −0.997 in the three heating substrates; for Beans-B toxin, corresponding values were −0.922, −0.998, and −0.976.

Table 3 shows the results of tests (Ostle and Mensing, 1974) to compare thermal inactivation curves between substrates. The first test of significance (F_1) resulted in the rejection of the hypothesis that the groups were equal. The second test (F_2) shows that the hypothesis, that individual regression lines in each pair are parallel, could not be rejected. The F values obtained for 73A are plotted against the various temperatures used for each substrate in Figure 1. A similar plot for Beans-B appears in Figure 2. Although

Table 3—Results from homogeneity of regression line tests

Toxin	Substrate	F_1^a (2,12)	F_2^b(1,12)
73A	Buffer pH 5.0 Buffer pH 6.05	177.80[c]	2.51
73A	Beef pH 6.05 Buffer pH 6.05	16.40[c]	0.09
Beans-B	Buffer pH 5.0 Buffer pH 6.05	28.63[c]	0.06
Beans-B	Beef pH 6.05 Buffer pH 6.05	78.38[c]	1.09

[a] Test to examine the hypothesis that two groups can be pooled.
[b] Test to examine the hypothesis that the two slopes of the regression line can be pooled.
[c] Significant at the α = 0.01 level.

the C_o for each strain varies slightly among substrates, these differences are insufficient to prevent meaningful comparisons between plots.

DISCUSSION

ACCORDING TO THE RESULTS of our heat-inactivation studies conducted in 0.1M acetate buffer (pH 5.0), it would appear that thermal stability among the three type A and between the two type B toxins differed considerably (Table 1). The difference is most apparent between the Beans-B and 999-B toxins. These differences are proportionately much greater than the small variance in initial concentrations of crude toxin, identically diluted from culture supernatant. It seems most unlikely that the dissimilar heat stabilities can be explained by the very minor variations in culture constituents that could exist. Our findings contradict earlier observations of Scott and Stewart (1950), who also used crude toxin in food systems, that inactivation rates for toxins produced by different strains of a given type were identical. Perhaps improvements in the thermal inactivation techniques used in these studies made strain differences more recognizable.

We have confirmed earlier observations by Licciardello et al. (1967b), Losikoff (1978), Ohye and Scott (1957), Scott (1950, Scott and Stewart (1950), Sommer and Sommer (1932) Woodburn et al. (1979) and Woolford et al. (1978) that the heat stability of botulinal toxins is greatest at low pH. Both toxins 73A and Beans-B are significantly more thermostable at pH 5.0 than at pH 6.05 (Tables 2 and 3, and Fig. 1 and 2).

On the basis of our results (Table 1), the projected temperatures that must be achieved to inactivate 3×10^4 mouse LD_{50} units of toxin in 1 min are 77.6, 79.0, 79.5, 77.9, and 74.6°C for toxins 62A, 73A, V141, Beans-B, and 999-B, respectively. Evidence indicates that such temperatures may never be achieved in the processing or cooking of convenience foods. In fact, Stevenson and Nicholas (1973) found that heating frozen pizzas in a household oven according to the manufacturer's directions was insufficient to reach this toxin's inactivation range in the pizza. The highest temperature reached at the slowest heating point (the interface of low-acid mushroom slices) was 48.9°C. It required 19 min of heating for that point to reach 79.4°C. The product was then overcooked. Woolford et al. (1978) inoculated meat pies with purified type A toxin and heated them according to the manufacturer's instructions. They found that the inactivation rate was nonlinear and that considerable toxin remained after cooking.

Our results indicate that both toxins are significantly more heat stable in beef and mushroom patties than in phosphate buffer at the same pH (6.05) (Table 3 and Fig. 1 and 2). In fact, the additional thermal stability conferred upon Beans-B toxin by the presence of beef and mushroom patty constituents is equal to the added protection provided to buffered toxin by reducing pH to 5.0 (Table 2, Fig. 2). A similar protective effect is shown by 73A toxin in beef patties, although to a more modest extent (Table 2, Fig. 1).

Our results demonstrating the protective effects of food constituents confirm the observations of Abrahamson et al. (1966), Licciardello et al. (1967b), Scott (1950), Scott and Stewart (1950) and Sommer and Sommer (1932), but contrast sharply with those of Woolford et al. (1978). The

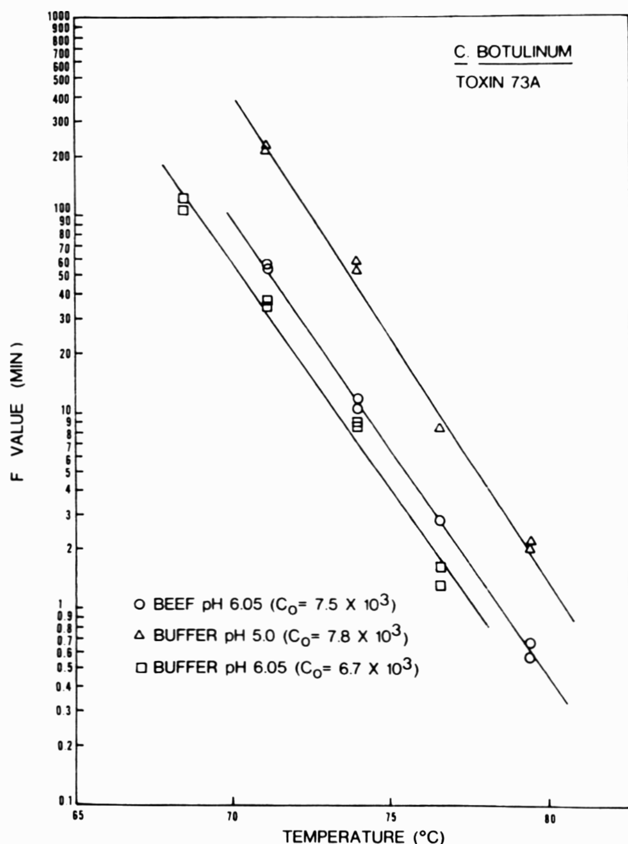

Fig. 1—Thermal inactivation curves for C. botulinum strain 73A toxin suspended in beef and mushroom patty, 0.1M phosphate buffer (pH 6.05), and 0.1M acetate buffer (pH 5.0). Initial concentration (C_o) in mouse LD_{50} units/0.5 ml.

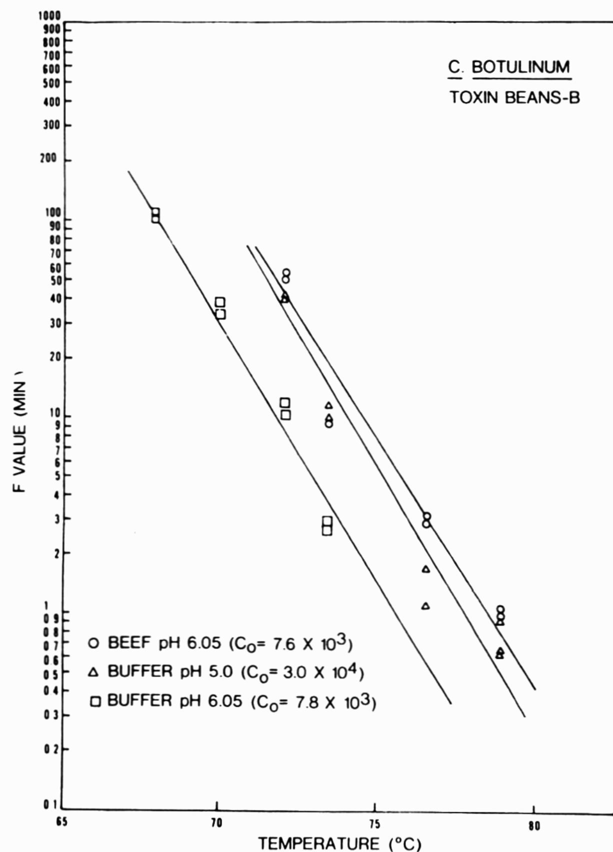

Fig. 2—Thermal inactivation curves for C. botulinum strain Beans-B toxin suspended in beef and mushroom patty, 0.1M phosphate buffer (pH 6.05), and 0.1M acetate buffer (pH 5.0). Initial concentration (C_o) in mouse LD_{50} units/0.5 ml.

latter reported little if any evidence of protective effects from food when they compared heat inactivation of pure botulinal type A toxin heated in beef pies and in phosphate buffer at the same pH 5.9. However, the research of Woodburn et al. (1979) indicates that the addition of 1% gelatin to botulinal toxins heated in buffer systems confers additional thermal stability. The protective effect was most marked with types A and B toxins in phosphate buffer at pH 6.8.

The present study shows (Table 2) that a product such as beef and mushroom patties (natural pH 6.05) would have to be cooked about 54 min at 71°C to inactivate 7.5 × 10^3 mouse LD_{50} units/0.5 ml of toxin 73A; at 77°C, it would take almost 3 min. When the internal temperature of the product is less than 66°C, it is probable that preformed toxin would not be destroyed if it were present (Fig. 1). On the other hand, heating for 1 min at an internal temperature of 82°C should be sufficient to inactivate botulinal toxin in convenience foods, provided the toxin concentration does not exceed 1 × 10^4 mouse LD_{50} units/0.5 ml (Fig. 1).

The protective effects of low pH and of beef and mushroom patty constituents on 73A and Beans-B toxins heated at 76.7°C, a process temperature frequently used for this type of convenience food, can be seen in Table 4. Based on our results (Table 2), the predicted time required to reduce C_o to one mouse LD_{50} unit/0.5 ml ranges from < 1 to approximately 8 min. These data demonstrate that lower pH and/or the presence of natural constituents found in ground beef will increase the time to inactivate toxin.

Table 4—Estimated time required at 76.7°C to inactivate C. botulinum toxins from initial concentration to one mouse LD_{50} unit in different heating substrates

Toxin tested	C_o[a]	Substrate[b]	Heating time (min)	99% CI[c]
73A	7.8 × 10^3	Buffer pH 5.0	8.10	7.30–9.00
	6.7 × 10^3	Buffer pH 6.05	1.60	1.30–1.90
	7.5 × 10^3	Beef pH 6.05	2.80	2.60–3.00
Beans-B	3.0 × 10^4	Buffer pH 5.0	1.70	1.10–2.70
	2.5 × 10^4	Buffer pH 6.05	0.58	0.48–0.70
	7.6 × 10^3	Beef pH 6.05	3.10	2.70–3.60

[a] Initial concentration in mouse LD_{50} units/ 0.5 ml.
[b] In 0.1M acetate buffer (pH 5.0), 0.1M phosphate buffer (pH 6.05), or beef and mushroom patty.
[c] The 99% confidence interval.

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Ms received 12/17/78; revised 3/10/79; accepted 3/18/79.

The authors express their gratitude to Mr. Dietrich Neumann for his excellent technical assistance.

HEAT INACTIVATION RATES OF BOTULINUM TOXINS A, B, E AND F IN SOME FOODS AND BUFFERS

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ABSTRACT

Inactivation of botulinum toxins was determined in selected acid and low acid foods and buffer systems. Heating at 74°C and 79°C gave a biphasic curve when the log of the inactivation of the toxins was plotted against the time of heating. At 74°C, the time for inactivation of 10^5 LD_{50} of type A toxin per gram of an acid food such as tomato soup to no detectable toxin by mouse assay was an hr. or more. At 85°C the inactivation was very rapid and approached exponential decrease with inactivation to no detectable toxin within 5 min. In general, the toxins were more stable in acid foods such as tomato soup at pH 4.2 than in low acid foods, such as canned corn at pH 6.2. Twenty minutes at 79°C or 5 min at 85°C is recommended as the minimum heat treatment for inactivation of 10^5 LD_{50} botulinum toxins per gram of the foods tested.

INTRODUCTION

Previous publications from this laboratory (Woolford et al., 1978; Woodburn et al., 1979) described the rates of heat inactivation of type A botulinum toxin in blended commercial beef pie, phosphate buffer and tomato soup and types A, B and E in canned salmon. Heating these foods and buffers containing toxin at temperatures of 60° and 68°C resulted in an extremely slow inactivation rate. Particularly in an acid formulated food such as tomato soup, 1–3 hr were required for inactivation of 10^5 LD_{50} of type A toxin per gram to a level safe for human consumption (no detectable toxin). At higher temperatures, 74° and 79°C, the rate of inactivation of the toxins in salmon paste was rapid and exponential at first, but tapered off quickly to form a biphasic reciprocal inactivation curve with a much slower rate. However, at 85°C the first phase was so rapid that the second phase was not apparent and the inactivation was, for all practical purposes, an exponential one. At any one temperature, the inactivation rate of types A and B toxins in a buffer at pH 4.2 or in an acid food such as tomato soup, pH 4.2, was considerably slower than in a food such as blended beef pie or salmon paste with a pH of about 6.

Cartwright and Lauffer (1958) working with type A toxin in buffers found after 30 min no inactivation at 40°C, a 2-log inactivation at 50°C, and a 3-log inactivation at 60°C. They also observed a biphasic curve of inactivation. Bradshaw et al. (1979) for types A and B, Scott (1950) and Losikoff (1978) for type A, and Yao et al. (1973), Abrahamsson et al. (1966) and Licciardello et al. (1967) for type E have published data on the heating times necessary to inactivate these toxins in foods at different temperatures, but without data on the inactivation rates.

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0022-1147/79/0006-1658$02.25/0
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This paper describes more detailed studies on the inactivation rates of the toxin types A, B, E and, to a limited extent, F, in some other common foods and in buffered solutions. The purpose was twofold: (a) to observe the nature of the inactivation curves, and (b) to determine the minimum heat treatment required to inactivate these toxins and avoid, as much as possible, the loss of quality and nutrients in the food due to heating.

MATERIALS & METHODS

CRYSTALLINE TYPE A TOXIN was prepared from the Hall strain of *Clostridium botulinum* as described by Duff et al. (1957). Partially purified type B (Okra strain) and type F (Langeland strain) were prepared following the purification schemes of DasGupta and Sugiyama (1976) and Yang and Sugiyama (1975), respectively, through the second ammonium sulfate precipitation. Type E (Alaska 43 strain) toxin was partially purified from cell extracts by precipitation with ammonium sulfate as described by Kitamura et al. (1968). The toxin crystals or one of the partially purified toxins was dissolved in 0.05M phosphate buffer at pH 6.2 or 0.05M acetate buffer at pH 4.2 and added to the food to give a concentration of about 10^5 mouse LD_{50} per gram or milliliter. One milliliter or gram of the food containing toxin was sealed in a 5 ml thin glass ampule (1.4 cm × 3.7 cm inside with a 4.7 cm neck). The heat treatment was carried out by immersing the ampules in a horizontal position in a thermostatically controlled water bath at 74°, 79° or 85°C (± 0.2°C) and withdrawing two or more for toxin assay at 0.5, 1, 2, 5, 8, 10, 15, and 20 min and 5 min intervals to 60 min. Upon removal from the bath, each ampule was cooled immediately in an ice water slurry. The come-up time from 23°C to 85°C for the liquid in the ampules, as indicated by a thermocouple, was from 5–6 sec and the cooling time less than 5 sec. Because these times were short, they were not considered in the inactivation times.

To determine if heating quantities of food was comparable to heating 1g in the thin glass ampules, 100g of canned corn or tomato soup contained in a 250 ml beaker was placed in a boiling water bath and rapidly stirred while being heated to 85°C. A concentration of 10^5 mouse LD_{50} of toxin per gram or milliliter was used because, on occasion, this amount of type A toxin has been found in foods (Woodburn et al., 1979 and other data from this laboratory).

Foods chosen were representative of acid foods (pH 4.6 or below) such as tomato soup (pH 4.2) and tomato juice (pH 4.1) and low acid foods (pH above 4.6) such as canned corn (pH 6.2), creamed corn (pH 6.3), canned string beans (pH 5.8) and canned mushrooms (pH 5.8). The corn, beans and mushrooms were crushed with a spatula and the pipettable liquid and solids were used for the experiments. The toxin was extracted from the food for mouse assay as described in *Bacteriological Analytical Manual for Foods* (FDA, 1976). One milliliter of 0.05M sodium phosphate buffer pH 6.2 was added to the ampule containing the food and toxin, mixed well to allow the toxin to diffuse from the solid particles and centrifuged at sufficient speed to give a clear or nearly clear supernatant fluid.

For studies on the rates of heat inactivation of the toxins in buffer solutions, types A, B and E toxins were dissolved at a concentration of about 10^5 LD_{50} per milliliter in 0.05M sodium phosphate buffer at pH 6.8, in 0.05M sodium citrate buffer at pH 5.5 and in 0.05M sodium acetate buffer at pH 4.2 with and without 1% gelatin. The buffers were treated in the same manner as foods containing toxin.

The determination of the LD_{50} for toxin in a food extract or buffer was carried out with white mice weighing 18–22g, essentially as described by Schantz and Kautter (1978). The mice were observed several times daily over a 72-hr period for signs of botulism prior to death. All experiments were run in duplicate for each point

on the curve using two mice on each of 3 or 4 serial dilutions over a range of all dead to no deaths. Experience with our mice using type A toxin has shown that this range coincides with a change in toxin concentration from 0.065 ng to 0.025 ng with 0.04 ng equivalent to one mouse LD_{50}. Variation in the mouse assay has been determined to be ± 15% of the mean (Schantz and Kautter, 1978). The term "no detectable toxin" is defined as the point where no deaths due to botulism occurred in the mice when challenged with 0.5 ml of the food extract, thus indicating a toxin concentration of less than 4 LD_{50} per gram of food.

RESULTS

THE HEATING TIMES for the inactivation of botulinum toxin types A, B and E at 74°, 79° and 85°C are presented in Table 1 for representative acid foods, tomato soup and tomato juice, and some representative low acid foods, canned corn, canned creamed corn, canned string beans and canned mushrooms. To show the general biphasic nature of the inactivation curves throughout the heating period without plotting each one graphically, Table 1 gives the time required to inactivate the toxin by 1-log, 3-logs and to the point where no toxin was detectable by the mouse assay. Figure 1 presents the heat inactivation curves for Type A and B toxins in tomato soup at its natural pH of 4.2 and for type A at pH adjusted to 6.2 with NaOH. Inactivation of type A toxin in soup at pH 4.2 required 60 min at 74°C, 15 min at 79°C, and 5 min at 85°C compared to 8 min at 79°C, and 2 min at 85°C at pH 6.2. The effect of pH in this case is clearly demonstrated. Figure 2 presents the heat

Table 1—Heating times in minutes at different temperatures to inactivate 10^5 LD_{50} of botulinum toxin per gram of food by 1-log, 3-logs and to no detectable toxin[a]

Food	pH	Temp °C	Type A			Type B			Type E		
			1	3	none	1	3	none	1	3	none
Tomato juice	4.1	74	2	10	60	2	7	40	–	–	–
		79	1	2	15	1	2	7	–	–	–
		85	1	2	5	1	2	5	–	–	–
Tomato soup	4.2	74	3	25	60	3	20	60	1	2	20
		79	1	4.5	15	1.5	4	10	1	2	5
		85	<1	1.5	5	1	1.5	5	1	1	2
Tomato soup	6.2[b]	79	1	2	8	–	–	–	–	–	–
		85	<1	1	2	–	–	–	–	–	–
String beans	5.1	74	1	4	25	2	15	30	<1	1	5
		79	1	1.5	10	1	2	10	<1	<1	2
		85	<1	1	2	1	1	1	<1	<1	<1
Mushrooms	5.8	74	1	5	60	–	–	–	–	–	–
		79	<1	3	20	–	–	–	–	–	–
		85	<1	1	2	–	–	–	–	–	–
Canned corn	6.2	74	<1	2	10	<1	1	10	<1	2	10
		79	<1	1	2	<1	1	2	<1	1	2
		85	<1	–	1	<1	–	1	<1	–	1
Creamed corn	6.3	79	<1	2	5	–	–	–	–	–	–
		85	<1	1.5	2	–	–	–	–	–	–

[a] The heating times for reduction of the toxin by 1-log and 3-logs are taken from the heat inactivation curves and are approximate. The endpoint or point of no detectable toxin is the actual heating time required.

[b] The tomato soup was adjusted to pH 6.2 with NaOH.

Table 2—Heating times in minutes at different temperatures to inactivate 10^5 LD_{50} of botulinum toxin per milliliter in various buffers by 1-log, 3-logs and to no detectable toxin[a]

Buffer	pH	Temp °C	Type A			Type B			Type E		
			1	3	none	1	3	none	1	3	none
Sodium acetate alone (0.5M)	4.2	74	<1	5	60	1	2	10	<1	1	2.5
		79	<1	1	20	<1	<1	2	<1	<1	1
		85	<1	<1	2	<1	<1	1	<1	<1	<1
Sodium acetate with 1% gelatin	4.2	74	1	15	60	1	3	10	<1	1	2.5
		79	<1	1.5	20	<1	<1	5	<1	<1	2
		85	<1	1	2	<1	<1	1	<1	<1	<1
Sodium citrate alone (0.05M)	5.5	74	<1	5	60	2	12	60	<1	1	10
		79	<1	2	20	<1	1.5	5	<1	<1	2
		85	<1	<1	2	<1	<1	1	<1	<1	<1
Sodium citrate with 1% gelatin	5.5	74	<1	5	60	2	12	60	<1	2	10
		79	1	2	20	<1	1.5	5	<1	<1	2
		85	<1	<1	2	<1	<1	1	<1	<1	<1
Sodium phosphate alone (0.05M)	6.8	74	<1	1.5	10	1	2	10	1	2	5
		79	<1	<1	2	<1	<1	1.5	<1	<1	1.5
		85	<1	<1	1	<1	<1	1	<1	<1	1
Sodium phosphate with 1% gelatin	6.8	74	1	5	40	1.5	6	40	1	1.5	5
		79	1	2.5	12	<1	1	5	<1	<1	2
		85	<1	<1	1	<1	<1	1	<1	<1	<1

[a] The heating times for reduction of the toxin by 1-log and 3-logs are taken from the heat inactivation curves and are approximate. The endpoint or point of no detectable toxin is the actual heating time required.

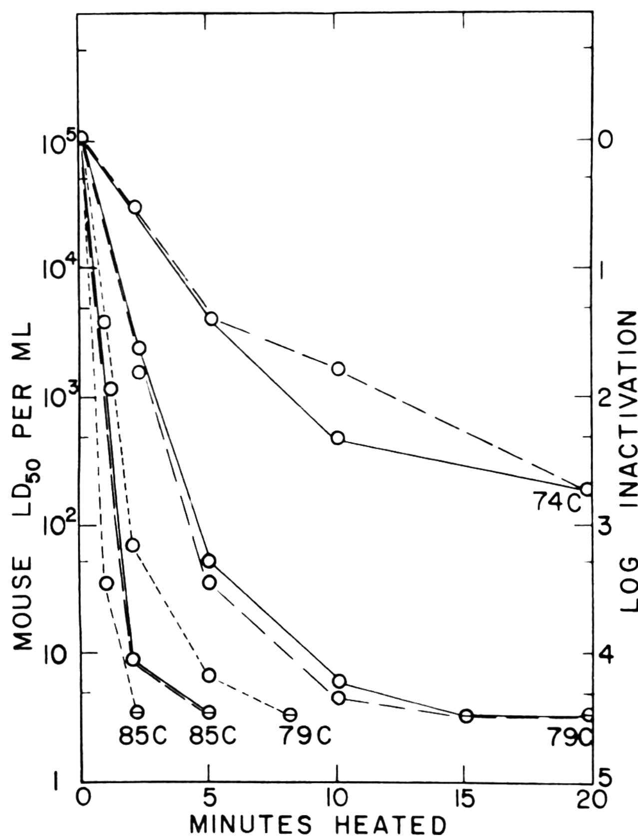
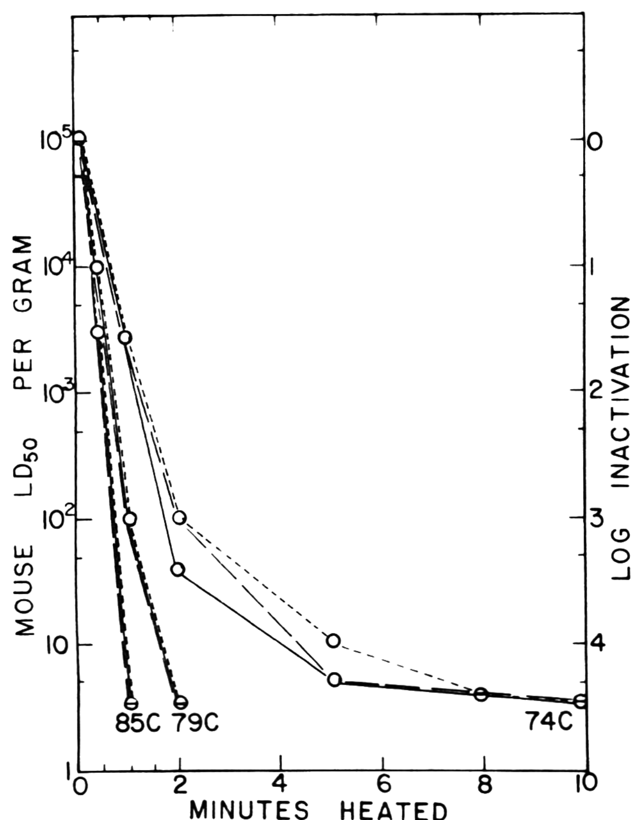


Fig. 1—Heat inactivation rates of types A and B botulinum toxins at different temperatures in a representative acid food, tomato soup at its natural pH of 4.2, and at a pH adjusted to 6.2 for type A. o——o Type A pH 4.2; o-----o Type A pH 6.2; o— — —o Type B pH 4.2; θ no detectable toxin.



inactivation curves for types A, B and E in canned corn at its natural pH of 6.2. In this food the inactivation times were considerably shorter than in tomato soup and approached an exponential inactivation at both 79° and 85°C. Although the data are not presented in Table 1, the inactivation rate for type F toxin in string beans, pH 5.1, was also studied and the results were very similar. At 74°C type F toxin was inactivated in 25 min, at 79°C in 10 min, and at 85°C in 1 min. Type A toxin in mushrooms, pH 5.8, was more stable than in string beans at pH 5.1 and compared well with toxin in tomato soup, pH 4.2.

Heating 100g of tomato soup (pH 4.2) to 85°C with stirring required 6 min for come-up time and an additional 4 min at this temperature to inactivate 10^5 LD_{50} of type A toxin per gram to no detectable toxin. Canned corn (pH 6.2) heated in the same manner required a similar time to reach 85°C but no toxin was detectable at that time.

The inactivation of toxin types A, B and E in the buffers, with and without gelatin, is shown in Table 2. Inactivation of type A toxin in 0.05M acetate buffer, pH 4.2, compared well with the inactivation in tomato soup and tomato juice; and in 0.05M phosphate buffer, pH 6.8, compared well with the inactivation in canned corn. Type E appeared to be less stable than types A and B in buffers and in foods. The effect of the addition of 1% gelatin to the buffers was most noticeable in the phosphate buffer at pH 6.8 in which the inactivation time for types A and B at 74°C was 10 min without gelatin and 40 min with gelatin. At higher temperatures, this difference became very small.

DISCUSSION

HEATING is the only practical means of inactivating botulinum toxins in foods and boiling has been the usual rule. It is important to establish the minimum heat treatment that inactivates the toxins in a practical period of time and thus conserves as much as possible the quality and essential nutrients in the foods. The kinetics of toxin inactivation appear to be the same for toxin added to the food as for that produced in the food (Woodburn et al., 1979). The rate at which the toxins are inactivated in a food depends first of all on the temperature. The time of inactivation is inversely proportional to the temperature but the rate at any one temperature decreases with time of heating, particularly in acid foods at 79°C and below. The observation that at the lower temperatures a portion of the toxin molecules appeared to become more resistant to heat should be further investigated. Cartwright and Lauffer (1958) proposed that two types of molecules were present, one being heat inactivated more readily than the other. However, other explanations should be considered such as aggregation of the toxin molecules or of the denatured molecules with toxin or changes in molecular shape. Experiments in our laboratory have shown that when heated and cooled toxin was reheated, the slow rate of inactivation continued.

The pH of the food is an important factor and its effect was observed at all temperatures but particularly at 79°C and below. To make a direct comparison on differences in pH in the same food, type A toxin in tomato soup was heated at its natural pH of 4.2 and at an adjusted pH of 6.2. Heating at 79°C and 85°C showed the toxin to be inactivated in approximately one-half of the time at pH 6.2. Many of our convenience foods contain low acid components that could contain toxins if inadequately processed or

Fig. 2—Heat inactivation of botulinum toxins at different temperatures in a low acid food, canned corn at its natural pH of 6.2. o——o Type A; o-----o Type E; o— — —o Type B; θ no detectable toxin.

stored. It these are added as ingredients to more acid mixtures such as those that include tomato products, the toxin would be protected against heat inactivation, especially at temperatures below 85°C.

Our research as well as that of others including Bradshaw et al. (1979) and Scott (1950) has indicated that there are other factors in foods that have an effect on the inactivation rate of toxin. For example, type A toxin in mushrooms, pH 5.8, was inactivated more slowly than toxin in string beans at pH 5.1 and compared well with the inactivation of the toxin in tomato soup at pH 4.2. Although we do not know what physical organization or compounds may lend protection to the toxins, the addition of 1% gelatin to phosphate buffer at pH 6.8 decreased the rate of inactivation of types A and B toxins (Table 2) and it is suggested that proteins and possibly other colloidal components in foods may be a factor. Gelatin in citrate buffer at pH 5.5 and acetate buffer at pH 4.2 gave no additional protection to the toxin, possibly because of the more favorable pH for toxin stability. In contrast, type E does not appear to be stabilized by a lower pH or by the presence of gelatin and was inactivated in tomato soup and string beans somewhat faster than types A and B, indicating that there may be differences in the inactivation of types under certain conditions. Bradshaw et al. (1979) have pointed out that types A and B toxin in beef patties are more stable than in phosphate buffer at pH 6.05, whereas Woolford et al. (1978) found very little if any difference between the inactivation rate of type A toxin in phosphate buffer at pH 5.9 and beef pot pie filling composed of several vegetables, modified food starch and some beef.

The differences in the heat treatment required to inactivate the toxin in acid and low acid foods, and the complication from the protective action of some foods, make it difficult to give a minimum heat treatment that is the same for all foods. However, a heat treatment at 79°C for 20 min or 85°C for 5 min has inactivated 10^5 LD_{50} of botulinum toxins (types A, B, E and F) in the foods tested in our laboratory and should serve as a guide.

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Ms received 4/4/79; revised 7/4/79; accepted 7/10/79.

The authors acknowledge the assistance of A.L. Woolford in the performance of a portion of this work.

Clostridium botulinum CONTROL BY SODIUM NITRITE AND SORBIC ACID IN VARIOUS MEAT AND SOY PROTEIN FORMULATIONS

J. N. SOFOS, F. F. BUSTA and C. E. ALLEN

ABSTRACT

Clostridium botulinum inoculated frankfurter-type mixtures formulated with different meats (mechanically deboned chicken meat, beef, and pork) and/or soy proteins (textured, concentrate, and isolate) were incubated at 27°C. The effects of sodium nitrite and/or sorbic acid on botulinal spore germination, outgrowth, and toxin production were determined at specified time intervals during the incubation period. Also, residual nitrite depletion and in some instances total microbial growth were measured. Sorbic acid alone (0.2%) or in combination with nitrite (80 μg/g) retarded spore germination and outgrowth, and delayed toxin production. These effects were of larger magnitude in mixtures formulated with both nitrite and sorbic acid and were greater in beef, pork, and soy proteins compared to mechanically deboned chicken meat. Nitrite (80 μg/g) alone was only slightly effective in delaying toxin production and of significance only in pork. The highest nitrite level (156 μg/g) tested in a meat-soy mixture was significantly inhibitory to toxin development. Botulinal germination, outgrowth, and toxin production were slower in all-soy, control (nitrite/sorbic acid-free) formulations than in all-meat, control samples. Residual nitrite depletion was slower in soy formulations, especially of the textured form, compared to all-meat treatments. Inclusion of nitrite and/or sorbic acid in the formulation did not significantly change the rate and extent of total microbial growth in meat-soy formulations.

INTRODUCTION

THE EFFECTIVENESS of sodium nitrite in delaying *Clostridium botulinum* toxin production in cured meat products has been established (Christiansen et al., 1973; 1974; Hustad et al., 1973). Generally, the effect of nitrite in controlling *C. botulinum* growth and toxin production increases with increasing nitrite concentration (Christiansen et al., 1973; 1974; Bowen et al., 1974). Nitrite levels above 50 μg/g were found to delay botulinal toxin production (Hustad et al., 1973; Bowen et al., 1974).

Recent investigations have shown that botulinal inhibition is possible even with lower nitrite levels (e.g. 40 μg/g) when sorbic acid or potassium sorbate is included in the formulation (Ivey et al., 1978; Ivey and Robach, 1978; Robach et al., 1978). Work performed in our laboratory has also indicated that a combination of nitrite (40 μg/g) and sorbic acid (0.2%) was effective in delaying *C. botulinum* growth and toxin production in mechanically deboned chicken meat frankfurter emulsions during temperature abuse (Sofos et al., 1979, a, b).

Variation in the inhibitory effect of nitrite against *C. botulinum* toxin production was reported by Tompkin et al. (1978a) for meats ranging from turkey breast to pork hearts. Our work (Sofos et al., 1979) indicated that nitrite effectiveness in mechanically deboned chicken meat frankfurters was lower than that expected in similar products formulated with red meats (beef and pork) under similar conditions.

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0022-1147/79/0006-1662$02.25/0
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Significant levels (25–30%) of soy proteins can successfully replace meat or fat in frankfurter-type products (Sofos and Allen, 1977; Sofos et al., 1977), and nitrite (156 μg/g) will successfully delay botulinal toxin production in these (meat-soy) formulations (Sofos et al., 1979c). Similar work with different soy protein forms showed that 156 μg/g nitrite retarded *C. botulinum* toxin production in all cases except for mixtures formulated with soy isolates (Sofos et al., 1979c).

The objective of the studies reported here was to determine the effects of nitrite and/or sorbic acid on *C. botulinum* growth and toxin production in frankfurter-type mixtures, and to compare these effects among various meats (chicken, beef, pork) and soy protein products (tex-

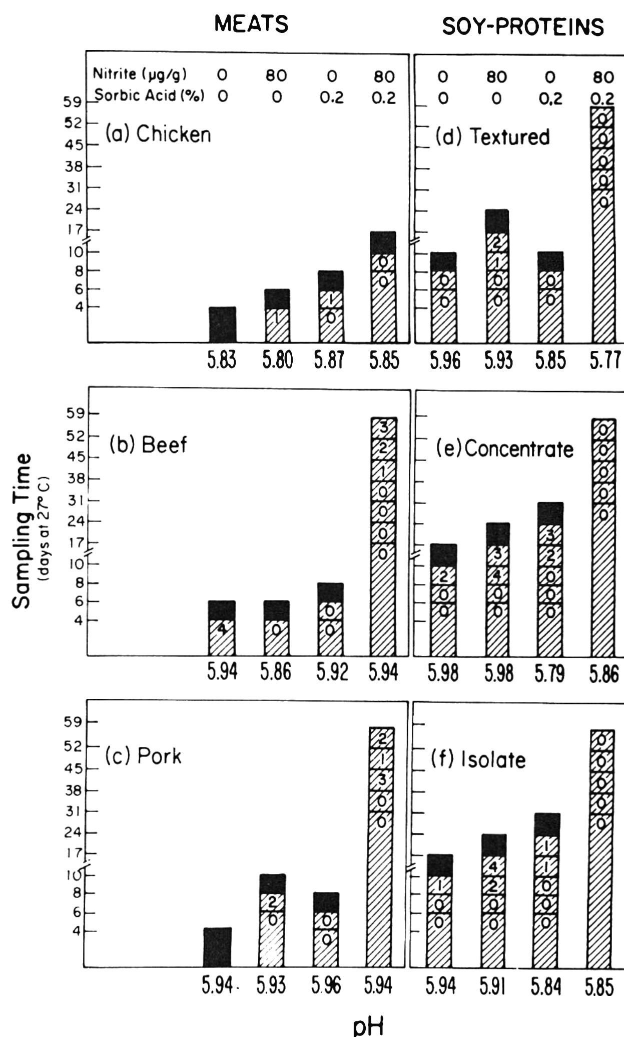

Fig. 1—Effects of sodium nitrite and/or sorbic acid on Clostridium botulinum toxin production in frankfurter-type mixtures formulated with different meats and soy proteins during temperature (27°C) abuse. Numbers indicate toxic samples out of five tested and dark bars demonstrate that all five samples tested were toxic.

tured, concentrate, isolate) during elevated temperature (27°C) abuse. Also, a meat (beef and pork backfat)-soy (textured and isolate) mixture was examined. Toxin production, spore germination (loss of heat resistance) and outgrowth, residual nitrite depletion and in some instances total microbial growth were measured.

MATERIALS & METHODS

FOUR TRIALS were conducted to study the effects of nitrite and/or sorbic acid on botulinal growth and toxin production in frankfurter-type mixtures formulated with different meats and soy proteins. One trial consisted of evaluating control (nitrite/sorbic acid-free) mechanically deboned chicken meat (MDCM), beef, pork, textured soy protein, soy protein concentrate, and soy protein isolate formulations. The effects of nitrite (80 µg/g), sorbic acid (0.2%), and a combination of both (80 µg/g nitrite–0.2% sorbic acid) were determined in second, third, and fourth trials, respectively. A fifth trial was conducted to study the effects of nitrite and/or sorbic acid in a beef-soy (textured and isolate)-pork backfat product. The fat content of the raw ingredients as determined by standard AOAC methods was: MDCM, 17.18%; ground beef, 20.93%; ground pork, 15.00%; ground pork backfat, 90.00%; textured soy protein, 0.38%; soy protein concentrate, 0.20%; and soy protein isolate, 0.26%. The dry textured soy protein was hydrated 1:2 (wt/wt, one part of soy product in two parts of water) before its use; the concentrate 1:3; and the isolate 1:4. The textured soy protein had a protein dispersibility index (PDI) of 17; the concentrate of 49; and the isolate of 88.

Commercial chicken frankfurters are of lower fat content (about 20%) than red meat wieners which generally contain near the maximum permitted fat level (30%). It seemed appropriate to compare the effect of nitrite and/or sorbic acid in formulations similar to commercial practice. Thus, pork backfat was used to increase the total fat content of the beef, pork, and soy formulations to 30%, while no additional fat was included in the MDCM (chicken) formulations. The meat-soy treatments of Figure 2 were formulated to contain 20% fat. Low-fat (15–22%) formulations were found more acceptable when increased levels of soy products were included in the mixtures (Sofos and Allen, 1977).

The total lean and fat content of the meat treatments was 93.63% and the remainder consisted of other ingredients (spices etc.). In the soy formulations, 30.00% was fat, 60.36% was hydrated soy, and 3.00% was lean pork, contributed from the pork backfat. The meat-soy treatments contained a total of 36.83% hydrated soy protein (31.83% hydrated textured soy protein and 5.0% hydrated soy protein isolate); 36.80% total lean; and 20.00% fat.

Common ingredients in all formulations were: salt, 2.50%; corn syrup solids (Star-Dri, 35C, A.E. Staley Manufacturing Co., Decatur, IL), 2.00%; dextrose (Mallinckrodt, Inc., St. Louis, MO), 1.00%; white pepper, 0.25%; nutmeg, 0.07%; ascorbic acid (Mallinckrodt, Inc., St. Louis, MO), 0.05%; and liquid smoke (Charoil, Red Arrow Products Co., Manitowoc, WI), 0.50%.

All formulations contained a total of 20% water and/or inorganic phosphate buffers. Part of the water was used to incorporate the spore inoculum and other ingredients (nitrite, etc.) into the mixtures. Inorganic phosphate solutions (0.37M KH_2PO_4, 1M K_2HPO_4) were used to adjust the pH of the treatments (Fig. 1) to similar levels. It was shown (Sofos et al., 1979b) that sorbic acid alone or in combination with nitrite (40 µg/g) was more effective in inhibiting botulinal growth and toxin production at lower pH values (<6.20). Thus, the pH of the meat and soy treatments was adjusted so that before incubation all treatments had similar (5.77–5.98) pH values (Fig. 1). The pH values of the meat-soy formulations (Fig. 2) were not adjusted.

A concentration of 40 µg of nitrite per gram of product was found ineffective in delaying botulinal toxin production in MDCM frankfurter products (Sofos et al., 1979). The Expert Panel on Nitrites, Nitrates and Nitrosamines has recommended lowering the ingoing nitrite levels to 100 or 120 µg/g for most cured meat products (Anon., 1977). Therefore, it seemed reasonable in these studies to determine the effect of 80 µg/g nitrite alone or in combination with sorbic acid (0.2%). Thus, depending on the treatment, formulations containing 80 µg of sodium nitrite (Mallinckrodt, Inc., St. Louis, MO) per gram of product and/or 0.2% (wt/wt) sorbic acid (Monsanto Co., St. Louis, MO) were prepared.

All treatments were inoculated with the same volume of a five type A and five type B composite *C. botulinum* heat-shocked (80°C, 15 min) spore suspension (Sofos et al., 1979).

Methods for product formation, inoculation, incubation, sampling, residual nitrite determination, and toxicity testing have been detailed by Sofos et al. (1979). Microbiological procedures to determine spore, spore plus vegetative cell, and total plate counts were previously described (Sofos et al., 1979b).

RESULTS & DISCUSSION

Botulinal germination, outgrowth, and toxin production

Substrate effects. Data in Figure 1 indicate that botu-

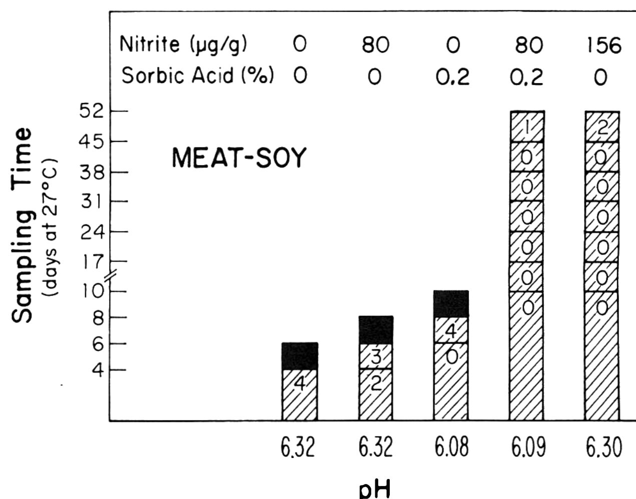

Fig. 2—Effects of sodium nitrite and/or sorbic acid on Clostridium botulinum toxin production in meat (beef and pork backfat)-soy protein (textured and isolate) frankfurter-type mixtures during temperature (27°C) abuse. Numbers indicate toxic samples out of five tested and dark bars demonstrate that all five samples tested were toxic.

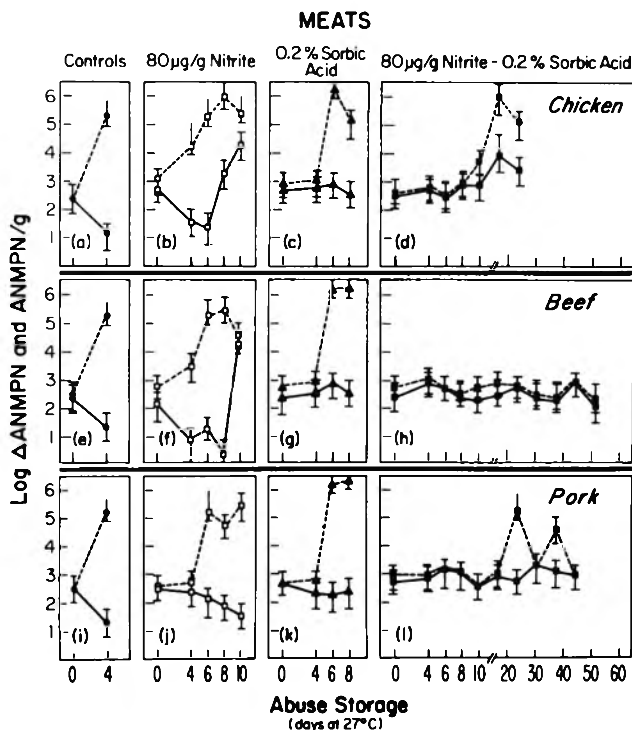

Fig. 3—Effects of sodium nitrite and/or sorbic acid on Clostridium botulinum spore germination (ΔANMPN, continuous lines) and outgrowth (ANMPN, broken lines) in frankfurter-type mixtures formulated with different meats during temperature (27°C) abuse.

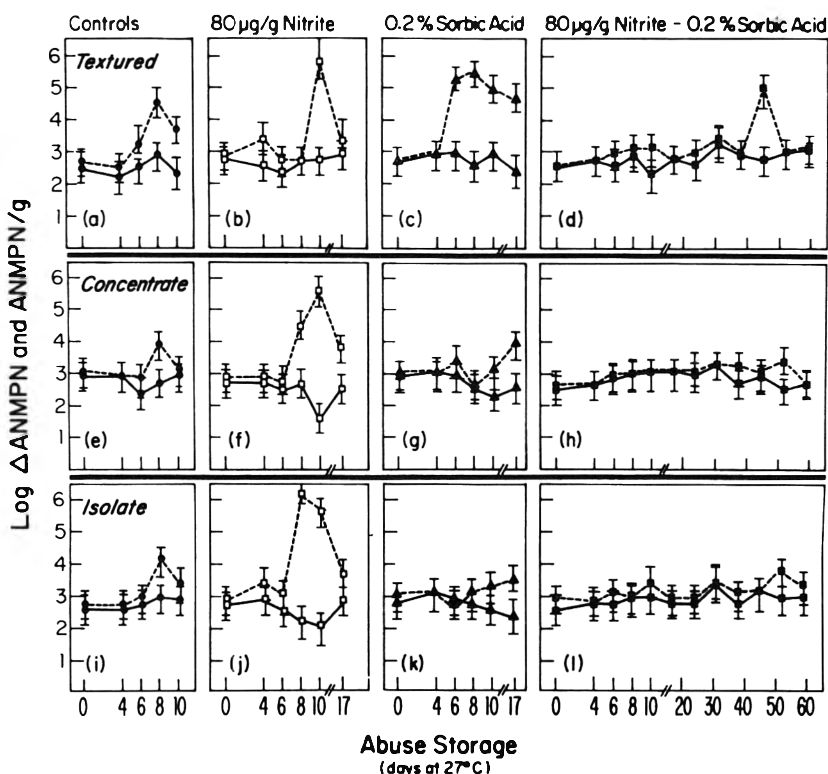

Fig. 4—Effects of sodium nitrite and/or sorbic acid on Clostridium botulinum spore germination (ΔANMPN, continuous lines) and outgrowth (ANMPN, broken lines) in frankfurter-type mixtures formulated with different soy proteins during temperature (27°C) abuse.

linal toxic samples (four or five out of five tested) were detected after 4 days of 27°C temperature abuse of frankfurter emulsions formulated with different meats (chicken, beef, and pork). Such rapid toxin production in control formulations was previously shown for MDCM (chicken) and beef emulsions (Sofos et al., 1979, b, c). The above authors have indicated that in control (nitrite/sorbic acid-free) MDCM (chicken) formulations botulinal spore germination and outgrowth occurred within 3–4 days of 27°C incubation. Results shown in Figure 3 (a, e and i) demonstrate that similar conclusions can be drawn and from products formulated with pork. Therefore, no major differences exist among meats (chicken, beef, pork) in botulinal germination, outgrowth and toxin production.

Results in Figure 1 demonstrate that *C. botulinum* toxin production was somewhat slower in soy-based, frankfurter-like mixtures. Samples formulated with textured soy protein, soy concentrate, and soy isolate (Fig. 1d, 1e and 1f) were found toxin-free at 8 days even though no nitrite or sorbic acid was added. Toxicity was detected at 10 days in all soy-based formulations. The delay in toxin production recorded for control, soy-formulated treatments compared to toxin production in similar meat formulations (Fig. 1) can be explained by the spore germination (loss of heat resistance) and outgrowth results of those treatments (Fig. 4a, 4e and 4i). No statistically significant observable spore germination occurred in the control, soy-containing treatments up to the incubation time that toxic samples were detected. However, the outgrowth results (Fig. 4a, 4e and 4i) would suggest that a low number of spores germinated, and growth occurred slowly to eventually toxic levels. The rate and the extent of outgrowth in the soy-based formulations (Fig. 4a, 4e and 4i) was much slower than that in meat-formulated products (Fig. 3a, 3e and 3i). It appears that some unknown component(s) of the soy products retarded botulinal spore germination and delayed outgrowth or that soy products are lacking some factor(s) essential for botulinal spore germination or growth. Information on the subject would be of interest and might facilitate the search for the mechanism of botulinal inhibition in cured meat products.

When a mixture of meat (beef and pork backfat) and soy proteins (textured and isolate) was tested, toxin production in the controls (Fig. 2) was as rapid as in all-meat, control formulations (Fig. 1a, 1b and 1c). In this treatment (meat-soy) the presence of meat apparently facilitated spore germination (Fig. 5a). Outgrowth on the other hand (Fig. 5a) was slightly delayed in comparison to all-meat (Fig. 3e), and the presence of soy proteins might have been responsible for this delay.

Nitrite effects. The results shown in Figure 1 from treatments with 80 µg/g nitrite demonstrate that nitrite is less effective in controlling *C. botulinum* toxin production in chicken emulsions than in beef or pork formulations. It appears that addition of 80 µg/g nitrite had only a very slight or negligible effect on the rate of toxin production in the chicken emulsion. In product formulated with beef, no toxic samples were detected at 4 days, while in the pork-based formulation toxin was not detected until after 6 days. The explanation for this difference among meats from different species is unknown. It was previously postulated (Sofos et al., 1979) that a possible higher available iron content in mechanically deboned chicken meat might have lowered nitrite effectiveness. As Tompkin et al. (1978a) postulated, differences in muscle pigmentation and in turn iron content and possibly other unknown factors might be responsible for differences in rates of growth and gas production.

The presence of 80 µg/g nitrite did not significantly affect the rapid rates of spore germination and outgrowth in

the chicken (MDCM) formulation (Fig. 3b). Similar results were found earlier in a similar product with 40 or 156 μg/g nitrite (Sofos et al., 1979a, b). After germination and outgrowth, sporulation and death of vegetative cells were observed. This was similar to results reported by Christiansen et al. (1978). Germination and outgrowth rates similar to those in the chicken emulsion were also found in the beef-based formulation (Fig. 3f). In pork (Fig. 3j) formulated with the same nitrite concentration (80 μg/g) the rates of germination and outgrowth were slower than those in chicken or beef. These slower rates of spore germination and outgrowth were parallel to the slower rate of toxin production recorded with pork samples (Fig. 1).

Generally, no delay in toxin production from inclusion of 80 μg/g nitrite in the formulation was observed in samples formulated with the soy proteins (Fig. 1). Toxic samples were detected after the same incubation period in all the soy (textured, concentrate, and isolate) formulations, and the rate of increase in the number of toxic samples was only slightly slower than that of similar control (nitrite/sorbic acid-free) soy formulations. The spore germination results for the soy-based, nitrite (80 μg/g)-containing formulations (Fig. 4b, 4f and 4j) were similar to those from soy-based, control (nitrite/sorbic acid-free) treatments (Fig. 4a, 4e and 4i).

The extent of outgrowth in soy mixtures formulated with nitrite appeared to be increased over that of control treatments. Such an increase could be due to trial variation, since the control trials were conducted at one time and the nitrite (80 μg/g) trials at another. A delay in onset of outgrowth was observed in the 80 μg/g nitrite, textured soy protein treatment (Fig. 4b) compared to that of soy concentrate or isolate treatments (Fig. 4f and 4j). Such a difference could explain the effectiveness of nitrite (156 μg/g) in delaying toxin production in textured soy protein formulations found in earlier studies (Sofos et al., 1979c). In those studies nitrite (156 μg/g) did not affect toxin production in soy isolate-based formulations.

The results of Figure 2, for the meat (beef and pork backfat)-soy (textured and isolate) formulation confirm the effectiveness of nitrite (156 μg/g) in delaying botulinal toxin production in such products (Sofos et al., 1979c). The level of 80 μg nitrite per g of product, however, was found ineffective.

Data found in Figure 5b indicate that the rates of spore germination and outgrowth in the meat-soy, 80 μg/g nitrite, mixture were similar to the control formulation (Fig. 5a). However, when the nitrite level in the meat-soy mixture was increased to 156 μg/g (Fig. 5c), spore germination and outgrowth were greatly retarded. A combination of factors could explain the results observed for the meat-soy mixtures. Soy proteins retarded spore germination and delayed outgrowth, both in the presence or absence of 80 μg/g nitrite (Fig. 4). Previous studies (Sofos et al., 1979c) have shown decreased rates of residual nitrite depletion with increased levels of soy proteins in the formulation. Residual nitrite was implicated by Tompkin et al. (1978a; 1978b) and Christiansen et al. (1978) as being the important consideration in botulinal control of cured meats. Therefore, a retarded germination rate due to the presence of soy and perhaps a higher nitrite (156 μg/g) concentration, a slower outgrowth rate due to soy, and the presence of increased residual nitrite concentrations due to soy could result in additive effects and explain the observed results.

The practical implications of the results in Figures 1 and 2 in relation to the effect of nitrite alone on *C. botulinum* toxin production are very important. A low ingoing nitrite concentration (e.g. 80 μg/g) without any supplementary means of protection would give formulations of marginal or no protection from botulism depending on the product and distribution of nitrite in the formulation.

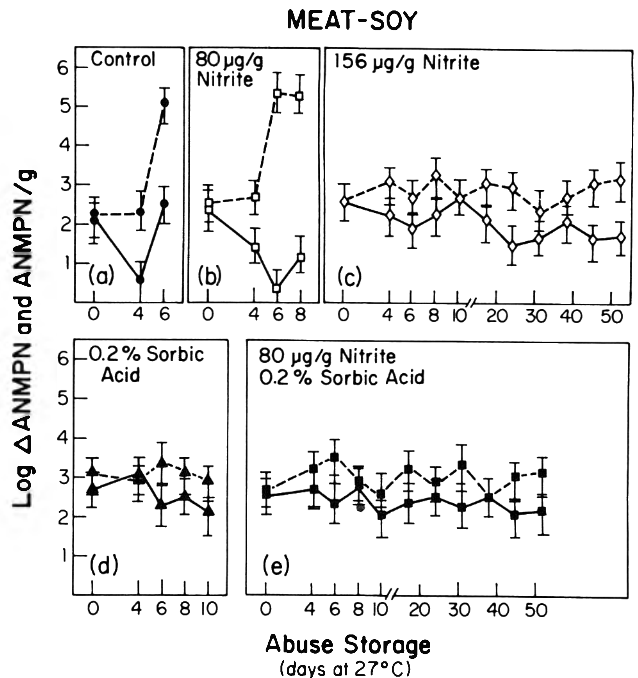

Fig. 5—Effects of sodium nitrite and/or sorbic acid on Clostridium botulinum spore germination (ΔANMPN, continous lines) and outgrowth (ANMPN, broken lines) in meat (beef and pork backfat)-soy protein (textured and isolate) frankfurter-type mixtures during temperature (27°C) abuse.

Sorbic acid effects. Data presented earlier (Sofos et al., 1979b) demonstrated that sorbic acid alone (0.2%) delayed toxin production in MDCM emulsions at appropriate (<6.0) pH levels. The results shown in Figure 1 demonstrate that similar conclusions are expected and from products formulated from other meat sources (beef and pork) and soy proteins (concentrate and isolate). No delay in toxin production was recorded for samples formulated with textured soy protein and 0.2% sorbic acid (Fig. 1d). The pH of all treatments was in the range of 5.77 to 5.98 and close to values (<6.0) previously shown to be effective in MDCM (chicken) formulations. The ineffectiveness of sorbic acid in delaying toxin production in textured soy protein, and its lower effect in chicken (MDCM) compared to other meats (beef and pork) cannot be explained by pH of the products. However, a reasonable postulation is that destruction of reactive site(s) in the highly heat-treated, textured soy protein and possibly an increased availability of important growth stimulants or lack of inhibitors in textured soy protein and MDCM (chicken) could be responsible for the differences observed. In the meat-based formulations, it appears that the sorbic acid effect was of similar (Fig. 1c) or greater (Fig. 1a and 1b) magnitude than that of 80 μg/g nitrite depending on the system. Sorbic acid (0.2%) also delayed toxin production in the meat-soy mixture tested at pH 6.08 (Fig. 2). Compared to the control formulation, twice as much time was necessary for toxin to be formed.

The germination and outgrowth data in Figures 3, 4 and 5 readily explain the toxicity results reported above. Inclusion of 0.2% sorbic acid in the formulation significantly ($p < 0.05$) retarded botulinal spore germination and delayed outgrowth in all meats tested (Fig. 3c, 3g and 3k). These results confirm previous conclusions with MDCM (Sofos et al., 1979b) and demonstrate that sorbic acid inhibits botulinal spore germination (loss of heat resistance) in beef and pork formulations as well. In the soy-based formulations

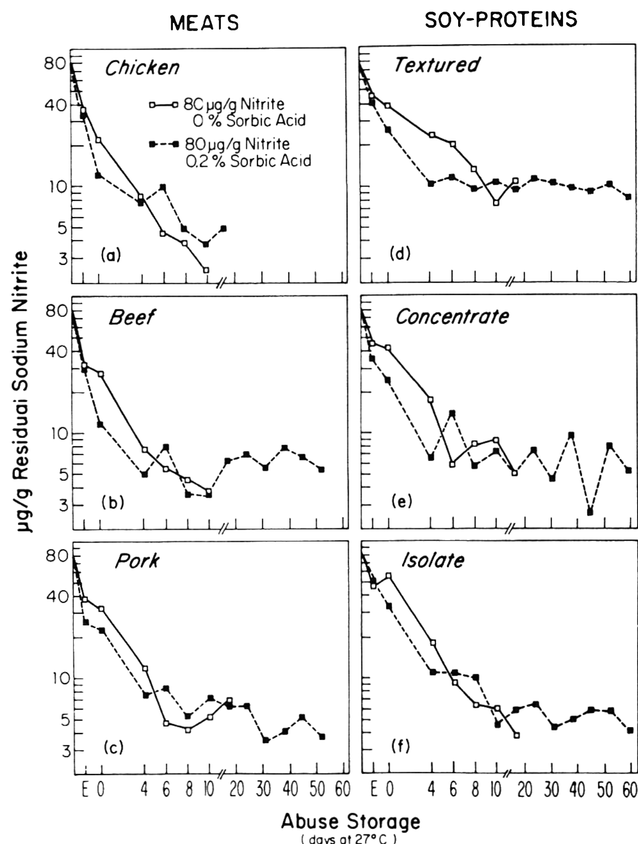

Fig. 6—Sodium nitrite depletion in frankfurter-type mixtures formulated with different meats and soy proteins during processing and temperature (27° C) abuse. E = uncooked emulsions.

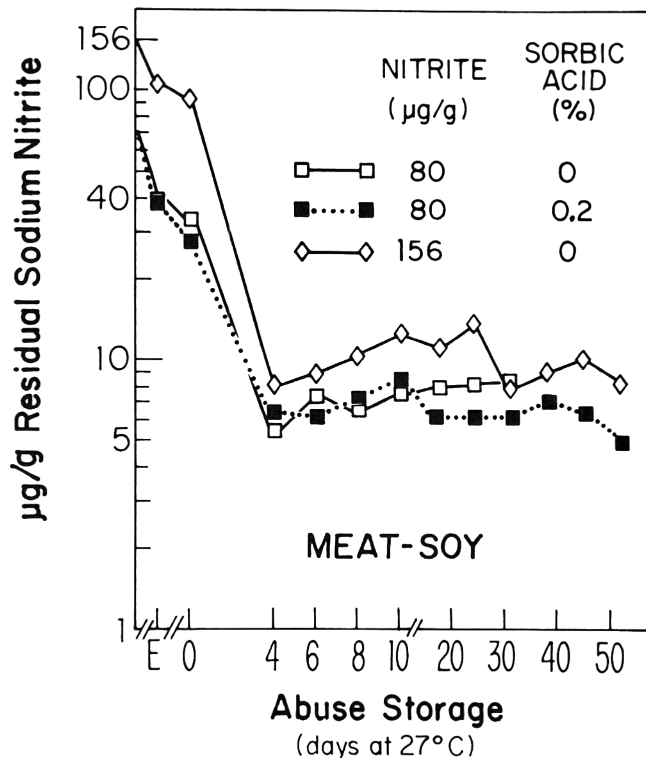

Fig. 7—Sodium nitrite depletion in frankfurter-type, meat (beef and pork backfat)-soy protein (textured and isolate) mixtures during processing temperature (27° C) abuse. E = uncooked emulsions.

the lack of measurable germination in control treatments (Fig. 4a, 4e, 4i) did not permit observation of any additional inhibition of germination by sorbic acid. One difference observed among the soys tested was that sorbic acid (0.2%) did not delay outgrowth in the textured soy form (Fig. 4c). In the meat-soy mixture (Fig. 5d) both germination and outgrowth were retarded by sorbic acid (0.2%).

Nitrite-sorbic acids effects. Although 0.2% sorbic acid alone delayed botulinal toxin production in most substrates, and 80 µg/g nitrite was effective in some substrates, the effects were of low magnitude and would probably be considered of minor practical value. However, when sorbic acid (0.2%) and nitrite (80 µg/g) were combined, a dramatic extension of the time necessary for toxin production was observed in all products tested (Fig. 1 and 2). The delay was not as long in meats as it was in soy proteins, in which no toxin was detected up to the last sampling time of 59 days at 27°C. In meats, the effect was similar in beef and pork where toxin was first detected after 45 days (Fig. 1b and 1c). In contrast, toxin production in chicken was faster since all five samples tested were toxic after 17 days of temperature abuse (Fig. 1a). The results of Figure 2 indicated that in the meat-soy mixture the ineffective level of 80 µg/g nitrite became significantly inhibitory when 0.2% sorbic acid was added to the formulation. The botulinal safety of this (80 µg/g nitrite-0.2% sorbic acid) formulation was similar to that of the 156 µg/g nitrite formulation where toxic samples appeared only after 52 days at 27°C.

The germination and outgrowth data for nitrite-sorbic acid combination treatments generally agree with the toxicity results. The combination of 80 µg/g nitrite and 0.2% sorbic acid significantly ($p < 0.05$) retarded spore germination in all substrates tested (Fig. 3d, 3h, 3l, 4d, 4h, 4l and 5e). Outgrowth was similarly retarded in all instances, except in the chicken (MDCM) formulation (Fig. 3d) where a significant increase in cell counts occurred after 10 days of temperature abuse. The reason for the scattered outgrowth peaks observed in some cases (Fig. 3l and 4d) was not evident.

It has been shown (Sofos et al., 1979b) that nitrite (40 µg/g) in conjunction with sorbic acid (0.2%) at appropriate pH levels (<6.20) inhibited spore germination and delayed outgrowth and toxin production in chicken (MDCM) emulsions. The results of the present studies demonstrate that a combination of 80 µg/g nitrite and 0.2% sorbic acid is also effective in retarding *C. botulinum* spore germination, outgrowth, and toxin production in systems formulated not only with chicken, but with red meats (beef and pork) and/or soy proteins of different forms.

Decreased nitrite levels in conjunction with sorbic acid or potassium sorbate have been found to greatly delay toxin production in various meat products or systems (Ivey and Robach 1978; Ivey et al., 1978; Robach et al., 1978). The results reported here confirm those findings and extend the conclusions to include emulsified products formulated with a variety of meats and soy proteins. The effects observed earlier with MDCM emulsions (Sofos et al., 1979a, b) have been confirmed and have been shown to be more dramatic for beef and pork, and especially soy proteins. Tompkin et al. (1978a) found 156 µg/g nitrite to be more inhibitory to botulinal growth in pork ham than in beef round or other types of meats. They suggested that differences in muscle pigmentation associated with increased levels of available iron in highly pigmented meats such as hearts, caused a loss of botulinal inhibition by nitrite in such meats. Such variables or other unknown parameters might have been responsible for the differences among the various meats studied here. Caution is advisable in extending conclusions from one type of product or one

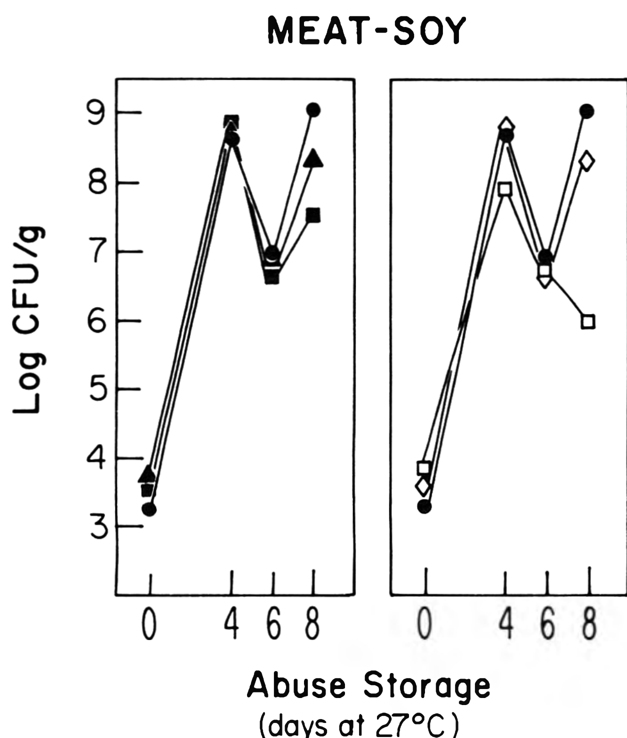

Fig. 8—Effects of sodium nitrite and/or sorbic acid on the total microbial growth (CFU) of frankfurter-type, meat (beef and pork backfat)-soy protein (textured and isolate) mixtures during temperature (27°C) abuse: ●, control; □, 80 µg/g nitrite; ◇, 156 µg/g nitrite; ▲, 0.2% sorbic acid; ■, 80 µg/g nitrite-0.2% sorbic acid.

source of raw materials to include other products or substrates.

Residual nitrite

The results presented in Figure 6 further demonstrate that nitrite depletion during processing and storage (27°C) was slower in products formulated with soy proteins than in all-meat treatments. Similar results were previously reported by Sofos et al. (1979c). It also appears that nitrite was depleted slower in the textured soy protein-based product (Fig. 6d) than in the concentrate and isolate (Fig. 6e and 6f). As previously discussed, the presence of higher residual nitrite levels for a longer time in meat-soy (textured and isolate) and in textured-based products could account at least in part for the longer botulinal safety of such treatments (Christiansen et al., 1978).

The previously reported delay in nitrite depletion in the presence of sorbic acid (Sofos et al., 1979, b) was not apparent in the results presented in Figure 6. However, trial variation could be a factor because the nitrite and nitrite-sorbic acid treatments reported here were conducted on different days whereas previously all the treatments were performed simultaneously.

All treatments of Figure 7 were conducted at the same time and appropriate comparisons in the rates of nitrite depletion are justified. It appears that in meat-soy mixtures, sorbic acid inclusion in the formulation did not result in any delay in residual nitrite depletion. The increased pH of soy-containing treatments; the delay in nitrite disappearance caused by soy; or just the presence of soy itself might have been responsible for the absence of additional delay in nitrite depletion compared to soy itself. However, delay in nitrite disappearance in the presence of sorbic acid could be unique to chicken (MDCM) formulations.

Total microbial growth

Previous results have shown that the presence of nitrite and/or sorbic acid did not significantly change the rate and extent of total aerobic growth in MDCM formulations of similar pH values (Sofos et al., 1979b). It was also reported that in the presence of nitrite (156 µg/g) such growth was similar in meat and/or soy mixtures (Sofos et al., 1979c). The results of Figure 8 confirm those findings and further demonstrate that sorbic acid (0.2%) alone or in combination with nitrite (80 µg/g) did not change the rate and extent of total growth (CFU) in meat-soy mixtures. Growth also was similar in the nitrite (80 and 156 µg/g) formulations.

SUMMARY

A LEVEL of 80 µg/g nitrite alone was ineffective in delaying botulinal toxin production in chicken and meat and/or soy frankfurter-type emulsions while it was only slightly effective in beef and pork formulations. These results would suggest the need for high (>80 µg/g) nitrite levels to control botulism from such products and especially in chicken-based formulations if no alternative means of preservation were advanced.

Sorbic acid (0.2%) alone delayed to some extent toxin production in most substrates tested, (chicken, beef, pork, soy concentrate and soy isolate), but no effect was observed in the textured soy protein.

The effectiveness of low nitrite (80 µg/g) levels in conjunction with sorbic acid (0.2%) on delaying *C. botulinum* toxin production was of a larger magnitude in frankfurter emulsions formulated with beef, pork and/or soy proteins than in chicken (MDCM) products. The results suggest that such nitrite-sorbic acid combinations would be of significant value in providing botulinal safety in emulsified products formulated from all the protein sources tested.

Botulinal spore germination and outgrowth were significantly retarded by sorbic acid (0.2%) and combinations of nitrite (80 µg/g) and sorbic acid (0.2%) in all meat sources tested. Similar effects were observed in soy-based formulations even when no nitrite or sorbic acid was added. The extent of these inhibitory effects coincided with the delay in toxin production in the various treatments. Residual nitrite disappeared slower in soy formulations and especially in the textured form. Total microbial growth in meat-soy mixtures was not affected by inclusion of nitrite and/or sorbic acid in the formulation. Of significant scientific and practical importance are the observed differences in the effects of nitrite and sorbic acid against botulism among meats and soy protein tested.

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EFFECT OF MATURITY AND MARBLING ON THE MYOFIBRIL FRAGMENTATION INDEX OF BOVINE LONGISSIMUS MUSCLE

F. C. PARRISH JR., C. J. VANDELL and R. D. CULLER

ABSTRACT

Myofibril fragmentation index (MFI), sarcomere length, total and soluble collagen, Warner-Bratzler shear and sensory tenderness were determined on loin steaks from carcasses that varied in marbling, maturity and composition. Loin steaks from 72 A, 11 B, 19 C and 22 E maturity beef carcasses were removed at 10–14 days of postmortem storage at 2°C. Steaks were double wrapped in freezer paper and stored at −29°C until the samples were used for analysis. MFI was significantly related to sensory tenderness scores as evidenced by the correlation coefficients of 0.66, 0.77, 0.75 and 0.71 for A, B, C and E maturities, respectively, and 0.76 for all maturities. MFI of loin steaks from A maturity were significantly different from MFI of loin steaks from B, C and E maturity carcasses. Differences in MFI among marbling groups within A maturity were found only at the lowest degree of marbling (practically devoid). Therefore, it seems that MFI could be useful as a criterion of quality for A, B, C and E maturity carcasses, and especially A maturity carcasses without being significantly affected by marbling degree or carcass composition. These results further substantiate the use of myofibril fragmentation tenderness as a term to describe a state of tenderness of conventionally aged bovine longissimus muscle.

INTRODUCTION

OUR PREVIOUS STUDIES have shown that Z-disk degradation and fragmentation of the myofibril of bovine longissimus muscle during postmortem storage were closely related (Olson et al., 1976). Furthermore, a measure of fragmentation of the myofibril during postmortem storage, the myofibril fragmentation index (MFI), was related to beef steak tenderness (Olson and Parrish, 1977; MacBride and Parrish, 1977; Culler et al., 1978). Therefore, the relationship between MFI and beef steak tenderness indicates that MFI offers the potential of objectively classifying carcasses and cuts into tenderness groups on the basis of the amount of myofibril fragmentation of bovine longissimus muscle. Before MFI can have widespread implementation, however, certain postmortem factors associated with a meat quality grading system require investigation. Two postmortem factors, degree of marbling and carcass maturity, are currently the major criteria used to determine quality grade of beef carcasses in commercial operations. Because intramuscular fat (marbling) and maturity are important criteria of beef quality grade, it would be of value to determine the effect of differences in marbling and maturity on MFI. Therefore, the purpose of this paper is to report the effect of marbling and maturity on MFI.

MATERIALS & METHODS

MFI WAS DETERMINED on 123 loin steaks from carcasses postmortem aged for 10–14 days at 2°C that had wide variation in marbling, maturity and composition according to the procedure reported by Culler et al. (1978). These loin steaks were part of a cooperative study with Texas A&M University, Colorado State University and ARS, Beltsville. Carcasses were selected from various commercial packing plants in the U.S. There were 71 A, 11 B, 19 C and 22 E maturity carcasses.

The procedures for source of steak, preparation and evaluation were reported by Culler et al. (1978). In brief, steaks were thawed to 5°C ± 2°C and cooked on preheated Model 450 A Farberware electric broilers (163 ± 17°C at grill surface) to an internal temperature of 70°C. Two sections were presented to each panelist (a ten-member descriptive attribute panel) and tenderness was scored according to an 8-point structured scale (8 = extremely tender). Warner-Bratzler shear, sarcomere length and total and soluble collagen were determined also by the procedures reported by Culler et al. (1978).

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0022-1147/79/0006-1668$02.25/0
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RESULTS & DISCUSSION

MYOFIBRIL FRAGMENTATION INDEX was found significantly different between loin steaks from A maturity carcasses and the other three maturity groups. There was, however, no difference in MFI of loin steaks among B, C and E maturity carcasses (Table 1). This suggests that chronological age reduces the amount of fragmentation that the myofibril undergoes during postmortem aging. However, the sample size is somewhat limited in the three advanced maturity groups, and because of the small number of samples, this may not be representative of the population. In an earlier study, (Olson and Parrish, 1977) little difference was found for MFI of loin steaks of A and C maturity carcasses.

The tenderness evaluation of these steaks by both the sensory panel and the Warner-Bratzler shear demonstrated that tenderness of loin steaks declined with chronological age. There was, however, a significant difference in tenderness of loin steaks from only A and E maturity carcasses. We were able to show earlier (Culler et al., 1978) that MFI of loin steaks could be used to differentiate between tender and tough groups. These groups, however, contained a combination of four maturity groups A, B, C and E. These data further emphasize that there are both tender and tough steaks within the same maturity and that maturity differences do not always signify tenderness differences. Thus, MFI offers a means of differentiating between tough and tender steaks within the same maturity. In addition, these results on a tenderness-maturity relationship are substantiated by the work of Goll et al. (1965), Breidenstein et al. (1968) and Norris et al. (1971). That is, large differences must occur in maturity before there is a recognizable difference in tenderness by a sensory panel.

No significant differences were found among maturity groups for sarcomere length, fat thickness and rib eye area. On the other hand, there were differences in soluble and total collagen among maturities. Amount of soluble collagen of loin steaks from A maturity carcasses was significantly greater than for the other three maturities. These results confirm earlier work by Goll et al. (1964) and Hill (1966) showing that collagen solubility decreased with increased chronological age.

Table 2 contains the data of the chemical, physical and sensory characteristics of loin steaks from A maturity carcasses among marbling classifications. Degree of marbling

significantly affected MFI only in the practically devoid classification. Thus, MFI is independent of the amount of intramuscular fat in the rib eye muscle and could be used as a tenderness measurement without quantity of intramuscular fat interfering with this measurement. A significant change in tenderness, measured by both panel and shear, occurred essentially only in the practically devoid level of marbling. In addition, no significant differences were detected in sarcomere length, rib eye area and soluble collagen within the eight groups of marbling. No significant difference in sarcomere length of different maturities (Table 1) and no difference in sarcomere length of different marbling classifications within A maturity confirm our earlier observations (Culler et al., 1978). This again seems to imply that sarcomere length is of importance to tenderness of prerigor excised muscle stored in the cold (Marsh and Leet, 1966;

Table 1—Certain physical, chemical and sensory characteristics of beef loin steaks from four carcass maturities[a]

Maturity group	Myofibril fragmentation index[b]	Sensory[c] tenderness score	Warner-Bratzler Shear (kg/cm²)	Sarcomere length (μm)	Fat thickness (cm)	Rib eye area (cm²)	Soluble collagen (%)	Total collagen (mg/g)
A	60.1a(71)[d]	6.18a(72)	2.46a(72)	1.78a(72)	1.09a(72)	70.3a(72)	6.44a(27)	3.45a(27)
	±1.4	±.12	±.09	±.01	±.08	±1.4	±.37	±.10
B	48.4b(11)	5.01ab(11)	3.47b(11)	1.78a(11)	0.81a(11)	69.4a(11)	3.07b(11)	5.60b(11)
	±5.4	±.58	±.51	±.03	±.24	±3.8	±.10	±.43
C	49.0b(19)	4.77ab(19)	3.24ab(19)	1.77a(19)	1.19a(19)	64.1a(19)	3.39b(19)	4.40c(19)
	±3.1	±.35	±.30	±.02	±.23	±4.5	±.17	±.48
E	45.6b(22)	3.96b(22)	3.68b(22)	1.78a(22)	0.71a(22)	64.1a(22)	3.30b(22)	3.32a(22)
	±2.5	±.29	±.22	±.02	±.12	±2.0	±.10	±.34

[a] Mean values and standard errors not followed by the same letter are significantly different at the 5% level of probability.
[b] Absorbance at 540 nm × 200
[c] Eight-point descriptive scale (8 = extremely tender and 1 = extremely tough)
[d] Numbers in parentheses are the number of carcasses sampled.

Table 2—Certain physical, chemical and sensory characteristics of beef loin steaks for eight marbling groups within A maturity carcasses[a]

Marbling group	Myofibril[b] Fragmentation Index	Sensory[c] tenderness score	Warner-Bratzler shear (kg/cm²)	Sarcomere length (μm)	Fat thickness (cm)	Rib eye area (cm²)	Soluble collagen (%)
Moderately abundant	62.8a(9)[d]	6.77a(9)	2.17a(9)	1.82a(9)	1.75a(9)	73.7a(9)	4.93a(5)
	±3.9	±.14	±.17	±.02	±.15	±3.4	±.29
Slightly abundant	59.8a(10)	6.65a(10)	2.08a(10)	1.77a(10)	1.73a(10)	71.1a(10)	7.63a(4)
	±2.9	±.22	±.14	±.02	±.18	±3.9	±.48
Moderate	63.62a(9)	6.44a(9)	2.17a(9)	1.76a(9)	1.09abc(9)	71.2a(9)	4.52a(2)
	±3.1	±.27	±.21	±.02	±.14	±4.3	±.94
Modest	60.6a(10)	6.32a(10)	2.34ab(10)	1.77a(10)	.96bcd(10)	66.1a(10)	7.82a(4)
	±5.1	±.30	±.35	±.02	±.16	±4.3	±1.14
Small	61.1a(8)	6.50a(8)	2.46ab(8)	1.76a(8)	.84bcd(8)	63.7a(8)	7.55a(3)
	±2.7	±.25	±.32	±.01	±.14	±2.9	±1.95
Slight	60.6a(8)	5.79abc(9)	2.70ab(9)	1.81a(9)	1.34abc(9)	75.6a(9)	6.13a(4)
	±2.6	±.31	±.24	±.03	±.29	±4.6	±.82
Traces	64.4a(7)	5.96abc(7)	2.62ab(7)	1.75a97)	.46bcd(7)	69.1a(7)	6.59a(2)
	±3.4	±.30	±.24	±.03	±.10	±2.8	±.48
Practically devoid	50.2b(10)	5.11bc(10)	3.11b(10)	1.77a(10)	.38d(10)	71.1a(10)	6.07a(3)
	±5.6	±.48	±.27	±.02	±.15	±4.4	±1.06

[a] Mean values and standard errors not followed by the same letter are significantly different at the 1% level of probability.
[b] Absorbance at 540 nm × 200
[c] 8 = extremely tender and 1 = extremely tough
[d] Numbers in parentheses are the number of carcasses sampled.

Table 3—Correlation coefficients between certain physical, chemical and sensory characteristics of loin steaks from A maturity bovine carcasses

	Myofibril fragmentation index	Sensory tenderness score	Warner-Bratzler shear	Sarcomere length	Fat thickness	Rib eye area
Sensory tenderness score	0.66**					
Warner-Bratzler shear	−0.58**	−0.73**				
Sarcomere length	0.24*	0.06	−0.10			
Fat thickness	0.17	0.16	0.21	0.34**		
Rib eye area	0.08	0.27*	0.06	0.05	0.35**	
Soluble collagen	−0.07	−0.07	0.18	0.00	−0.19	−0.28

* $p < 0.05$
** $p < 0.01$

Herring et al., 1967), but is not of significance to tenderness of conventionally handled carcasses. There was, however, a significant difference in amount of fat over the rib eye between the 12th and 13th rib, but these values were inconsistent with amount of marbling.

Data in Tables 3, 4, 5 and 6 are correlation coefficients between the various physical, chemical and sensory characteristics of loin steaks from A, B, C and E maturity carcasses, respectively. The most significant and meaningful relationships were between MFI and tenderness measurements and between sensory panel and Warner-Bratzler shear. Correlation coefficients of 0.66, 0.77, 0.75 and 0.71 were

Table 4—Correlation coefficients between certain physical, chemical and sensory characteristics of loin steaks from B maturity bovine carcasses

	Myofibril fragmentation index	Sensory tenderness score	Warner-Bratzler shear	Sarcomere length	Fat thickness	Rib eye area
Sensory tenderness score	0.77**					
Warner-Bratzler shear	−0.79**	−0.94**				
Sarcomere length	0.32	0.50	−0.62*			
Fat thickness	0.66*	0.66*	−0.62*	0.27		
Rib eye area	0.38	0.46	−0.58	0.71**	0.20	
Soluble collagen	0.08	0.24	0.00	−0.22	0.41	0.10

* $p < 0.05$
** $p < 0.01$

Table 5—Correlation coefficients between certain physical, chemical and sensory characteristics of loin steaks from C maturity bovine carcasses

	Myofibril fragmentation index	Sensory tenderness score	Warner-Bratzler shear	Sarcomere length	Fat thickness	Rib eye area
Sensory tenderness score	0.75**					
Warner-Bratzler shear	−0.72**	−0.87**				
Sarcomere length	0.00	0.07	−0.10			
Fat thickness	0.08	0.11	0.26	0.14		
Rib eye area	0.06	0.16	0.05	−0.20	0.28	
Soluble collagen	0.06	0.04	−0.19	0.21	−0.23	0.01

* $p < 0.05$
** $p < 0.01$

Table 6—Correlation coefficients between certain physical, chemical and sensory characteristics of loin steaks from E maturity bovine carcasses

	Myofibril fragmentation index	Sensory tenderness score	Warner-Bratzler shear	Sarcomere length	Fat thickness	Rib eye area
Sensory tenderness score	0.71**					
Warner-Bratzler shear	−0.66**	−0.85**				
Sarcomere length	0.32	0.11	0.00			
Fat thickness	−0.09	−0.21	0.11	0.08		
Rib eye area	0.16	−0.04	0.06	0.11	0.12	
Soluble collagen	−0.31	−0.41	0.37	−0.07	−0.03	0.06

* $p < 0.05$
** $p < 0.01$

Table 7—Correlation coefficients between certain physical, chemical and sensory characteristics of loin steaks from A, B, C and E maturity bovine carcasses

	Myofibril fragmentation index	Sensory tenderness score	Warner-Bartzler shear	Sarcomere length	Fat thickness	Rib eye area
Sensory tenderness score	0.76**					
Warner-Bratzler shear	−0.72**	−0.86**				
Sarcomere length	0.18*	0.12	−0.16			
Fat thickness	0.22*	0.21*	−0.16	0.23**		
Rib eye area	0.18*	0.10	−0.12	0.06	0.30**	
Soluble collagen	0.26*	0.34**	−0.25	0.10	−0.01	0.04

* $p < 0.05$
** $p < 0.01$

found between MFI and sensory tenderness score for A, B, C and E maturities, respectively. Correlation coefficients between MFI and W-B shear were similar to those between MFI and sensory tenderness score, with the major exception that they were of a negative relationship. These values are similar to the ones that we have reported earlier (Olson and Parrish, 1977; MacBride and Parrish, 1977; Culler et al., 1978). There were some significant relationship between other variables, but in general they were of such small magnitude that they would not be of much practical value. In only one instance was sarcomere length related to tenderness measurements (Table 4). A significant correlation coefficient of −0.62 was observed between sarcomere length and Warner-Bratzler shear values of loin steaks from B maturity carcasses (11 samples).

MFI was found to be either highly significantly, or significantly, related to the variables studied for all maturities (Table 7). A correlation coefficient of 0.76 and −0.72 was found between MFI and sensory tenderness score and Warner-Bratzler shear, respectively, for all maturities. Although the other variables were significantly related to MFI, and in some instances to other variables, they were of too small a magnitude to be of much practical use. Consequently, these results show that MFI (1) accounted for the largest amount (about 50%) of the variation in loin steak tenderness of the several factors investigated in this study within A, B, C and E maturity carcasses, and (2) that MFI could be useful in differentiating between tender and tough carcasses within these four maturities. In addition, these results add further support to the term "myofibril fragmentation tenderness" (MacBride and Parrish, 1977) to describe a state of tenderness in conventionally aged bovine longissimus muscle.

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Ms received 3/1/79; revised 5/19/79; accepted 5/27/79.

Journal Paper No. J-9450 of the Iowa Agriculture and Home Economics Experiment Station, Ames, IA, Project 2049. The technical assistance of Kim Bless and Diane Kuhlers and the statistical analysis by Dr. David F. Cox are gratefully acknowledged. This research was supported in part by grants from the USDA Meat Science Research Laboratory, Agricultural Research Service, Beltsville, MD and the Iowa Beef Industry Council, Ames, IA.

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Ms received 3/10/79; revised 6/6/79; accepted 7/1/79.

Presented at the 38th Annual Meeting of the Institute of Food Technologists, St. Louis, MO, June 10—13, 1979.

Paper No. 10,721, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, MN 55108.

This research was supported in part by a gift from Monsanto Industrial Chemicals Co. We thank K. Bhothipaksa for technical assistance; J. Bauermann and H.W. Longacre, Inc. for providing mechanically deboned chicken meat and technical advice; B. Link of Cargill Inc. for providing soy protein samples; R.B. Tompkin and L.N. Christiansen of Swift and Co. for *C. botulinum* cultures and helpful advice; and the Center for Disease Control for the antitoxins.

ACTION OF PROTEOLYTIC ENZYMES ON BOVINE MYOFIBRILS

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ABSTRACT

The effect of cathepsin D from muscle and spleen on bovine myofibrils has been examined under postmortem pH conditions using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis, scanning electron microscopy (SEM) and electron spin resonance spectroscopy (ESR). Cathepsin D causes a degradation of Z disks of the myofibrils and has a relatively selective action on the myofibrillar proteins in comparison to the plant derived enzyme papain. The heavy chain of myosin (200,000 daltons) was degraded to fragments of about 170,000, 150,000, and 80,000 daltons at 25°C. Degradation becomes more extensive at 37°C. ESR studies on spin-labeled myofibrils indicated that the proteolytic attack of cathepsin D occurred more distal to the globular region of the myosin when compared to papain or trypsin. Although there appears to be little proteolytic effect on α-actinin or actin, changes in the gel electrophoresis pattern below 42,000 daltons indicate alterations of the regulatory complex and myosin light chains.

INTRODUCTION

THE CHEMICAL and structural changes occurring in the postmortem aging of muscle are directly related to the degradation and alterations of myofibrillar structure of the muscle. These changes at the myofibrillar level involve a drop of ATPase activity and of pH from 7.1 to 5.2 in the muscle followed by subsequent disappearance of Z disks (Stromer et al., 1967; Davey and Gilbert, 1969), fragmentation of myofibrillar structure into smaller components with appearance of a 30,000 dalton fragment (Olson and Parrish, 1977; Olson et al., 1977), and increase in the dissociation of actomyosin complex (Fujimaki et al., 1965; Goll and Robson, 1967).

Many of these postmortem changes in myofibrillar proteins have generally been assumed to arise from the release and activity of endogenous muscle proteases active at postmortem pH. Since skeletal muscle cells contain lysosomes (Canonico and Bird, 1970) several groups have investigated the effect of the lysosomal proteases, such as cathepsins, on myofibrils. Eino and Stanley (1973a, b) observed that a preparation of muscle cathepsin D incubated with myofibrils under postmortem pH conditions produced structural changes similar to those observed during postmortem aging of muscle. Robbins and Cohen (1976) treated bovine myofibrils at pH 5.3 with an extract of bovine spleen, which contained a high concentration of cathepsin D in addition to other catheptic enzymes and also observed selective degradation of the Z disk region. In treatment of the muscle tissue with the spleen extract these authors further observed that both the sarcolemma and stroma of muscle appeared to be degraded. Recently, Schwartz and Bird (1977) and Arakawa et al. (1976) have also observed the breakdown of myofibrillar proteins by catheptic enzymes.

It should be mentioned here that several publications (Olson et al., 1977; Penny, 1974; Dayton et al., 1976) have shown that a calcium activated protease that exists intracellularly in the muscle tissue, designated as calcium activated factor (CAF), can degrade myofibrillar proteins. This protease, CAF, possesses optimum proteolytic activity at pH 7.5 and decreases rapidly below pH 6.5 or above pH 8 (Dayton et al., 1976), and was shown to remove Z disks from intact myofibrils, degrade troponin, tropomyosin and C-protein components of muscle. When troponin complex was incubated with CAF, troponin T was degraded to a 30,000 dalton residue. These observations thus suggest that CAF may play a significant role in the breakdown of muscle tissue, although it is unlikely that at the postmortem pH conditions (pH 5.2), CAF would be reasonably active.

In view of the above observations, it seemed that for a clear understanding of the role of catheptic enzymes on the postmortem conditioning of muscles, selective analytical techniques which examine integral structure changes must be investigated. In this report, we present the results of our on-going efforts on the action of muscle and spleen cathepsin D on bovine myofibrils (Robbins and Cohen, 1976; Chatterjee et al., 1977). For comparison, the effect of trypsin and several plant proteases on bovine myofibrils has been evaluated. These studies include evaluation of morphological changes in myofibrillar structure by scanning electron microscopy (SEM), specific proteolysis of myofibrillar protein components revealed by SDS polyacrylamide gel electrophoresis, and evaluation of environmental and conformational states of spin-labeled myofibrils under proteolytic condition by electron spin resonance (ESR) spectroscopy.

MATERIALS & METHODS

Materials

The nitroxide spin labeling reagents, 2,2,6,6-tetramethyl piperidino-1-N-oxyl-4-iodoacetamide (ISL), and 2,2,6,6-tetramethyl piperidino-1-N-oxyl-4-maleimide (MSL) (Fig. 5A) were obtained from Syva, Palo Alto, CA. Cathepsin D was prepared from bovine spleen and bovine muscle (see below); trypsin was obtained from Nutritional Biochemicals, Cleveland, OH; bromelain, ficin and papain were obtained from EM Reagents (E. Merck), Darmstadt, Germany. The commercial enzymes were used without further purification.

Isolation of myofibrils

Bovine myofibrils were isolated from pre-rigor bovine longissimus dorsi following the method of Perry (1953). Scissor-minced muscle from which surface fat and exposed connective tissue was removed was suspended in 5 volumes of cold (4°C) relaxing buffer (25 mM KCl, 39 mM $Na_2B_2O_7$, and 5 mM EGTA, pH 7.2). The muscle tissue was homogenized with five bursts of 15 sec duration each in a Waring Blendor. After each homogenization the sediment was examined by phase contrast microscopy to monitor the extent of fibril fragmentation. The homogenate was centrifuged at 500 × G for 5 min at 4°C and the pellet suspended in 5 volumes of fresh relaxing buffer. Depending on the results of microscopy, the suspended fibrils were rehomogenized. Usually three brief homogenizations were sufficient. Myofibrils were generally used immediately after preparation; however, when storage was required, the myofibrils were suspended in relaxing buffer containing 20% (V/V) gly-

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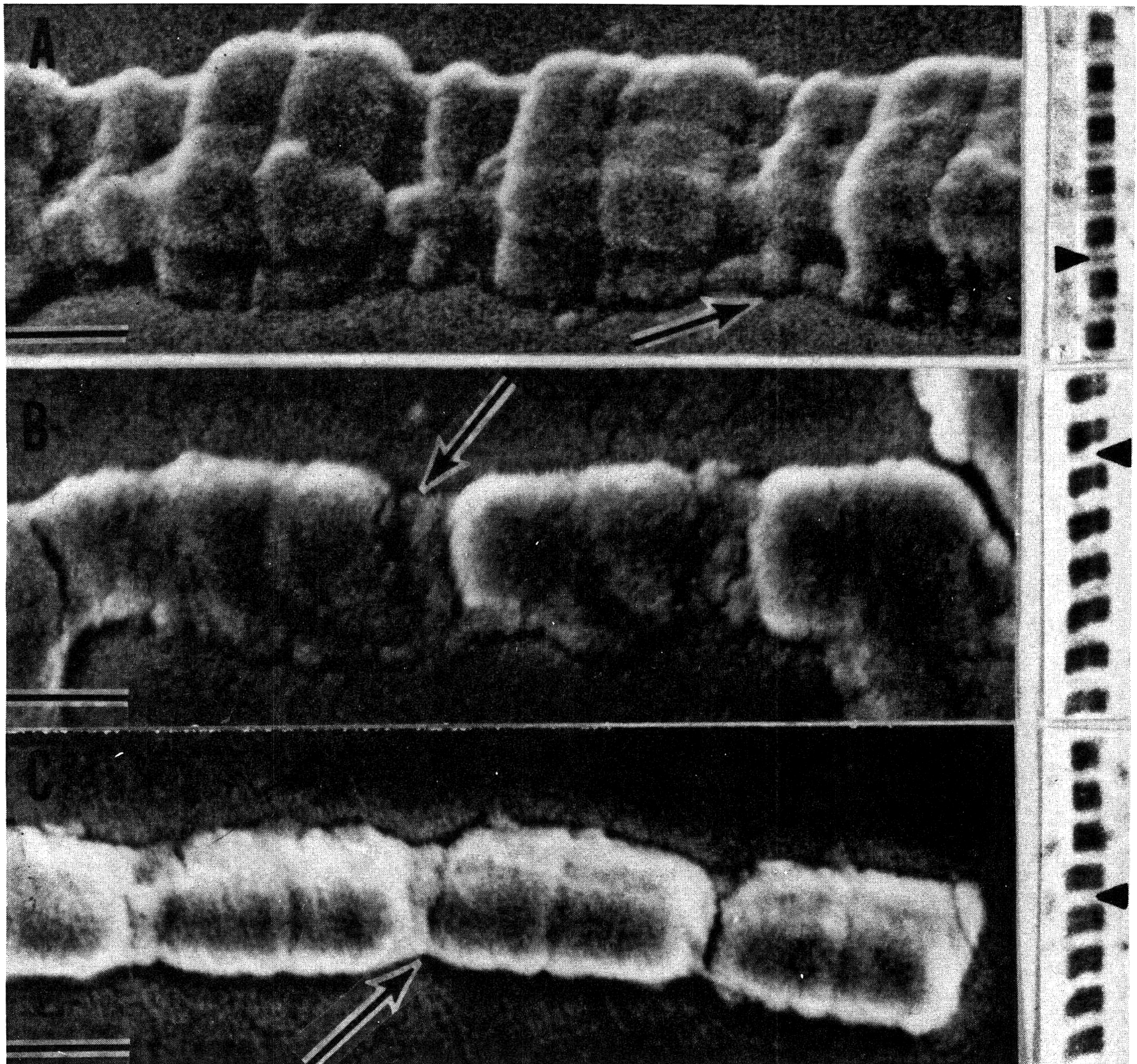

Fig. 1–SEM micrographs of myofibrils (left) and light micrographs (920X) (right): (A) Control; (B) after 24 hr treatment with Cathepsin D (spleen) from bovine spleen; (C) after 24 hr treatment with Cathepsin D (muscle). Arrows show the location of Z-bands.

cerol and 0.001% sodium azide and frozen at –40°C. Storage times never exceeded 1 wk.

Incubation of myofibrils with cathepsin D

Myofibrils were incubated at 4°C in relaxing buffer which had been previously adjusted to pH 5.2–5.3 with HCl. The myofibrils were spun out and resuspended in the relaxing buffer containing either spleen or muscle cathepsin D. The enzyme activity of the solution was in units per milliliter of sedimented myofibrils (3.5–4.0 mg protein). Incubation was at 25°C and 37°C at pH 5.2–5.3 and the total time of reaction was 24 hr. Samples were withdrawn at intervals for analysis by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. Control myofibrils, containing no enzyme, were incubated and analyzed under the same conditions.

Sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis

The enzymatically treated myofibrils were washed extensively with relaxing buffer to remove enzyme and finally solubilized in relaxing buffer containing urea (8M), dithiothreitol (DTT) (1%) and SDS (1%). Gel electrophoresis of the solubilized myofibrils was performed following the method of Weber and Osborn (1969) using 5% gels. Prior to application, the urea-DTT-SDS treated myofibrils were heated at 100°C for 3 min to insure complete reaction. A calibration curve for the relative mobility-molecular weight relationship was made using the following proteins: serum albumin, ovalbumin, chymotrypsinogen and myoglobin. Densitometry of the gels was performed on a Gilford Model 2400 spectrophotometer equipped with a linear transport. Gel electrophoresis of myofibrils treated with plant proteases was carried out by the method of Rattrie and Regenstein (1977).

Preparation of Cathepsin D

Partially purified spleen and muscle cathepsin D were prepared from acetone powders of fresh bovine spleen and pre-rigor bovine semimembranous muscle as previously described for the spleen enzyme (Robbins and Cohen, 1976). In the case of muscle, the ground tissue was extracted twice with four times its weight of distilled water prior to treatment with acetone. The enzymes were further purified by affinity chromatography on support bound hemoglobin.

Attempts to purify the enzymes using a column of hemoglobin coupled to cyanogen bromide activated Sepharose 4B as described by Smith and Turk (1974) were unsuccessful in our hands because of leaching of hemoglobin from the column and co-elution with the

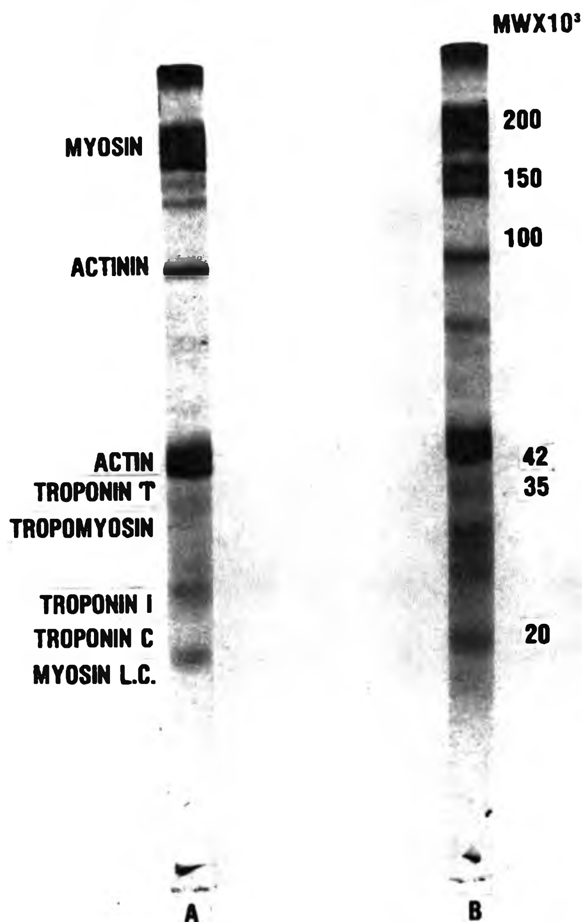

Fig. 2–SDS gel electrophoresis of bovine myofibrils (5% gels), after treatment with cathepsin D (muscle) at 25°C: (A) No enzymes; (B) After treatment with enzyme.

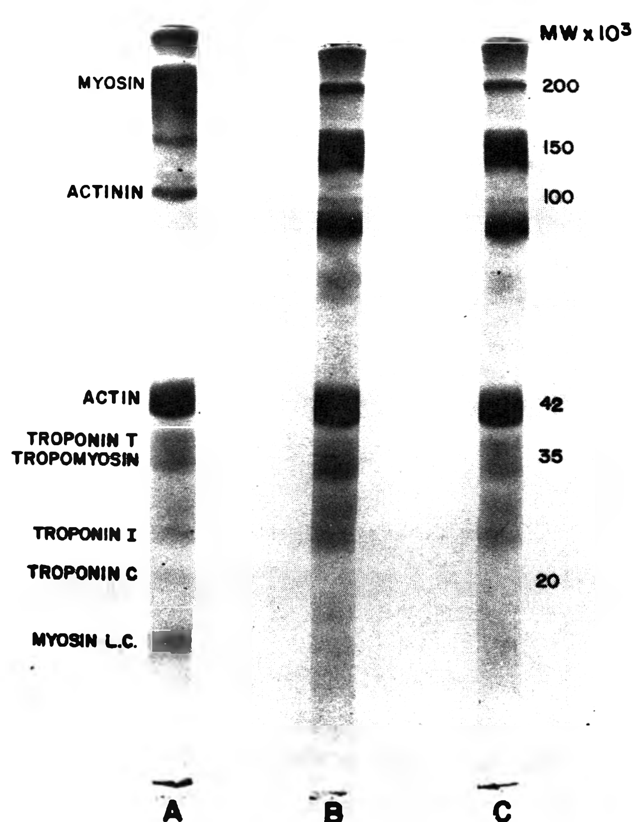

Fig. 3–SDS electrophoresis of bovine myofibrils (5% gels): (A) No enzyme (control); (B) After treatment with Cathepsin D (muscle); (C) After treatment with Cathepsin D (spleen). T = 37°C.

enzyme under the conditions of chromatography. This difficulty was circumvented by carrying out the affinity chromatography step on a column of hemoglobin coupled to deacetylated chitin with glutaraldehyde (F.M. Robbins, to be published) under the conditions described by Smith and Turk (1974) for Sepharose 4B coupled hemoglobin. These steps resulted in about a 250-fold purification of each enzyme.

Cathepsin D activity was determined using hemoglobin as substrate as described by Smith and Turk (1974). One unit of activity corresponds to the release of 24 microequivalents of tyrosine/hr (soluble in trichloroacetic acid) at 37°C. The specific activity (units/mg protein) was 34 and 8.6 for muscle and spleen cathepsin, respectively.

Spin labeling of myofibrils

Isolated bovine myofibrils were spin labeled on the thiol groups of the myosin heavy chains (S_1 and S_2 thiol groups) using nitroxide spin label I and II following the procedure (see Fig. 5A) of Seidel et al. (1970). For each spin labeling experiment, 2–5 ml of compacted myofibrils in relaxing buffer pH 7.2 were used. Spin label dissolved in ethanol was diluted with relaxing buffer to give a concentration of 1–2 mg per ml of spin label and an ethanol concentration of 0.1%. An equal volume of this solution was added to an equal volume of compacted myofibrils, and the mixture allowed to react for 24 hr at 4°C. In spin labeling experiments containing ATP (10 mM), the pH of the relaxing buffer was adjusted to pH 7.2 before and after addition of myofibrils and spin labels; the mixture was then equilibrated at that pH for 6–8 hr. Spin labeled myofibrils were freed from unreacted nitroxide spin labels by successive centrifuging (6000 × G) and washings with the relaxing buffer until the washings gave no ESR signal of the free nitroxide. The labeled myofibrils were then resuspended in either pH 7.2 or pH 5.2–5.3 relaxing buffer for equilibration before enzyme treatment.

Electron spin resonance (ESR) studies

ESR studies were performed at 25°C in relaxing buffer adjusted to pH 5.2–5.3 for cathepsin D, and the plant proteases and at pH 7 for trypsin. ESR experiments with cathepsin D were carried out by mixing 100 μl of myofibril suspension (400 μg protein) in relaxing buffer with 40 μl of the enzyme preparation. The total enzyme activity was 0.128 and 0.412 units for the muscle and spleen enzyme, respectively. In experiments with plant proteases, the reaction volumes were 200 μl of myofibrils (800 μg protein) and 40μl enzymes. The total enzyme activity of each plant protease in Anson units (Anson, 1938) was papain 0.028, bromelin 0.016, and ficin, 0.031. The reaction of myofibrils with trypsin was carried out using 100 μl of myofibril suspension and 20 μl of enzyme solution containing 3.7 N.F. units (National Formulary, 1965).

ESR spectral studies were performed using a Varian V-4500-10A spectrometer equipped with a V-FR-2503 field regulator, magnet power supply, a 9 in. electromagnet and a 100 KC field modulation unit. In all the experiments, the magnetic field was calibrated using an aqueous solution of nitroxide II (10^{-3}M). A V-4531 rectangular microwave cavity equipped with a V-4557 variable temperature accessory was used and microwave frequency of 9.5 GHz was used in all the experiments. ESR spectra were taken using a Varian E248-1 micro aqueous flat cell.

Ultra structure examination

Samples were prepared for light microscopy for screening purposes, and scanning electron microscopy (SEM) for more detailed analysis of myofibrillar structure.

A drop of suspension containing myofibrils was put onto a glass slide and a coverslip placed over the drop. Excess fluid was blotted using bibulous paper and the slide examined with phase contrast optics in a Zeiss ultraphot camera microscope. Representative samples were photographed with Polaroid type 55 P/N film so that the negatives could be used at a later date for making prints.

The myofibrillar suspension was first photographed as described above and then prepared for SEM analysis using the procedures described by Cohen (1976).

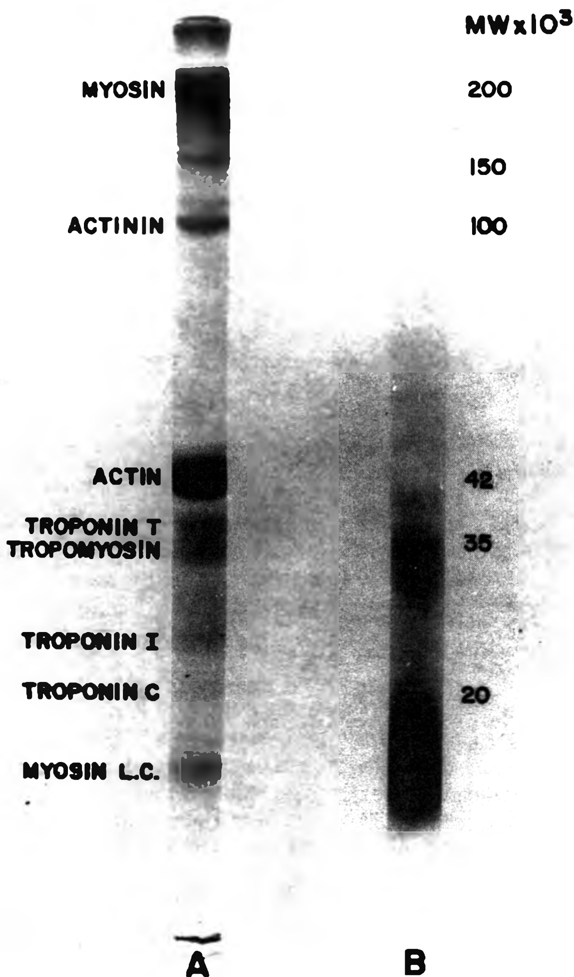

Fig. 4–SDS gel electrophoresis of bovine myofibrils (5% gels), treated with papain: (A) No enzyme; (B) After treatment with papain (10 min), at 37°C.

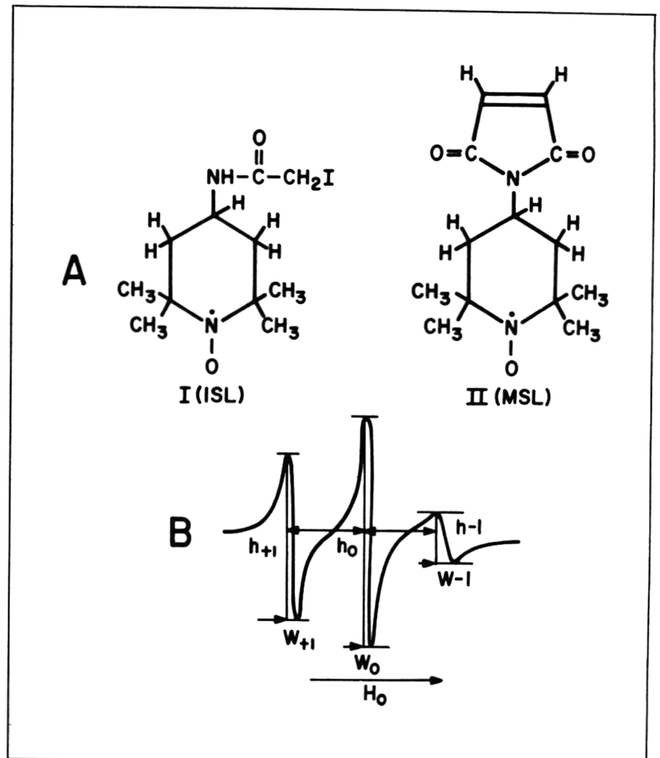

Fig. 5–(A) Structure of nitroxide free radicals: 2,2,6,6-tetramethylpiperidino-1-N-oxyl-, -4-iodoacetamide (I), and -4-maleimide (II); (B) ESR spectra of partially immobilized nitroxide free radicals I and II showing the line separations (gauss), line width (W) and intensities (h) of low (h_{+1}), middle (h_0) and high (h_{-1}) field lines at magnetic field (H_0) for N^{14} nucleus.

The slide was placed coverslip down on dry ice, and the coverslip flipped off with a single-edge razor blade, then put into 50, 70, 90, 95 and 100% ethyl alcohol successively for 5 min each. The samples were then critical point dried using CO_2, mounted on specimen stubs with silver paste, sputter coated with gold palladium and examined in a Coates and Welter scanning electron microscope, Model 100.

RESULTS

Microscopy of myofibrils

Prior to analysis of cathepsin D treated myofibrils by gel electrophoresis, samples of the myofibrils were monitored by light microscopy, for disappearance of Z disks. It was found under the conditions of the enzyme incubation at 25°C or 37°C, the degradation of the Z disk structure was apparently complete within 40 min to 1 hr. Figure 1 shows the light and SEM micrographs of myofibrils treated with cathepsin D from spleen and muscle.

Gel electrophoresis

Samples of myofibrils incubated with cathepsin D removed at intervals for 40 min to 24 hr showed progressive changes in their protein patterns. The gel electrophoresis patterns of the control and enzyme treated myofibrils after 24 hr incubation at 25°C and 37°C are shown in Figures 2 and 3. The data show an increase of components at 25°C having molecular weights of about 170,000 and 150,000 daltons, and 80,000 daltons which arise from the degradation of the 200,000 dalton component of the myosin heavy chains. At 37°C the degradation of the myosin heavy chain is more pronounced than at 25°C and components of about 150,000, 95,000 and 80,000 daltons were generated. The other major protein of the myofibrils, actin, is apparently unaffected by cathepsin D.

At 25°C there is a discrete band present in the control gel at about 190,000 daltons which does not appear to be affected by enzyme treatment. At 37°C this band is absent in the control and enzyme treated gels. The identity of the component(s) giving rise to this band is unknown.

The band at about 100,000 daltons due to α-actinin does not appear to be significantly affected by the enzyme at 25°C; however, at 37°C there is a decrease in its intensity indicating degradation at the higher temperature.

Changes are also evident in the protein patterns occurring in the lower molecular weight region of the gels (below 42,000 daltons) which may arise from tropomyosin, the troponin complex, or the myosin light chains. However, due to the build-up in this region of peptide components resulting from the breakdown of higher molecular weight species, it is difficult to detect changes in these proteins unambiguously. Gel patterns of the 25°C experiments show some loss of tropomyosin and troponin T and a build-up of a 30,000 dalton component. At 37°C there appears to be a degradation of troponin I and myosin light chain-2 (determined from 10% gels not shown) as well as the alterations in the tropomyosin and troponin T components seen at 25°C.

In comparison with the cathepsin D effects, the gel electrophoresis pattern of papain-treated myofibrils shows that this enzyme leads to extensive degradation of the proteins of the myofibrils, and formation of peptides with molecular

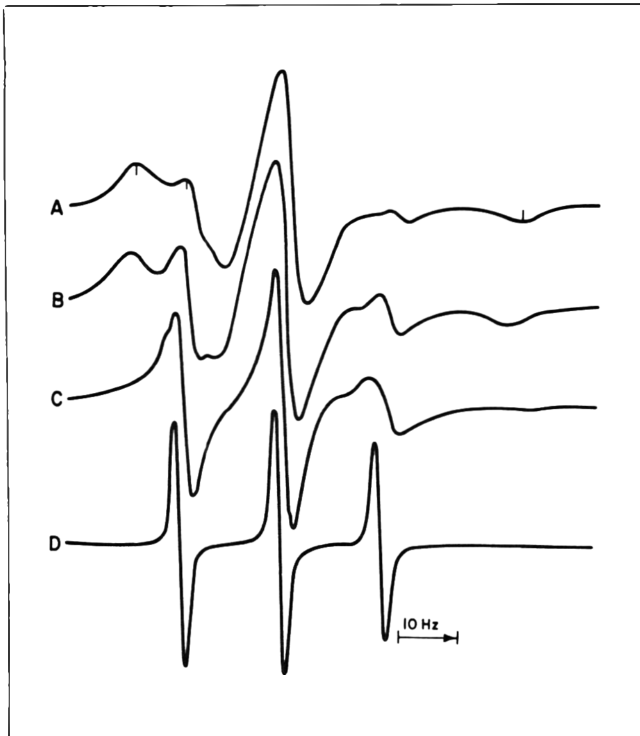

Fig. 6—ESR spectra of spin-labeled bovine myofibrils in relaxing buffer, labeled with nitroxide (I), (II): (A) II, in tne presence of 10 mm ATP; (B) II, in the absence of ATP; (C) Spectrum of spin-labeled (I, II) myofibrils after treatment with proteases: (A) Papain; (B) Bromelain; (C) Ficin; (D) Trypsin.

weights less than 20,000 daltons (Fig. 4). This agrees with the observations of Rattrie and Regenstein (1977) on the effect of papain on myofibrils from chicken muscle.

ESR spectral studies of spin-labeled myofibrils

The technique of spin labeling (Berliner, 1976) provides a simple method for probing the physicochemical and environmental changes in proteins and other biomolecules. The method involves binding of a nitroxide free radical (spin label) (Fig. 5A) to the appropriate site in the protein molecule. The ESR spectrum of the free radical reflects the changes in the mobility of the bound spin label (McConnell and McFarland, 1970).

Studies were made to spin label isolated myofibrils at the reactive thiol sites in the head region of myosin (Seidel et al., 1970) to probe possible changes induced by the action of cathepsin D on the myofibrillar structure.

Although the interpretations of ESR spectra of nitroxide spin labels covalently bound to a large biomolecule and experiencing anisotropic motions are generally made theoretically by the application of quantum mechanics (Nordio, 1976), here, direct analyses have been used to evaluate the changes in anistropic mobility of the spin probe in relation to the environmental and conformational changes (Morrisett and Bromfield, 1971). For a free tumbling nitroxide radical in a low viscosity medium, the observed ESR spectra show an average value of the anisotropic contribution; in this case, a sharp three-line spectrum from the interaction of the magnetic moment of the unpaired electron with the magnetic moment of the N^{14} nucleus is observed (Fig. 6D). This type of spectrum shows equal line width (W) and line heights (h) with equal spacing (Fig. 5B). Anisotropic motions of the nitroxide free radicals are observed in the ESR spectra where the above three sharp lines are inhomogeneously broadened due to the restricted mobility of the radical in space. Experimentally the changes in the ratios of the height (h) (Fig. 5B) at high field (h_{-1}), mid field (h_0) and low field (h_{+1}) can be used as a direct measure of the conformational or other "changes" taking place in the biomolecule when a precise determination of the molecular motion and rotational correlation time is not warranted. The ESR spectra of spin-labeled myofibrils were analyzed by measuring the ratios of the high field (h_{-1}) and mid field (h_0) lines.

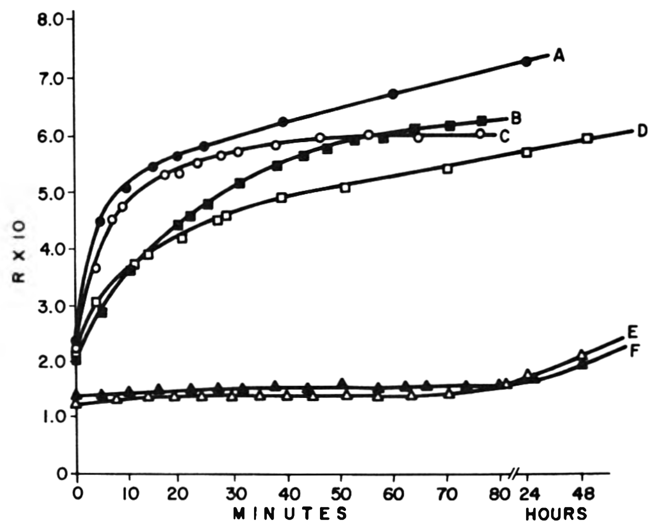

Fig. 7—Action of proteases on nitroxide (I and/or II) labeled bovine myofibrils, in relaxing buffer (See materials and methods for conditions): (A) Papain; (B) Bromelain; (C) Ficin; (D) Trypsin; (E) Cathepsin D (spleen); (F) Cathepsin D (muscle).

ESR spectra of myofibrils spin labeled with nitroxide (Fig. 6A) in the presence of ATP showed strongly immobilized components relative to those myofibrils labeled in the absence of ATP (Fig. 6B). The outer extrema in both cases were 64 Hz. Myofibrils labeled with nitroxides I showed a partially immobilized spectrum (Fig. 6C) with three basic lines and anisotropic broadening. Figure 6D shows the isotropic ESR spectrum of both nitroxide I, and nitroxide II labeled myofibrils after treatment with proteolytic enzymes A-D (Fig. 7). Here the restricted motion of the free radical in the myofibrillar system was destroyed due to extensive proteolysis. The effect of the proteolytic enzymes on spin-labeled myofibrils are shown graphically in Figure 7, where the ratio R of the high field line (h_{-1}) to the midfield line (h_0) is plotted against time of proteolysis $R=h_{-1}/h_0$.

The data of Figure 7 show that a relatively rapid rise occurs in the ratio, R, corresponding to an increase in mobility of the spin label, when the myofibils are incubated with the plant proteases, or trypsin. This results from the extensive proteolysis of the myofibrils produced by these enzymes (Rattrie and Regenstein, 1977). By comparison, the action of cathepsin D on the labeled myofibrils produces essentially no change in the environment of the spin label, and only after 24 hr incubation is a slight increase in mobility observed (Fig. 7E and F).

DISCUSSION

THE RESULTS of this study show that cathepsin D from muscle or spleen degrades myofibrils under postmortem pH conditions (5.1–5.3) causing an alteration of Z disk structure and breakdown of myosin heavy and light chains as well as effecting changes in the troponin-tropomyosin complex. Some degradation of α-actinin is evident at 37°C.

Our finding that myosin is degraded by cathepsin D is supported by two reports which appeared while this work

was in progress in which essentially the same results were found. Schwartz and Bird (1977) reported that muscle and liver cathepsin D degraded the heavy chains of isolated myosin (200,000 daltons) to fragments of about 175,000 daltons to 150,000 daltons and then subsequently to fragments of 100,000 daltons which appeared to be resistant to further degradation. Arakawa et al. (1976) reported that a catheptic enzyme from rabbit muscle with properties similar to cathepsin D degraded the heavy chains of myosin present in myofibrils to fragments of 150,000 daltons and 70,000 daltons.

The relatively selective action of cathepsin D on the myosin component of myofibrils is in contrast to the extensive breakdown of this protein which occurs when myofibrils are incubated with trypsin or papain (Rattrie and Regenstein, 1977). Trypsin and papain initially degrade myosin to light and heavy meromyosins, and total rods and globular heads, respectively (Lowey et al., 1969). This attack is followed by more extensive breakdown with time to low molecular weight fragments. This extensive breakdown is reflected in the ESR studies with spin-labeled myofibrils, which, when treated with a series of plant-derived proteases, showed a rapid increase in mobility of the spin label located on the globular head of myosin indicating a change in structure or conformation in the vicinity of the label due to the hydrolysis. Cathepsin D, by contrast, produces essentially no change in the ESR signal associated with the spin label on the myosin head even after incubation for up to 24 hr. This finding suggests that the attack of cathepsin D on the myosin heavy chains occurs at the distal portion of the helical tail region rather than near the globular head of myosin, which binds the F actin filaments, and that attack on the latter protein is limited or does not occur (see below).

The origin of the changes leading to the alteration of Z disk structure when myofibrils are treated with cathepsin D is uncertain, and it is difficult to rationalize this breakdown with the degradation in myofibrillar proteins observed in this study. If changes in Z disk are caused by the degradation of myosin or the disruption of the troponin-tropomyosin complex, it would have to be the result of an indirect effect since these proteins are not located in this region. On the other hand, our data show that little or no degradation of α-actinin, the main protein present in the Z disk region, occurs at 25°C, although there is evidence for some breakdown at 37°C.

Some recent observations on the breakdown of myofibrillar proteins by lysosomal proteases, however, provide a possible explanation for the observed changes in the Z disk structure. Schwartz and Bird (1977) reported that cathepsin D degrades isolated F actin at about 10% the rate of attack on myosin, and Spanier et al. (1977) found that a mixture of lysosomal proteases from muscle degraded actin and myosin in myofibrils. Recent experiments in our laboratory have also demonstrated that lysosomal proteases from spleen degrade actin in myofibrils, and in isolated actomyosin complex (J. Walker, unpublished observations). The breakdown of actin with or without a concomitant breakdown of α-actinin could explain the loss of Z disk structure in myofibrils since it is probable (Stromer and Goll, 1972; Suzuki et al., 1976) that α-actinin binds to the F actin thin filaments of adjacent sarcomeres in this region. We have not observed a loss of actin in myofibrils treated with cathepsin D in this study. However, if the degradation of the F actin thin filaments were to occur initially in the region adjacent to the Z disks as suggested by Penny (1972) and subsequently proceeded toward the distal end of the filaments located near the actin binding sites on the myosin heads, the initial breakdown of a few G actin subunits would result in disruption of the Z disk structure, but their loss would be difficult to detect by SDS-polyacrylamide gel electrophoresis, or from the ESR spectra of spin-labeled myofibrils.

Olson et al. (1977) observed a breakdown of myofibrillar proteins from myofibrils isolated from muscle conditioned at 2°C and 25°C for various times, but reported no degradation of myosin. They observed, as in the present study, a decrease in troponin T, the appearance of a 30,000 dalton fragment which increased with time of conditioning, and little if any breakdown of α-actinin. They concluded on the basis of these results and the similarity of the breakdown of the myofibrillar proteins by the endogenous muscle protease CAF (Dayton et al., 1976) that this enzyme was responsible for the changes occurring during conditioning of muscle. In a similar study, Penny (1974) assessed the effect of CAF on myofibrillar proteins. He found it difficult to reconcile the action of this enzyme on myofibrils with the changes occurring during postmortem conditioning, finding little or no change in α-actinin during conditioning although Z disk structure was reduced or disappeared completely. Since CAF releases α-actinin from myofibrils, the content of this protein should have been significantly reduced during conditioning.

The studies with CAF demonstrated that this endogenous muscle protease can degrade certain of the myofibrillar proteins. The present study, as well as other work referred to above, demonstrates that another endogenous protease, cathepsin D, is also capable of acting on certain myofibrillar proteins. The relative contribution of these two proteases to the postmortem conditioning process is unclear. CAF with is maximum activity at physiological pH (7.5) would not be expected to play a significant role in myofibrillar breakdown under postmortem pH conditions, although cathepsin D which is active under postmortem pH conditions would be expected to play a major role.

Advantage has been taken of the relative selectivity of cathepsin D toward the proteins of the myofibrils by utilizing it as an exogenous meat tenderizer. In a study by Robbins and Cohen (1976) it was demonstrated that enzyme extracts prepared from bovine spleen, a rich source of cathepsin D and other catheptic enzymes which may also degrade muscle proteins were capable of simulating the natural aging of muscle. The texture of muscle treated with these extracts is observed to be similar to that of naturally aged muscle, in contrast to the "mush" texture resulting from the indiscriminate degradation of the myofibrillar proteins by commercial tenderizers containing papain (Robbins et al., unpublished observations). Recent studies in our laboratory (Cohen et al., 1979) have also demonstrated that spleen extracts have a significant effect in increasing the rehydratability and tenderness of pre-cooked, freeze-dried beef slices, which have been subjected to long term storage. These results suggest that spleen extracts have an excellent potential for use as exogenous muscle tenderizers, because of their selectivity of action and their ready availability from spleen, a cheap by-product of the meat industry.

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EFFECTS OF COOKING METHODS ON THE PROTEIN QUALITY OF MEATS AS DETERMINED USING A *Tetrahymena pyriformis* W GROWTH ASSAY

ESSI EVANS, SUSAN C. CARRUTHERS and RALPH WITTY

ABSTRACT

T. pyriformis relative nutritive value (RNV) bioassay can be used to compare protein quality of meats. Experiments were conducted to assess effects of standard cooking procedures on RNV and amino acid composition of various meats. Boiled tissue meats and processed meat showed higher RNV and levels of essential amino acids than fried or microwave cooked or uncooked samples. However, in organ meats, cooking did not change total protein content or total essential amino acid contents relative to uncooked organ meats.

INTRODUCTION

IT HAS BEEN SHOWN that the *T. pyriformis* relative nutritive value (RNV) bioassay can be used in comparisons of the protein quality of meats (Butrum and Kramer, 1977; Evans et al., 1977). This experiment was conducted to assess the effects of standard cooking procedures on the RNV and amino acid compositions of a variety of meats and meat products.

EXPERIMENTAL

Sample preparation

Fresh samples of tissue meats, organ meats and processed meats were cut into cubes with sides 1–2 cm. Subsamples were either fried at 160°C for 3 min, boiled for 10 min, cooked in a microwave oven for 70 sec/100g (700 Watts/hr) or retained raw. Meats were freeze dried and pulverized subsequent to cooking. Aliquots of the dried, powdered samples were rinsed with hexane until all fat was removed, using a Soxhlet apparatus. The protein contents of the fat-extracted samples were determined through the use of the macro-Kjeldahl procedure (AOAC, 1970). Amino acid compositions were determined using a Beckman model 121 Amino Acid Analyzer after hydrolysis with 6N HCl for 22 hr at 110°C.

RNV determination

The stock culture of *T. pyriformis* (*furgasoni*) W was obtained from the American Type Culture Collection, Rockville, Maryland. Cultures were maintained in peptone-tryptone broth and transfered semi-weekly.

The procedure of Stott et al. (1963) was employed in the determination of RNV with the following modifications. The basal media were modified by substituting dextrin for glucose and by elevating the phosphate buffer concentration (Evans et al., 1979). All meat samples were ground to pass through a 0.3 mm screen, and digested for 4 hr at 56°C with papain, using the procedure outlined by Shorrock (1976), prior to inclusion in the media.

Statistical analyses

Treatment means were compared by using a complete block analysis of variance as described by Steel and Torrie (1960). Tukey's ω procedure was used to compare individual treatment means (Steel and Torrie, 1960).

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0022-1147/79/0006-1678$02.25/0
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Table 1—Effects of cooking procedure on the relative nutritive values (RNV) of meats and meat products[a]

Sample	RNV, % of casein control			
	Uncooked	Fried	Boiled	Microwave
Tissue meats				
Beef loin	110.3	101.4	118.5	105.4
Beef rump	121.3	97.3	116.3	111.6
Beef chuck	110.2	87.9	114.6	86.0
Pork loin	109.1	105.5	111.3	106.3
Pork ham, fresh	113.6	112.9	120.1	115.2
Pork shoulder	107.5	110.4	116.2	109.7
$\bar{X}$	112.0b,c	102.6a	116.2c	105.7a,b
Organ meats				
Beef heart	97.8	99.4	113.4	115.3
Beef liver	107.4	94.8	104.6	101.2
Pork heart	110.1	90.4	108.4	105.0
Pork liver	101.9	96.6	98.6	102.2
$\bar{X}$	104.3b	95.3a	106.3b	105.9b
Processed meats				
Salami	74.0	68.2	73.5	70.5
Pepperoni	75.7	69.1	77.5	72.6
Bologna (Product 1)	95.5	97.9	98.8	88.4
Bologna (Product 2)	105.2	106.8	115.6	94.2
Wieners	100.7	92.4	102.1	90.3
Luncheon ham	107.9	110.1	112.9	106.8
Smoked picnic	105.0	83.2	106.7	96.3
Smoked ham	123.5	119.0	122.0	120.9
$\bar{X}$	98.4a,b	93.3a	101.1b	92.5a

[a] Means within each row bearing different letters differ significantly ($P < 0.05$).

RESULTS & DISCUSSION

RNV RESULTS for the various meat samples are shown on Table 1. Tissue meats and processed meats that had been boiled showed higher ($P < 0.05$) RNV than did meats from the same classifications that had been either fried or cooked by microwave. For all categories of meats, samples that had been boiled produced slightly higher RNV than those attained with uncooked samples, but the differences between raw and boiled meats were not statistically significant ($P > 0.05$). Frying reduced ($P < 0.05$) the RNV of tissue and organ meats relative to samples that had not been cooked.

The mean protein contents of boiled tissue and processed meats were higher ($P < 0.05$) than they were for raw samples in the same groupings (Table 2). Because the values shown were determined on dried, fat-extracted samples, differences in protein contents due to cooking could not be attributed either to rendering of fat or to moisture loss. Levels of total essential amino acids (Table 3) were also elevated somewhat with tissue and processed meats that had been boiled as compared to their uncooked counterparts.

It would appear that for meats composed primarily of nonvisceral tissues, boiling increased the removal of low molecular weight, low nitrogen compounds (increasing the percentage of protein) as well as soluble nonamino acid nitrogenous compounds (increasing the proportion of essential amino acids within the remaining crude protein fraction). The data shown on Table 4 is supportive of this

hypothesis. It can be seen that, with tissue meats and processed meats, concentrations of individual nonessential as well as essential amino acids generally increased with boiling.

Table 2—Effects of cooking procedure on the protein contents of meats and meat products[a]

Sample	Protein, % of sample[b]			
	Uncooked	Fried	Boiled	Microwave
Tissue meats				
Beef loin	91.8	92.0	94.3	89.9
Beef rump	90.1	92.1	93.1	92.3
Beef chuck	89.2	88.2	91.9	90.9
Pork loin	90.5	91.7	90.4	92.8
Pork ham, fresh	89.8	89.8	91.4	89.9
Pork shoulder	90.0	90.4	92.4	90.6
$\bar{X}$	90.2b	90.7b,c	92.3c	91.1b,c
Organ meats				
Beef heart	80.9	83.8	84.7	83.9
Beef liver	76.7	76.2	78.6	78.7
Pork heart	82.7	82.5	85.2	84.6
Pork liver	80.7	79.6	78.5	81.0
$\bar{X}$	80.3	80.5	81.8	82.1
Processed meats				
Salami	64.4	64.0	64.6	64.6
Pepperoni	67.0	68.7	65.1	64.3
Bologna (Product 1)	61.5	62.7	66.0	68.8
Bologna (Product 2)	59.0	60.3	61.9	63.1
Wieners	60.0	59.3	63.6	58.6
Luncheon ham	83.6	79.7	84.1	82.3
Smoked picnic	82.0	83.3	88.7	83.6
Smoked ham	85.5	78.5	83.3	82.2
$\bar{X}$	70.4b,c	69.6b	72.2c	70.9b,c

[a] Means within each row bearing different letters differ significantly ($P < 0.05$).
[b] Protein contents of samples as determined after freeze drying and fat extracting.

Table 3—Effects of cooking procedure on the total essential amino acid (TEAA) contents of meats and meat products[a]

Sample	TEAA, % of total protein[b]			
	Uncooked	Fried	Boiled	Microwave
Tissue meats				
Beef loin	45.64	45.31	47.46	45.45
Beef rump	46.39	45.53	46.88	46.62
Beef chuck	44.45	45.28	46.14	46.37
Pork loin	45.51	46.06	46.87	45.95
Pork ham, fresh	43.67	44.55	46.74	46.20
Pork shoulder	43.95	45.89	47.18	45.93
$\bar{X}$	44.94b	45.44b,c	46.88c	46.09b,c
Organ meats				
Beef heart	42.25	43.05	42.48	44.42
Beef liver	42.16	42.45	42.23	42.52
Pork heart	42.77	41.64	42.95	43.58
Pork liver	41.97	41.63	41.91	42.16
$\bar{X}$	42.28	42.19	42.39	43.17
Processed meats				
Salami	36.45	35.78	37.98	35.49
Pepperoni	35.19	35.23	38.84	36.75
Bologna (Product 1)	36.15	36.39	39.65	34.09
Bologna (Product 2)	40.82	37.93	38.97	37.93
Wieners	38.75	36.96	39.02	38.02
Luncheon ham	40.58	42.37	46.47	41.15
Smoked picnic	40.87	42.28	42.51	41.94
Smoked ham	43.32	42.20	41.80	40.73
$\bar{X}$	39.02b,c	38.64b,c	40.66c	38.26b

[a] Means within each row bearing different letters differ significantly ($P < 0.05$).
[b] Totals shown are exclusive of tryptophan and semiindispensible amino acids.

The losses in nonamino acid nitrogen help to explain the improvement in RNV with boiled tissue meats and processed meats relative to those that had been fried and microwave treated. Sample aliquots are added to the media for *T. pyriformis* on a nitrogen weight, rather than on a total weight basis, providing a higher proportion of essential amino acids per unit of nitrogen with the boiled samples. When considered on a before-cooking, sample weight basis, it is likely that no change in protein nutritional value would accompany the boiling of meats. While Donoso et al. (1962) noted a reduction in the nutritive value of pork subsequent to boiling for 24 hr, it is unlikely that meats would be subjected to such drastic cooking procedures.

Cooking did not change ($P > 0.05$) the total protein

Table 4—Effects of cooking procedure on the amino acid compositions of various groups of meats and meat products

Amino acid	Group	Amino acid, % of total protein			
		Uncooked	Fried	Boiled	Microwave
Alanine	Tissue	6.17	6.12	6.28	6.29
	Organ	6.20	6.14	6.16	6.21
	Processed	6.07	6.08	6.33	6.00
Arginine	Tissue	6.82	6.95	7.23	7.17
	Organ	6.91	6.94	6.92	7.02
	Processed	6.07	6.01	6.38	5.96
Aspartic acid	Tissue	9.30	9.43	9.75	9.66
	Organ	9.24	9.13	9.30	9.28
	Processed	8.95	8.90	9.42	8.86
Glutamic acid	Tissue	17.42	17.59	18.10	17.80
	Organ	15.94	15.68	15.77	15.82
	Processed	16.31	16.26	16.99	16.17
Glycine	Tissue	5.04	4.86	4.90	4.86
	Organ	5.79	5.66	5.65	5.68
	Processed	6.50	6.46	6.64	6.38
Histidine	Tissue	4.39	4.17	3.88	4.15
	Organ	2.81	2.79	2.80	2.88
	Processed	3.16	3.12	3.06	3.04
Isoleucine	Tissue	4.50	4.60	4.75	4.68
	Organ	4.05	4.03	4.15	4.21
	Processed	3.95	3.89	4.16	3.89
Leucine	Tissue	8.30	8.43	8.71	8.61
	Organ	8.99	8.96	9.06	9.20
	Processed	7.64	7.62	8.06	7.58
Lysine	Tissue	9.14	9.29	9.58	9.43
	Organ	7.90	7.71	7.94	8.10
	Processed	7.43	7.25	7.67	7.19
Methionine	Tissue	2.96	3.02	3.20	3.05
	Organ	2.77	2.59	2.66	2.68
	Processed	2.31	2.31	2.49	2.32
Phenylalanine	Tissue	4.19	4.33	4.62	4.36
	Organ	4.53	4.74	4.40	4.55
	Processed	4.30	4.27	4.45	4.22
Proline	Tissue	4.57	4.40	4.48	4.90
	Organ	5.10	5.00	5.03	5.02
	Processed	5.16	5.08	5.26	5.21
Serine	Tissue	4.45	4.45	4.57	4.55
	Organ	4.99	4.88	5.04	5.01
	Processed	4.34	4.33	4.61	4.36
Threonine	Tissue	4.62	4.65	4.89	4.79
	Organ	4.46	4.44	4.49	4.53
	Processed	4.17	4.17	4.37	4.07
Tyrosine	Tissue	3.60	3.65	3.76	3.66
	Organ	3.56	3.46	3.52	3.62
	Processed	3.19	3.17	3.39	3.18
Valine	Tissue	4.93	4.90	5.06	5.03
	Organ	5.39	5.28	5.49	5.42
	Processed	4.19	4.23	4.41	4.16

contents or the total essential amino acid contents of the organ meats, relative to the uncooked organ meats. The lack of differences between cooked and raw organ meats with respect to these parameters indicates that the nitrogen fraction is probably lower in readily soluble components than it is with striated muscle tissue. Thus, it is most likely because the amino acid profiles of the sample aliquots included in the media for *T. pyriformis* did not change, that RNV was not altered when the organ meats were either boiled or cooked by microwave.

The noticable reduction in the RNV of meats with frying indicates that this cooking procedure damages a portion of the protein. The data in Table 4 emphasize the fact that such damage to protein quality cannot be detected from the amino acid compositions of foodstuffs, and illustrates the sensitivity of the RNV assay. Bodwell and Womack (1978) noted losses in the protein nutritional value of some foods with cooking that were detectable with rat feeding experiments but not with the amino acid profiles of the foods. The surface damaged by the short frying time with these small samples would be comparable to that affected by longer frying times with larger samples.

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Ms received 3/16/79; revised 6/13/79; accepted 7/1/79.

We acknowledge the technical assistance of Mr. John Kostick during this investigation.

RATE OF pH DECLINE IN BEEF MUSCLE STORED AT ABOVE- AND BELOW-FREEZING TEMPERATURES

R. J. WINGER, O. FENNEMA and B. B. MARSH

ABSTRACT

A highly significant linear relationship exists between pH decline and lactic acid accumulation during development of rigor mortis in beef sternomandibularis muscle at both −3°C and 15°C. No acceleration of the "overall" rate of rigor development was observed in these muscles as the storage temperature was reduced from 0 to −3°C. It was observed, however, that the rate of rigor development over the first 5–7 hr of storage was greater at both 0 and −2°C than at 15°C. The complex, nonlinear rates of pH decline, especially at 0 and −2°C, were mathematically evaluated using spline analysis to define the rates of rigor development during storage.

INTRODUCTION

DESPITE the current use of freezing as a means of preserving meat, and the knowledge that a variety of changes can occur in biological systems at high subfreezing temperatures (Fennema et al., 1973), surprisingly little work has been done on changes occurring in meat during freezing and frozen storage. An important area of concern is that of rigor development in frozen meat. Although rigor mortis is known to occur in beef at high subfreezing temperatures (Marsh and Thompson, 1958; Partmann, 1963; Behnke et al., 1973) there is some disagreement as to the rate of rigor development. The reasons for this disagreement are partly attributable to differences in experimental procedures and partly to the various parameters measured (e.g., pH, lactic acid, ATP) at high subfreezing temperatures. Although it has been conclusively shown that predictable chemical and physical relationships exist for all types of rigor development at above-freezing temperatures (Bendall, 1973), it is not necessarily valid to assume that chemical relationships occurring in nonfrozen meat will remain unchanged in frozen meat.

The purposes of this investigation were to establish the relationship between changes in pH and lactic acid during rigor development in beef muscle at −3°C and to evaluate some possible reasons for discrepancies in rates of rigor mortis observed in beef muscle held at high subfreezing temperatures.

EXPERIMENTAL

Procedures involving the relationship between pH and lactic acid at −3°C

The right and left sternomandibularis muscles each 15–30 cm long and 150–200g were excised from four cutter cows (2–5 yr of age) 30–60 min after animal death, wrapped in aluminum foil and frozen in crushed dry ice (solid carbon dioxide, −78°C) for 45–75 min. Samples cooled from 0 to −5°C (center temperature) in less than 8 min, and ultimately attained temperatures of −75 to −78°C. These muscles were roughly cracked (by striking them sharply with a metal object) into six pieces (15–30g each) which were individually vacuum-packaged in double 4-mil polyethylene bags and stored at −3 ± 0.2°C in an agitated ethanol-water bath.

At appropriate times, samples were removed from storage, unpackaged and immediately immersed in liquid nitrogen for at least 20 min. Each sample, together with about 200 ml liquid nitrogen, was ground to a fine powder in an Osterizer blender. Both pH and lactic acid were determined on the resulting powder.

Lactic acid was enzymatically determined according to Bergmeyer (1974). The reported lactic acid value for a given muscle sample is the mean of triplicate assay determinations on duplicate samples of pulverized muscle powder (i.e. the mean of six determinations).

Muscle pH was measured according to the recommendations of Bendall (1973). The reported pH value is a mean of the pH readings on triplicate 0.5–1g samples of muscle powder, each homogenized in a separate 10-ml volume of iodoacetate-KCl solution (5 mM iodoacetate, 0.15M KCl, pH 7) and measured at room temperature.

Procedures involving the effect of storage temperature on rate of pH decline

The right and left sternomandibularis muscles were excised from five cutter cows (2–5 yr of age) approximately 30 min after animal death. The muscles were packaged in polyethylene bags and immediately transferred to a water bath at 15 ± 1°C for transportation to the Department of Food Science.

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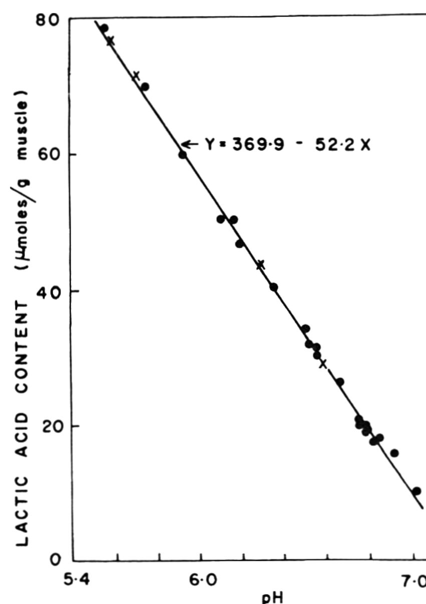

Fig. 1—Relationship between lactic acid content and pH of beef sternomandibularis muscles stored at −3°C. Each lactic acid value is a mean based on duplicate samples of pulverized muscle powder, each assayed in triplicate (i.e. a mean of six determinations). Each pH value is a mean of triplicate determinations based on three samples of pulverized muscle powder. Five animals were used in this study. Correlation coefficient for the regression line is 0.994. Data for −3°C storage (•) and 15°C storage (x) are superimposed on the regression line.

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0022-1147/79/0006-1681$02.25/0
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Upon arrival at the Department of Food Science (30–45 min post-excision), each muscle pair was trimmed of fat and connective tissue and cut into about 28 pieces (each 5–10g). Samples stored above freezing were immediately vacuum-packaged in double 4-mil polyethylene bags and transferred to either an agitated water bath at 15 ± 0.2°C, or an ice-water bath at 0°C. Samples to be stored below their freezing points were immediately immersed in liquid nitrogen for 15 min. Individual pieces were vacuum-packaged in double 4-mil polyethylene bags and transferred to agitated ethanol-water baths at either −2 ± 0.2°C, or −3 ± 0.2°C.

The muscle pH was measured at room temperature following homogenization of either 0.5–1g (for samples stored at 15 or 0°C) of muscle in 10 ml of iodoacetate-KCl solution or 5g (for samples stored at −2 or −3°C) of muscle in 50 ml of iodoacetate-KCl solution.

RESULTS & DISCUSSION

Relationship between pH and lactic acid content of beef sternomandibularis muscles at −3°C

A plot of pH vs lactic acid content in beef muscles held at −3°C is shown in Figure 1. The correlation coefficient of 0.994 indicates that a highly significant relationship exists between pH and lactic acid content of beef sternomandibularis muscle stored at −3°C.

In a separate study involving a single animal, it was found that the relationship between pH and lactic acid (both slopes and absolute values) at 15°C was identical to that at −3°C. Thus, relationships between lactic acid and pH derived from beef sternomandibularis muscles at 15°C

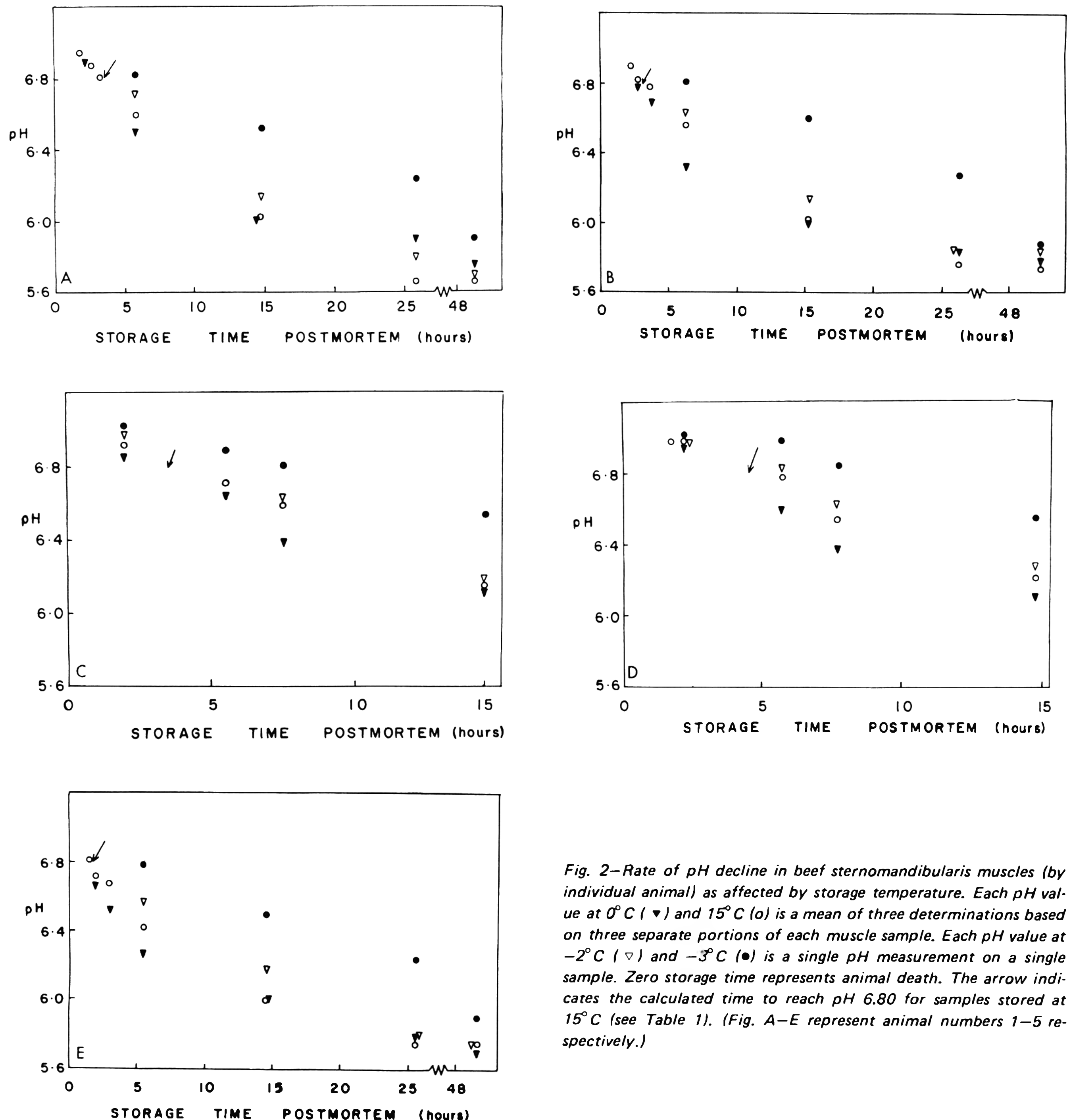

Fig. 2—Rate of pH decline in beef sternomandibularis muscles (by individual animal) as affected by storage temperature. Each pH value at 0°C (▼) and 15°C (o) is a mean of three determinations based on three separate portions of each muscle sample. Each pH value at −2°C (▽) and −3°C (●) is a single pH measurement on a single sample. Zero storage time represents animal death. The arrow indicates the calculated time to reach pH 6.80 for samples stored at 15°C (see Table 1). (Fig. A–E represent animal numbers 1–5 respectively.)

can be justifiably applied to similar muscles stored at −3°C, or vice versa. Reported discrepancies with respect to the temperature dependence of various physiological changes (e.g. Partmann, 1963; Behnke et al., 1973) cannot, therefore, be attributed to a temperature dependence of the pH-lactic acid relationship.

Rates of pH decline as affected by storage temperature

The pH decline in muscles from five animals stored at various temperatures are shown in Figure 2 (A–E). It is clear that the general patterns of pH decline with time of storage are similar for all muscles. The results, however, are sufficiently different, quantitatively, that combination of the data from Figure 2 (A–E) is not feasible. This situation is not surprising since muscles from different animals differ with respect to time postmortem at which the rapid pH decline commences. It was found, however, that the pattern of pH decline among muscles from the five animals at any given storage temperature was almost identical if the lag phase of the pH decline was ignored and the steep portions of the curves were superimposed by lateral movement of the plots (for example, the 15°C storage curves would be simply "moved" along the abscissa axis until the data intersected at, say, pH 6.80). Knowing this, it was considered appropriate to regard the 15°C data for each animal as the "control" data, and to determine the time at 15°C for each animal to reach an arbitrary pH value of 6.80. This time was denoted as "zero storage time" and was calculated using polynomial regression analysis (pH 6.80 was chosen because this was the highest pH value which could be interpolated for every animal from the experimental data). These postmortem times for each of the five animals, shown in Table 1, were then used to adjust the experimental data for each animal at each storage temperature. Using animal #1 and 0°C as an example, the adjustment was made by subtracting the time to reach pH 6.80 at 15°C (3.33 hr) from all sampling storage times at 0°C. Note that by using this correction technique, only the 15°C data intersect pH 6.80 at "zero storage time." The pH values of muscle samples from animal #1 at "zero storage time" were

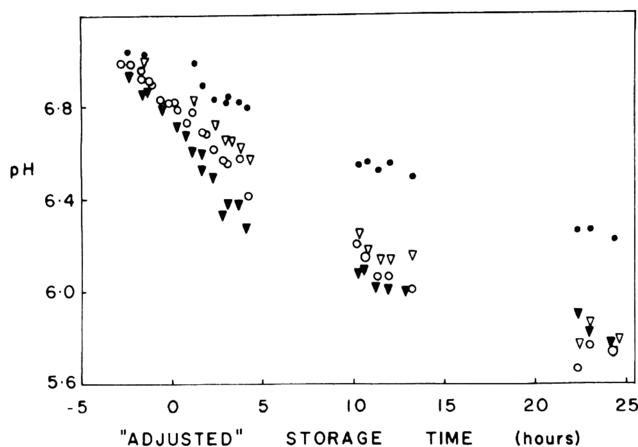

Fig. 3—Standardized data for rates of pH decline in beef sternomandibularis muscles as affected by storage temperature. The storage times for each animal in Fig. 2 (A–E) were adjusted in accordance with the postmortem times at 15°C required for samples from a given animal to reach pH 6.80 (Table 1). Thus, "zero storage" time in this figure represents the calculated time for samples from each animal stored at 15°C to reach pH 6.80. Each pH value at 0°C (▼) and 15°C (o) is a mean of triplicate determinations based on three separate portions of each muscle. Each pH value at −2°C (▽) and −3°C (•) is a single pH measurement on a single sample. The combined data from five animals are shown in this figure.

Table 1—Postmortem times required for beef sternomandibularis muscles at 15°C to reach pH 6.80[a]

Animal no.	Figure no.	Time to reach pH 6.80 (15°C) (hr)
1	2 A	3.33
2	2 B	3.14
3	2 C	3.66
4	2 D	4.58
5	2 E	1.26

[a] Determined from polynomial regression lines calculated to fit data shown in Figure 2 (A–E).

approximately 6.91 at −3°C, 6.87 at −2°C, 6.77 at 0°C, and 6.80 at 15°C. Following this standardization procedure, all data at a given temperature were pooled and the results appear in Figure 3. To assist in perceiving trends in these data, visually determined lines of best fit were drawn through the data and are shown in Figure 4.

It is well-known that plots of pH vs time, over the entire time of pH decline postmortem, are usually nonlinear regardless of the plotting technique used (Bate-Smith and Bendall, 1949). Attempts to attain linear plots for all, or part, of the postmortem period have in fact led to diverse results and considerable confusion in the literature. A far better approach is to use matrix mathematics to indicate changes in the first differential of the data—a technique called 'spline analysis.' This technique (Reinsch, 1967, 1971) does not involve an assumption that the data fit a particular equation. Thus, the differential is calculated sequentially between adjacent pairs of data (pH values at two adjacent sampling times) and does not depend on choosing a suitable equation to describe the entire set of data.

The mathematical bases for the computer program are described by Reinsch (1967) and Wahban and Wold (1975). Briefly, given a set of data (x_i, y_i), $i = 0, ..., n$, and $x_0 < x_1 < .. < x_n$, the spline function $f(x)$ to be constructed will,

(1) minimize $I = \int_{x_0}^{x_n} g''(x)^2 \, dx$ among all functions $g(x)$ such that

(2) $J = \sum_{i=0}^{n} \left(\frac{g(x_i) - y_i}{\delta y_i} \right)^2 \leqslant S$

for $\delta y_i > 0$ and $0 \leqslant S > \infty$.

The function I is a measure of the smoothness of the

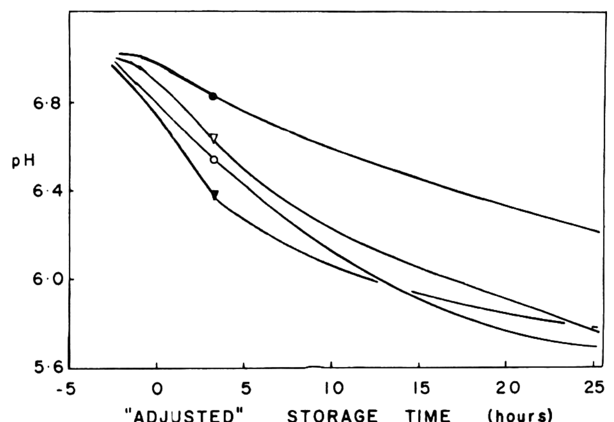

Fig. 4—Data from Fig. 3 approximated by lines (visually determined): ○, 15°C; ▼, 0°C; ▽, −2°C; ●, −3°C.

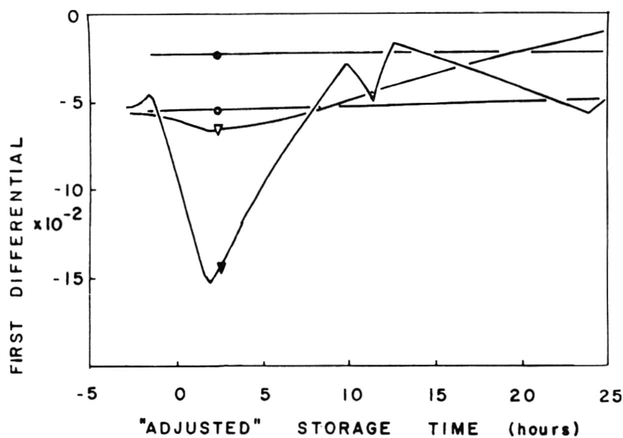

Fig. 5—Changes in the first differential calculated from the rates of pH decline in beef sternomandibularis muscles stored at various temperatures. First differentials were calculated from the data in Fig. 3 using spline analysis (described in the text). Zero time represents the calculated time required for samples stored at 15°C to reach pH 6.80: ○, 15°C; ▼, 0°C; ▽, −2°C; ●, −3°C.

spline function. The function J is a measure of the proximity of the spline function to the discrete values x_i and y_i. The value δy_i accounts for the uncertainty (error) in the y_i data. S is a measure of the "stiffness" of fit of the spline to the data. For example, large values of S (e.g. 1000) approximate a straight line, whereas small values of S (e.g. less than 1) approximate a spline which passes through every point in a very jagged pattern. For any set of data, there is only one S value which results in the best representation of the data. This unique S value is calculated from the equations above—in effect these equations minimize the mean square error (sum of the squares of the difference between actual and calculated data) of the data. For any given S value (unless S values are large (e.g., 1000)) there is often no single equation that will represent all the data, i.e. there may be sub-sections of the data that conform to linear, curvilinear, asymptotic, or even sinusoidal curves. The spline analysis results will, however, indicate the point at which the data change from one type of relationship (e.g. linear) to another (e.g. curvilinear).

The first differentials of the data shown in Figure 3 appear in Figure 5. The first differentials are constant for the −3°C and 15°C data (S value 500), indicating that both sets of data do, in fact, conform to a straight-line relationship over the entire time studied, and that the rate of pH change is approximately constant for the period of study.

At −2°C (S value 104), the initial rate of pH decline (up to about 7−9 hr beyond "zero storage time" in Fig. 5) is actually greater than the rate of pH decline at 15°C. This rate of pH decline increases up to about 2−4 hr beyond "zero storage time" and then decreases substantially and progressively so that the −2 and 15°C rates coincide at 8−9 hr beyond "zero storage time." Thereafter, the rate of pH decline at −2°C is less than that at 15°C.

Rates of pH decline at 0°C (S value 190) do not follow an orderly pattern. The very large increases in rate during the first 4−5 hr of storage at 0°C (starting with −2 hr storage in Fig. 5), followed by the large decreases in rate during the 2−10 hr storage period in Figure 5, are particularly noteworthy. It is also interesting that during most of the early storage period, the sample held at 0°C exhibited a much greater rate of pH decline than the sample at 15°C. It is obvious that single rates should not be used to characterize pH declines in muscles held at 0°C, or −2°C.

The greater rate of pH decline at 0°C than at 15°C could be related to the phenomenon of "cold shortening." This expression was coined by Locker and Hagyard (1963) who observed that excised muscles of prerigor beef sternomandibularis contracted dramatically at temperatures near 0°C. In contrast to "rigor shortening," which occurs in muscles held at all temperatures of storage above freezing, cold shortening commences in muscle very early postmortem (ATP level is still quite high) upon exposure to chilling temperatures (Locker and Hagyard, 1963; Cassens and Newbold, 1966; Buege, 1975; Jeacocke, 1977). This early postmortem shortening is reflected in a concomitant decline in the amount of ATP present in the muscle, an accumulation of lactic acid and a decline in the pH. The cold-shortening phenomenon in beef sternomandibularis muscle is completed within about 2−4 hr storage at0 −5°C (Cassens and Newbold, 1966; Buege, 1975). Upon completion of this shortening, the turnover of ATP is reduced, causing the rate of decline in pH to diminish. These chemical changes have been described in detail by Newbold (1966) and Newbold and Scopes (1967).

It is interesting that, during the early storage period, the rates of pH decline at 0°C and −2°C are greater than the rate at 15°C (Fig. 5). This is in accord with events associated with cold shortening. At −3°C, the rate-depressing effect of the low temperature, per se, apparently overcomes the effects of cold shortening.

Reactions representing the progress of rigor mortis (pH decline, lactic acid accumulation, ATP depletion) in frozen muscle tissues have been studied in a variety of animals by various investigators. In beef, Partmann (1963) reported that the rates of ATP depletion in extensor digitorum muscles decreased sharply as the temperature was lowered from 0°C to −8°C. On the other hand, Behnke et al. (1973) studied beef sternomandibularis muscles and observed that rates of ATP depletion and lactic acid accumulation were greater at temperatures between 0°C and −3°C than at higher and lower temperatures.

In tissues other than beef, equally divergent results have been obtained. Smith (1929a, b) and Smith and Moran (1930) studied frog muscle and observed accelerated rates of lactic acid accumulation at temperatures of −1°C to −4°C. Similar accelerations have been observed in chicken by Behnke et al. (1973) and Khan et al. (1963) and in fish muscles by Sharp (1934, 1935), Bito and Amano (1960), Partmann (1963), Tomlinson et al. (1963), and Nowlan and Dyer (1969). Other studies, however, failed to indicate a tendency for the rate of lactic acid accumulation to accelerate at temperatures near freezing [Partmann (1963) with chicken muscle; Vickery (1930) with rat muscle]. It is difficult to adequately compare all these data because of the variety of species and muscles used. In some instances, however, it is clear that discrepancies do exist between studies involving the same species and muscle (e.g. Behnke et al., 1973, and this study), indicating that the reasons for these discrepancies are not necessarily related to the muscle or specie used.

A possible cause for these discrepancies is the method used to evaluate rates. As shown in this report, use of a single expression for the rate of rigor mortis development (especially at 0°C and −2°C) is a questionable procedure and may lead to inappropriate conclusions.

A second possible cause for the discrepancies in the rate of rigor development is related to the phenomenon of "thaw shortening" ("thaw rigor"). A muscle which is frozen prerigor and then rapidly thawed undergoes pronounced shortening, rapid depletion of ATP, rapid accumulation of lactic acid and a corresponding rapid decline in pH (see Newbold, 1966). Acceleration of these activities is much greater during thaw shortening than during cold shortening. Muscles which are frozen prerigor and then held

at high subfreezing temperatures reportedly undergo all the aforementioned changes associated with thaw shortening and cold shortening, except that contraction of the muscle cannot occur because of the ice crystal matrix (Marsh and Thompson, 1958; Lawrie, 1968).

It is possible that researchers who observed accelerated rates of rigor mortis development at high subfreezing temperatures may have used treatments or conditions that triggered the thaw shortening phenomenon. Those researchers, on the other hand, who did not observe this acceleration phenomenon at high subfreezing temperatures may have induced cold shortening but not thaw shortening in their experiments. This possibility is difficult to evaluate because of the paucity of information concerning the two phenomena.

In separate studies (data not shown) it was found that patterns of pH decline in beef sternomandibularis muscles at 15°C and −3°C were not affected in any consistent manner by animal age (about 2–5 yr) time postmortem (45–100 min) at which freezing commenced, or rate of freezing (dry ice vs liquid nitrogen freezing treatments), although variations in animal age and freezing treatments tended to increase the variability of the results.

CONCLUSIONS

THIS STUDY, along with the results of Behnke et al. (1973), indicate that various chemical processses which occur during the development of rigor mortis at temperatures above freezing (0–37°C) also occur at high subfreezing temperatures. Excellent relationships among pH, lactic acid and ATP exist at high subfreezing temperatures, just as they do at nonfreezing temperatures. Thus, the standard approaches used to evaluate rigor development at nonfreezing temperatures can also be used at subfreezing temperatures.

There is some disagreement in the literature as to the rate of rigor development in beef muscle at high subfreezing temperatures. This is not too surprising since the apparent rate is highly dependent on the method used to define it. Moreover, the importance of cold shortening, thaw rigor, freezing rate and freezing depth may differ from one study to another and these factors may have a large influence on the observed rate of rigor development.

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Ms received 10/23/78; revised 6/5/79; accepted 7/1/79.

Research supported by the College of Agricultural and Life Sciences, University of Wisconsin, Madison, WI.

IONIC, pH, AND TEMPERATURE EFFECTS ON THE BINDING ABILITY OF MYOSIN

D. G. SIEGEL and G. R. SCHMIDT

ABSTRACT

The peak force required to separate pieces of meat at a binding junction was measured with the Instron Universal Testing Machine. These measurements were used to study the effects of salt, phosphate, pH, cooking temperature and protein concentration on the ability of crude myosin to bind meat pieces. Results showed salt and phosphate to linearly increase the ability of myosin to bind meat pieces primarily by solubilizing the protein. Temperature and the level of protein had increasing effects on binding ability in the range 45–80°C and 0–8%, respectively. Scanning electron micrographs of the ultrastructure of gels formed by myosin showed a three dimensional network of overlapping fibers in the presence of salt and phosphate and a sponge-like network in their absence. Since the ultrastructure of the gel formed by myosin heavy chains was comparable to that of myosin, it was concluded that the heavy chain core of the myosin molecule plays an important role in the heat-induced gelation of myosin. The relationship between the formation of a three dimensional network of fibers by the heavy chains of myosin and the mechanism of binding meat pieces was discussed.

INTRODUCTION

BINDING PROPERTIES in meat products are produced by extracting protein from the meat to serve as a binder between the meat pieces. Myosin has been shown to be the most capable of the muscle proteins for developing adequate binding properties in not only sectioned and formed meat (Macfarlane et al., 1977; Ford et al., 1978; Siegel et al., 1978a,b) but emulsified and comminuted sausage products as well (Fukazawa et al., 1961a,b,c; Samejima et al., 1969; Nakayama and Sato, 1971a,b,c). Extracting myosin from the muscle requires salt and phosphate levels of at least 2% and 0.3%, respectively. Therefore, adding myosin as the binding agent offers a method for improving binding quality without using the high levels of salt and phosphate.

Ford et al. (1978) have shown that when crude myosin or a mixture of crude myosin and sarcoplasmic proteins, with little or no added salt, are used as the binding agent in restructured meat products, adequate binding properties are produced. They showed that panelists prefer the texture of restructured steaks prepared with a myosin binder.

Macfarlane et al. (1977) compared the binding strengths of myosin, actomyosin and a mixture of myosin with sarcoplasmic proteins. They found that the mixture exerted the highest binding strength in the absence of salt and that the binding strength of the myosin was greatest in the presence of salt up to 1M. At concentrations greater than 1M the binding strength of myosin was not significantly higher than that of actomyosin.

Earlier work in this laboratory compared the binding strength of crude myosin with that of a nonprotein control, a muscle homogenate and a muscle homogenate freed of sarcoplasmic protein and fat. It was found that crude myosin had the greatest binding strength and that under the same extracting conditions crude myosin fractions with higher mole ratios of myosin to actin possessed higher binding strengths (Siegel and Schmidt, 1979). This work led to an interest in studying physical and chemical effects on the molecular properties of myosin and how this relates to its ability to bind meat pieces.

The objective of this study was to determine the effects salt, phosphate, pH, cooking temperature and protein concentration have on the ability of crude myosin to bind together meat pieces. The effects of salt and phosphate on the ultrastructure of the gel formed by myosin and how this relates to the mechanism of the binding between meat pieces will also be discussed.

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0022-1147/79/0006-1686$02.25/0
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MATERIALS & METHODS

Crude myosin preparation

Crude myosin was extracted from prerigor bovine trapezius muscle according to Siegel and Schmidt (1979) using the Guba-Straub solution (0.3M KCl, 0.15M K-phosphate, pH 6.5). Briefly, 400g muscle was homogenized with 1200 ml of the extracting solution in a Waring Blendor and centrifuged at 1000 × G for 1 hr. The supernatant was retrieved and diluted with water to precipitate the myosin which was collected by centrifugation (12,000 × G for 1 hr).

After collecting the crude myosin, it was divided into three portions and the pH was carefully adjusted to 6, 7, or 8 using cold 0.2M NaOH or 0.2M HCl. Salt, phosphate and protein were adjusted to give levels of 0, 2, 4, or 6% for salt, 0 or 0.5% for phosphate and 3% for protein. The salt and phosphate were dissolved in water and the pH was adjusted prior to their addition in order to prevent any effect they would have on the pH of the protein. In order to examine the effects of salt, phosphate and pH, a 4 × 2 × 3 factorial arrangement of treatments was established with the four levels of salt (0, 2, 4 and 6%), the two levels of sodium tripolyphosphate (0 and .5%) and the three levels of pH (6, 7 and 8).

The effect of temperature was determined by measuring the binding ability of a crude myosin preparation containing 5% protein, 3.5% NaCl and 0.5% sodium tripolyphosphate. Binding was initiated by cooking to internal temperatures of 40, 45, 50, 55, 60, 65, 70, 75, 80, 85, 90 and 95°C.

The effect of protein concentration was determined by measuring the binding ability of crude myosin containing 3.5% NaCl and 0.5% sodium tripolyphosphate over protein concentrations of 0, 2, 4, 6 and 8%.

Myosin, actomyosin and myosin heavy chain preparation

Myosin was prepared according to Perry (1955) with the Hasselbach-Schneider extracting solution (0.6M KCl, 0.1M potassium phosphate, 1.0 mM $MgCl_2$, 10 mM pyrophosphate, pH 6.4). After precipitating the crude myosin, it was further purified by removing the actomyosin at an ionic strength of 0.3μ and reprecipitating the myosin twice.

The actomyosin used in these studies was obtained from the actomyosin removal step described above.

Heavy chains were isolated from twice precipitated myosin prepared as described above. The procedure of Gaetjins et al. (1968) was used to separate the light and heavy chains of myosin. Briefly, a myosin solution of 0.5% protein was incubated at 0°C for 30 min with 2 mM ATP, 1 mM DTT, 0.1M KCl, 2M LiCl, 0.1M glycine buffer, pH 11.1. The solution was made to 0.8M potassium citrate by adding 2.5M potassium citrate, pH 7.0. This precipitated the myosin heavy chains which were collected by centrifuging at 12,000 × G for 20 min. They were washed twice with 10 volumes of distilled water and twice with 10 volumes of 0.04M KCl and col-

lected by centrifuging at 12,000 x G for 45 min. At the appropriate steps during the preparation of the heavy chains, samples of actomyosin and twice precipitated myosin were saved. Protein was measured in each of the preparations with the Kjeldahl method. Binding abilities and gel ultrastructures of each protein at a concentration of 3% were examined in the presence and absence of 6% NaCl and 2% sodium tripolyphosphate.

Binding ability

Binding ability was measured on each binder as described by Siegel and Schmidt (1979).

Scanning electron microscopy

Scanning electron microscopy was used to examine the effect of salt and phosphate on the ultrastructure of gels formed by myosin, and to compare this with the ultrastructure of gels formed by the heavy chains of myosins. The gels were formed by immersing test tubes containing 3g of the 3% protein solution into a 90° water bath for 10 min. The gels so formed were cryofractured and prepared for scanning electron microscopy using the procedure outlined by Theno et al. (1978).

Statistical analysis

All effects of the various treatments on binding abilities were determined by analysis of variance for a factoral arrangement of treatments in a completely randomized design. (Steel and Torrie, 1960).

RESULTS & DISCUSSION

Effect of salt, phosphate and pH

Salt and phosphate both linearly increased binding ability ($P < 0.0001$) while pH was not found to have any significant effect (Table 1). Salt was expected to increase binding ability due to its ability to dissolve myosin by enhancing electrostatic repulsions (Hamm, 1960) and to dissociate myosin aggregates (Huxley, 1963). These effects would increase the number of molecular interactions initiated by heating resulting in the formation of a firmer gel. Also, the ability of salt to increase the water-holding capacity of both the myosin binder and the muscle surfaces being bound minimizes the negative effect free water has on binding.

Hamm (1970) and Brotsky and Everson (1973) attribute the effect of phosphate on muscle protein to increased pH, increased ionic strength and a specific polyphosphate protein interaction. However, the effect of polyphosphate on pH was eliminated in this experiment and the increase in binding ability was greater than would be expected due to the increased ionic strength offered by 0.5% sodium tripolyphosphate. Therefore, the increased binding strength of myosin incurred by polyphosphate may be attributed primarily to the specific interaction between polyphosphate and the protein. This effect causes increased myosin solubility by dissociating the actomyosin contaminating these crude myosin preparations.

The absence of any significant effect of pH on binding ability may be explained by the buffering effect of the muscle surfaces on the myosin binders. Before the binders are measured for their binding ability, they are allowed to equilibrate with the muscle. Therefore, the buffering capacity of the muscle could have eliminated any differences in pH.

Effect of temperature

The ability of myosin to bind meat pieces was absent at temperatures lower than 45°C. Increasing temperature up to 80°C had a linear effect ($P < 0.01$) on binding ability (Fig. 1). These results are comparable to those of Acton

Table 1—Effects of salt, phosphate and pH on the binding ability (B.A.) of crude myosin (3% protein)

Level of salt (%)	B.A.[a] (g)	Level of phosphate (%)	B.A.[b] (g)	Level of pH	B.A.[c] (g)
0	46.6	0	67.3	6	81.1
2	41.2	0.5	102.5	7	92.8
4	106.5			8	80.9
6	145.4				

[a] Values are the mean of two replicates averaged over the levels of phosphate and pH. Standard error of the mean = 8.70.
[b] Values are the mean of two replicates averaged over the levels of salt and pH. Standard error of the mean = 6.16.
[c] Values are the mean of two replicates averaged over the levels of salt and phosphate. Standard error of the mean = 7.54.

Table 2—Comparisons of myosin heavy chains, myosin and actomyosin for their binding abilities (B.A.) in the presence and absence of salt and sodium tripolyphosphate

Protein (3%)	B.A. (g) 6% NaCl, 2% STP	B.A. (g) 0% NaCl, 0% STP
Heavy chains	250	0
Myosin	253	68
Actomyosin	306	60

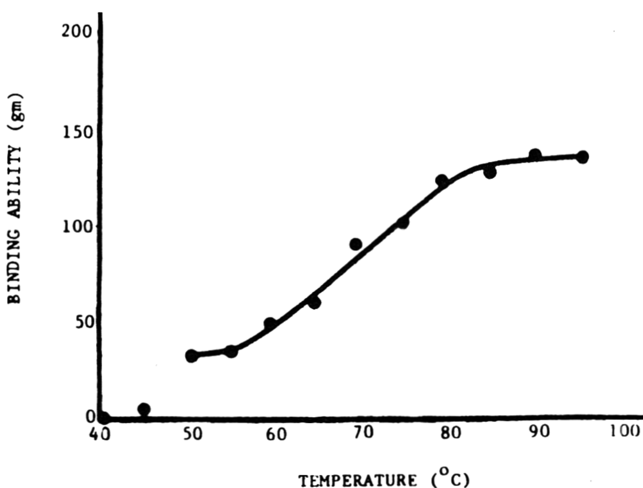


Fig. 1—Effect of the final internal temperature on the binding ability of crude myosin (5% protein, 3.5% NaCl, and 0.5% sodium tripolyphosphate).

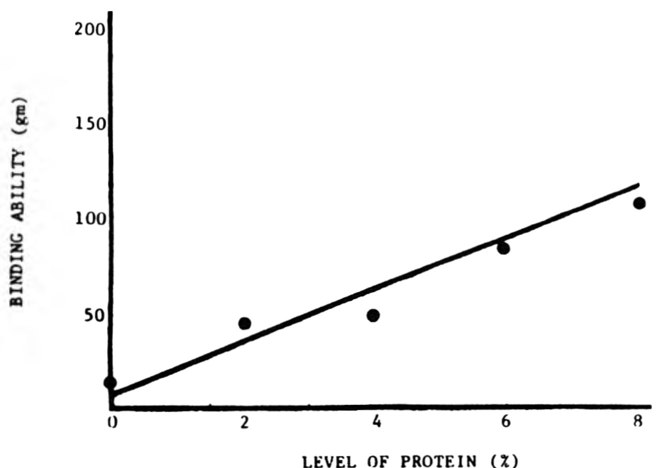
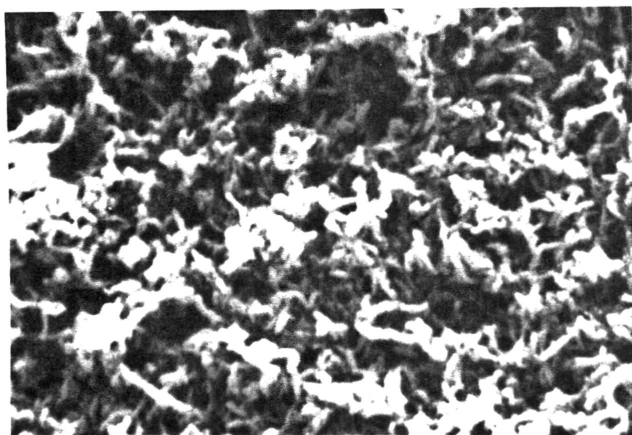
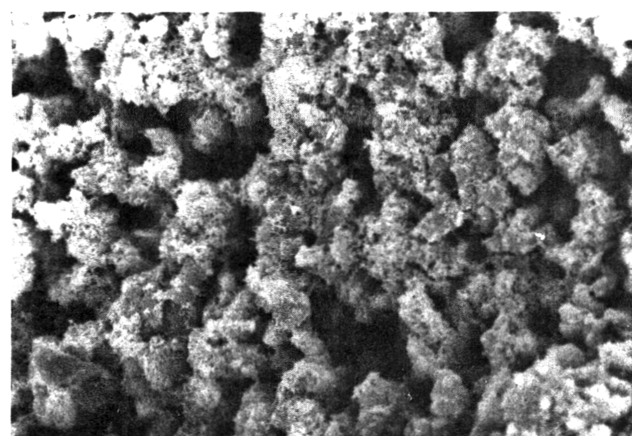
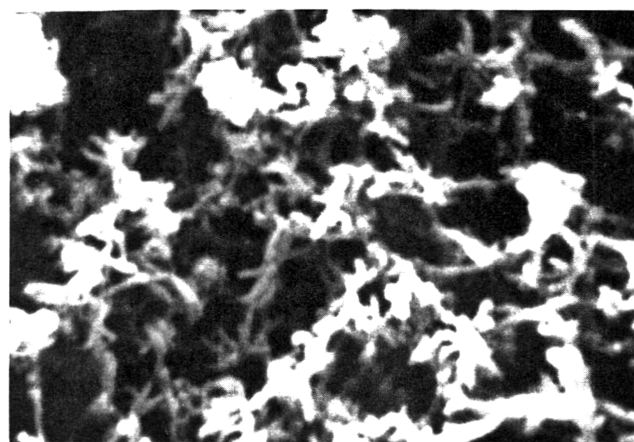


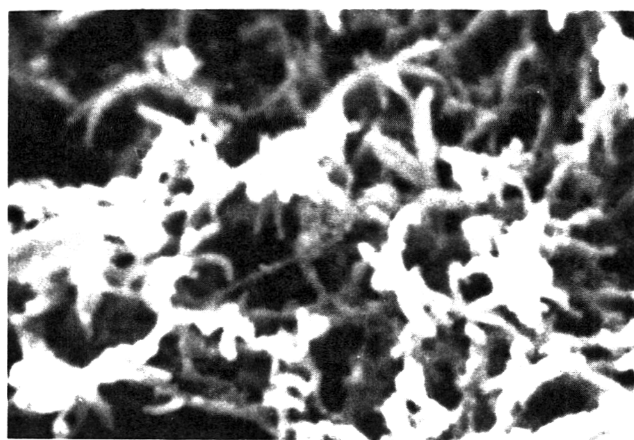
Fig. 2—Effect of the level of protein on the binding ability of crude myosin (3.5% NaCl, 0.5% sodium tripolyphosphate).





Figs. 3&4—Scanning electron micrographs showing the ultrastructure of 3% protein gels formed by myosin in the presence (Fig. 3, top, magnification ≈6200X) and absence (Fig. 4, bottom, magnification ≈1200X) of 6% NaCl and 2% tripolyphosphate.





Figs. 5&6—Scanning electron micrographs of the ultrastructure of 3% gels formed in the presence of 6% NaCl and 2% tripolyphosphate by myosin heavy chains (Fig. 5, top, magnification ≈6200X) and by actomyosin (Fig. 6, bottom, magnification ≈6200X).

(1972) who found that the binding strength of poultry loaves increases in the range 35–82°C.

Differential scanning calorimetry studies by Wright et al. (1977) showed that at high ionic strength (1.0μ) myosin undergoes transitions at temperatures of 42°C, 49.5°C and 60.5°C. These transition temperatures reflect conformational changes occurring in myosin which free reactive groups to allow their interaction with reactive groups on other myosin molecules. The result is an increasing number of gel stabilizing molecular interactions. However, binding strength increases at higher temperatures than the last transition temperature of myosin. Therefore, the ability of myosin to bind meat pieces is dependent upon the effect heating has on the proteins constituting the muscle surfaces being bound in addition to its effect on myosin.

Effect of protein concentration

Figure 2 shows that the level of protein has an increasing effect on the binding ability of crude myosin. This effect was found to be linear ($P < 0.01$). These results may be attributed to a tighter matrix of interweaving fibers formed from the higher concentration of protein.

Gel ultrastructure

The binding abilities of myosin heavy chains, actomyosin and myosin were not found to be significantly different, although they were increased ($P < 0.001$) by the presence of salt and phosphate (Table 2). By examining the ultrastructure of the heat-set gels formed by myosin with and without salt and phosphate, the effect of salt and phosphate may be explained on the basis of molecular interactions. The structure of a gel formed by crude myosin in the presence of salt and phosphate (Fig. 3) shows that the protein has coagulated to form a three-dimensional network of protein fibers typical of protein gels as described by Paul and Palmer (1972) and Fennema (1976). This three dimensional network of protein fibers contributes to the harboring of water as well as the strength of the gel due to the occurrence of a greater number of molecular interactions. When salt and phosphate are absent, myosin coagulated to form a spongelike framework atypical of protein gels (Fig. 4). This type of structure is less suited to harbor water or attain an appreciable gel strength. Thus, the solubilization of the protein, as produced by salt and phosphate is required to allow for the molecular interactions that are initiated by heating.

Figure 5 shows the ultrastructure of a gel formed by myosin heavy chains. This structure is comparable to that formed by myosin (Fig. 3) as evidenced by its three dimensional network of interweaving filaments. The ultrastructure of actomyosin (Fig. 6) also showed the three dimensional network of protein fibers. These similarities in gel structure help explain the inability to detect differences in binding ability between actomyosin, myosin and myosin heavy chains.

The dimensions of the spindle-shaped fibers shown in Figures 3, 5 and 6 are $0.1-0.2\mu$ wide by $0.6-1.0\mu$ long.

Since the myosin molecule is much smaller and the salt concentration was high enough to dissolve myosin filaments, heating must have caused the myosin molecules to aggregate into filaments. Dimensions of synthetic thick filaments reported by Huxley (1963) are comparable in length, but only one-tenth the width of these. However, Huxley (1963) also reported that light meromyosin, which comprises most of the heavy chain core of the myosin molecule, is capable of forming filaments of an indefinite length and diameter. Since myosin dissociates into its light and heavy chains at only 40°C (Dreizen and Richards, 1973) it is possible that the higher temperatures initiate the aggregation of the free heavy chains into super thick filaments. During the formation of these filaments they bond together to produce the network of overlapping fibers. Since the results of Samejima et al. (1969) showed that the light and heavy meromyosins form gels of poorer quality than the parent molecule, and scanning electron micrographs presented here show that myosin heavy chains form super thick filaments, it appears as though intact myosin heavy chains are involved in the mechanism of myosin gelation.

CONCLUSIONS

SALT AND PHOSPHATE increase the ability of myosin to bind meat pieces primarily by solubilizing the protein. This allows for the molecular interactions that are necessary to produce a three dimensional network of protein fibers, which gives the myosin gel greater strength and a higher water holding capacity. Salt effects are primarily ionic and act to solubilize myosin while phosphate acts to dissociate actomyosin, both of which free myosin to participate in a greater number of molecular interactions upon heating.

Temperature has an increasing effect on the ability of myosin to bind meat pieces in the range 45–80°C, and the level of protein has an increasing effect on the binding ability of myosin in the range 0–8%.

Heating of myosin most likely causes the heavy chain cores of myosin to aggregate into super thick filaments which bond with each other to form a three dimensional network of overlapping fibers. From these results the mechanism of the binding between meat pieces involves the interaction of the super thick synthetic filaments formed by the intact heavy chain cores from the extracted myosin with those formed by the heavy chain cores of myosin located within muscle cells on or near the surfaces of the pieces of meat. Salt is required to solubilize the myosin prior to heating so that the heavy chains can be freed from the parent molecule at the lower temperatures of heating, and their subsequent super thick filament formation is possible at the higher temperatures.

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Ms received 3/11/78; revised 6/1/79; accepted 6/9/79.

RIPENING CHANGES AND FLAVOR DEVELOPMENT IN MICROBIAL ENZYME TREATED CHEDDAR CHEESE SLURRIES

V. K. SOOD and F. V. KOSIKOWSKI

ABSTRACT

Cheddar cheese slurries from salted curds were studied to determine an initial level of microbial protease and lipase that potentially might be incorporated optimally into Cheddar cheese to accelerate ripening without developing flavor defects. Soluble protein, free volatile fatty acids values and total bacteria counts of microbial enzyme treated slurries were considerably higher than in controls. Slurry pH decreased slightly during initial ripening but then rose gradually, with the rate of rise, particularly marked in microbial enzyme treated slurries. Electrophoretic illustrations indicated more degradation of αs_1 and β-caseins in microbial enzyme treated slurries than in the control slurry. Bitter peptides formed in microbial enzyme treated slurries were transitory. Microbial enzymes used in slurries were inactivated at 80°C.

INTRODUCTION

RIPENING, involving flavor development, in Cheddar cheese is a complex biochemical phenomenon related to enzyme concentration.

Richardson and Nelson (1968), following the work of Kristoffersen et al. (1967), suggested cheese slurries to rapidly evaluate the role of added microbial enzymes in the accelerated ripening of cheeses and Kosikowski and Iwasaki (1975) used Cheddar cheese slurries layered in sterile petri dishes–packed in aluminum foil bags under carbon dioxide–to screen various enzyme preparations for Cheddar cheese application. Later microbial lactase behavior in Cheddar cheese ripening was studied by its incorporation into the cheese slurries (Woodward, 1977).

In the present investigation, selected fungal protease and lipase preparations were added at various levels to slurries to predict levels for optimum acceleration of Cheddar cheese ripening and flavor development.

MATERIALS & METHODS

Slurry preparation

Heat treated whole milk (65°C for 15 sec) was obtained from the Cornell Dairy Plant. The Cheddar cheese curds were prepared by standard cheesemaking practices as described by Kosikowski (1977). Slurries were prepared by mixing 200g of fresh salted Cheddar cheese curd with 100g of sterilized saline water (4.5% NaCl) in a Waring Blendor. Some of the slurry was treated with specific amounts of proteolytic and lipolytic enzyme preparations and packaged in polyethylene Whirl-pak bags (Fisher Scientific, PA). These were inserted into foil-laminated Flex-Vac pouches (Standard Packaging Corp., Chicago), vacuum sealed and incubated at 37°C for 7 days.

Sources and activities of enzymes

Food grade enzymes [fungal protease 31000 from *Aspergillus oryzae* (Miles Laboratory, Elkhart, IN), microbial protease P-53 of *Bacillus subtilus* (Rohm and Haas, Philadelphia, PA), and Lipase-MY from *Candida cylindracea* (Meito Sangyo Co., Nagoya, Japan)] were used.

Activities of the three microbial enzymes are listed below.

(a) Fungal protease 31000: Activity – 31000 HV, where 1 HV of enzyme liberates 0.0447 mg of nonprotein nitrogen from 1.6% solution of hemoglobin in 30 min at 4°C and pH 4.7.
(b) Rhozyme P-53 conc: Activity – 1000 HV, where enzyme has an activity of 1000 HV per gram if 11.18 mg of it produces an increase in soluble nitrogen of 5.00 mg from 0.417g of hemoglobin in 5 hr at 4°C and pH 4.7.
(c) Lipase-My: Activity – 30000 units/g, where one enzyme unit is the amount that can liberate 1 μmole of fatty acid in 1 min under the condition described in the technical service bulletin for the enzyme Lipase-My published by Meito Sangyo Co. Ltd. 2-41 Sasazuka, Nishi-Ku, Nagoya, Japan.

Enzyme optimization studies

A Response Surface analysis (RSA) was devised for optimizing the concentration of enzymes likely to produce fully ripened flavor in young Cheddar cheese. The slurries with varying amounts of enzymes were evaluated for acceptable cheese flavor by a taste panel of 12 graduate students at the Food Science Dept., Cornell University. An incomplete randomized block design was adopted so that every panelist tasted only six samples at one time.

A quadratic equation of the type

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_1{}^2 + \beta_4 \beta_2{}^2 + \beta_5 X_1 X_2$$

(where Y is the flavor response, X_1 and X_2 represent the enzyme levels and the β_1, β_2 etc. the regression coefficients) described the response surface for the flavor of slurries. A two variable central composite design with 12 replicates at the center point was fitted to obtain the precise location of maxima. The path of steepest ascent was followed. A Hewlett Packard (Model 9820A) calculator performed the multiple regression and drew the contour maps.

Table 1–Regression coefficients for enzyme optimization studies

b_0	3.90869
b_1	–0.06098
b_2	–0.03049
b_{11}	–0.14073
b_{22}	–0.33604
b_{12}	–0.06250

Analysis

Total solids, fat, pH, total and soluble protein, and free volatile fatty acids in slurries were determined according to the procedure described by Kosikowski (1977); microbial count was conducted on standard plate count agar in APHA methods (1967). All analyses were performed in duplicate.

Casein proteolysis in slurries was observed following the gel electrophoresis procedure of Kiddy (1974). 0.2g of slurry was dissolved in 1.3 ml of Poulak working buffer. Fat was extracted with 0.5 ml of ether and κ-casein was demonstrated using mercaptoethanol. The samples were incubated at 37°C for 30–60 min before application to the gel slots in a vertical gel electrophoresis cell (E-C Apparatus Co., Philadelphia, PA).

RESULTS

FROM RSA DATA, the optimal amounts of microbial enzymes required for flavor acceleration in Cheddar cheese slurries was fungal protease (Miles) 0.0015%, and fungal

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0022-1147/79/0006-1690$02.25/0
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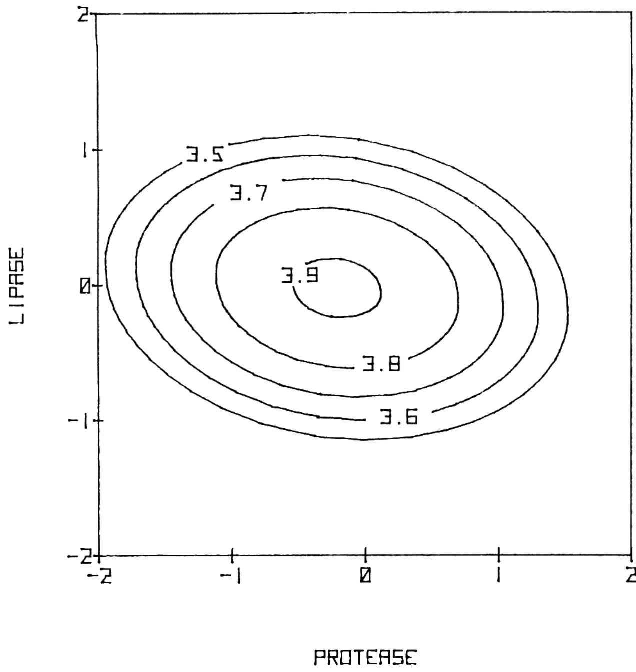

Fig. 1—Final response surface for acceptability of Cheddar cheese slurries treated with different levels of protease and lipase preparations. (0 levels: Protease, 0.0015%; lipase, 0.003%; each unit for protease and lipase: 0.0005%)

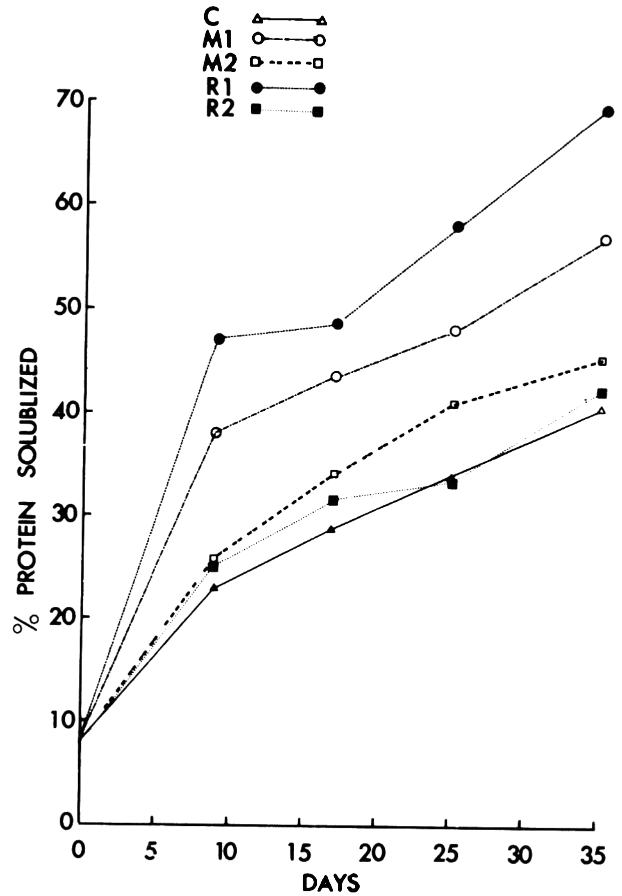

Fig. 2—Soluble protein at different time intervals in various cheese slurries.

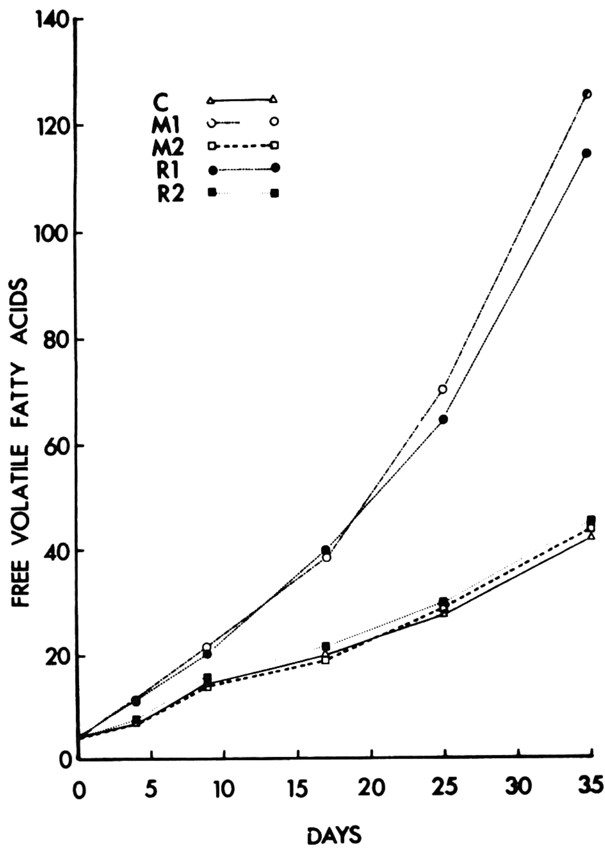

Fig. 3—Free volatile fatty acids of various Cheddar cheese slurries.

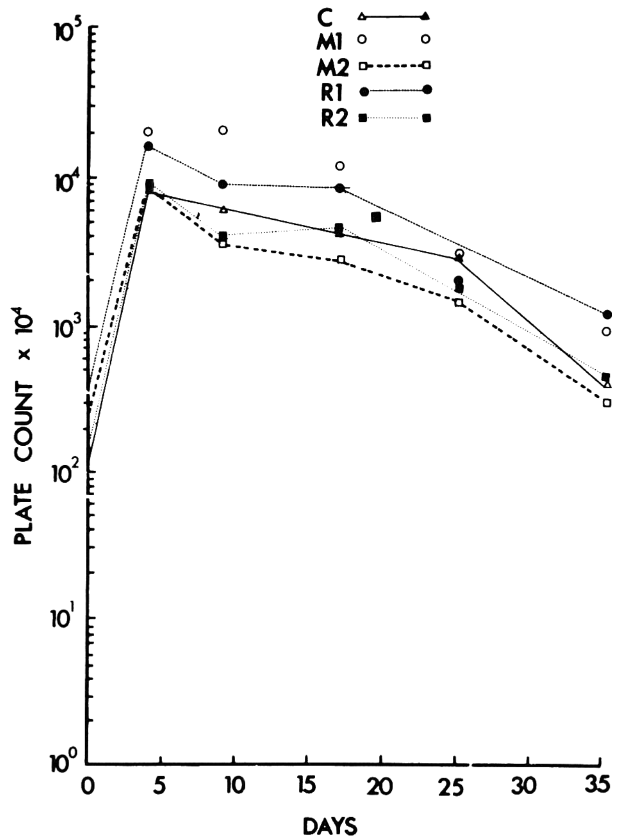

Fig. 4—Colony counts of various Cheddar cheese slurries.

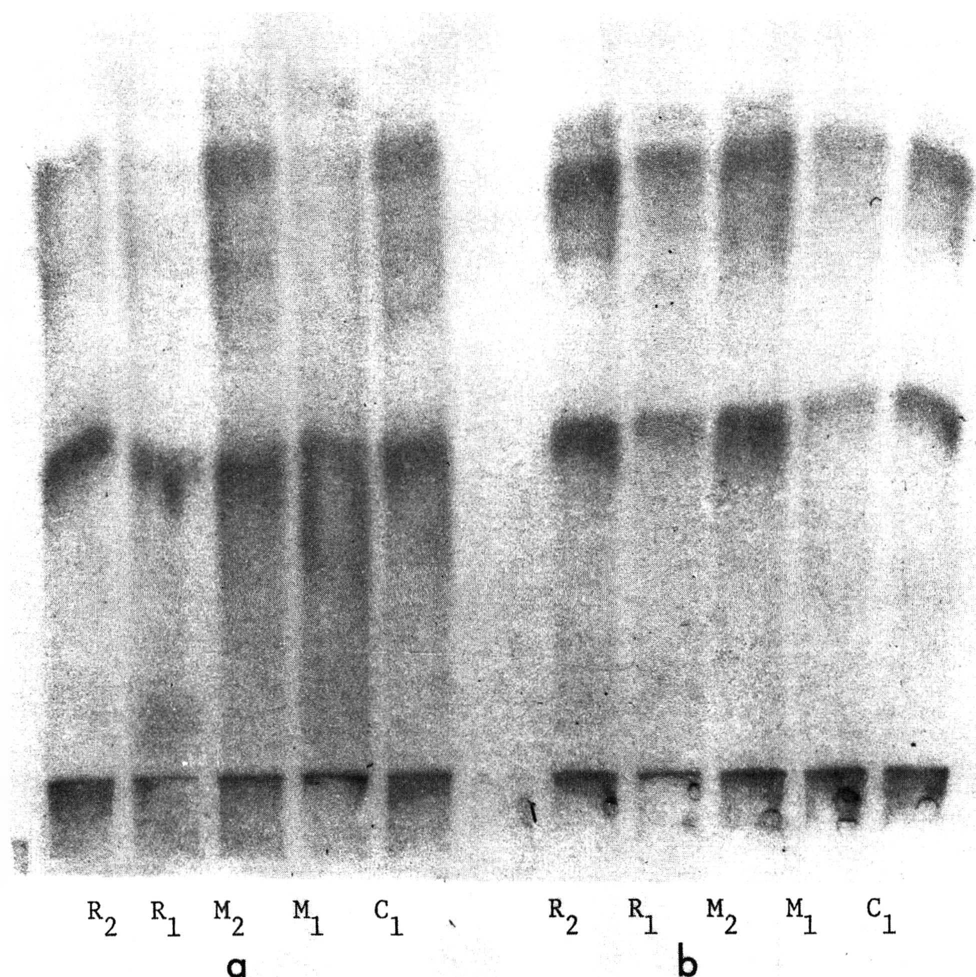

Fig. 5—(a) Gel electrophoretic patterns of fresh cheese slurries; (b) of 9-day old slurries.

lipase (Meito) 0.003%. The regression coefficients of the independent variables in the multivariate analysis (Table 1) and the flavor response surface derived from the regression model are illustrated in Figure 1. Basic flavor of acceptable slurries was cheesy but included at times some overtones of free fatty acids along with mild saltiness.

In a continuing experiment, five Cheddar cheese slurries were treated with enzyme preparations as follows:

- C_1 Control—no enzymes added
- M_1 0.005% protease (Miles) + 0.00005% lipase (Meito) added
- R_1 0.0035% protease (Rohm & Haas) + 0.00005% lipase (Meito) added
- M_2 0.005% protease (Miles) + 0.00005% lipase (Meito) suspended in water, heated to 80°C in a water bath, and then immediately added to slurry
- R_2 0.0035% protease (Rohm & Haas) + 0.00005% lipase (Meito) suspended in water, heated to 80°C in a water bath, and then immediately added to slurry.

Each slurry was divided into six polyethylene Whirl-pak bags, packed and incubated at 37°C. One bag of each treatment was analyzed after 0, 4, 9, 17, 25 and 35 days of incubation.

The gross composition of different slurries was similar (Table 2). Soluble protein of slurries increased markedly with time, particularly during the initial ripening period (Fig. 2). After 9 days, 38% and 47% of the total proteins were solubilized in M_1 and R_1 slurries, and 23% in the control slurry. Free volatile fatty acids of the control slurry increased relatively uniformly in 35 days from 4.37 to 41.98 ml NaOH/100g (Fig. 3). Addition of a microbial lipase enzyme preparation to the slurries accelerated free volatile fatty acids production, particularly after 9 days of ripening. The final concentration of free volatile fatty acid

Table 2—Composition of slurries made with microbial enzymes[a]

Sample	Moisture (%)	Fat (%)	Protein (%)
Control C_1	58.9	21.7	15.1
Enzyme treated M_1	59.1	22.3	15.0
Enzyme treated R_1	58.8	22.2	15.1

[a] M_1 0.005% Protease (Miles) + 0.00005% Lipase-MY R_1 0.0035% Protease (Rohm + Haas) + 0.00005% Lipase-MY

Table 3—pH changes in various slurries made with microbial enzymes

	Ripening period					
	0	4	9	17	25	35
Sample	Days					
C_1-Control	5.03	4.95	5.00	5.12	5.10	5.18
M_1-Enzyme	5.02	4.95	5.08	5.19	5.25	5.65
M_2-Enzyme	5.03	4.92	5.02	5.12	5.20	5.30
R_1-Enzyme	5.02	4.99	5.10	5.23	5.51	5.80
R_2-Enzyme	5.03	4.90	5.00	5.11	5.13	5.20

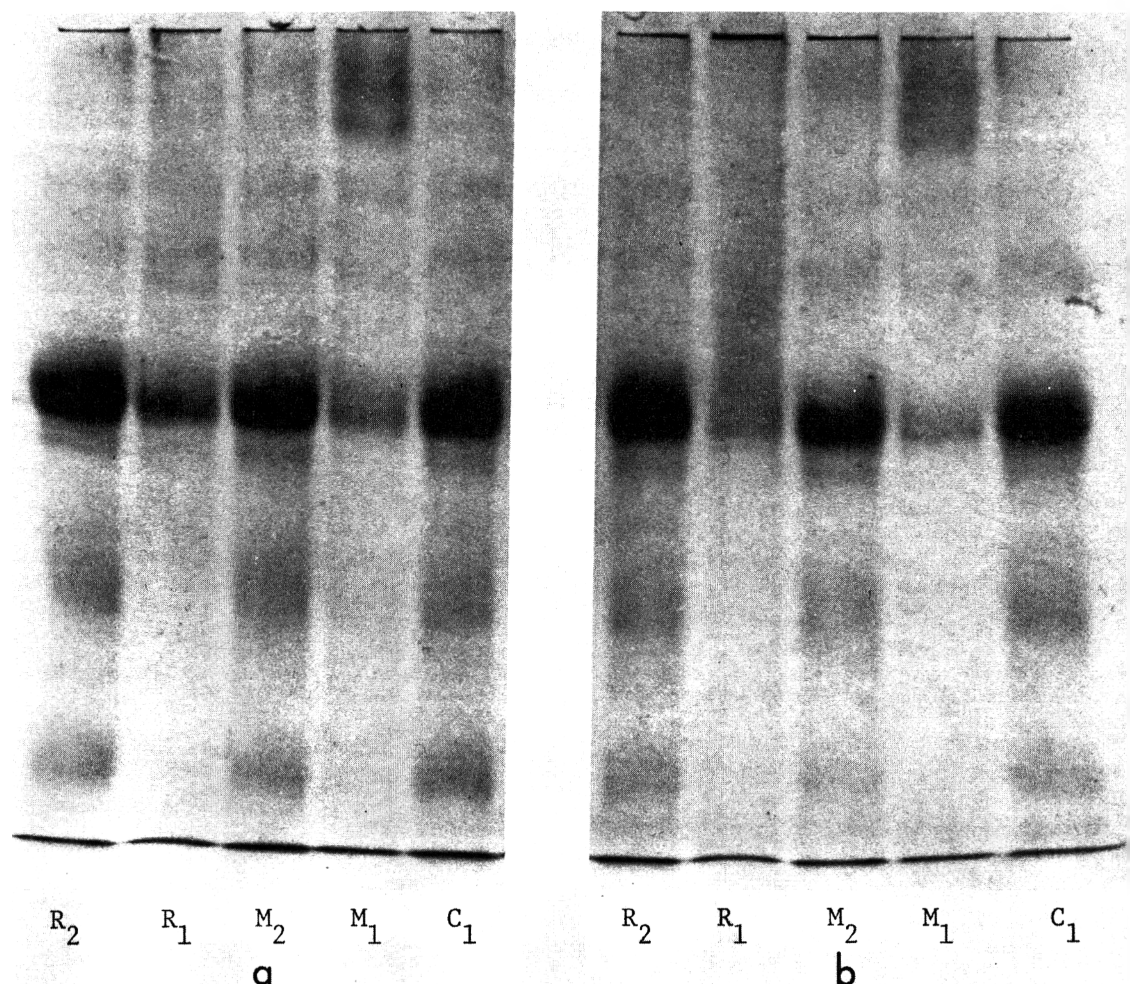

Fig. 6—(a) Gel electrophoretic patterns of 17-day old slurries; (b) of 25-day old slurries.

in lipase treated slurries ranged from 115 to 126 ml NaOH/100g—about 2.5 times higher than in the control slurry.

All slurries showed a slight decrease in pH during the initial ripening period, followed by a gradual rise which was particularly marked in the enzyme treated slurries (M_1 and R_1), Table 3. The pH of the C_1, M_1 and R_1 slurries after 35 days of incubation was 5.18, 5.65 and 5.80, respectively. In general, an increase of more than one log cycle in total microbial count in slurries occurred during the initial 4 ripening days, followed by a gradual decline during the remainder of the ripening period (Fig. 4). Enzyme treated slurries (M_1 and R_1) showed higher total counts than the control slurry (C_1).

Changes in the soluble protein, free volatile fatty acids, pH and total microbial count of slurries treated with enzyme preparations previously heated in water to 80°C (M_2 and R_2) were similar to the control slurry (C_1).

In gel electrophoresis patterns, fresh slurries showed partial conversion of αs_1 to αs_1-I casein, (Fig. 5a). After 9 days, M_1 and R_1 slurries contained less αs_1 casein than the C_1 slurry and more αs_1-I casein, Figure 5b. In 25-day old M_1 and R_1 slurries, αs_1 and αs_1-I caseins were virtually absent (Fig. 6b).

β-casein degradation was minimum in fresh and 9-day old slurries, Figures 5a and 5b. However, in 17-day old M_1 and R_1 slurries, Figure 6a, β-casein breakdown was higher than in the corresponding C_1, M_2 and R_2 slurries. After 35 days, enzyme treated slurries M_1 and R_1, Figure 7b, displayed little β-casein but it was essentially intact in slurries C_1, M_2, and R_2.

Bitter flavor

Cheddar cheese slurries were treated with 0.1% of protease enzyme, incubated at 37°C for 7 days and freeze dried. Bitter fractions were isolated from the freeze-dried slurries following the procedure of Halwalkar and Elliot (1965). Further fractionation was carried out on a Sephadex G-25 column (2.5 × 90 cm) using 0.1N NH_4OH as an eluant. The bitter fraction was further separated on a column of silica gel G (Merck, type 60) with n-propanol:water (7:3) eluant, as described by Vissar et al. (1975). The bitter fraction which included more than one peptide on thin-layer chromatogram was eluted from the G-25 column and treated a second time with 0.05% microbial protease enzyme preparation 31000 (Miles), and incubated at 45°C for 24 hr. This bitterness disappeared suggesting that bitter peptides formed in slurries were intermediate in nature. Similar results were obtained with 0.05% microbial protease enzyme preparation P-53 (Rohm and Haas).

DISCUSSION

THE OPTIMIZATION STUDIES with slurries adopting the RSA identified an initial level of enzymes that could potentially be incorporated into Cheddar cheese to achieve accelerated ripening without developing flavor defects.

Both proteolytic enzymes studied reacted similarly in the slurries. Enzyme P-53 was more concentrated, and pro-

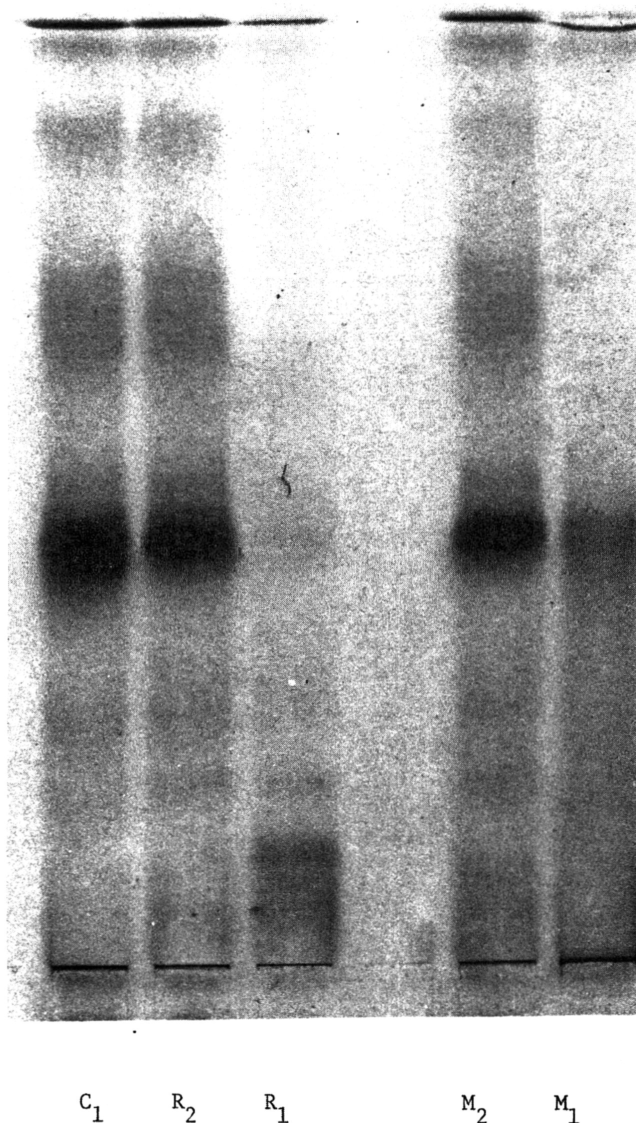

Fig. 7—Gel electrophoretic patterns of 35-day old slurries.

duced higher proteolysis on a weight-weight basis with level of soluble proteins formed in the first day in M_1 and R_1 slurries being comparable to those found in medium sharp Cheddar cheese (Kristoffersen et al., 1967).

Added microbial proteases degraded αs_1 casein much faster than β-casein, comparable to native milk proteases and to the residual rennin in ripening Cheddar cheese (Ledford et al., 1967). As a result, the proteolysis of αs_1 and αs_1-I caseins in M_1 and R_1 slurries was more rapid and complete. Furthermore, as slurry ripening advanced, β-casein decreased faster in M_1 and R_1 slurries than in others, but TS, γ and R peptides levels remained constant. Since the low molecular weight, slower moving peptides (TS, γ, R) are derived from β-casein (Gordon et al., 1972), they apparently were being simultaneously hydrolyzed into still smaller peptides and free amino acids.

A pH drop during the initial period of slurry ripening, also observed by Kristoffersen et al. (1967), presumably was due to the conversion of residual lactose to lactic acid by bacteria. However, the pH of slurries rose during the last 10 ripening days apparently due to accumulated ammonia and related compounds produced from the continuous proteolysis of caseins. Furthermore, the increased levels of lower molecular weight peptides and free amino acids available in enzyme treated slurries (M_1 and R_1) apparently increased microbial populations.

Since 'bitter' starters lack the protease enzyme that degrades the bitter peptides (Visser et al., 1975; Visser, 1977), it is suggested that added microbial proteases, of the type used in the present study, when tried with the 'bitter' starter at low levels, may lead to cheeses free of bitterness.

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Ms received 3/10/79; revised 7/4/79; accepted 7/10/79.

PHYSICO-CHEMICAL STUDIES OF MODEL MEAT EMULSIONS IN RELATION TO THE PREPARATION OF STABLE SHEEP AND GOAT MEAT SAUSAGE

D. K. CHATTORAJ, A. N. BOSE, M. SEN and P. CHATTERJEE

ABSTRACT

The capacity of water and salt-soluble proteins obtained from the meat of goat, sheep, chicken and pork to emulsify peanut oil was compared at different protein concentrations in an aqueous medium. At a given concentration, the maximum phase-volume ratio (ϕ_m) of oil in the emulsion for water-soluble proteins was in the order: goat≈chicken≫sheep≈pork. For pure actomyosin, the order of ϕ_m was the following: goat≈sheep≫pork≈chicken. The same order for the actomyosins was also verified by viscometric experiments. From gel electrophoretic analysis, the number and nature of the polypeptide chains of pure actomyosins prepared from various meat sources were found to be different from each other. At higher protein concentrations, ϕ_m was observed to exceed 0.6 both in the presence of salt extracted proteins and meat slurries. Melted sheep and goat fat formed very unstable emulsions in the presence of their respective meat slurries. GLC experiments indicated that the poor dispersibility of the sheep and goat fat was due to the presence of excessive quantities of saturated fatty acids so that the melting point was too high. When sheep fat was mixed with 50% peanut oil, then mixed fat formed excellent emulsions with the sheep meat slurry and ϕ_m was observed to exceed 0.7. Using model meat emulsions, sheep meat sausage was prepared and found to be acceptable by a taste panel.

INTRODUCTION

IN RECENT YEARS, attempts have been made to investigate the physico-chemical properties of model meat emulsions because of the importance of such investigations for the preparation of sausage. Swift et al. (1961, 1968) have studied the emulsifying capacity of model emulsions in which all factors except a single variable were kept constant. The properties of various meat proteins to stabilize fats and oils have subsequently been investigated for determining ideal conditions for meat emulsion preparations (Hegarty et al., 1963; Carpenter and Saffle, 1964; Trautman, 1964). The emulsifying capacity of water- and salt-soluble meat proteins is determined as a function of various parameters, such as protein concentration, pH, temperature, ionic strength and the nature of the neutral salt in the aqueous media (Swift and Sulzbacher, 1963; Helmer and Saffle, 1963; Saffle, 1968). The stabilities of sausage emulsions during treatment by heat have been studied by Meyer et al. (1964), Saffle et al. (1967) and others (Schut, 1976; Saffle, 1968). The plausible mechanisms for emulsification of fat globule by actomyosins have also been discussed (Schut, 1976). Christian and Saffle (1967) have further investigated the extent of emulsification of various fats, oils and fatty acids in model emulsion systems in the presence of meat protein extracts as emulsifiers. Acton and Saffle (1972) have recently shown that the emulsifying capacity of model systems must be expressed in terms of the phase-volume ratio of the emulsion. The physico-chemical properties of model meat emulsions and its relation to protein hydration have been reviewed in detail by Saffle (1968) and Schut (1976).

Most of these studies on model emulsions have, however, been made by mixing pork fat with beef or pork meat since these meat sources are popular in the Western World. These sources are highly unpopular to the various religious groups of people living in India, Asia and Africa. In these latter countries, meat from sheep or goat are frequently accepted as nonvegetarian protein food sources. Thus, the present study was undertaken to investigate the physico-chemical properties of model emulsions using these meat sources. A system for preparation of sausage using these meat sources has also been explored in this investigation.

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0022-1147/79/0006-1695$02.25/0
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MATERIALS & METHODS

Preparation of actomyosin

Actomyosin from the meat of sheep, goats and chickens was prepared following the method of Herring et al. (1969). Washed meat pieces were blended for 3 min in the presence of three volumes of water. The mixture was then centrifuged in the cold at 7000 × G and the residue was extracted with 0.6M KCl in 0.01M $NaHCO_3$ for 6–8 hr with slow stirring. The extracted solution was then centrifuged for an hour at 15000 × G and the collected supernatant was mixed with two volumes of cold water with stirring so that the actomyosin precipitated out. The precipitate was dissolved in 3M KCl and actomyosin was reprecipitated three times following the procedure of Herring et al. (1969). Actomyosin thus prepared was washed with cold distilled water. The precipitate was then dissolved in 2.0M NaCl solution and the protein concentration of the solution was determined by the Kjeldahl method. The heterogeneity of actomyosin was determined by the gel electrophoresis technique of Weber and Osborn (1969) and the number of bands in the gel for the different actomyosins were measured directly.

Preparation of protein extract and meat slurry

Ten grams of meat from goat, sheep or pork were mixed and blended for 5 min in an electric blender with 100 ml of either 0.5M NaCl, NaBr or Na_2SO_4 solutions. The slurry was used for emulsification. In some cases, the slurry obtained was centrifuged and the protein in the supernatant was used for emulsification. In a few experiments, the meat was blended with water and the slurry was centrifuged. The water-extracted protein present in the supernatant was used for emulsification. Protein contents of the meat slurry, salt extract or water extract were determined by the Kjeldahl method.

Oil and fat

Double-refined peanut oil was purchased from the market and used directly for emulsification. Fat associated with the meat of goats, sheep or pork was procured from the local market and melted carefully at high temperatures. The melted fat was filtered through clean cloth and was added directly to the meat slurry or protein extracts during emulsification.

In some cases melted fat of sheep or goats was mixed with hot peanut oil at volume ratios 25:75, 35:65 and 50:50. The fat-oil mixtures were added to the protein solution at 80°C during emulsification.

Fatty acid composition of fat

The fatty acid content of the fats from different meat sources were determined by gas-liquid chromatography using the method of Luddy et al. (1968) as described by Dutta et al. (1968). An F and M model 700-R-dual column analytical gas chromatograph with dual flame ionization detector was used. The peaks of the chromato-

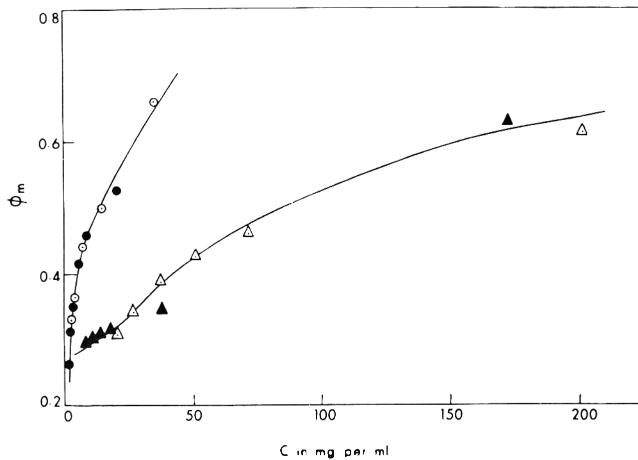

Fig. 1—Plot of maximum phase volume ratio (ϕ_m) vs concentration (C) of water extracted meat proteins for peanut oil emulsion: △ —Pork meat; ▲—Sheep meat; ⊙—Goat meat; ●—Chicken meat.

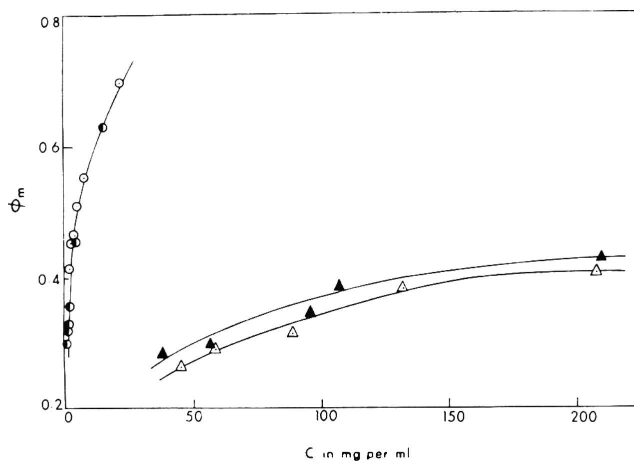

Fig. 2—Plot of maximum phase-volume ratio (ϕ_m) vs concentration (C) of actomyosins for peanut oil emulsion: NaCl concentration—2.0 (M): ⊙ —Goat actomyosin; ◑—Sheep actomyosin; △ —Chicken actomyosin; ▲—Pork actomyosin.

grams were identified by comparing with known reference standards.

Phase-volume ratio

The emulsifying capacity expressed in terms of the phase-volume ratio of the emulsion was measured by a modification of the method of Swift et al. (1961). A measured volume of double refined oil at ordinary temperature or melted fat or oil-fat mixture at 80°C was added in to a definite volume of actomyosin solution, meat slurry or meat protein extract contained in a specially designed blender. Blades of the blender were fixed at the bottom of the shaft covered by a perforated socket. The addition of the oil to the aqueous phase was continued until the emulsion became saturated and the system showed oil separation at the top. At this stage, the emulsifying capacity or the maximum phase volume ratio (ϕ_m) of the oil was calculated directly from the formula below (Acton and Saffle, 1972; Becher, 1957):

$$\phi_m = \frac{\text{ml of oil}}{\text{ml of water + ml of oil}} \quad (1)$$

ϕ_m was determined for various protein concentrations after addition of appropriate amounts of water to the system.

Viscosity of the emulsion

Apparent non-Newtonian viscosity of the meat protein emulsion was measured using a co-axial rotating type of cylindrical model V.M. viscometer (Ferranti, England) precalibrated for known viscosities of standard liquids. A definite volume of an aqueous solution of known protein concentration was placed in a beaker and a measured volume of peanut oil was added so that the phase-volume ratio (ϕ) of the oil in the system could be calculated using the expression on the right hand side of Eq (1). The oil and water system was then blended and the apparent viscosity (η) of the resulting emulsion was instantly measured by dipping the viscometer into the system and noting the scale reading (Sherman, 1968). The phase-volume ratio (ϕ) for the (unsaturated) emulsion was increased by addition of further amount of oil and measurement of η was continued.

Method of laboratory scale preparation of meat emulsion

Sheep meat was boned and then passed through a meat mincer. 60 ml of melted sheep fat were mixed with 60 ml of peanut or mustard oil. The meat slurry and fat-oil mixture prepared in this manner was blended in the presence of additives in an electric mixer for 10 min at a constant speed. The meat emulsions thus prepared were stuffed into sausage casings (salted washed sheep intestine). The sausage was cooked at 80°C for 30 min, boiled at 100°C for 30 min, and then subjected to deep fat frying.

The sheep meat sausages prepared in this manner with various types of cooking ingredients were served to a taste panel comprising five persons who had previous experience in taste panel work. For each variety of sausage, five independent batches were made and their average scores for color, odor, flavor, texture and acceptability were rated by the members of the taste panel.

RESULTS & DISCUSSION

THE EFFICIENCY of emulsification of a protein at a given concentration is expressed in terms of its maximum ability to emulsify oil (or fat) in the dispersed aqueous phase under standard conditions. The amount of oil emulsified may be expressed in various units (Saffle, 1968). However, Saffle et al. (1972) have recently concluded that the emulsification capacity should be expressed in terms of the maximum phase-volume ratio (ϕ_m) of the oil in the emulsion for the correct physico-chemical interpretation.

Meat proteins involved in emulsification can be broadly divided as "water soluble" or "salt soluble." Salt-soluble proteins are mostly actomyosins. Since peanut oil is widely utilized in India for cooking and frying food materials, this oil was generally used in the present study for preparing the model emulsions. In Figure 1, the phase-volume ratio (ϕ_m) of the saturated oil-water emulsion is plotted against concentration (C) of the total protein extracted from various types of meat in the presence of pure water. By comparison of the curves, it is evident that the emulsification efficiency of water-soluble goat meat and chicken meat proteins at various protein concentrations is practically identical. Pork and sheep meat proteins were also nearly the same. At a given concentration of protein, say 50 mg per ml, ϕ_m for goat and chicken protein stabilized emulsions was 0.70, whereas, for pork and sheep proteins it was about 0.45. In the latter cases, ϕ_m approached 0.6 if protein concentration exceeded 200 mg per ml. From these results, it may be concluded that the water extracted proteins of goat and chicken are considerably better emulsifiers than those of sheep and pork proteins. From the classical theories of emulsion, it may be mentioned here that the value of ϕ_m for an oil in water emulsion may become 0.74 provided the size of the different emulsion droplets is standardized. The particle size of the emulsion in the present case was examined microscopically after diluting the emulsion and found to be somewhat evenly distributed. The phase volume ratio may even exceed 0.74 under ideal conditions (Becher, 1957), if the particle size of the emulsion is widely distributed.

In Figure 2, ϕ_m for peanut oil emulsions have been plot-

Table 1—Gel electrophoresis of different actomyosins

Actomyosin source	No. of heavy bands	No. of light bands
Goat	3	5
Chicken	3	7
Sheep	2	5

ted against the concentration of actomyosin prepared from goat, sheep, pork and chicken meat. Actomyosin is insoluble in water but acts as an emulsifier on being dissolved in 2.0M salt solution. ϕ_m in all cases increased directly with increasing concentrations (C) of protein (mg per ml) in the aqueous medium. However, the rate of increment of ϕ_m with C was very sharp with sheep and goat actomyosin, whereas, that with chicken and pork actomyosin is relatively small. At a given concentration, ϕ_m for different proteins were as follows:

$$\text{goat} \approx \text{sheep} \gg \text{pork} \approx \text{chicken}.$$

The emulsifying capacity of goat and sheep proteins was considerably higher than that of pork and chicken proteins. The difference in emulsifying capacity indicates that the protein composition and the related surface activity of the actomyosin from the various species differed. The number of protein bands of actomyosin from goat, sheep and chicken are observed to be significantly dependent on the protein source (Table 1). During gel electrophoresis, the mobility of individual fractions of actomyosin of one kind was not found to be exactly the same, possibly because of inherent small differences in the primary structure of the proteins. The color intensities of the gel bands of the different protein fractions were also found to be dependent on the source of actomyosin. These results may explain the differences in emulsifying capacity for various actomyosin preparations as being due to differences in the surface activity of the proteins. Schut and co-workers (1976) suggested that one of the salt-soluble proteins may become preferentially adsorbed on the interface of the emulsion droplets so that the stability of meat emulsion may be considerably increased. The preferential adsorption for different actomyosin fractions may be responsible for the difference in the ϕ_m versus C curves presented in Figure 2.

In Figure 3, the viscosities of the peanut oil emulsions stabilized by various actomyosins were plotted against the phase-volume ratio (ϕ) of the emulsion. The viscosity of the emulsion increased with more and more dispersion of oil until at a critical value for the phase-volume ratio, the viscosity fell sharply. This critical value of ϕ and the height of the maximum in each curve for viscosity are intimately related to the saturation states of the emulsion, as well as to the size distributions of the emulsion particles in the system. Values for the phase-volume ratio were maximum for goat myosin and least for chicken myosin. These results are verified by Figure 2.

Figures 1 and 2 show that the emulsifying capacity of the water-soluble proteins of chicken, sheep and pork meat at a given protein concentration are higher than those of their respective myosins. In the case of goat meat, both the water extract and salt-soluble actomyosin may act as highly efficient emulsifiers for the preparation of peanut oil emulsions. These observations lead to the conclusion that both water-soluble proteins and salt-soluble actomyosin may act as highly effective emulsifiers in the preparation of model meat emulsions in agreement with observations made earlier (Saffle, 1968).

The crude salt extract of a meat sample contains both salt-soluble actomyosin and water-soluble proteins. In Figure 4, the phase-volume ratio of a peanut oil emulsion was observed to increase directly with total protein concentration of the salt extract from either goat or sheep meat. At a given value for C, ϕ_m of goat meat remained unaltered when NaCl in the aqueous medium was replaced by NaBr, but it increased significantly when NaCl was replaced by Na_2SO_4. The latter salt is known to increase hydration (Bull and Breeze, 1970; Mitra et al., 1977) of protein. The dependence of the emulsifying capacities of the water-soluble proteins and salt-soluble actomyosins (obtained

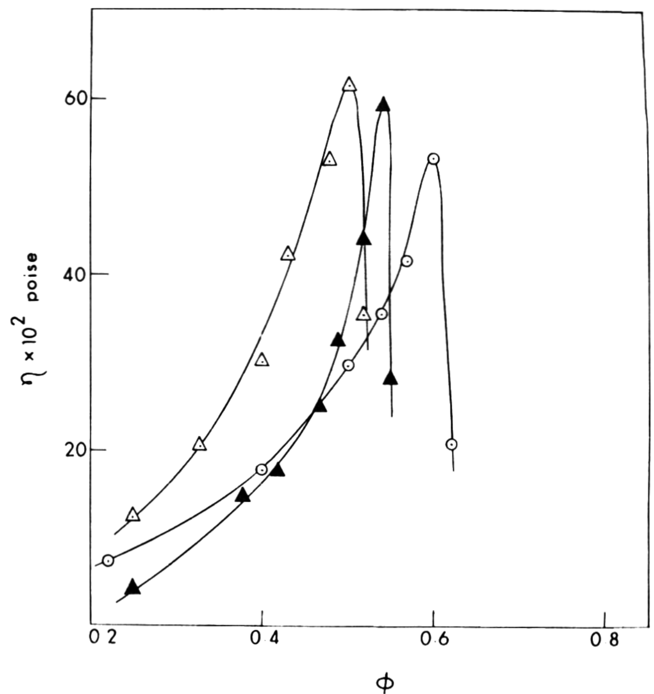

Fig. 3—Plot of apparent viscosity (η) vs phase-volume ratios (ϕ) of peanut oil emulsion in the presence of 2.0M NaCl solution: ⊙—Goat actomyosin (Protein conc 10.47 mg/ml); ▲—Sheep actomyosin (Protein conc 10.8 mg/ml); △—Chicken actomyosin (Protein conc 51.8 mg/ml).

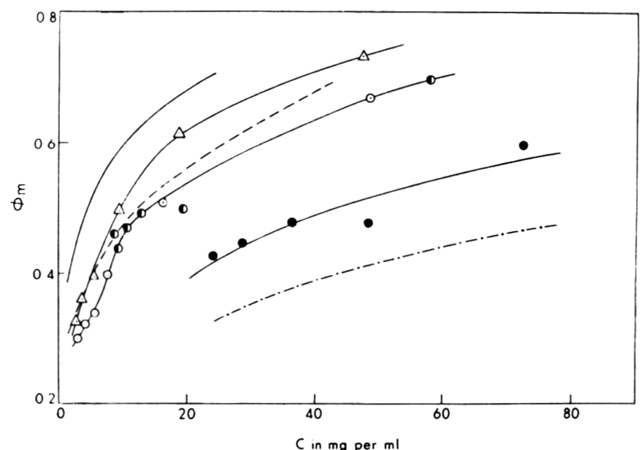

Fig. 4—Plot of maximum phase-volume ratio (ϕ_m) vs protein concentration (C) of different extracts of goat and sheep meat for peanut oil emulsion: ⊙ —0.5(M) NaCl extract of goat meat; △ —0.5(M) Na_2SO_4 extract of goat meat; ◐ —0.5(M) NaBr extract of goat meat; ●—0.5(M)NaCl extract of sheep meat; — — — Goat water extract; — Actomyosin of goat or sheep; -●-·-●-Sheep water extract.

from animal sources other than sheep or goats) on the nature of inorganic salts was studied in detail by Swift and Sulzbacher (1963). From Figure 4, it also appears that a given value of C, ϕ_m for salt extract is always considerably less than that of actomyosin from goat and sheep meats. Under these conditions, ϕ_m of water extract at a given value of C is close to that of the salt extract of goat meat. The phase-volume ratio of the water extract of sheep meat is considerably less than that of the corresponding salt extract. Thus actomyosin of goat and sheep in the salt extract may behave as active and efficient emulsifiers for oils and fats to be dispersed in the aqueous phase. Swift and Sulzbacher (1963) reached the same conclusion for other sources of meat proteins. The difference observed in the value of ϕ_m at a given C value for salt extracted goat and sheep proteins (Fig. 4) may be related to the preferential adsorption of the two proteins having different surface activities as was suggested by Schut et al. (1976).

In Figure 5, the apparent viscosities (η) of emulsions stabilized by salt-extracted water-soluble and salt-soluble proteins of various types of meat have been plotted against their respective phase-volume ratios (ϕ). With gradual dispersion of the oil and subsequent conversion of oil into smaller and smaller droplets, the emulsion viscosity increases directly with ϕ until η reaches a maximum value (η_{max}). The maximum value relates to the saturation capacity of the emulsion, with minimum or optimum size of the emulsions (Sherman, 1968). Beyond this maximum, the viscosity is observed to fall sharply as a result of the coalescence of emulsion droplets and phase separation due to the emulsion breaking. The phase-volume ratio for maximum viscosity is greatest with goat extract followed by sheep, chicken and pork extracts, respectively. Goat protein is, therefore, very efficient for emulsification in terms of maximum phase-volume ratio. However, at ϕ equal to 0.6, the viscosity of the chicken meat extract is considerably greater than those of other extracts. This indicates that oil droplets are dispersed into very small sizes in this emulsion system, whereas, in all other emulsions the particle size is considerably larger.

We have also studied the maximum emulsifying capacity of peanut oil and sheep fat in the presence of sheep meat slurries as emulsifiers. The results for oil and oil-fat mixtures are presented in Figure 6. Figure 6 shows that ϕ_m of peanut oil in the presence of the sheep meat slurry may reach a value as high as 0.6, if the overall protein concentration in the meat slurry is nearly 600 mg per ml in the aqueous phase. Figure 4 shows that ϕ_m of a peanut oil emulsion attained a value 0.6 when the concentration of the salt extracted sheep protein solution is close to 7.0 mg per ml of solution. This means that the stabilizing efficiency for the protein slurry is quite satisfactory, providing the total protein concentration in the system is high. The higher values for protein concentration in production of a saturated emulsion may indicate that a considerable amount of proteins other than actomyosins and water-soluble fractions are present in the insoluble state (Schut, 1976). These insoluble connective tissue proteins have a negligible effect on the emulsifying capacity for oil.

In contrast, the sheep meat slurry was observed to have the lowest efficiency in emulsifying and dispersing melted sheep fat in the aqueous phase containing 0.5M sodium chloride solution. Melted sheep fat at 80°C was quickly added to the meat slurry kept at ordinary temperature and the mixture was blended for 5 min. The value of ϕ_m for this emulsion was observed to be 0.33 at a protein concentration of 390 mg per ml. The emulsion thus prepared becomes unstable within a short period of time. For peanut oil, ϕ_m equals 0.6 (Fig. 6) at this protein concentration. Similarly goat fat was observed not to be dispersed well in

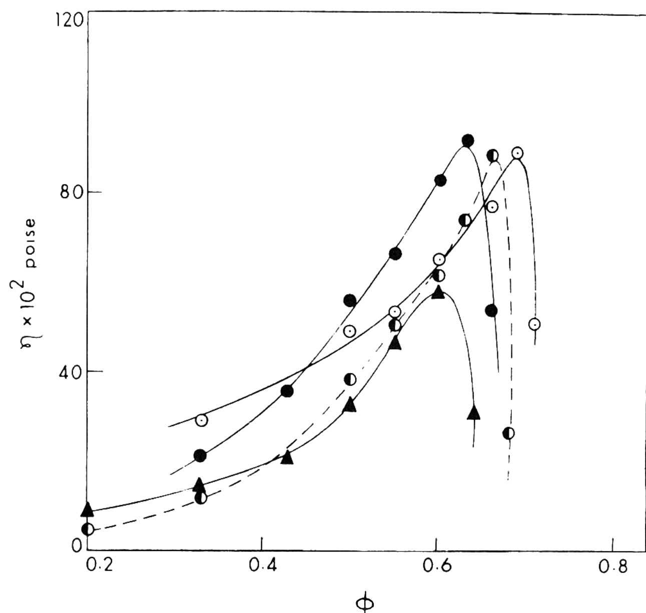

Fig. 5—Plot of apparent viscosity (η) vs phse-volume ratio (ϕ) of peanut oil emulsion stabilized by meat proteins extracted by 1.0 molar NaCl solution: ⊙—Goat meat (protein conc 13.9 mg/ml); ●—Chicken meat (protein conc 14.6 mg/ml); ▲—Pork meat (protein conc 56 mg/ml); ◐—Sheep meat (protein conc 57 mg/ml).

goat meat slurry at 0.5M NaCl and here again ϕ_m did not exceed 0.3. Under the same condition pork fat could easily be dispersed in the presence of a pork slurry with ϕ_m for pork fat being as high as 0.56. We have already noted that sheep and goat myosins possess considerably higher capacities for emulsifying oil compared to that of pork myosin. Further, the sheep and goat meat slurries containing myosins and other proteins were observed to disperse well in peanut oil (Fig. 6). The inability of the sheep and goat meat slurries to disperse sheep or goat fat, therefore, lies in the chemical nature of the animal fat rather than in the chemical properties of the animal proteins.

To resolve this point, the fatty acid composition of the fats of goat, sheep and pork were determined by gas-liquid

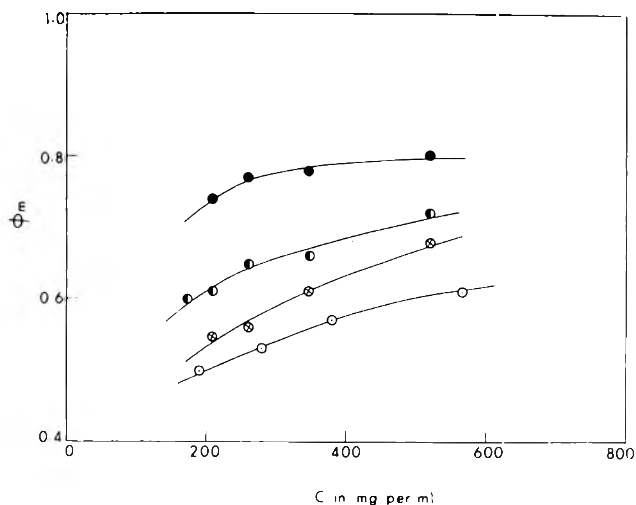

Fig. 6—Plot of maximum phase-volume ratio (ϕ_m) of sheep fat-peanut oil mixed emulsion at 80°C vs (C) in sheep protein slurry (NaCl conc 0.05M): ⊙—100% oil; ◐—75% oil + 25% fat; ⊗—65% oil + 35% fat; ●—50% oil + 50% fat.

Table 2—Fatty acid analysis of fats of pork, goat and sheep

No. of C atoms in fatty acid	No. of double bonds in fatty acid	Percent of fatty acid present		
		Pork	Goat	Sheep
10	0	–	–	1.30
12	0	–	–	1.20
14	0	0.80	1.50	8.90
14	1	Trace	Trace	1.70
16	0	11.50	20.20	23.80
16	1	Trace	Trace	Trace
17	0	Trace	Trace	Trace
18	0	9.80	39.00	26.50
18	1	50.00	37.00	34.70
18	2	25.00	2.30	1.70
18	3	0.50	–	–
20	0	2.00	–	–
Total:		99.60	100.00	99.80

Table 4—Sensory rating of cooked sausage

Sample	Color[a]	Flavor[b] and odor[c]	Texture[d]	Overall accept-ability[e]	Average score
A	2.0	1.5	1.0	1.5	1.50
B	3.0	2.0	2.0	2.0	2.25
C	3.0	3.0	4.0	3.0	3.25
D	4.0	5.0	4.0	4.0	4.25

[a] Color: Reddish brown = 5; light red = 4; light reddish brown = 3; light brown = 2; light red = 1.
[b] Flavor: Characteristic meat flavor = 5; slight meat flavor = 4; just recognizable meat flavor = 3; no meat flavor = 2; soft = 1.
[c] Odor: Characteristic meat odor = 5; slight meat odor = 4; just recognizable meat odor = 3; no meat odor = 2; off odor = 1.
[d] Texture: Excellent (firm) = 5; very good = 4; good = 3; fair = 2; soft = 1.
[e] Acceptability: Like extremely = 5; like moderately = 4; like slightly = 3; neither like nor dislike = 2; dislike = 1.

chromatography. From the results of GLC analysis given in Table 2, it is noted that 76% of fatty acids in pork fat contain one, two or even three double bonds. As a result of the occurrence of the unsaturation in pork fat, it becomes soft and its melting point (Swift, 1968) is nearly 40°C, so that this fat (in melted state) like peanut oil can be easily dispersed in the protein-water system by rapid blending. In contrast to this, only 39 and 36% of the goat and sheep fats contain double bonds, respectively (Table 2), so that these meat fats have very high melting points. These hard fats are difficult to disperse in the meat slurry kept at ordinary temperatures during blending. This suggests that the poor quality of the sheep and goat meat emulsions, originates from the hardness of the fat.

An attempt was, therefore, made to improve the dispersion of sheep fat by changing the pH of the sheep meat slurry to 11.0. However, improvement was marginal. In the melted sheep fat, addition of oleic acid (containing double bonds) further improved emulsification at relatively high temperatures. In all these cases, the process of dilution of the protein in the emulsion resulted in fat separation. In another approach, peanut oil was mixed with the melted sheep fat in 50:50, 65:35, 75:25 volume ratios, respectively. Considerable improvement in emulsification resulted. The analysis presented in Figure 6 indicates that ϕ_m approaches a value as high as 0.80 when sheep fat and peanut oil are mixed in a 50:50 ratio. Further, at given value of C, ϕ_m for the oil-fat system mixed in ratios 75:25 or 65:35 are significantly lower (vide Fig. 6) than that obtained for an oil-fat mixture present in 50:50 ratio. We also noted that the efficiency of the fat dispersion and emulsification considerably decreased when the volume ratio composition of the oil-fat mixture was 30:70. The ideal oil-fat volume ratio in the mixture for the preparation of the most stable emulsion may therefore be taken as 50:50.

The viscosity of various fat emulsions at a fixed protein concentration for different meat slurries was also measured. Viscosity was observed to increase with dispersion of more and more fat until the maximum phase volume ratio stage was reached after which the viscosity began to fall. For pure fat, breakdown of the emulsion corresponded to a low value of ϕ_m equals 0.3. However, when the dispersed phase consisted of a fat and oil mixture in 50:50 volume ratio, the emulsion became very stable and the maximum phase-volume ratio approached 0.70 or higher. At this high phase-volume ratio, the viscosity of the system increased to such a high value that the measurement of viscosity became impossible.

Utilizing this information, an attempt was made to prepare sausage on a laboratory scale using sheep fat and sheep meat mixed with peanut or mustard oil in the presence of various other additives. The characteristics of the different types of sausages thus prepared are given in detail in Table 3. The panel scores on the various properties of these preparations are presented in Table 4. The lowest score was given to sample A in which the fat used was the mixture of sheep fat and peanut oil. The sheep fat contains 1.3% saturated fatty acid having 10 carbon atoms. This fraction may become the source of undesirable odor. The double refined oil mixed with sheep fat cannot fully remove the odor of lower fatty acid from the sausage. Probably this was the reason for the unacceptability of products A and B. Addition of spices, ginger and garlic in product C may be the reason for the partial removal of the undesirable odor. The group of panel testers gave the highest score to the spiced product D in which the fat originally used was the mixture of sheep fat and mustard oil. The improvement of flavor in this product was possibly due to the masking of C_{10} acid flavor by pungent flavor of allylisothiocyanate present in mustard oil and also by the flavoring components present in spices.

Continued on page 1702

Table 3—Properties of cooked sausage using sheep fat and sheep meat

Composition of the cooking ingredients	Texture of sausage prior to cooking	Shrinkage of sausage	Fat sepn during cooking	Flavor	Odor
A. 100g meat; 100 ml H_2O; 10g salt: 120 ml 50:50 fat-peanut oil mixture	Porous	Significant	High	Unattractive due to peanut oil	Of peanut oil
B. 200g meat; 100 ml H_2O; 5g salt; 120 ml 50:50 fat-peanut oil mixture	Much less porous	Significant	High	Same as above	Of peanut oil
C. 200g meat; 40 ml H_2O; 10g salt: 120 ml 50:50 fat-peanut oil mixture	Compact	Negligible	Low	Peanut oil flavor exists	Of peanut oil
D. 200g meat; 40 ml H_2O; 10g salt; 120 ml 50:50 fat-mustard oil mixture; spices (5g garlic + 2g black pepper)	Compact	Negligible	Low	Good	No disagreeable odor

OCCURRENCE AND DETERMINATION OF N-NITROSOPROLINE AND N-NITROSOPYRROLIDINE IN CURED MEAT PRODUCTS

J. W. PENSABENE, J. I. FEINBERG, E. G. PIOTROWSKI and W. FIDDLER

ABSTRACT

N-Nitrosopyrrolidine (NPYR) has been detected and confirmed at the ppb level in a significant number of fried bacon samples. N-Nitrosoproline (NPRO) has been assumed to be the primary precursor of NPYR, but there are conflicting reports about its precise role. A method was developed for determining NPRO and a survey of 60 cured meat samples was conducted. No NPRO was detected in uncooked, conventionally cured bacon, Canadian bacon, ham, salt pork, pork roll or pastrami. NPRO was detected and confirmed in 1 of 7 dry cured bacon samples (106 ppb), 8 of 12 dry cured ham samples (18–604 ppb), and 5 of 6 samples of pork side meat (86–411 ppb). The results suggest the NPRO may not be the main precursor of NPYR in bacon, but may have a role in nitrosamine formation in dry-cured products.

INTRODUCTION

THE CONSISTENT occurrence of N-nitrosopyrrolidine (NPYR) in fried bacon has led to an intensive search for both the precursors and mechanism that could account for the formation of this nitrosamine. The precursors of NPYR have been associated with bacon adipose tissue (Fiddler et al., 1974; Mottram et al., 1977). The fact that N-nitroso-3-hydroxypyrrolidine also has been found occasionally in bacon at the low ppb level (Janzowski et al., 1978; Lee et al., 1978; Sen et al., 1976a) suggests that collagen may be the precursor of the two nitrosamines, since collagen consists primarily (ca 46%) of proline, hydroxyproline and glycine. Free proline is present in pork bellies at a concentration of approximately 20 ppm (Lakritz et al., 1976). This amount of proline is sufficient to account for the NPYR normally detected in bacon at the ppb level (Hwang and Rosen, 1976). In a recent report we proposed that free proline could react with nitrite, which is present in the cure solution, to form N-nitrosoproline (NPRO), and then decarboxylate to yield NPYR under conditions normally recommended for frying (Kushnir et al., 1975). Sen et al. (1976b), however, report that preformed NPRO is not the primary precursor of NPYR, as shown by the fact that ascorbyl palmitate, when added to raw bacon, inhibits the formation of NPYR. Hansen et al. (1977) also claim that NPRO is not the main precursor of NPYR in fried bacon. Nakamura et al. (1976) and more recently Coleman (1978) claim that NPYR is formed from pyrrolidine reacting with nitrite and not by the decomposition of NPRO. Bills et al. (1973) demonstrated that putrescine and spermidine also can form NPYR under model system conditions simulating bacon frying. The reason for this controversy is the lack of a sensitive and reliable method for the detection of NPRO. Therefore, because of the conflicting reports describing the precise role of NPRO in bacon, we are reporting in this paper a survey of 60 cured meat samples examined for the presence of NPRO by a new method that is more sensitive than the procedure we previously reported (Kushnir et al., 1975).

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EXPERIMENTAL

Materials

NPYR, NPRO and N-nitrosopipecolic acid (NPIC) were synthesized and purified as reported previously (Pensabene et al., 1972). Acetonitrile, dioxane, ethyl acetate and hexane were "Distilled in Glass" solvents purchased from Burdick & Jackson Laboratories and used without further purification; whereas, diethyl ether and methanol were distilled prior to use. Diazomethane was prepared from Aldrich N-methyl-N-nitroso-p-toluenesulfonamide as directed. The Dowex 21K anion exchange resin was pretreated by successive washings with 50 ml water, 50 ml of 1N NaCl and 250 ml water.

Determination of NPRO

Cured meat samples were ground twice in a mixer equipped with a 1/8 in. plate and thoroughly mixed prior to analysis. A 50-g sample of the ground meat was spiked with 1 ml of a methanol solution containing either 2.5 μg/ml or 10 μg/ml NPIC, mixed well and allowed to stand for 30 min to assure complete penetration of the spike. The fat was removed from the sample by refluxing it with 150 ml of hexane for 15 min. The solvent was decanted from the sample and 150 ml of cold ether was added to the meat residue in the flask to remove any remaining fat. After standing 15 min the ether was decanted and the remaining ether was evaporated from the sample in a stream of nitrogen. The defatted sample was homogenized for 2 min with 150 ml of a mixed solvent system comprised of 1N HCl, acetonitrile and dioxane (2:1:1). The sample was centrifuged for 15 min at 5000 rpm in a refrigerated centrifuge at 2–6°C. The supernatant (pH < 2) was filtered through glass wool into a 500 ml separatory funnel. The residue was rehomogenized as described above to insure complete extraction. The combined filtrates were extracted 3 times with 125 ml of ethyl acetate, and the combined extracts were transferred to a round bottom flask and dried on a vacuum rotary evaporator in a 35–40°C water bath. The residue in the flask was redissolved in 10 ml of a methanol-water (1:1) solution and transferred to a 20 × 300 mm chromatographic column packed with 10g of 50–100 mesh Dowex 21K anion exchange resin. Artifacts were removed from the column with 75 ml of water. The sample was then eluted with 175 ml of 0.5N HCl solution. The acidic eluent was extracted 3 times in a separatory funnel with 100 ml of ethyl acetate, the combined extracts were passed thru anhydrous Na_2SO_4 held in a coarse fritted glass funnel, then dried on a rotary evaporator. The residue in the flask was dissolved in 1 ml of absolute methanol and transferred to a 16 × 145 mm test tube. Three ml of an ether solution containing ca. 1% diazomethane was added, and the tube was heated and shaken for 20 min at 33°C as described by Wolfram et al. (1977). The sample was transferred from the tube to a 4 ml concentrator tube with ether and concentrated to 1 ml prior to gas-liquid chromatography-Thermal Energy Analyzer (GLC-TEA) detection and quantitation.

Determination of volatile nitrosamines

A representative portion of each cured meat sample was fried for 4–6 min in a preheated Presto Teflon-coated electric frying pan calibrated at a temperature of 177°C (350°F). The edible portion and all rendered drippings were retained for subsequent nitrosamine analysis. The samples were analyzed for nitrosamines by procedures described previously (Fiddler et al., 1978).

Detection and quantitation

Volatile nitrosamines were determined directly (Fiddler et al., 1978), and NPRO and NPIC, the internal standard, were detected as their volatile methyl esters by GLC-TEA. The TEA conditions were similar to those described previously (Fiddler et al., 1978). The Varian Aerograph Model 2700 gas chromatograph was equipped with a 9 ft × 1/8 in. stainless steel column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas Chrom P, operated isothermally at 215°C. Meat samples were spiked with either 50 ppb or 200 ppb

NPIC, and the average recoveries of NPIC were 74% and 77%, respectively. The minimum detectable level of NPRO was 10 ppb. The nitrosamine results reported are the average of two determinations per sample.

GC-Mass spectral confirmation

NPYR and NPRO methyl ester were confirmed by use of the following system: a Varian Aerograph Model 2700 gas chromatograph was interfaced with a Varian Mat 311A mass spectrometer. The gas chromatograph was equipped with a 6 ft × 1/8 in. glass column packed with 15% Carbowax 20M-TPA on 60–80 mesh Gas Chrom P, the helium flow rate was 15 ml/min, and the temperatures used were: detector, 200°C; injection port, 200°C; GLC-mass spectrometer interface, 180°C; and column programmed from 150° to 190°C at 4°/min for NPYR and operated isothermally at 180°C for NPRO methyl ester. The mass spectrometer was operated in the peak matching mode adjusted to a resolution of 1 in 10,000 or 12,000. The mass spectra were measured at an ionizing voltage of 70 ev and an ion source temperature of 150°C. The mass-to-charge ratios (m/e) of 100.06366 for NPYR and 158.069136 for NPRO methyl ester were determined by use of the m/e 99.99361 and m/e 154.992011 perfluorokerosene reference peaks, respectively, by measuring the difference in m/e.

RESULTS & DISCUSSION

THE METHOD for the detection of NPRO described in this paper was applicable, without modification, to all of the various cured meat products analyzed. A typical chromatogram is shown in Figure 1. The nitrosamines can also be quantitated on a gas chromatograph equipped with an alkali flame ionization detector (AFID) and a three-way valve, which is used to direct the solvent from the detector. The use of this valve results in a more sensitive and reproducible signal than would normally be obtained without it. However, even with this valve the AFID chromatogram is not as clean as that obtained by the TEA detector, but it still can be used for quantitation.

Sixty cured meat samples were analyzed by our method for the presence of NPRO, with the results shown in Table 1. Contrary to our earlier report (Kushnir et al., 1975), no NPRO was found in any of the 17 raw bacon samples tested, despite the fact that all the sample had confirmable levels of NPYR in the fried product (5–63 ppb). Our results support the conclusions reached by other investigators (Hansen et al., 1977; Sen et al., 1976b) that free NPRO is not present in sufficient quantities in bacon to account for the high levels of NPYR found in fried bacon.

No NPRO or NPYR was detected in Canadian bacon, conventionally cured ham, or other cured meat products such as salt pork, pork roll, pastrami or corned beef. NPRO (106 ppb) was detected in 1 of 7 samples of dry cured bacon even though all of the bacons had higher NPYR values than conventionally cured bacon after frying (34–89 ppb). The nitrite Safety Council has also reported high levels of NPYR in dry cured bacon (Food Chemical News, 1978). NPRO (18–604 ppb) was also detected and confirmed in 8 of 12 dry cured ham samples and the corresponding fried products all had confirmable levels of NPYR (3–54 ppb); however, high levels of NPYR were not always correlated with high levels of NPRO. Occasionally, N-nitrosodimethylamine (NDMA, 1–5 ppb) was detected in fried dry cured bacon and ham. The presence of NPYR in these dry cured products may result from the long exposure of the nitrite containing curing salts with meat components that serve as the nitrosamine precursor.

In 5 of 6 samples of pork side meat obtained from three producers, NPRO (86–411 ppb) was found. Pork side meat is a dry cured, pepper-coated product similar to back fat, used for flavoring and cooking purposes. This commercial product, although a specialty product not in common usage, contained high levels not only of NPYR (19–149 ppb), but also NDMA (12–51 ppb) and N-nitrosopiperidine (NPIP, 5–35 ppb) after frying. Previous research by Gough and Goodhead (1975) and Sen et al. (1973) showed that cure pre-mixes containing spices, such as black pepper and paprika, can form NDMA, NPIP and NPYR. These spices could then serve as a source of nitrosatable amine when added to cured meat products such as pork side meat. Eisenbrand et al. (1976) reported the occurrence of NDMA, NPIP and NPYR in a peppered ham product. This evidence suggests that spice-coated cured meats have the potential for forming nitrosamines in high concentrations. Therefore, these products should be surveyed more extensively in the future for the presence of volatile and non-volatile nitrosamines.

Our results indicate that NPYR is formed in cured meat products which do not contain detectable levels of NPRO

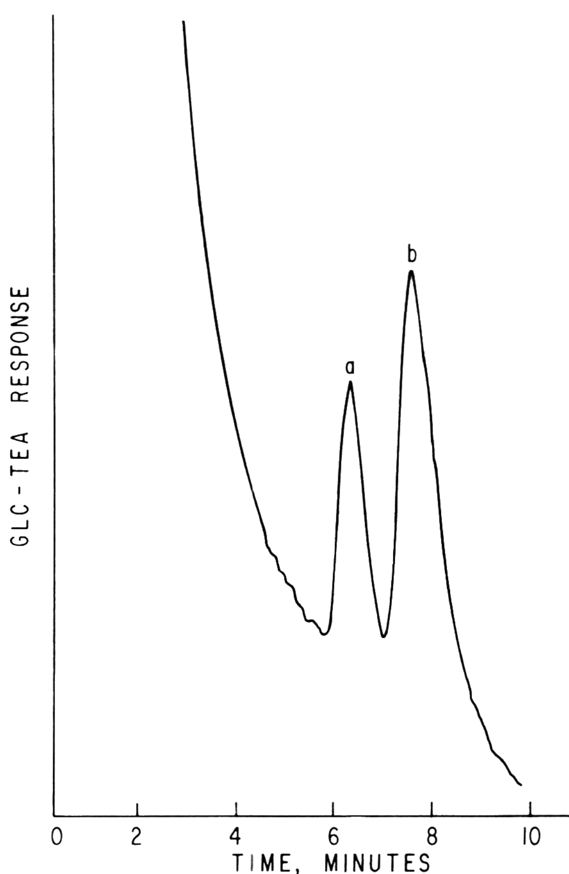

Fig. 1—GLC-TEA chromatogram of an extract from pork side meat with 50 ppb N-nitrosopipecolic acid added: (a) N-nitrosopipecolic acid methyl ester (80% recovery as free acid); (b) N-nitrosoproline methyl ester (63 ppb as free acid).

Table 1—Nitrosamines in cured meat products

	NPRO[a,b,c,d]		NPYR[a,b,e]
Sample type	No. positive/total	μg/kg	μg/kg
Bacon	0/17	N.D.[f]	5–63
Dry cured bacon	1/7	106	39–89
Canadian bacon	0/5	N.D.	N.D.
Ham	0/5	N.D.	N.D.
Dry cured ham	8/12	18–604	3–54
Pork side meat	5/6	86–411	19–149 (12–51 NDMA; 5–31 NPIP)
Other cured meat products	0/8	N.D.	N.D.

[a] Corrected for recovery of internal NA standards
[b] Confirmed by M.S.
[c] Minimum detectability 10 μg/kg
[d] Uncooked samples
[e] After frying
[f] N.D.—none detected

by our method. It is likely therefore, that NPRO is not the main precursor for NPYR in bacon. However, NPRO may play a significant role in NPYR formation in dry cured meat products based on our results with dry cured products. Nitroso derivatives of amino acids found in food products still require further investigation to determine their role in nitrosamine formation.

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Note: Precaution should be exercised in the handling of nitrosamines since they are potential carcinogens.

The authors thank Dr. James Wolfram for his technical assistance and the National Cancer Institute for the loan of a Thermal Energy Analyzer under contract No. N01-CP-55715.

Reference to a brand or firm name does not constitute endorsement by the U.S. Department of Agriculture over others of a similar nature not mentioned.

SHEEP/GOAT SAUSAGE MODEL MEAT EMULSIONS . . . From page 1699

It is clear that our detailed physico-chemical study of meat emulsions may lead to the ideal conditions for preparing meat sausages using solely ingredients of sheep and goat meat. The sausage prepared from these sources will be useful as vital and convenient protein foods for consumption by people, where sheep and goat meats are used in abundance in place of pork and bovine meats. Further, sheep and goat meat sausages may be used by those people who do not eat pork and beef meat because of religious restrictions. Sausage meat prepared on a large scale in this manner may utilize the fat from sheep and goats which otherwise may be wasted in large amounts in Eastern countries.

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We are grateful to Mr. J. Dutta of Bose Research Institute, Calcutta, for fat analysis by GLC method and for many helpful discussions. Financial assistance received from the Ministry of Defence, R&D organization, Government of India during the active period of this research program is acknowledged with thanks.

INFLUENCE OF TIME AND CONDITIONS OF STORAGE ON TECHNOLOGICAL AND NUTRITIONAL PROPERTIES OF A DRY BEAN (Phaseolus vulgaris, L.) VARIETY ROSINHA G2

PEDRO L. ANTUNES and VALDEMIRO C. SGARBIERI

ABSTRACT

The influence of storage on dry bean was studied for 6 months under three different conditions of temperature and relative humidity (RH): A, 12°C, 52% RH; B, 25°C, 65–70% RH; and C, 37°C, 76% RH. The hydration capacity of the seeds remained constant under condition A, increased slightly under condition C and decreased to less than 50% of the original under condition B. Changes in the percentages of hardshell beans were inversely proportional to the hydration capacity. The texture (hardness) of the cooked beans increased from 200 to 250 kg force in the Instron apparatus for the conditions A and B but it reached values greater than 500 kg after 4 months of storage under condition C. Cooking time increased from 60 min to 95, 116 and 300 min for the conditions A, B and C, respectively. The PER value dropped from 1.01 to 0.66, 0.43 and 0.10 under storage conditions A, B and C, in this order. Biological availability of methionine dropped from 46.3% to 43.1, 38.2 and 27.6%, whereas cysteine availability decreased from 51.6% to 45.8, 43 and 30% under the conditions A, B and C, respectively. Protein digestibility changed from 62.4% to 58.9, 57.1 and 54.4% for the conditions A, B and C, in the same order. Addition of methionine to the beans stored for 6 months under the conditions A, B and C, raised the PER from 0.66, 0.43 and 0.10 to the values 2.45, 2.46 and 2.40, respectively, without significantly affecting the digestibility.

INTRODUCTION

DRY BEAN (*Phaseolus vulgaris*) contains over 20% protein and about 65% carbohydrate in addition to several essential minerals and vitamins. It constitutes, together with cereals, roots and tubers, the most important staple in Latin America.

Brazil is the major producer as well as the largest consumer of *Phaseolus vulgaris* in the world. Beans are harvested twice a year, December–January and May–June and, therefore, to maintain a constant supply to the population they have to be stored for at least 6 months.

The yearly production of beans in Brazil remained essentially constant in the decade 1965–1975 whereas the per capita availability dropped from 28.2 to 21.0 kg/yr. There have been two explanations for the decrease in availability in addition to the increased population: (1) lack of satisfactory storage conditions to maintain harvested beans with good technological, organoleptic and commercial properties; and (2) a rapid increase in the cultivation of soybean in areas previously used for cultivation of common bean.

Controlled storage conditions are essential for the preservation of bean quality. Moisture content of the seeds or relative humidity of the air and storage temperature are the most critical parameters (Morris and Wood, 1956; Muneta, 1964; Kon, 1968; Burr et al., 1968). The cooking qualities of the beans deteriorate very rapidly with elevation of the temperature, particularly at moisture contents above 10%.

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0022-1147/79/0006-1703$02.25/0
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Burr et al. (1968) demonstrated that Pinto bean stored for 7 months at 32°C showed a 14-fold increase in the cooking time, i.e. from 24–340 min, whereas storage of the beans with moisture content below 10% and at 8°C did not affect cooking time even after 2 yr.

The mechanism underlying the loss of cookability depending on storage time and conditions is still not understood.

Very little has been reported about the possible changes in nutritive value of beans upon storage. In this work, changes in both technological and nutritive properties were studied for a Brazilian variety of bean stored under different conditions of relative humidity and temperature.

EXPERIMENTAL

Bean variety and storage conditions

The bean variety (Rosinha G2) was obtained locally from the Agronomic Institute of Campinas, S.P., Brazil. The lot received was divided into three sub-lots which were stored under the following conditions: (1) in a fiber bag under the normal conditions of the laboratory (25°C, 65–70% RH); (2) fiber bag in a refrigerated and dehumidified storage room (12°C, 52% RH) and; (3) incubator chamber (37°C, 76% RH). The last condition was obtained by storing the seeds in glass sealed containers (desiccators) maintained inside of an incubator at 37°C. The relative humidity was maintained constant at 76% by a sulfuric acid solution of suitable concentration placed under the bean seeds (bottom of the desiccators).

Samples were withdrawn from all three sub-lots at zero and after 2, 4 and 6 months of storage for the physical and chemical determinations and at zero and after 3 and 6 months for the biological assays.

Preparation of the bean samples

For texture measurements in the Instron apparatus the beans were first soaked in distilled water for 12 hr, then cooked under atmospheric pressure for 30 min and cooled to 30°C before measurements.

The samples utilized for most of the chemical analyses and for the biological assays were soaked in distilled water for 12 hr and subsequently cooked in an open kettle (atmospheric pressure) to the right texture. Prior to cooking, the soaking water was discarded and a volume of fresh distilled water equal to the bean volume was added. The adequate cooking for each storage time and condition was determined subjectively, in separate experiments (3 replicates), by an expert panel of six members (3 men and 3 women, 20–40 yr of age). The cooked samples were freeze-dried and ground to pass a 60-mesh screen before use.

Chemical and physical determinations

Crude protein, ash, total lipid and crude fiber were determined in the cooked samples according to the procedures described in the AOAC (1975). Chemical determinations were all done in duplicate. Percent carbohydrate was calculated by difference.

Total methionine and cysteine were determined in the raw sample at zero time and in the cooked bean samples. Methionine was determined by the colorimetric procedure of Lunder (1973) and cysteine (1/2 cystine) by the method of Spackman et al. (1958) after acid hydrolysis (6N HCl, 105°C, 22 hr).

Hydration capacity of beans stored under different conditions was determined by soaking in distilled water at room temperature (25°C) for various periods of time. Percent water absorption was calculated by comparing the drained weight of the soaked bean with that of the beans before soaking.

Percentage of hardshell beans was determined by counting the seeds which did not absorb water at 25°C, after 6 hr soaking in distilled water.

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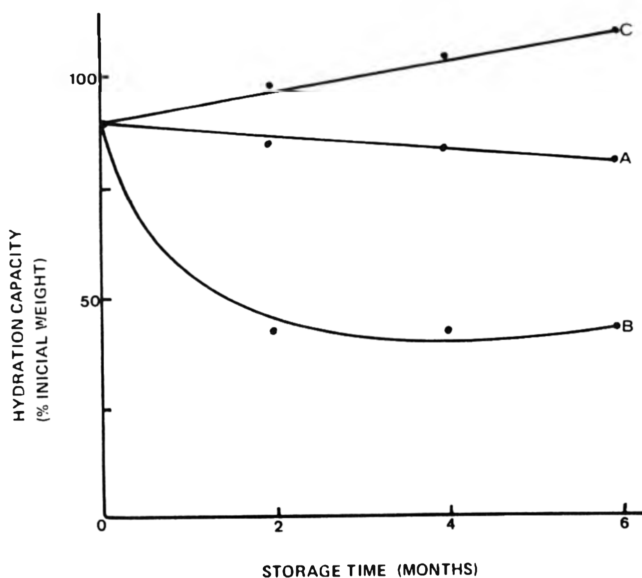

Fig. 1—Variation of the hydration capacity of a dry bean (Phaseolus vulgaris) variety Rosinha G2 as a function of storage time and conditions: A—12°C, 52% RH; B—25°C, 65–70% RH; and C—37°C, 76% RH.

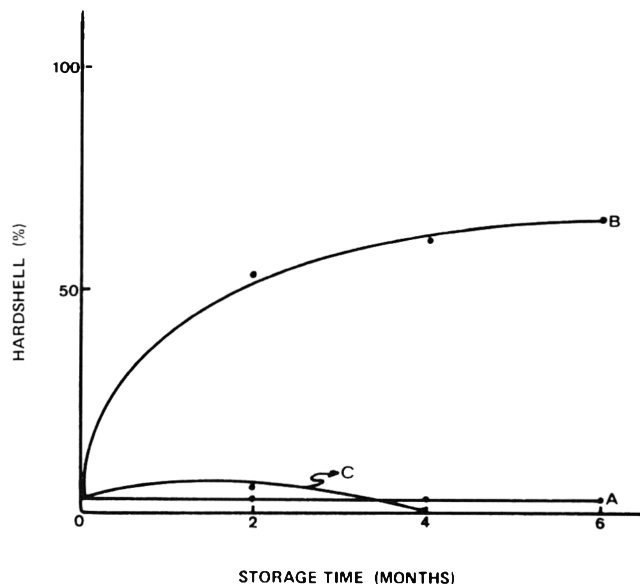

Fig. 2—Changes in the percentage of hardshell of a dry bean (Phaseolus vulgaris) variety Rosinha G2, as a function of time and storage conditions: A—12°C, 52% RH; B—25°C, 65–70% RH and C—37°C, 76% RH.

Texture hardness was determined, in cooked beans, by extrusion force in the Instron apparatus using extrusion cell of 7 × 7 cm containing holes of 0.6 cm in diameter and extrusion velocity of 10 cm/min. The results were expressed in kilogram-force.

The results of hydration capacity, percentages of hardshell beans and texture hardness are mean values of three replicates.

Procedures employed for nutritional evaluation

Changes in the nutritional properties of the bean proteins were evaluated by PER, apparent digestibility and available methionine and cysteine. All biological assays were done on beans cooked under atmospheric pressure (100°C) for various times to reach the most acceptable texture by the panel.

Protein efficiency ratio (PER) was determined by the procedure described in the AOAC (1975) and apparent digestibility by measuring total nitrogen intake and total excretion in the feces of the rats submitted to the PER assay during the second and third weeks of feeding the experimental diets. Digestibility was calculated from the ratio of absorbed and ingested nitrogen.

The percentages of available methionine and cysteine were determined biologically using rat assay. For each assay, 20 male weanling rats of the Wistar strain were divided into four groups of approximately the same body weight. The animals received a basal diet which contained 10% protein exclusively from beans. All the other nutrients were added as necessary to make the diet adequate for the rat. For the methionine assay one of the groups received only the basal diet and to the diets of the other three groups DL-methionine was added at 0.3, 0.6 and 0.9% of the protein. L-cysteine was added to all four diets at 1% of the protein to spare methionine.

For the determination of available cysteine, the same general procedure was followed; the basal diet was made sufficient in methionine with 0.6% of the DL-amino acid, whereas the other three diets received L-cysteine at 0.2, 0.4 and 0.6% of the dietary protein.

The amount of available methionine or cysteine from the bean protein was read directly from the straight line plot of the growth responses (y-axis) and the percent methionine or cysteine added (x-axis). In such a plot, the growth response for the animal group on the basal diet (zero addition of the amino acid in question) was laid on the y-axis. The growth responses were clearly a linear function in the ranges 0–0.6% DL-methionine and 0–0.4% L-cysteine. In view of this linearity, the line was extrapolated back to the actual zero methionine or cysteine addition (x-intercept). This interception point represented the amount of the amino acid from bean protein actually available.

By establishing the proportion between the total amino acid in the bean (basal diet) and the amount estimated graphically to be available to the growing rat, the percent availability of methionine or cysteine could be calculated.

Table 1—Proximate percent composition of a dry bean (Phaseolus vulgaris) variety Rosinha G2

Component	Percentage
Protein	23.7
Lipid (P.E. extract)	1.8
Ash	2.9
Crude fiber	4.6
Water	10.0
Carbohydrate (difference)	57.0

RESULTS & DISCUSSION

PROXIMATE PERCENT composition of the bean under study is shown in Table 1. The content was within the range considered adequate for good stability during storage.

The changes in the hydration capacity of the grains during 6 months of storage under the three different conditions are plotted in Figure 1. The variation in the percentage of hardshell beans is shown in Figure 2. By comparison of the data of Figure 1 with those of Figure 2 it becomes apparent that the hydration capacity is inversely proportional to the formation of hardshell beans. In treatment A (12°C, 52% RH) both the hydration capacity (% of bean weight) and percentage of hardshell grains remained essentially constant. Treatment B (25°C, 65–70% RH) decreased the hydration capacity rapidly and significantly in the first 2 months of storage which then remained essentially constant for the rest of the storage period. On the other hand, the percentage of hardshell beans increased proportionally along the same periods. Treatment C (37°C, 76% RH) did not show any hardshell bean after 4 months of storage and showed a small increase in the hydration capacity with storage time. Although no statistical data are provided, a negative correlation is apparent between hydration capacity and percentage of hardshell beans for the sample stored under condition B. Figure 3 shows the changes in texture of bean samples stored under the three different conditions, as measured by extrusion forces in the Instron apparatus. The extrusion force increased from approximately 200–250 kg

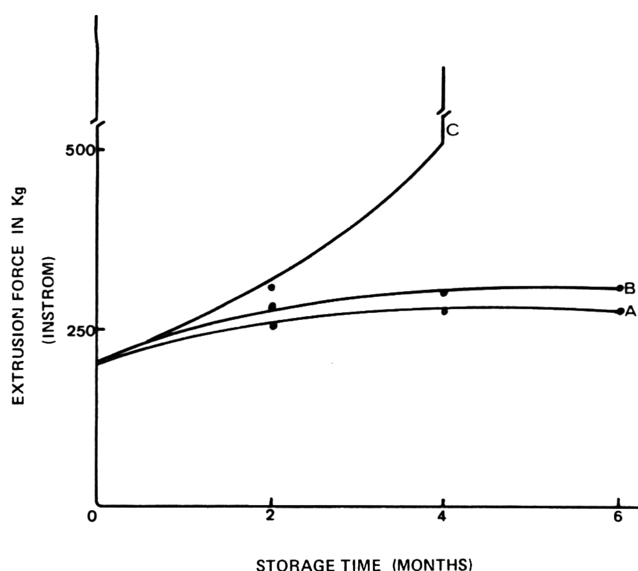

Fig. 3—Textural changes of beans stored for 6 months under different storage conditions: A—12°C, 52% RH; B—25°C, 65–70% RH; and, C—37°C, 76% RH.

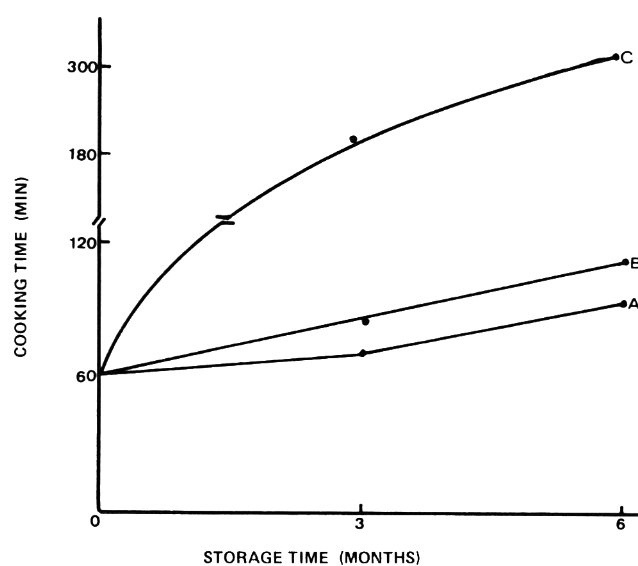

Fig. 4—Variation in the cooking time of a dry bean (Phaseolus vulgaris) stored under different conditions: A—12°C, 52% RH; B—25°C, 65–70% RH; and, C—37°C, 76% RH.

for the beans stored under conditions A and B. However, for the beans stored at 37°C and 76% RH, the increase in the extrusion force was very great after the second month of storage reaching the end of the scale of the apparatus (500 kg) by the fourth month.

The increase in cooking time as a result of storage is shown in Figure 4. The initial cooking time was 60 min which increased to 95 and 116 min for the beans stored under conditions A and B, respectively. For treatment C (37°C, 76% RH) the cooking time was 300 min after 6 months of storage. Even after this period of cooking the texture was not as soft as in the other treatments.

Total methionine, total cysteine and the results of biological evaluation of cooked beans, which had been submitted to different storage conditions for various periods of time, are shown in Table 2. Cooking times for the samples utilized for methionine and cysteine determination and for biological assays are shown in Figure 4. In all samples cooking was sufficient to completely inactivate trypsin and chymotrypsin inhibitors and the hemagglutinating activity. All parameters studied decreased with storage time even under the mildest storage condition A. PER, methionine and cysteine availability dropped significantly with time as the conditions of storage became more drastic. PER decreased from 1.01 to 0.66, 0.43 and 0.10 after 6 months of storage under the conditions A, B and C, respectively. Methionine availability dropped from 46.3 to 43.1, 38.2 and 27.6% whereas available cysteine decreased from 51.6 to 45.8, 43.0 and 30.1%, for the conditions A, B and C, respectively, for the same storage period. Apparent digestibility dropped from 62.4% to 58.9, 57.1 and 54.4%, respectively, for the conditions A, B and C.

The decrease in availability of methionine and of cysteine is quite consistent with the decrease in PER.

Total methionine and total cysteine in the raw bean were 1.23 and 0.95g/16g N, respectively. The methionine and cysteine data of Table 2 show that the concentration of these amino acids dropped considerably in the cooked bean samples. This was attributed primarily to elimination of free amino acids with the soaking water, although, some methionine and particularly cysteine could have been degraded during cooking and the acidic condition of hydroly-

Table 2—Effect of time and conditions of storage on PER, digestibility, total methionine, total cysteine, methionine and cysteine availability from a cooked dry bean (Phaseolus vulgaris) protein

Determination \ Storage time and conditions (months)	Zero time (starting material)	A[a]		B[b]		C[c]	
		3	6	3	6	3	6
PER[d]	1.01(a)	0.98(b)	0.66(c)	0.75(d)	0.43(e)	0.21(f)	0.10(g)
Digestibility (%)	62.4	62.4	58.9	61.9	57.1	59.7	54.4
Total methionine (g/16g N)	0.85	0.85	0.85	0.84	0.81	0.85	0.77
Available methionine (%)	46.3	44.7	43.1	41.5	38.2	36.6	27.6
Total cysteine (g/16g N)	0.60	0.60	0.58	0.62	0.59	0.57	0.52
Available cysteine (%)	51.6	47.7	45.8	45.8	43.0	42.0	30.1

[a] 12°C, 52% RH
[b] 25°C, 65–70% RH
[c] 37°, 76% RH
[d] Standard deviation for PER: (a) ± 0.248; (b) ± 0.264; (c) ± 0.235; (d) ± 0.236; (e) ± 0.283; (f) ± 0.214 and (g) ± 0.210. PER for casein 3.36 ± 0.265.

Table 3—Effect of methionine and cysteine supplementation on PER and protein digestibility of cooked dry bean (Phaseolus vulgaris) stored for 6 months under different condition

Storage conditions[a]	PER ± SD Without addition	PER ± SD Added[b] met + cysH	Digestibility (%) Without addition	Digestibility (%) Added[b] met + cysH
A	0.66 ± 0.235	2.45 ± 0.241	61.10	62.23
B	0.43 ± 0.283	2.46 ± 0.220	61.22	60.51
C	0.10 ± 0.210	2.40 ± 0.200	58.91	59.62

[a] A–12°C, 52% RH; B–25°C, 65–70% RH; C–37°C, 76% RH.
[b] Added met. 3% and cysH 2% on protein basis.

sis. Soaking water was discarded prior to cooking because this is custumary in domestic preparation of beans in Brazil. There was no decrease on the methionine and cysteine contents with storage time, except for the beans stored 6 months under condition C. The slightly lower contents of sulphur amino acids in these samples could have been the result of a much longer cooking time to which they were submitted.

Molina et al. (1975) reported an increase in available lysine and in total methionine during a six months storage study of black beans. On the other hand the PER values for the corresponding material decreased significantly.

The results reported in this paper for PER are in agreement with those of Molina et al. (1975), whereas their data on total methionine are in disagreement with ours since we did not find an increase in the amount of this amino acid with increased storage time. This discrepancy could be attributed to varietal differences or to the different method employed for the determination of methionine. They employed a microbiological assay whereas we used a chemical determination.

The cited authors interpreted the lack of correlation between increase in methionine contents and PER values as a possible drop in the availability of methionine during storage and processing.

Evans et al. (1974) indicated that the degree of availability of the sulphur-containing amino acids for either beans or soybeans correlated with PER values, which is in perfect agreement with our findings.

Further evidence that the low biological value of the proteins in the beans stored for various periods was due to methionine and cysteine unavailability was obtained by a PER assay in which methionine and cysteine were added at 3% and 2% of the protein, respectively. The diets contained the beans stored under conditions A, B and C as the only source of protein. The results of PER and apparent digestibility for these assays appear in Table 3. The PER of all three stored samples increased essentially to the same value by the addition of the sulphur-containing amino acids. The digestibility remained essentially unaffected by the addition of methionine and cysteine.

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Ms received 12/31/78; revised 5/12/79; accepted 5/20/79.

FURTHER STUDIES ON THE ENRICHMENT OF LIME-TREATED CORN WITH WHOLE SOYBEANS

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ABSTRACT

A series of experiments were performed to study further the effect of supplementing corn with whole soybeans processed in a batch home process and in a continuous industrial operation on chemical composition, presence of antiphysiological substances and protein quality of tortillas. Variables studied with the conventional home cooking process included levels of whole soybeans from 0–20%, cooking times at atmospheric pressure and 96°C from 0–90 min at a constant corn-to-soybean ratio of 85:15 and lime concentration of 0, 1 and 2%. In the industrial process a 90:10 mixture of corn to whole soybeans was used, and processing conditions were those normally employed for corn alone. Although addition of whole soybeans to corn increased total protein and fat content, both the PER and weight data suggest that the optimum amount of soybeans to add to maize falls between 8 and 12%. Levels of soybeans above 12%, did not significantly improve PER any further. Antiphysiological factors were inactivated after 30 min of cooking independent of lime concentration in the home process. These factors were also inactivated in the industrial process as measured chemically and by biological assays. The study demonstrated the feasibility of producing an enriched tortilla flour at the industrial scale, which besides containing a higher protein content and quality, also provides an increased amount of calories to the consumer.

INTRODUCTION

THE BASIC CEREAL of many Latin American countries is corn, consumed in a relatively large variety of forms. In most of the Central American countries and Mexico, the main form of consumption is the well-known tortilla, made either at home or industrially by cooking maize with lime (Bressani et al., 1972; Katz et al., 1974; Bressani, 1972). Even though "tortillas" are flat-round cakes, the size, thickness and appearance preference vary among countries.

In the northern countries of South America corn consumption is in the form of "arepas," made by cooking degermed corn flour with water (Bressani et al., 1972). Besides these two main forms of consumption, corn is used to make drinks called atoles and foods such as "tamales" and the like.

Corn proteins are known to be deficient in the essential amino acids lysine and tryptophan (Bressani and Marenco, 1963). Many efforts have been made to improve their quality by genetic means as in the case of Opaque-2 corn, by adding the deficient amino acids or by protein supplementation (Bressani et al., 1972). This last approach offers various nutritional advantages such as increasing protein content besides improving its quality. Many protein supplements have been tested, and among them soybean protein has received much attention (Bressani and Marenco, 1963; Bressani and Villarreal, 1963; Del Valle and Pérez-Villaseñor, 1974; Franz, 1975; Del Valle et al., 1976; Green et al., 1976, 1977).

Studies carried out in our laboratories with experimental animals have suggested that optimum protein quality is obtained when whole corn flour, either raw or cooked, is supplemented with 4–5g % of soybean protein (Bressani et al., 1974). The improved quality has been confirmed by nitrogen balance studies in children (Bressani et al., 1972). In these studies, nitrogen retention values were similar to those from milk, and significantly above those obtained with common corn flour.

One of the problems in implementing these results is the fact that many population groups still transform maize into tortillas at home, although tortilla flour is industrially produced and available in most corn-consuming countries in Latin America. With the idea of exploring possible ways to implement the above finding in the rural areas, which would also be capable of industrial implementation, various sources of soybean proteins were examined, including whole soybeans (Bressani et al., 1974). With this background information, results from further studies on the uses of whole soybeans as a supplement to corn will be presented.

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0022-1147/79/0006-1707$02.25/0
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MATERIALS & METHODS

Pilot plant studies

Whole corn and whole soybeans produced at INCAP's experimental farm were used in the studies carried out in the pilot plant.

Lots of 10 kg samples with 0, 4, 8, 12, 16 and 20% whole soybeans replacing equivalent amounts of corn by weight were processed in pilot plant facilities by a standard lime-cooking treatment described previously (Bressani and Scrimshaw, 1958; Bressani et al., 1958). After cooking and washing excess lime, the material was ground wet, dried in a forced draft oven at 60°C overnight, ground in a hammer mill into a fine flour and analyzed for protein by the Kjeldahl method and for fat (AOAC, 1970). These samples were then subjected to biological assay as will be described later.

In a second study, samples of 85% maize and 15% whole soybeans were cooked for 0, 15, 30, 45, 60, 75 and 90 min. During the cooking process samples were withdrawn to determine water uptake in the components and mixture. After cooking, the material was converted into a dry flour as described above. An additional lot was cooked for 60 min, and converted into a dough and then into tortillas. These were air-dried at 60°C in a tray drier, and then ground. All these samples were assayed for protein quality in diets made of 85% of the mixture supplemented with minerals (4%), cod liver oil (1%), maize starch to adjust to 100%, and a B-vitamin mixture. A casein diet was used as control. The samples were analyzed for residual trypsin inhibitor activity by the method of Kakade and Evans, 1966.

In order to obtain additional information on the effects of lime-cooking on the activities of urease and trypsin inhibitors in soybeans, mixtures of maize (85%) and soybeans (15%) were cooked at 96°C in water with 0, 1 and 2% $Ca(OH)_2$. Samples were withdrawn every 15 min up to 90 min and the soybeans were manually separated from the maize, dried at room temperature and assayed for urease (Caskey and Knapp, 1944) and trypsin inhibitors (Kakade and Evans, 1966).

Industrial studies

These were carried out with an industrial enterprise (Asesoría de Empresas S.A.) in Monterrey, México. In this case, 10% whole soybeans was used in a continuous lime-cooking process. Duplicate samples were withdrawn at specific intervals from various places in the

process, representing different cooking times. These samples were used to measure the distribution of the two grains during the process, water absorption in the individual grains and mixtures, protein and fat content in the mixture, and urease and trypsin inhibitor activity as well as protein quality as described before. Lots of lime-cooked maize flour, and two lots of the cooked maize/soybean blends were also fed to rats for an 85-day feeding period in order to detect possible adverse effects.

Biological assays

In all animal assays weanling white rats of the Wistar strain from the INCAP colony were used. Eight animals per experimental group were placed in individual all-wire screen cages and fed ad libitum, with water available all the time. Weight gain and food intake were measured every 7 days. The PER assays lasted the conventional 28-day period, while the long-term feeding assay lasted 85 days. In some experiments the animals were sacrificed and the pancreas excised and weighed.

Three types of diets were fed: isocaloric with variable protein content (Diets A); isocaloric and isoproteic (Diets B), and isoproteic with variable energy content (Diets C). A casein control diet containing 11.2% protein and 5% oil was also fed. The PER values were not adjusted to that of the casein diet.

RESULTS

THE MAIZE FLOUR with the various levels of soybeans added before processing were tested biologically using three sets of diets as described previously. The partial composition of the diets are presented in Table 1. As shown in the upper part of the Table, both protein and fat content in the cooked flour increased as whole soybeans percentage in the mixture increased. From the weight gain and protein efficiency ratio data shown in Table 2, it appears that between 8 and 12% whole soybeans produce maximum quality in maize, even though the dietary conditions imposed affected weight gain more than PER. Levels above 12% did not improve further protein quality. This is clearly observed when comparing experiments 1 and 3 with 2.

Information of the rate of water uptake in maize, soybeans and mixture 85:15 during cooking are summarized in Table 3. For maize, water uptake doubled during the first 15 min of cooking and then increased slowly with respect to time; for soybeans, however, the increase during the first 15 min was 5 times as high as the initial value and then increased slowly with respect to time, reaching higher levels as compared to maize. Protein content in the mixture at 10% moisture was 13.2% and fat 7.8%, as compared to 8.9 and 5.2%, respectively, in whole cooked maize.

As shown in Table 4, the PER values increased from 1.15 in the raw maize:soybeans 85:15 mixture to values between 2.41 and 2.51 after cooking periods longer than 15 min. Increasing cooking time or the transformation of the

Table 1—Proximate composition of flours and formulation of diets used in the rat experiments

		% Whole soybeans in blend with maize					
		0	4	8	12	14	20
Moisture in flour (%)		7.0	7.6	7.1	7.5	6.4	6.6
Protein (%)		9.7	10.6	11.6	12.5	14.3	14.8
Fat (%)		3.5	4.6	5.0	6.1	7.1	7.4
Diets A[a]	Ingredient[b]						
	Maize + soybeans	90.0	90.0	90.0	90.0	90.0	90.0
	Cottonseed oil	3.6	2.6	2.2	1.2	0.3	–
	Maize starch	1.4	2.4	2.8	3.8	4.7	5.0
	% protein in diet[c]	8.8	9.1	10.0	11.5	12.6	13.0
Diets B[a]	Ingredient						
	Maize + soybeans	90.0	80.0	72.0	65.0	60.0	55.0
	Cottonseed oil	3.6	3.0	3.1	2.7	2.4	2.6
	Maize starch	1.4	12.0	19.9	27.3	32.6	37.4
	% protein in diet[c]	8.8	8.4	8.4	8.5	8.5	8.5
Diets C[a]	Ingredient						
	Maize + soybeans	95.0	95.0	95.0	95.0	95.0	95.0
	% protein in diet	8.5	9.5	10.7	11.9	13.0	13.8
	% fat in diet	4.3	5.4	5.8	6.8	7.7	8.0

[a] Diets A – Constant fat, variable protein; Diets B – Constant fat, constant protein; Diets C – Variable fat; variable protein
[b] All diets were supplemented with 1.0% cod liver oil, 4% mineral mixture and a complete B vitamin mixture.
[c] Diets were adjusted to 6.7% oil content.

Table 2—Weight gain and PER in rats fed different blends of maize and whole soybeans

	% Whole soybeans in blends with maize					
	0	4	8	12	16	20
Diets A[a]						
Avg weight gain, g[b]	31 ± 7.4[c]	57 ± 4.6	80 ± 4.9	110 ± 2.9	114 ± 8.0	139 ± 5.8
PER	1.25 ± 0.27	1.79 ± 0.06	2.11 ± 0.09	2.26 ± 0.06	2.18 ± 0.09	2.41 ± 0.05
Diets B[a]						
Avg weight gain, g[b]	31 ± 7.4	39 ± 3.6	57 ± 3.4	69 ± 2.8	69 ± 4.9	86 ± 5.4
PER	1.25 ± 0.72	1.54 ± 0.11	2.04 ± 0.05	2.29 ± 0.13	2.32 ± 0.13	2.45 ± 0.13
Diets C[a]						
Avg weight gain, g[b]	25 ± 3.9	70 ± 6.9	96 ± 3.6	101 ± 10.2	128 ± 10.3	111 ± 11.3
PER	1.02 ± 0.13	2.13 ± 0.18	2.21 ± 0.08	2.22 ± 0.19	2.30 ± 0.24	2.08 ± 0.27

[a] See Table 1. [Casein diet (10% protein): PER, 2.77[b] ± 0.23. PER values for experimental diets were not adjusted to casein PER.]
[b] Average initial weight, 43g
[c] Standard error

flour into tortilla did not change the quality of the product. It is also of interest to point out the complete destruction of trypsin inhibitor activity (Table 4). These observations were confirmed by pancreas weight of the animals at the end of the 28-day experimental period.

The effects of cooking and lime concentration on urease and trypsin inhibitor activity are shown in Table 5. The raw soybeans showed an average trypsin inhibitor activity of 33 units. The results suggest little, if any, effects of lime concentration in inactivating urease and trypsin although urease activity disappeared faster when lime concentration was 2%. Thus the inactivation is due to temperature and time more than to the other processing conditions.

With respect to the samples taken from the industrial plant, Table 6 shows the weight distribution of maize and soybean, a possible problem in the adaptation of the technology since the process was a continuous operation. In theory, the mixture subjected to processing was a 90:10, maize:soybeans mixture. The distribution taken at various times showed a variation of 7.0–26.0% soybeans. It can be concluded that, apparently, even with the variation found, this is not a problem even though some changes will have to be made to insure more consistent results. Analyses of moisture in samples of maize, soybeans and the mixtures as shown in Table 6, indicate a more rapid uptake of moisture by soybeans than by maize, as previously found in the pilot plant studies. Further evidence on the distribution based on chemical analysis is also shown in Table 6. It can be seen that the chemically determined values for protein and fat in the mixture confirm those based on the quantities found individually in maize and soybeans, since there is a 2-point increase in fat from 4 to 6 and a 3.6 point increase in protein content. These data were taken to indicate that the distribution of corn and soybean kernels was as found, that is, slightly higher than originally set, 90:10. The quality improvement is shown in Table 7. The raw mixture had a PER value of 0.46 which increased to 2.04 in the final flour. For comparative purposes the Table includes the PER of the maize flour itself (1.20). The improvement in quality observed is slightly less than the predicted value as established from other studies; however, it may be due to the individual protein quality of the two components.

Analysis of urease and trypsin inhibitor activity indicates again a disappearance of these antiphysiological substances, also corroborated by pancreas analysis, indicated by the IPC values (Elías et al., 1976).

The long-term feeding test results are summarized in Table 8. They show normal development of the animal; no gross pathological signs were observed.

DISCUSSION

PREVIOUS RESULTS reported by various groups (Del Valle and Pérez-Villaseñor, 1974; Franz, 1965; Del Valle et al., 1976; Bressani et al., 1974) on the subject presented in this paper, showed that the addition of soybean protein to maize increases the protein quality and total protein content of the latter. As shown in this and other reports, addition of whole soybeans also provides additional amounts of

Table 3—Water uptake with respect to cooking time in maize, whole soybeans and in 85:15 mixture

Cooking time (min)	Maize	Whole soybeans	Maize:whole soybeans 85:15
0	13.8	10.0	13.4
15	26.1	54.4	31.8
30	31.2	59.3	39.9
45	37.1	59.5	42.0
60	38.3	60.0	43.8
90	42.7	60.6	47.4
Tortilla	39.1	61.4	45.2
% Protein (10% moisture)	8.96	41.21	13.24
% Fat	5.2	22.2	7.8

Table 4—Effect of cooking time on protein quality of maize:whole soybeans blend (85:15) and trypsin inhibitor activity

Cooking time[a] (min)	Avg wt gain, g[b]	PER	TUI/ml[c]
0	30 ± 3.9	1.15 ± 0.11	10.3
15	96 ± 3.8	2.36 ± 0.07	4.4
30	103 ± 5.9	2.46 ± 0.07	0.0
45	90 ± 4.5	2.41 ± 0.08	0.0
60	107 ± 7.6	2.51 ± 0.11	0.0
90	97 ± 4.6	2.43 ± 0.08	0.0
Tortilla	97 ± 6.8	2.34 ± 0.11	0.0
Casein	117 ± 6.6	2.79 ± 0.11	–

[a] Protein in diets: 10.4
[b] Average initial weight, 48g
[c] TUI: Trypsin Units Inhibited

Table 5—Effect of lime concentration on trypsin inhibitor and urease activity of whole soybeans cooked with maize[a]

Cooking time (min)	Lime concentration					
	0		1%		2%	
	TUI[b]	Urease	TUI	Urease	TUI	Urease
0	29.9	+	36.4	+	38.8	+
15	28.8	+	18.3	+	37.1	+
30	4.8	+	2.8	+	2.8	+
45	4.0	+	0.0	+	2.8	+
60	0.0	–	0.0	–	0.0	–
75	0.0	–	0.0	–	0.0	–
90	0.0	–	0.0	–	0.0	–

[a] Analysis on soybeans only
[b] Trypsin units inhibited

Table 6—Grain distribution, water uptake, protein and fat content of samples withdrawn at various times during industrial processing of maize:soybeans mixture

Cooking time (min)	% distribution (d.w.)[a]		Water uptake % moisture			In mixture[b]	
	Maize	Soybeans	Maize	Soybeans	Mixture	Protein, %	Fat, %
0	87.2	12.8	12.6	8.8	12.2	12.1	6.2
15	74.0	26.0	29.4	42.0	32.6	–	–
30	92.3	7.7	35.0	51.0	35.8	12.3	5.6
45	90.0	10.0	38.1	57.0	40.0	–	–
60	93.0	7.0	38.3	53.0	40.0	11.4	5.5

[a] d.w.: dry weight. Average distribution: 87.3% maize, 12.7% soybeans.
[b] Air-dried weight basis

Table 7—Protein quality, urease and trypsin inhibitor activity of maize:soybeans samples withdrawn at different times during industrial processing

Product	Avg wt gain, g[a]	PER[b]	Urease	TUI/mlc[c]	ICP[d]
Raw mixture	10 ± 2.1	0.46 ± 0.10	+	6.2	0.328
15 min cooked	41 ± 4.1	1.52 ± 0.08	+	4.4	1.009
30 min cooked	48 ± 3.7	1.65 ± 0.11	–	2.0	1.692
Final product (flour)	61 ± 2.7	2.04 ± 0.06	–	0.8	2.029
Cooked maize (flour)	32 ± 3.1	1.20 ± 0.08	–	–	1.601
Casein	99 ± 5.3	2.82 ± 0.12	–	–	2.714

[a] Average initial weight: 50g
[b] Average protein in diets: 8.5%
[c] Trypsin units inhibited
[d] Pancreas growth index (Average wt gain/pancreas wt X 100

Table 8—Long term rat feeding test with lime cooked maize and maize:whole soybeans

Lime-cooked product	Avg wt gain, g[a]	Food efficiency[b]	Pancreas wt/100g rat	ICP[c]
Maize flour	102 ± 4.8	9.7	0.155	4.330
Maize: soybean flour	126 ± 7.6	8.7	0.199	3.589
Maize: soybean flour	169 ± 5.9	7.3	0.171	4.516
Casein	170 ± 9.3	7.4	0.227	3.410

[a] 85 days – average initial weight: 50g
[b] g food consumed/g weight gain; (See Table 6)
[c] Protein in diets: 4.8%

energy as fat (Bressani et al., 1974). The fat content increases on the average from a value of 3.7% on tortilla flour to 5.1% on whole soybeans-supplemented flour, thus increasing the energy density of the food. It is common to find in Guatemalan rural areas (Bressani et al., 1972) an intake of 14–22 tortillas/person/day, which is equivalent to an intake of 12.9–20.3g fat which increases to 17.8–28.0g when the corn is supplemented with 12–15% whole soybeans, thus increasing the total energy as well as total protein intake of the individual, with a more efficient utilization. Children would benefit less because of a lower tortillas intake; however, a greater nutritional benefit would result if such a food was offered as a gruel or drink.

The alkaline cooking process can be carried out at home as well as by using the industrial process and as the results showed, there is a complete inactivation of the antiphysiological factors present in soybeans. Of particular concern was the possibility that with a continuous process as the industrial processing is, the two grains would separate. On the average, raw corn kernels weigh 0.325 g/grain while raw whole soybeans weigh 0.152 g/grain. Furthermore, corn kernel density is around 0.730 g/ml, while that of soybeans is 0.680 g/ml. Due to the higher rate of water uptake by soybeans as compared to corn, however, a more or less constant distribution is maintained, as the material proceeds along the cooking, washing, drying and grinding processes. It is also important to indicate that cooking time used in the preparation of tortillas is also adequate to cook soybeans.

From the results of the studies, it may be concluded that enrichment of corn with not less than 8% whole soybeans increases protein content and protein quality as well as energy content of tortillas. Furthermore, both processes inactivate the antiphysiological factors present in the whole soybeans added to corn. This, as well as the increases in nutrient content and quality, are of great nutritive significance to corn-consuming populations in Latin America.

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Ms received 2/23/79; revised 5/29/79; accepted 6/5/79.

Presented at the 37th Annual Meeting of the Institute of Food Technologists, Philadelphia, PA, June 5–8, 1977.

This research was carried out with funds from the Research Corporation, New York, NY (Grant-in-aid INCAP PN-740).

ESTIMATION OF ZINC AND COPPER IN RAW AND COOKED LEGUMES: AN INTERLABORATORY STUDY OF ATOMIC ABSORPTION AND X-RAY FLUORESCENCE SPECTROSCOPY

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ABSTRACT

Zinc and copper are important nutrient elements in dry beans. The precision and accuracy of two instrumental methods, atomic absorption and x-ray fluorescence spectroscopy, were compared and evaluated for the estimation of total copper and zinc in raw and cooked legume products. After calibration against National Bureau of Standards reference samples, corrected values obtained by both methods were essentially identical and in good agreements with values reported in the literature for random, analogous materials. Cooked standard and quick-cooking beans both contained nutritionally significant amounts, but less zinc and copper than inedible, raw, dry beans.

INTRODUCTION

RECOGNITION of the importance of mineral elements in maintenance of optimum nutrition and health, as well as formal requirements for information to be used in nutritional labeling, has emphasized the need for convenient, rapid, accurate and precise values for individual elements in human foods. Interest in the nutritional importance of zinc and copper (Mertz and Cornatzer, 1971; Prasad, 1976); and concurrent interests of the Western Regional Research Center and the Nutrition Institute in food grain legumes and analytical methodology served as a mutual basis for conducting a collaborative study on the accuracy and precision of atomic absorption and X-ray fluorescence for measurements of zinc and copper in various legume food products. Atomic absorption and X-ray fluorescence spectroscopy techniques each have their own unique advantages and limitations. Equipment for atomic absorption spectroscopy is less expensive and more generally available. The more sophisticated X-ray fluorescence technique permits automatic nondestructive, repetitive, computerized measurements on multiple samples and elements.

Identical authentic samples of various bean products and National Bureau of Standards reference samples were analyzed for zinc and copper using the atomic absorption facilities at the Nutrition Institute, and X-ray fluorescence equipment at the Western Regional Research Center. The two elements were determined in a variety of raw, cooked standard and quick-cooking products prepared from four commercial legume cultivars, and the corrected values compared for accuracy and precision.

EXPERIMENTAL

Dry beans

Blackeye beans (*Vigna unguiculata*), 1970 crop, and pink beans (*Phaseolus vulgaris*, c.v. Gloria), 1974 crop, were isogenetic strains grown at the University of California, Davis. The large lima beans (*Phaseolus lunatus*, c.v. Ventura), 1973 crop, were obtained from established commercial sources in May, 1974. The garbanzo beans (*Cicer arietinum*), 1970 crop, also were obtained from commercial sources in February, 1971.

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Table 1—Processing conditions for quick-cooking beans

	Bean variety			
Processing variable	Large lima	Blackeye	Garbanzo	Pink
Bean weight, g	150	150	150	150
Hydravac time, min (5 min on/off)	50	–	–	–
Steam blanch, min	–	–	–	3
Salt solution				
Volume, ml	450	450	450	450
Sodium chloride, %	1.0	2.0	2.0	2.0
Sodium tripolyphosphate, %	0.5	1.0	0.0	0.1
Sodium bicarbonate, %	0.45	0.45	0.375	0.45
Sodium carbonate, %	0.15	0.15	0.125	0.15
Soaking time, hr	6	24	24	24
Subjective cooking time for salt-soaked beans, min	8	10	15	11
Subjective cooking time for water-soaked beans, min	45	25	120	50

Sample preparation

Standard, water-soaked beans were prepared by adding 150g of whole dry beans to 450 ml of distilled water, allowing the beans to imbibe water, and hydrate at 21°C for 24 hr. Half of the drained beans or about 150g of the raw, hydrated product was slurried with an equal volume of distilled water in a Waring Blendor. The slurry was lyophilized and equilibrated in ambient air at about 50% relative humidity for 48 hr to minimize changes in moisture content during subsequent handling. The second portion of raw, hydrated beans was cooked in 4 wt vol of boiling, distilled water until tender. Cooking times for individual types of beans are shown at the bottom of Table 1. The cooked beans were drained, slurried with an equal volume of water, lyophilized and equilibrated in the same way as the raw products.

Quick-cooking, salt-soaked beans were prepared from whole, raw dry beans as shown in Table 1. Hydration in the salt solution of large lima beans was facilitated by Hydravac treatment (Rockland and Metzler, 1967). Steam blanching was employed to accelerate hydration of pink beans. No special treatment was employed for blackeye and garbanzo beans which hydrate rapidly at ambient temperature. As indicated in Table 1, beans were soaked in 3 wt vol of the appropriate salt solution for the specified hydration period, drained and rinsed lightly. One-half portion of about 150g of each hydrated bean sample was slurried with an equal volume of water, lyophilized and equilibrated in ambient air. The second portion of each sample was cooked in 3 wt vol of fresh boiling water in an open stainless steel saucepan for the time period indicated in Table 1. Subjective cooking times were estimated as described by Rockland and Metzler (1967). Cooked beans were drained, rinsed, slurried with an equal volume of water, lyophilized and equilibrated in air.

All raw and cooked samples of beans were ground to pass a 20 mesh screen in a Micro Wiley Mill.

Proximate analysis

Proximate analyses were determined by the standard methods of AOAC (1975).

X-Ray fluorescence spectrometry

Cooked, dried bean powder samples were prepared for analysis by compressing a 300 mg portion of each into a self-supporting pellet, 1 mm thick and 32 mm diameter, using a circular die and a hydraulic press at 16,000 psi. Pellets of the less cohesive raw bean powders were prepared similarly after blending 100 mg of Whatman DF 11 Cellulose powder, used as a binder, with 200–400 mg of

ground raw beans in a SPEX mixer-mill (Spex Industries, Inc., Metuchan, NJ). Each pellet was placed into a rotating sample holder in the sample changer chamber and evacuated for immediate analysis; or it was stored temporarily in a Millipore petri slide (Millipore Corp., Bedford, MA) as described by Reuter and Reynolds (1974).

A Finnigan Model 8 Energy Dispersive Fluorescence Spectrometer (Finnigan Corp., Sunnyvale, CA), equipped with a 30 mm² Si (Li) detector collimated to 12 mm², was employed for analysis. The exciting spectrum from a Rh targeted X-ray tube was filtered through 50.8 μm Rh foil. The tube voltage was 40 kV and the running time was 1000 sec. Tube current was adjusted to 0.4 ma for 400 mg samples and 1.0 ma for all other samples. Observed detection limits were 0.1 mg/100g for both zinc and copper.

Data reduction was performed on an IBM 1800 computer, employing elemental X-ray intensitites from the sample, relative X-ray production probabilities determined from thin film standards, elemental X-ray attenuation coefficients, and density of the sample (mg/cm²). This mathematical treatment of the data accounts for the absorption effects of a cellulose matrix, and deviations of the matrix effect caused by the measured elements (Reuter, 1975).

National Bureau of Standards Reference Material 1571, (Orchard Leaves) was analyzed to verify calibration of the procedure.

Table 2—Analyses of National Bureau of Standard's Reference Materials

Material	Zinc (μg/g) Observed[a]	Zinc (μg/g) NBS	Copper (μg/g) Observed[a]	Copper (μg/g) NBS
Orchard leaves SRM 1571 (X-ray fluorescence)	29.4 ± 0.8	25 ± 3	12.1 ± 1.2	12 ± 1
Bovine liver SRM 1577 (atomic absorption)	128.5 ± 2.3	130 ± 10	211.0 ± 9.5	193 ± 10

[a] Average of three replicate determinations

Atomic absorption spectrometry

Triplicate samples of 0.5 – 1.0g were weighed into glass test tubes (15 × 150 mm). One milliliter of concentrated sulfuric acid (Ultrex, J.T. Baker, Phillipsburg, NJ) and, 1.0 ml metal-free distilled water was added and the samples heated gently at less than 50°C (Multi-

Table 3—Comparison of atomic absorption and X-ray fluorescence analyses of copper in various bean products[a]

Product	Condition	Copper, mg/100g: A Atomic absorption Avg[b]	A Std. Dev. σ	B X-Ray fluorescence Avg	B Std. Dev. σ	Difference A – B
Large lima	Raw, dry	0.58	–	0.79	0.03	–0.21
	Cooked, standard	0.53	0.17	0.65	0.11	–0.12
	Cooked, quick-cooking	0.77	0.03	0.73	0.09	0.04
Blackeye	Raw, dry	0.85	0.11	1.06	0.04	–0.21
	Cooked, standard	0.82	0.06	0.66	0.10	0.16
	Cooked, quick-cooking	0.80	0.02	0.63	0.11	0.17
Garbanzo	Raw, dry	0.92	0.02	1.18	0.13	–0.26
	Cooked, standard	0.86	0.03	0.90	0.01	–0.04
	Cooked, quick-cooking	0.98	0.02	1.09	0.10	–0.11
Pink	Raw, dry	0.70	0.19	0.98	0.03	–0.28
	Cooked, standard	0.78	0.04	0.93	0.09	–0.15
	Cooked, quick-cooking	0.65	0.03	0.57	0.10	0.08
Average			0.06		0.08	–0.08

[a] Moisture free basis. Values represent three replicate samples except for cooked, quick-cooking garbanzo using X-Ray fluorescence which represents nine replicate samples.

[b] See text. Correction of –9% of the observed value was applied based on deviation from SRM 1577 (Bovine Liver).

Table 4—Comparison of atomic absorption and X-ray fluorescence analyses of zinc in various bean products[a]

Product	Condition	Zinc, mg/100g: A Atomic absorption Avg	A Std. Dev. σ	B X-Ray fluorescence Avg[b]	B Std. Dev. σ	Difference A – B
Large lima	Raw, dry	2.96	0.03	2.77	0.38	0.19
	Cooked, standard	2.65	0.32	2.55	0.17	0.10
	Cooked, quick-cooking	2.57	0.09	2.25	0.15	0.32
Blackeye	Raw, dry	3.68	0.30	3.04	0.16	0.64
	Cooked, standard	3.69	0.09	3.22	0.60	0.47
	Cooked, quick-cooking	3.12	0.11	3.33	0.14	–0.21
Garbanzo	Raw, dry	3.40	0.09	2.91	0.12	0.51
	Cooked, standard	3.67	0.15	3.34	0.13	0.33
	Cooked, quick-cooking	3.21	0.11	3.02	0.12	0.19
Pink	Raw, dry	2.29	0.41	2.43	0.09	–0.14
	Cooked, standard	2.69	0.12	2.32	0.08	0.37
	Cooked, quick-cooking	1.81	0.08	1.39	0.03	0.42
Average			0.16		0.18	0.27

[a] Moisture free basis. Values represent three replicate samples except for cooked, quick-cooking garbanzo using X-ray fluorescence which represents nine replicate samples.

[b] See text. A correction of –17.5% of the observed value was applied based on deviation from SRM 1571 (Orchard Leaves).

temp Block #2093, Labline Instrument Co., Melrose Park, IL) for 1 hr. One milliliter of 50% hydrogen peroxide was carefully added until evolution of bubbles ceased and additional aliquots of H_2O_2 were added until the solution became clear. The cooled solution was filtered into 15 ml plastic graduated tubes through medium filter paper (Whatman #40) prewashed with dilute HCl. The precipitate was washed with water and the solution made to 10.0 ml. If excessive precipitate were noted, concentrated nitric acid was substituted for the sulfuric acid in the digestion procedure. Aliquots of the filtered solution were diluted by a factor of three or four for analysis by flame atomic absorption spectrometry using a Perkin-Elmer 503 Atomic Absorption Spectrometer equipped with triple slot burner head, air-acetylene flame, standard operating conditions (Zn 2319Å, Copper 3247Å). Triplicate samples of National Bureau of Standards Reference Material 1577 (Bovine Liver) were run with each set of unknown samples.

RESULTS & DISCUSSION

EACH LABORATORY applied its own procedure and standards for the analyses of various bean products. Biological Standard Reference Materials (SRM) were used for calibration purposes. These SRM analyses, listed Table 2, required a −9% correction to observed values for copper by atomic absorption analysis and a −17.5% correction to the observed values for zinc by X-ray fluorescence analysis. No corrections were necessary for the X-ray fluorescence values for copper or for the atomic absorption values for zinc since each value for the respective SRM was essentially identical with the certified value. The use of a primary standard, such as an SRM, in an interlaboratory comparison permits identification of systematic errors and use of secondary standards.

Comparisons of the analytical data from atomic absorption and X-ray fluorescence analyses of the various bean products are presented in Table 3 for copper and Table 4 for zinc.

The average standard deviation observed for copper in 12 samples estimated by atomic absorption was 0.06 mg/100g compared to 0.08 mg/100g for the same samples estimated by X-ray fluorescence. The difference between the two methods ranged from a −0.28 to a +0.17 mg/100g with an average difference of only −0.08 mg/100g. The average standard deviation observed for zinc in 12 samples was 0.16 mg/100g using atomic absorption and 0.18 mg/100g using X-ray fluorescence analysis. The average difference between the two methods for the 12 samples was 0.27 mg/100g or slightly less than the sum of the average standard deviation observed for both methods.

The copper content of 7 samples of isogenetic lima beans, grown in the same location during a 12-yr period, ranged from 0.47 to 1.11 mg/100g and averaged 0.81 mg/100g (Table 5). The zinc content of the same samples ranged from 2.84 to 3.73 mg/100g and averaged 3.23 mg/100g. The ratio of zinc to copper was about 4:1 for the raw dry seeds. Variations in the copper and zinc content were more variable than the protein, fiber and ash but less variable than the lipids in the same samples (Table 6). There were small, but no consistent differences between raw seeds and either cooked standard or quick-cooking beans with respect to zinc or copper content, except possibly for the cooked, quick-cooking pink beans which contained 30–40% less zinc and copper than the raw seeds (Tables 3 and 4). The zinc and copper levels of raw or cooked large lima and pink beans were about 30% lower than garbanzo or blackeye beans.

The averages of closely agreeing atomic absorption and X-ray fluorescence values for copper and zinc compared

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Table 5—Variations with crop year of copper and zinc in isogenetic raw, large lima beans, estimated by X-ray fluorescence soectrometry[a]

Crop year	Amount, mg/100g	
	Copper	Zinc[b]
1958	0.71	2.90
1959	0.47	2.84
1960	1.09	3.73
1961	0.71	3.25
1964	0.94	3.25
1965	1.11	3.70
1972	0.67	2.97
Average	0.81	3.23
Std. Dev. (σ)	0.23	0.36

[a] Moisture-free basis; Single determinations
[b] Corrected as described in text

Table 6—Variations with crop year in proximate composition of isogenetic raw, dry large lima beans[a]

Crop year	Protein (N X 6.25) (%)	Lipid (%)	Fiber (%)	Ash (%)
1958	22.8	0.9	4.5	5.7
1959	22.7	1.1	5.4	5.0
1960	27.3	1.0	5.4	5.7
1961	25.0	0.7	5.3	5.8
1964	24.9	0.9	5.1	5.5
1965	23.8	2.0	5.5	6.0
1972	22.9	0.9	5.1	6.1
Average	24.2	1.1	5.2	5.7
Std. Dev. (σ)	1.7	0.4	0.3	0.4

[a] Moisture-free basis

Table 7—Copper and zinc in raw and cooked beans: Comparison of authors' data with literature values[a]

Product	Condition	Copper, mg/100g			Zinc, mg/100g		
			Literature			Literature	
		Authors[b]	Meiners[c]	Other	Authors[b]	Meiners[c]	Other
Large lima	Raw, dry	0.69	0.92	0.83–0.87[d]	2.84	3.11	2.69–2.97[d]
	Cooked, standard	0.59	0.97	–	2.60	3.41	–
Blackeye	Raw, dry	0.96	1.09	0.86–0.93[d]	3.36	3.45	2.49–3.22[d]
	Cooked, standard	0.74	1.14	–	3.46	4.27	–
Garbanzo	Raw, dry	1.05	1.06	0.97–1.18[e]	3.16	3.51	2.51–3.32[d]
	Cooked, standard	0.88	1.12	–	3.51	4.15	–

[a] Moisture-free basis
[b] Values represent average obtained from atomic absorption and X-ray fluorescence estimations.
[c] Meiners et al. (1976), moisture-free basis
[d] McCarthy et al. (1977), as-is basis
[e] Tiwari et al. (1977), moisture-free basis

TEXTURIZATION OF SUNFLOWER/SOY FLOUR MIXTURES: CHEMICAL AND NUTRITIVE EVALUATION

ENRIQUE YÁÑEZ, MAGALY VÁSQUEZ and FERNANDO SÁNCHEZ

ABSTRACT

The thermoplastic extrusion technique using a Wenger X-25 extrusion cooker was applied to mixtures of sunflower flour and soy flour in the ratios 1:1 (TSP_1) and 3:1 (TSP_2). The processing conditions were as follows: extrusion moisture 18%, feed rate 2750 g/min and extrusion temperature 145°C. Proximate analyses were performed on TSP_1 and TSP_2. The amino acid content of sunflower flour and of TSP_1 were determined in a Perkin-Elmer amino acid analyzer after acid hydrolysis of the proteins. The protein content of TSP_1 and TSP_2 was 46 and 48%, respectively, and both samples were tested for their PER in rats. The values obtained were 1.96 for TSP_1 and 1.63 for TSP_2. The casein control diets gave PERs of 2.54 and 2.48, respectively. Supplementation of TSP_1 with 0.15% DL-methionine improved its PER to 2.32 ($P < 0.05$), but 0.30% of the same amino acid failed to produce a further increase in protein efficiency ratio. Supplementation of TSP_2 with 0.1% L-lysine improved the PER to 2.09 but this value was not as good as methionine-supplemented TSP. Apparent digestibilities shown by these texturized, high protein flour mixtures may be considered satisfactory.

INTRODUCTION

THERE IS MUCH interest today in a greater use of texturized vegetable protein (TSP) as a meat-extender in the human diet. The texturized products resemble meat in some characteristics such as appearance, chewability, flavor and color. Incidents that may have contributed to this process were the meat shortage during 1973–74 and the continued world population growth (Wosje, 1976). At present soybean is relevant in this field but the literature shows us that the investigations have continued including other oilseeds (de Man, 1976). In this particular case the addition of defatted sunflower flour to defatted soy flour in order to obtain a textured vegetable protein has been considered. It is well known that soy protein is rich in lysine and low in sulphur amino acids (Mustakas, 1971; Shelef and Morton, 1976). On the other hand, sunflower protein is poor in lysine and has a moderate content of sulphur amino acids (Smith, 1976; Gheyasuddin et al., 1970). For this reason both proteins should complement each other. One advantage that sunflower seed appears to have is that no toxic components have been reported thus far (Ballester et al., 1967; Eklund et al., 1971). However, the chlorogenic acid present in sunflower seeds presents a disadvantage when the flour is processed since a dark green color develops at an alkaline pH (Cater et al., 1972; Fleming et al., 1974).

Our country can account for reasonable quantities of sunflower presscake with a protein content of 40–50% (Yáñez et al., 1969). Therefore, we became interested in studying blends of these two proteins to obtain an extruded product that could be used as a meat extender.

The objectives of the present investigation were to study the partial substitution of soy flour by sunflower flour to obtain a satisfactory meat extender using the thermoplastic extrusion technique and secondly, to evaluate the nutritional value of the textured protein in rats.

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0022-1147/79/0006-1714$02.25/0
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EXPERIMENTAL

Materials

A batch of sunflower presscake (SP_1) from the variety Klein A was obtained from a local edible oil industry. This material was sieved in a Rotab sifter and then milled in a Pascall mill in order to obtain the sunflower flour (SFF_1). The defatted soy flour (SF) was purchased from Archer Daniels Midland Co. Two mixtures of sunflower flour and soy flour were mixed in the ratios 1:1 and 3:1. They were called TSP_1 and TSP_2 respectively.

The blended sunflower flour/soy flours 1:1 were extruded through a 1/4 in. die in a Wenger X-25 expander. The chemical composition of the raw materials and texturized blends is shown in Table 1. The operating conditions are shown in Table 2.

Chemical tests

Official methods of the AOAC (1965) were used in determining fat (ether extractables), moisture, ash, fiber and protein by Kjeldahl determination. Atomic absorption spectrophotometry was used to determine calcium and iron (Perkin-Elmer, 1973). Phosphorus was analyzed according to Tausky and Shorr (1953). For amino acid analyses, each sample was hydrolyzed for 22 hr in 6N hydrochloric acid at 110°C (Kohler and Palter, 1967) and the hydrolysate analyzed with a Hitachi Perkin-Elmer amino acid analyzer (model KLA-3B) based on the principle of Spackman et al. (1958). Tryptophan was not determined. The essential amino acid content of TSP_1 was compared with the FAO/WHO amino acid scoring pattern (1973).

Biological tests

The protein biological values of TSP_1 and TSP_2 were determined by the protein efficiency ratio (PER) method (Chapman et al., 1959) using 21-day-old weanling rats of the Charles River strain. The basal diet consisted of 80% cornstarch, 10% corn oil, 5% nonnutritive cellulose (Alphacel), 4% salt mixture (USP XIV) and 1% vitamin mixture (Chapman et al., 1959). The textured proteins were added to the basal diet to the 10% protein level at the expense of cornstarch. A 10% protein casein diet was used as a standard. All diets were isonitrogenous and isocaloric. Ten animals, randomly assigned to groups were housed in individual, screen-bottomed cages in an air-conditioned room (25 ± 2°C). Food and water were offered ad libitum for 28 days. Body weight and food intake were recorded for each rat at regular 7-day intervals. TSP_1 was tested alone and supplemented with 0.15 and 0.30% DL-methionine. TSP_2 was supplemented with 0.1 and 0.2% L-lysine.

The apparent protein digestibility (D) was measured during the same experiment. The following formula was used for the calculation.

$$D = \frac{\text{Ingested nitrogen} - \text{fecal nitrogen}}{\text{Ingested nitrogen}} \times 100$$

The feces were collected for each rat during 3 wk. The samples were dried, weighed, milled and homogenized for nitrogen determination by the Kjeldahl procedure (AOAC, 1965).

Statistical analysis

Biological data were analyzed by the analysis of variance (Snedecor and Cochran, 1967) and Duncan's multiple range test (1955).

RESULTS & DISCUSSION

THE DRY EXTRUDED product (TSP_1) consisted of light, tan-colored pellets 1–2 cm long. TSP_2 formed small, dark greenish pellets partially disintegrated.

The chemical analysis of TSP_1 and TSP_2 as given in Table 1 showed that the textured proteins contained less crude fiber and phosphorus than sunflower flour. The protein content of the textured product is comparable to the protein content of soy texturates (Shelef and Morton, 1976). The chlorogenic acid present in sunflower flour was no doubt responsible for the dark color that developed in the sunflower flour/soy blend when passed through the X-25.

The amino acid composition of the sunflower presscake and of TSP_1 are compared with the FAO/WHO amino acid scoring pattern (1973) in Table 3. The sulphur-containing amino acids are limiting in TSP_1. Lysine and threonine also appear defficient. The mixture of sunflower protein with soy protein contributes to a better amino acid pattern in TSP_1 in which lysine increases from 3.12 to 3.90 and threonine from 2.37 to 3.38 (g/16g N), respectively, even though these values still remain below the FAO/WHO pattern in Table 3. The cystine content of sunflower presscake and TSP_1 was not determined.

The growth data for the young rats are shown in Table 4. Results showed that the PER value of 1.96 for TSP_1 approached that for defatted soy reported by Cotton (1974) and Kellor (1974). The value of 1.96 also meets the standards for textured vegetable protein of the Food and Nutrition Service of USDA (Bird, 1975). TSP_1 supplemented with 0.15% DL-methionine improved in protein efficiency ratio to 2.32 ($P < 0.05$) and rat weight gain to 73.9 ($P < 0.01$). However, the supplemented diet did not reach the PER value obtained for casein ($P < 0.05$). The level of 0.30% did not produce a further increase in protein quality as determined in this biological study thus suggesting that the added level of 0.15% completely corrects the methionine deficiency. It is suggested that supplementation of TSP_1 with lysine further improves the protein quality. Considering that beef has an acceptable methionine and lysine content (Orr and Watt, 1966; Shelef and Morton, 1976), a synergistic effect on TSP_1, may be realized when combined with beef in a complementary ratio.

As expected, TSP_2 showed a lower protein quality than TSP_1 (Table 5) when related to growth of rats and protein efficiency ratio. Unsupplemented TSP_2 gave a PER of 1.63. Supplementation with 0.1% L-lysine improved both parameters, but the level of 0.2% L-lysine failed to produce a further increase in protein quality. Supplemented TSP_2 did

Table 1—Proximate composition and mineral contents of sunflower presscake (SP_1), sunflower flour (SFF_1) soybean flour (SF), TSP_1 and TSP_2 (Dry basis)

Product	Moisture (%)	Protein (N X 6.25) (%)	Ether extract (%)	Crude fiber (%)	Ash (%)	P (mg/100g)	Ca (mg/100g)	Fe (mg/100g)
SP_1	14.6	47.6	6.4	13.2	8.8	1623	446	26
SFF_1	8.2	46.5	8.1	10.6	8.7	1616	506	35
SF	8.4	56.2	1.5	3.4	6.3	725	235	34
TSP_1	11.8	45.9	5.9	6.1	9.4	12.93	683	51
TSP_2	9.0	48.5	4.1	6.5	9.3	1378	531	47

Table 2—Experimental conditions used during thermoplastic extrusion of TSP_1 and TSP_2

Product	Formulation (%)		Feed rate (g/m)	Extrusion moisture (%)	Extrusion temp °C	Additive
TSP_1	Sunflower flour	50	2750	18.0	145	CaO
	Soy flour	50				
TSP_2	Sunflower flour	75	2300	19.8	155	CaO
	Soy flour	25				

Table 3—Essential amino acid composition of sunflower presscake, TSP_1 as compared to the FAO/WHO amino acid scoring pattern (1973)[a]

Amino acid	Sunflower presscake	TSP_1	FAO/WHO pattern 1973
Lysine	3.12	3.90	5.44
Threonine	2.37	3.38	4.00
Valine	4.54	4.22	4.96
Methionine + cystine	2.00[b]	1.38[b]	3.52
Isoleucine	3.76	3.88	4.00
Leucine	5.47	7.21	7.04
Phenylalanine + tyrosine	6.11	7.71	6.08
Tryptophan	—[c]	—[c]	0.96

[a] g per 16g N
[b] Only methionine
[c] Tryptophan was not determined

Table 4—Growth data of young rats fed with TSP_1 experimental diets for 28 days[a]

Treatment	Avg initial wt (g)	Avg wt gain (g)	Food intake (g)	Protein efficiency ratio (PER)[b]	Fecal nitrogen[c] (g)	Apparent digestibility (%)
TSP_1	36.8	60.Ca	267.3ac	1.96	0.869	78.5a
TSP + 0.15% DL-methionine	36.8	73.9bc	283.5bc	2.32b	0.923	78.1a
TSP_1 + 0.30% DL-methionine	36.8	77.4b	293.2b	2.33b	1.022	77.0a
Casein	38.6	65.2ac	246.5a	2.54c	0.327	86.3b

[a] Means within the same column with the same letter are not significantly different ($P < 0.05$).
[b] Protein efficiency ratio (PER) = Weight gain (g)/protein intake (g).
[c] The data are from 3-wk trials.

Table 5—Growth data of young rats fed with TSP_2 experimental diets for 28 days[a]

Treatment	Avg initial wt (g)	Avg wt gain (g)	Food intake (g)	Protein efficiency ratio (PER)[b]	Fecal nitrogen[c] (g)	Apparent digestibility (%)
TSP_2	40.9	41.7a	236.9a	1.63a	0.989	78.8a
TSP_2 + 0.1% L-lysine	41.0	68.6b	290.9b	2.09b	0.985	78.5a
TSP_2 + 0.2% L-lysine	40.8	71.0b	311.9b	2.18b	1.045	78.0a
Casein	40.2	75.5b	293.1b	2.48c	0.525	86.9b

[a] Means within the same column with the same letter are not significantly different ($P < 0.05$).

[b] Protein efficiency ratio (PER) = Weight gain (g)/Protein intake (g).

[c] The data are from 4-wk trials.

not achieve the value of 2.32 of TSP_1 plus methionine.

The protein digestibility of TSP_1 and TSP_2 in Table 4 and 5 may be considered satisfactory but showed lower values than that obtained with casein.

From the nutritional standpoint the protein content of sunflower presscake is relatively high although lower than that of soy flour (Table 1). The analysis of seven varieties of sunflower defatted kernel meals gave a mean value of 3.77g of lysine (g/16g N) (Earle et al., 1968). This value is certainly lower than the 6.25g of the same amino acid (g/16g N) contained in defatted soy flour (Kellor, 1974). But what makes sunflower a valuable material from the nutritional standpoint is its content of sulphur amino acids which amounts to 3.73 g/16g N (Earle et al., 1968), as compared to 1.27g for soy flour (Kellor, 1974). Since the protein values of most human diets are limited by their content of the sulphur amino acids (Miller and Donoso, 1963), sunflower can make a significant contribution to improving the protein nutritional status of man, especially in countries where this oilseed is a major crop, such as those of Eastern Europe, Chile and Argentina.

CONCLUSIONS

THE PRESENT STUDY showed that it is possible to texturize sunflower flour/soy flour mixtures using the thermoplastic extrusion technique. The sunflower flour/soy flour blend (1:1) designated TSP_1 has a useful protein content. The essential amino acid pattern of sunflower protein was improved when mixed with soy protein as shown by the amino acid analysis. The protein quality of TSP_1 measured as growth and protein efficiency ratio showed values comparable to those reported in the literature for texturized soy flour. TSP_2 failed to obtain a good biological quality and did not achieve a good texture.

In summary, the blend sunflower flour/soy flour in the ratio 1:1 did not change the biological quality of soy protein, Thus suggesting that sunflower flour may be used as a partial substitute for soy flour in the production of texturized meat extenders.

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This research was partially financed by a grant from the Servicio de Desarrollo Científico y de Creación Artística of the University of Chile.

Part of this paper was presented at the meeting of the 11th International Congress of Nutrition, Rio de Janeiro, Brasil, 1978.

SENSORY EVALUATION OF TEXTURED SUNFLOWER/SOY PROTEIN

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ABSTRACT

A blend of sunflower flour/soy flour in the ratio 1:1, intended as a meat extender, was extruded in a Wenger X-25 extrusion cooker. The effect of adding textured sunflower/soy flour (TSP) to ground beef was determined by evaluating sensory properties and quality. After preliminary analyses of TSP, sensory tests were performed on a mixture of TSP/beef (30:70), using beef as a control. Panelists detected significant differences at the 1% level for texture and flavor, but could not decide which sample they liked better. Appearance, flavor, texture, juiciness and overall quality of TSP/beef were also evaluated. Appearance and flavor of TSP/beef was significantly lower (at the 5% level) than of beef, but texture of TSP/beef was significantly better. No significant differences were found for juiciness or overall quality between samples.

INTRODUCTION

VEGETABLE PROTEINS are being increasingly used as fresh meat-extenders. In the USA, approximately 60 million pounds (wet wt.) were sold to schools for the National School Lunch Program, in the 1973–74 school year (Bird, 1975). In Chile textured plant protein has also been recently introduced. Among textured vegetable proteins soybeans have been most prominent and many studies on its nutritional and eating quality have been reported (Rakosky, 1974; Hamdy, 1974; Bressani, 1975; Bowers and Engler, 1975; Williams and Zabik, 1975; Kotula et al., 1976).

In Chile, sunflower is used as a source of edible oil. The cake left after oil extraction contains about 40% protein (Yáñez et al., 1965) and is used primarily as an animal feed. The national production of this crop amounts to an average of 15,000 tons per year. Sunflower protein is low in the essential amino acid lysine, but contains an adequate concentration of the sulphur amino acids, methionine + cystine. This has been confirmed by nutritional studies with rats (Yáñez et al., 1969; Eklund et al., 1971). On the other hand, soybean protein is rich in lysine but low in methionine + cystine (Sosulski and Fleming, 1977). These facts indicate that sunflower and soy proteins should complement each other thus producing a better balance of amino acids in the resulting blend.

Since no information concerning textured sunflower flour is available, however, we were interested in obtaining a textured sunflower/soy flour product. The objective of this study was to determine the effect of textured sunflower flour/soy flour (TSP) on sensory properties and quality when mixed with ground beef.

EXPERIMENTAL

A BLEND of sunflower flour/soy flour in the ratio 1:1 (TSP) obtained by thermoplastic extrusion in a Wenger X-25 extrusion cooker (Yáñez et al., 1979) and commercially available ground beef were used in this study. Initially TSP was evaluated by the following tests.

pH measurements

The pH of TSP and ground beef was measured in 10% distilled water suspensions (w/v) homogenized with a Waring Blendor.

Microbiological count

For the total aerobic plate count Petri dishes with total plate count agar (Difco) were prepared and incubated at 37°C for 18–24 hr. For molds and yeasts determination the basal medium used was "Difco" malt agar (pH 5.5). The following dilutions were made: initially 10g of TSP were suspended in 90 ml of sterile 0.9% saline and homogenized in a Waring Blendor for 1 min. For the second dilution, 1 ml of the first suspension was diluted with 9 ml of saline. The third suspension was made by diluting 1 ml of the second suspension with 9 ml of sterile saline solution. Finally, 1 ml from each dilution was added to the basal medium in Petri dishes and incubated at 28°C for 72–120 hr.

For the sensory evaluation tests, textured protein was washed with hot tap water (80°C) and squeezed by hand to remove the "herbaceous" flavor and obtain a bland product.

In the next experiment, TSP blended with beef was submitted to sensory evaluation tests. For the difference and preference tests, the mixture contained 30 parts of hydrated and washed TSP, 70 parts of ground beef and 2 parts of salt. Higher levels of TSP proved difficult to mask.

Sixty grams of TSP/beef were fried at 230°C for 2 min with stirring using 15 ml of vegetable oil. Beef was used as a control. For the quality tests, both TSP/beef (30:70) and ground beef were spiced. To 100g of product, 0.08g of each of the following spices: garlic, onion, parsley, cumin seed and paprika, and 0.05g of oranigum were added.

All the sensory tests were performed by the paired-comparison method. The panel was formed by eight individuals trained in food texture evaluation. The sensory tests were conducted in an especially equipped sensory evaluation laboratory with individual booths with day-light illumination, in a quiet environment. The samples to be tested were presented in dishes with three-digit random numbers. The test sample was presented together with the control sample.

The difference tests for texture and flavor (Larmond, 1970) included four replications and the presentation order was balanced. The preference test (Amerine et al., 1965) included three replications. This was intended as an exploratory test on a laboratory basis due to the small number of judges and the fact that they were individuals with sufficient training.

The quality test included three replications and the factors of appearance, flavor, texture, juiciness and overall quality were considered (Amerine et al., 1965). A 7-point rating scale was used (7 = excellent, 1 = very poor). The order of presentation of the samples to the panel was randomized. The score obtained for juiciness was not analyzed statistically because the scale used ranged from 5 to 1. In addition, a descriptive method was employed to obtain information regarding the sensory characteristics of this novel product.

The statistical significance of the difference and preference tests was calculated by the Chi square test, and the experimental design for the quality study was a randomized complete block, in which the test products corresponded to treatments and the panelists to blocks. The quality ratings were examined by the analysis of variance (Dawson and Harris, 1951; Amerine et al., 1965; Sidel and Stone, 1976).

RESULTS & DISCUSSION

TEXTURED VEGETABLE PROTEIN, when hydrated with tap water, increased its weight in the ratio 1:2.7. Hydrated TSP resembled cooked meat, was brown in color, and had

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0022-1147/79/0006-1717$02.25/0
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fibrous texture and "herbaceous" flavor. Due to its color, it was easily detected when mixed with raw ground beef.

The pH value of ground beef was 5.4 and that of TSP was 6.5. The pH of blended TSP/beef (30:70) was 5.7, similar to values obtained with meat analogs and ground beef by Hegarty and Ahn (1976).

Table 1—Judgements obtained in the difference test for TSP/beef (30:70) and beef

Test	No. of judgements	Difference judgements	X^2 calculates	X^2 Tabulated[a] 5%	1%
Difference	32				
Texture		30	11.39**	3.84	6.64
Flavor		27	8.89**		

[a] See Table IV in Fisher and Yates (1963)
** Significant at the 1% level

Table 2—Judgements obtained in the preference test for TSP/beef (30:70) and beef

Test	No. of judgements	Preference judgements	X^2 calculated	X^2 Tabulated[a] 5%	1%
Preference	24				
All beef		15	1.04ns[b]	3.84	6.64
TSP/beef (30:70)		9			

[a] See Table IV in Fisher and Yates (1963)
[b] ns = not significant

Table 3—Sensory characteristics of TSP/beef (30:70) and beef detected by the evaluation panel

Factor	TSP/beef (30:70)	Beef
Appearance	Typical of beef 54%; characteristics: dark, lack of rosy hue, musty.	Typical of beef 92%; characteristics: greasy
Flavor	Typical of beef 83%; characteristics: tasteless and herbaceous.	Typical of beef 75%; characteristics: balanced.
Texture	Typical of beef 79%; characteristics: tender, grainy and elastic.	Typical of beef 67%; characteristics: elastic, stringy and tough.
Juiciness	Moderate; Characteristics: semi-juicy	Moderate; Characteristics: semi-juicy
Aroma	Like meat and spices	Like meat and spices

Table 4—Mean values of sensory panel ratings of TSP/beef (30:70) and beef

Factor	TSP beef	Beef	Significance of F-value: Product (P)	Set (S)	Judges (J)	PxS	PxJ	SxJ
Appearance[a]	5.3	5.8	*	ns	ns	ns	ns	ns
Flavor[a]	5.4	5.9	*	ns	ns	ns	ns	ns
Texture[a]	5.9	5.4	*	ns	*	ns	ns	ns
Juiciness[b]	4.3	4.3	—[d]	—	—			
Overall quality[a]	5.6	5.7	ns[e]	*	*	ns	ns	ns
Total score[c]	26.5	27.1						

[a] Highest possible score 7.0
[b] Highest possible score 5.0
[c] Highest possible score 33.0
[d] Analysis of variance was not done
[e] ns = not significant
* Significant at the 5% level

Microbial count

The textured protein sample contained 600 total aerobic microorganisms/g sample, below the USA standard value of 5000 (Hamdy, 1974). The yeast and mold count was less than 50 microorganisms/g sample, the accepted USA maximum. Heating during extrusion apparently leads to a low microbial load.

Difference and preference tests

The sensory panel judgements of TSP/beef (30:70) and beef for texture and flavor are presented in Table 1. TSP/beef differed significantly in texture and flavor at the 1% level. However, no significant preference for TSP/beef (30:70) or beef was noted (Table 2). Sensory characteristics of TSP/beef and beef detected by the panel are summarized in Table 3.

Quality test

Sensory panel ratings for TSP/beef (30:70) and beef are shown in Table 4. Appearance was significantly lower at the 5% level for TSP/beef than for beef; the dark color of TSP/beef (Table 3) may have contributed to its lower rating. Flavor of TSP/beef also rated significantly lower than beef at the 5% level, probably due to its tasteless and "herbaceous" characteristics (Table 3). Texture of TSP/beef was significantly better than beef at the 5% level, however. The textured protein was tender and elastic (Table 3). Similarly, Huffman and Powell (1970) found that ground beef patties blended with 2% soy were more tender than those without soy. Overall quality of TSP/beef and beef, as judged by appearance, flavor, texture and juiciness, were equal. Possibly more spicing of TSP/beef improves its flavor and gives it the same rating as beef. Similarly, Kotula et al. (1976) noted that soy protein patties had better flavor, aroma, tenderness and overall acceptability scores when served with condiments. TSP/beef and beef did not differ in juiciness. Similarly beef patties do not significantly differ in juiciness from patties containing 20 or 30% textured soy protein (Smith et al., 1976).

The textured vegetable protein made from sunflower and soy flour (1:1) was used as a partial substitute of beef and compared to beef in sensory evaluation tests. The sensory analysis showed that TSP/beef (30:70) is equal to or better than beef in texture, juiciness and overall quality, but it is lower than beef in appearance and flavor. These sensory evaluation tests show that it is possible to partially substitute texturized sunflower/soy flours for beef. This may be advantageous in countries that do not cultivate soybeans, but grow sunflowers. The introduction of sunflower in human nutrition would make a significant contribution as a protein source, alone or in combination with other vegetable proteins such as soy as shown by Yáñez et al. (1979). Sunflower presents the additional advantage of its low price, since its presscake sells in Chile for about (U.S.) $0.15/kg while soy flour sells for (U.S.) $0.60/kg.

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This research was partially financed by a grant from the Servicio de Desarrollo Científico y de Creación Artística of the University of Chile.
Part of this paper was presented at the meeting of the 11th International Congress of Nutrition, Río de Janeiro, Brasil, 1978.

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favorably with values reported in the literature for raw and cooked samples of large lima, blackeye and garbanzo beans (Table 7).

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Supported, in part, by the California Dry Bean Advisory Board. The authors are indebted to Carl Tucker, University of California, Davis for samples of authentic bean cultivars.
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SOURCE OF ANTIOXIDANT ACTIVITY OF SOYBEANS AND SOY PRODUCTS

DAN E. PRATT and PAULA M. BIRAC

ABSTRACT

Soybeans, defatted soy flour, soy protein concentrates, and soy isolates possess appreciable antioxidant activity in lipid-aqueous systems. The antioxidant properties of soybeans, defatted soy flour, and soy protein concentrates are due primarily to polyphenolic compounds. Antioxidant activity of soy protein isolates are also due to polyphenolic compounds but other compounds also exert a marked antioxidant effect. Polyphenolic antioxidants of soy were found to be isoflavones, chlorogenic acid isomers, caffeic acid, and ferulic acid. These compounds occurred primarily as glycosides. The isoflavone aglycones were identified as genistein (5,7,4'-trihydroxyisoflavone), daidzein (7,4'-dihydroxyisoflavone), and glycitein (7,4'-dihydroxy-6-methoxyisoflavone. The compounds have antioxidant activity but not to the same extent as flavonoids with the dihydroxy configuration in either the α or β ring.

INTRODUCTION

SOYBEANS POSSESS several naturally occurring phenolic compounds that have been shown to have lipid antioxidant properties (Pratt, 1972, 1976; Naim et al., 1976; Hammerschmidt and Pratt, 1978). Work in our laboratory has demonstrated that hot water and methanolic extracts of fresh and dried soybeans, defatted soy flours, and soy protein concentrates have appreciable antioxidant activity (Pratt, 1972). Commercial textured vegetable (soy) proteins and soy protein isolates have also been shown to possess effective lipid antioxidants for aqueous-beef and aqueous-fat systems. At least nine phenolic acids, including syringic, vanillic, caffeic, ferulic, p-coumaric, and p-hydroxybenzoic acids have been identified and isolated from soybeans and defatted soybean flour (Arai et al., 1966). Chlorogenic and isochlorogenic acids have also been found in soybeans in the ratio of approximately 10:1. Such hydroxy cinnamic acid derivatives have appreciable antioxidant activity in lipid-aqueous systems (Pratt, 1965).

Many flavonoids have strong antioxidant characteristics in lipid-aqueous and lipid food systems. The low solubility of these compounds in lipids is often considered as a serious disadvantage if an aqueous phase is also present. However, flavonoids suspended in the aqueous phase of a lipid aqueous system offer appreciable protection to lipid oxidation. Also certain flavonoids are effective antioxidants when suspended in fats. The primary flavonoids of soybeans are glycosides of isoflavones. The main glycosides appear to be 7-glucosides of three isoflavones (Fig. 1).

The present investigation was initiated to study the antioxidant activity of soybeans particularly related to flavonoids and phenolic acids.

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0022-1147/79/0006-1720$02.25/0
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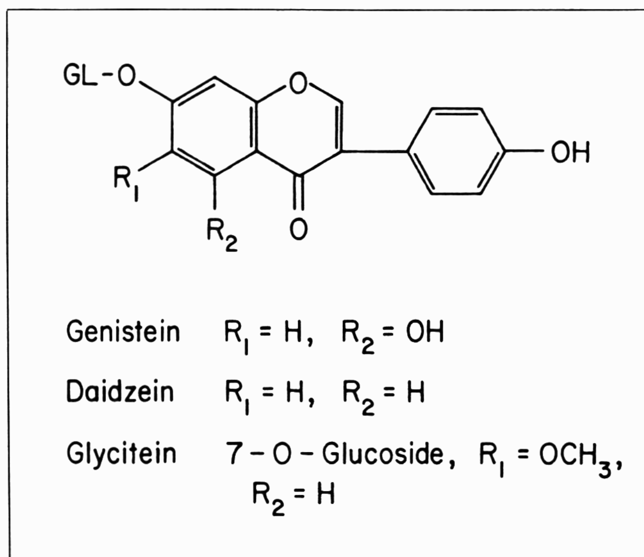

Fig. 1—Structure of isoflavone glycosides.

EXPERIMENTAL

METHANOL and water-soluble components were extracted from dried fresh and dried stored Corsoy variety soybeans (Glycine max L), soybean flours, and concentrates. Pulverized samples were defatted with hexane prior to extraction. Samples of 100g each were soaked for 16 hr in methanol or water. The materials were homogenized in the soaking medium in a Waring Blendor for 2 min at high speed. The mixtures were refluxed for 90 min, filtered, and the residue washed with 400 ml of methanol or water, as appropriate. The insoluble residue was discarded and the filtrate concentrated to 25 ml on a rotary evaporator at 40°C. Extracts were stored at −20°C until used.

Antioxidant activity was determined by measuring the coupled oxidation of carotene and linoleic acid as previously described (Hammerschmidt and Pratt, 1978). The rate of bleaching of β-carotene was determined by the difference in spectral absorbance reading, at 470 nm, between the initial reading (0 time) and the last reading for which the bleaching remained essentially linear (usually 30 or 45 min) divided by time. The antioxidant index is the ratio of the rate of bleaching of the control (system with no added test compound) to the rate of bleaching when a test compound was in the system.

A carotene spray solution was used for detecting antioxidants on thin-layer and paper chromatograms. The preparation and use of the carotene spray solution is described in detail by Hammerschmidt and Pratt (1978). Glycosides possessing antioxidant activity were hydrolyzed according to the procedure reported by Hammerschmidt and Pratt (1978). After fractionation in diethyl ether and water, the upper layer (ether) was removed and evaporated to dryness in a water bath. The residue was redissolved in methanol (Spectral grade). The low layer (water) was evaporated to near dryness on a rotary evaporator and the residue redissolved in 0.5 ml water. The water phase was tested for sugars by paper chromatography.

Precoated polyamide 6 TLC plates were used for the initial fractionation of extracts. Methanolic extracts were streaked on 20 × 20 cm plates and developed using methanol:acetic acid:water (90:5:5). Bands were eluted with methanol and rechromatographed on polyamide using chloroform:methanol:methyl ethyl ketone (12:2:1). Bands were eluted with methanol. When mixtures of glycosides were

hydrolyzed, aglycones were fractionated on silica gel G TLC plates. Extracts were streaked as described and developed in ethyl acetate: petroleum ether (3:1) and then in ethanol:chloroform (1:1). Bands were detected on duplicate plates using appropriate chromogenic sprays or with a UV lamp at 366 nm.

Co-chromatography in several developing systems, UV spectrophotometric analysis, HPLC, and GLC were used to identify glycosides, aglycones, and cinnamic acids. GLC and TLC were used to identify sugars obtained by hydrolysis. GLC was performed on trimethylsilyl derivatives using a 2m × 0.5 cm column of 1.5% SE 30 on Chromosorb W. GLC analysis was carried out at 230°C for flavonoids and programmed from 100 to 260°C for cinnamic acids.

Table 1—Antioxidant activity of aqueous extracts of soybeans and soy products

Extract	Soybean in extract (g/100 ml)	Antioxidant index[a]	Peroxide no.[b]
Soybeans (fresh)	0	1.0	1000
	10	8.7	62
	20	11.2	41
Soybeans (dried)	0	1.0	1000
	10	10.3	55
	20	14.5	37
Soy concentrate	0	1.0	1000
	10	7.2	57
	20	10.0	49
Soy protein isolate	0	1.0	1000
	1	4.7	128
	3	8.0	42

[a] Coupled oxidation of carotene and linoleic acid. Antioxidant index = rate of bleaching of control/rate of bleaching of β-carotene in test solution. Bleaching rate measured at 470 nm.

[b] Peroxide number (meq/kg) of linoleic acid incubated at 37°C with 1% extract for 5 days. Method: Dahl and Holman, Anal. Chem. 33: 1960 (1961).

RESULTS & DISCUSSION

THE ANTIOXIDANT INDICES of some unfractionated aqueous soybean extracts are given in Table 1. Data for soybean flour and textured vegetable (soy) protein (TSP) are similar to dried soybeans. As may be seen from these data, different soy products possess antioxidants. Apparently, antioxidants of soybeans, soy flour, soy protein concentrates, and commercial TSP are primarily isoflavones and cinnamic acid derivatives. The combined concentration of these compounds account for nearly all of the antioxidant activity. Soy protein isolates have antioxidant activity due to other compounds although they do possess small quantities of flavonoids and cinnamic acids.

Characteristics of isoflavones isolated from methanolic extracts are given in Tables 2 and 3. Glucosides of 5,7,5′-trihydroxyisoflavone (genistein) and 7,4′-dihydroxyisoflavone (daidzein) accounted for more than 80% of the flavonoids of soybeans. These were present as 7-0-glucosides.

Text continued on page 1722

Table 2—Characterization of soybean isoflavone

Compound	GLC[a]	TLC	UV irradiation at 366 nm
Genistein (5,7,4′-trihydroxyisoflavone)			
Authentic	1	Same on	Purple
Isolated	1	6 solvents	Purple
Daidzein (7,4′-trihydroxyisoflavone)			
Authentic	0.84	Same on	Fl. green
Isolated	0.84	6 solvents	Fl. green
Glycitein (7,4′-dihydroxy-6-methoxyisoflavone)			
Isolated	1.72		Fl. blue

[a] GLC analysis of trimethylsilyl derivatives using 2m × 0.5 cm column of 1.5% SE 30 on Chromosorb W at 230°C.

Table 3—Spectrophotometric characteristics of isoflavone of soybeans

Compound	λmax, nm: Methanol	λmax, nm: $AlCl_3$/HCl	Conc moles/kg
Aglycone			
Genistein	260,326*	271,370	4.0×10^{-5}
Daidzein	237*,247,303*	240*,247,301*	0.7×10^{-5}
Glycitein	227,256,315	227*,258,309	Trace
Glycoside			
Genistein	260,327*	271,307*,370	3.5×10^{-3}
Daidzein	255,310*	257,262*	1.0×10^{-3}
Glycitein 7-0-Glucoside	226*,254,316	226*,259,310	0.6×10^{-3}

* Shoulder

Table 4—Characteristics of soybean cinnamic acids

Compound	Fluorescence color: 366 nm	Fluorescence color: $+NH_3$	R_f Value in solvent[a]: 1	2	3	λmax, nm	GLC[b]	Conc moles/kg
Chlorogenic acid								
Authentic	Blue	Green	0.00	0.00	0.41	328	1.0	
Isolated	Blue	Green	0.00	0.00	0.41	328	1.0	2.8×10^{-2}
Caffeic acid								
Authentic	Blue	Blue-Green	0.22	0.32	0.72	321	0.58	
Isolated	Blue	Blue-Green	0.21	0.32	0.71	320	0.58	1.1×10^{-4}
p-coumaric acid								
Authentic	Faint	Violet	0.36	0.49	0.69	311	0.43	
Isolated	Faint	Violet	0.36	0.48	0.69	311	0.43	Trace
Ferulic acid								
Authentic	Blue-Violet	Green	0.39	0.51	0.74	314	0.62	
Isolated	Blue-Violet	Green	0.37	0.50	0.74	314	0.62	Trace

[a] Solvent 1= Benzene:Dioxane:Acetic Acid (90:25:4); 2 = Benzene:Methanol:Acetic Acid (90:16:8); 3 = Butanol:Acetic Acid:Water (5:1:4) upper phase

[b] GLC analysis of trimethylsilyl derivatives using 2m × 0.5 cm column of 1.5% SE 30 on Chromosorb W at 230°C.

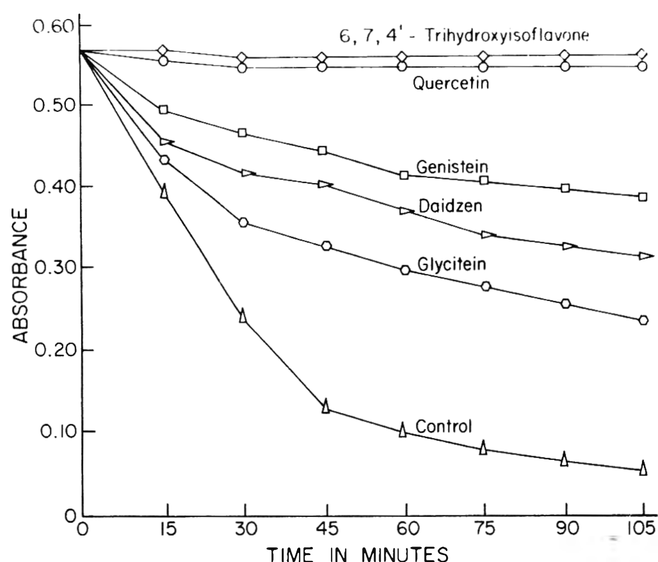

Fig. 2—Antioxidant activity of flavonoids (5 x 10^{-4} M conc) determined spectrophotometrically by the coupled oxidation of β-carotene and linoleic acid.

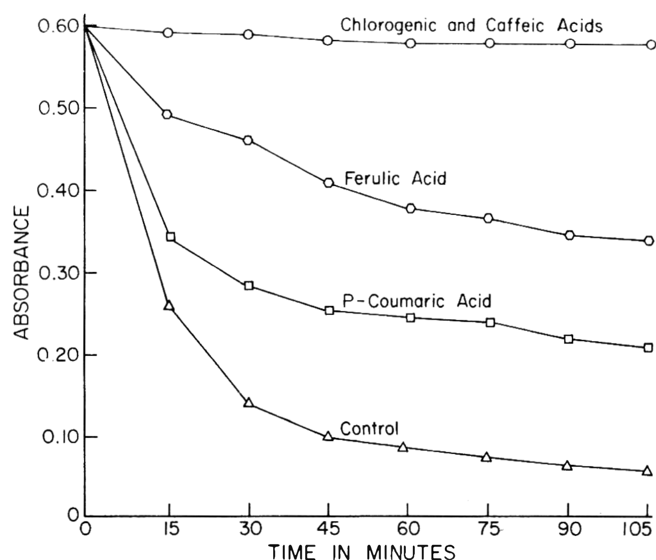

Fig. 3—Antioxidant activity of cinnamic acid (5 x 10^{-4} M conc) determined spectrophotometrically by the coupled oxidation of β-carotene and linoleic acid.

Only one other isoflavone glycoside was found, apparently, 7,4′-dihydroxy-6-methoxyisoflavone 7-0-Glucoside (glyciterin 7-0-glucoside). No authentic sample of this compound was available; due to the similarity between our data and those of Naim et al. (1976), the structure was assumed. TLC, HPLC, and GLC analyses failed to demonstrate the presence of 6,7,4′-trihydroxyisoflavones in any sample. The compound has been shown in several processed soybean products by others. As may be seen, this compound is a superior antioxidant to any isoflavone of soybeans.

Characteristics of cinnamic acids fractionated from extracts are given in Table 4. Of the several cinnamic acids found, only four were present in significant amounts or possess sufficient antioxidant properties to be considered. The major cinnamic acid derivative was found to be chlorogenic acid. This compound and its isomer isochlorogenic acid possessed appreciable antioxidant activity. Other cinnamic acids possessing antioxidant activity were caffeic, p-coumaric, and ferulic acids. These were present in minor concentrations compared to chlorogenic acid and the latter two possessed very little antioxidant activity.

The antioxidant activities of isoflavone and cinnamic acid derivatives are summarized in Figure 2 and 3. The activities of isolated compounds are compared to those of quercetin and 6,7,4′-trihydroxyisoflavone (authentic sample). These are known to possess antioxidant activity. The compounds, used to measure activity, were adjusted to 5 x 10^{-5} M. As may be seen in Figure 2, quercetin and 6,7,4′-trihydroxyisoflavone possessed considerably more antioxidant activity than isolated isoflavone. This demonstrates that ortho-dihydroxylation is important for antioxidant activity, as previously reported.

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Ms received 1/28/79; revised 5/18/79; accepted 5/27/79.

Purdue University Agricultural Experiment Station Journal Paper No. 7460.

DIFFERENTIATION OF THE AROMA QUALITY OF SOY SAUCE BY STATISTICAL EVALUATION OF GAS CHROMATOGRAPHIC PROFILES

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ABSTRACT

Stepwise discriminant analysis (SDA) and principal component analysis (PCA) were applied to quantitative gas chromatographic (GC) profiles of soy sauce volatiles in order to compare soy sauce quality evaluated by sensory evaluation with GC data. Although the brands of soy sauce were roughly separated into four groups on the basis of canonical score with the same tendency of the sensory rank, SDA correctly classified each sample into the proper group of the 8 groups. The six PCs among 8 factors extracted from the selected 39 peaks by the PCA showed significant relationships with the sensory evaluation in multiple regression analysis. The first PC showed a 55% of contributing proportion. The importance of harmonious balance of each aroma compound for the aroma quality was evident from a comparison of the six PCs.

INTRODUCTION

THE AROMA of soy sauce has been the object of scientific research in Japan for several decades, Yokotsuka (1960). Many compounds have been identified as aroma components of soy sauce, but no one component has been found to correlate well with the characteristic aroma of soy sauce, Goto (1973) and Nunomura et al. (1976). The reason is that the characteristic aroma cannot attributed to a single substance. Therefore, we considered soy sauce aroma was ascribed to an integrated response of numerous aroma compounds and applied statistical analyses to this problem by analyzing the relationship between the sensory data and gas chromatographic (GC) volatile profiles, we found a statistically significant relation between the two as previously reported, Aishima and Nobuhara (1976, 1977).

Use of computers to accomplish correlation of aroma quality with GC data has been reported successfully by Powers and Keith (1968), Biggers et al. (1969), Young et al. (1970), Bednarczyk and Kramer (1971, 1975). In the ACS Symposium on flavor quality, Lindsay (1977), Carter and Cornell (1977), and Williams et al. (1977) reported practical utilization of objective measurement for beer, orange juice, and apples, respectively. In the same symposium, Jennings (1977) reviewed general approaches and problems in such statistical studies.

In this paper, we applied discriminant analysis and principal component analysis to GC data of soy sauce aroma in order to investigate the significant factors for aroma quality included in GC data and the relationships between GC profiles and quality differentiation by sensory tests.

EXPERIMENTAL

Sensory analysis

Purely fermented soy sauce samples consisted of eight different brands (A, B, –, H) were purchased in the market monthly for 20 months. Therefore, the sum of used samples amounted to 160. The eight samples were evaluated by experienced sensory test panel for soy sauce in Kikkoman Shoyu Co., Ltd. using sniff test. The eight samples were ordered from one to eight according to odor preference. The number of panel members varied from 106–165 and, therefore, a sum of order for each sample divided by the number of panel members and the resulting average order was used as the sensory score in the following multivariate analysis.

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0022-1147/79/0006-1723$02.25/0
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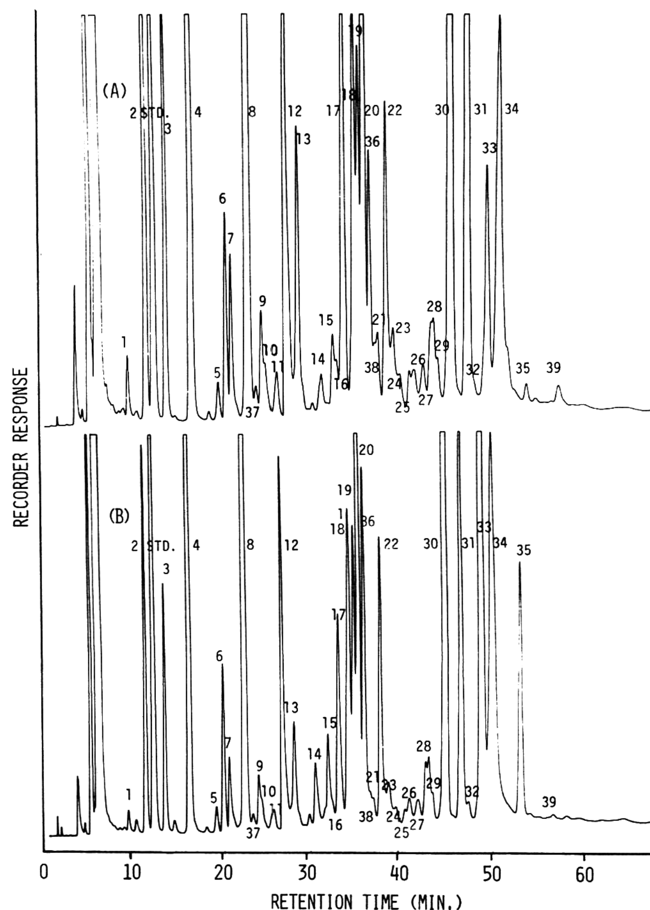

Fig. 1—Gas chromatograms of soy sauce aroma concentrates. (A) and (B) show the chromatograms each of brand A and E, respectively. n-Undecane peak is indicated as STD.

Sample preparation and GC analysis

Fifty ml of each sample in a 300 ml two-necked flask was distilled under a reduced pressure, 15 Torr, and 45°C with blowing nitrogen gas into the soy sauce sample through a glass capillary. The resulting distillate condensed in each of ice-water and dry ice-acetone cooled traps was combined and then 25 ml of the combined distillate was treated with 5 ml of dichloromethane and one ml of dichloromethane containing 80 ppm of n-undecane. The dichloromethane extract was concentrated to a volume of ca. 10 μl under a reduced pressure, 30 Torr, and two μl of the concentrate was injected into JEOL JGC-1100 gas chromatograph. GC analysis was performed under the conditions as follows. The columns were glass, 3 mm i.d., 3m length, packed with 20% PEG 20M on Chromosorb W

AW-DMCS, 60–80 mesh. Nitrogen was used as carrier gas and the flow rate was 40 ml/min. Inlet and detector temperature was 250°C. Column temperature was elevated from 50–210°C at the rate of 3°C/min. Each gas chromatogram was recorded by a JEOL JR-252A two pen recorder and the ratio of two pens settled as one to ten. The peak selected as variables for the following multivariate analysis were numbered as shown in Figure 1 and the height of these peaks was measured.

Mass spectrometry

The aroma concentrate was analyzed in a combined gas chromatograph-mass spectrometry Shimadzu-LKB 9000. The gas chromatographic separation was made with 50m glass capillary column coated with FFAP. Mass spectra were recorded at 20 eV. The separator temperature was 250°C and the ion source temperature was 270°C. The compounds in the concentrate were identified by comparing their MS spectra with reference spectra and GC retention times with authentic compounds.

Statistical analysis

Multivariate analyses were performed by a UNIVAC 1108 computer system. Prior to computer analysis, each peak height was converted into per cent value based on the whole chromatogram and then ratio of each peak to the sum of whole peak was transformed logarithmically by a FORTRAN program.

Stepwise discriminant analysis (SDA)

A discriminant function is expressed as:

$$Z_i = a_1 x_1 + a_2 x_2 + - + a_i x_i + - + a_m x_m \quad (1)$$

Z_i is calculated in order to make the ratio of between-group variation and within-group variation maximum. Therefore, Eq (1) can efficiently differentiate and classify individual cases into correct groups by calculating generalized distance, Mahalanobis D^2. This analysis was performed by a BMD 07M program, UNIVAC manual (1972a).

Principal component analysis (PCA)

Principal components (Z_i) were computed from Eq (2):

$$Z_i = l_{i1} x_1 + l_{i2} x_2 + - + l_{ik} x_k + - + l_{im} x_m \quad (2)$$

L_{ik} (i=1, 2, –, p, k=1, 2, –, m) and variance in Z_i are called the eigen vector and eigen value, respectively. At first, Z_1 and l_{1k} are computed on the basis of correlation matrix among GC peaks in order to maximize the variance in Z_1 among all simple equations. Then l_{2k} is calculated in order to maximize the variance in Z_2 among all simple equations except for Z_1 and l_{1k}. Therefore, the correlation coefficient between Z_1 and Z_2 becomes zero. However, computation can be continued until (m-1)th PC is calculated; usually this analysis is stopped before eigen value becomes less than 1.0. This analysis was performed by a BMD 01M program, UNIVAC manual (1972a).

Multiple regression analysis

A multiple regression model is generally expressed as:

$$Y = \beta_1 X_1 + \beta_2 X_2 + - + \beta_i X_i + - + \beta_m X_m + \alpha + e \quad (3)$$

In the equation, X_i and Y denote PCs and sensory score, respectively. Regression coefficients, β_i, and a constant, α, are calculated by a linear least squares method under the conditions of larger sample number than PC number, m. Contributing proportion (P_i), relative importance of each PC for aroma quality, was calculated by Barylko-Pikielna's method (4) (Barylko-Pikielna and Metelski, 1964).

$$P_i = \frac{|s_i r_{iy} \beta_i|}{\Sigma |s_i r_{iy} \beta_i|} \times 100R^2 \quad (4)$$

Where, s_i and r_{iy} represent standard deviation of each PC and a correlation coefficient between each variable and sensory score. This analysis was performed by a BMD 03R program, UNIVAC manual (1972b).

Table 1—Average sensory score, standard deviation, maximum, and minimum score in each brand

Brand	Avg	Std dev	Maximum	Minimum
A	2.56	0.23	2.89	2.09
B	2.60	0.24	3.01	2.24
C	3.42	0.27	3.97	2.77
D	3.96	0.31	4.54	3.37
E	4.16	0.69	5.92	3.23
F	6.16	0.38	6.86	5.49
G	6.25	0.58	7.05	5.20
H	6.88	0.22	7.23	6.30
Whole	4.50	1.65	7.23	2.09

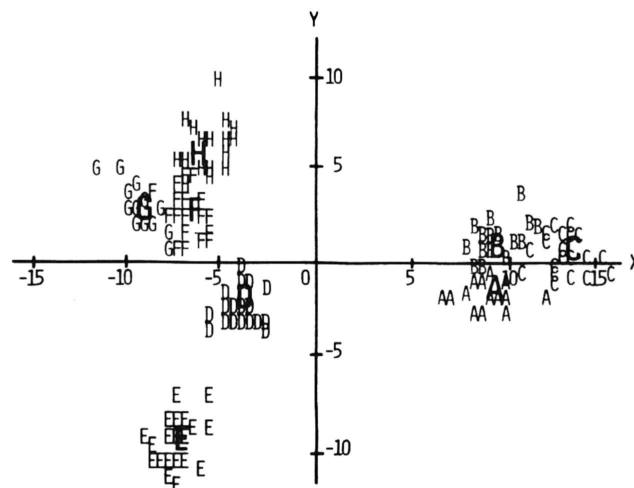

Fig. 2—Canonical plot of eight brands of soy sauce samples on the basis of GC data. X and Y indicate the first and second canonical variables, respectively.

RESULTS & DISCUSSION

Sensory analysis

The best and worst sensory scores among 160 samples were 2.09 and 7.23, respectively. The average score, standard deviation, maximum and minimum scores for each brand are shown in Table 1.

GC analysis

Thirty-nine peaks were selected as the variables for the following multivariate analyses. About fifty peaks were commonly observed on every chromatogram and the difference in GC profiles among the eight brands was mainly ascribed to the quantitative variation in each peak as shown in Figure 1. Two chromatograms in Figure 1 can be easily discriminated by comparing peaks as 3, 33, 35, and 36 etc.

Stepwise discriminant analysis

At first canonical variables were calculated on the basis of 39 GC peaks in order to examine discrimination among all eight brands as shown in Figure 2. X and Y indicate the first and second canonical variables, respectively. Although the eight brands were roughly classified into four groups, this scattergram shows the difficulty in the complete discrimination of all brands on the basis of two axes. In the sensory tests, brands A, B, and C were rated superior, brands D and E were average and brands F, G, and H were inferior. Highly significant difference in GC profiles among the eight brands were observed on the basis of F-values calculated from the analysis of variance at step 38 as shown in Table 2. Then actual discrimination between each of two brands on the basis of GC data was tested as shown in Table 3. Except for one sample that misclassified between brand A and B, all other samples were unambiguously assigned to the correct brands. Therefore, the discrimination of samples

Table 2–F-Matrix among the eight brands, calculated at step 38 in a stepwise discriminant analysis

	Brand						
Brand	A	B	C	D	E	F	G
B	9.33**						
C	11.91**	8.29**					
D	44.45**	52.48**	78.38**				
E	75.90**	81.30**	105.60**	36.59**			
F	63.16**	68.58**	91.56**	28.38**	46.20**		
G	76.80**	75.88**	107.10**	25.22**	38.58**	13.42**	
H	68.47**	55.99**	85.36**	37.17**	53.00**	28.39**	20.11**

** $P < 0.01$, $F(38, 115; 0.01) = 1.76$

belonging to different brands by comparing GC profiles is confirmed statistically.

Classification examples on the basis of Mahalanobis distance between two brands are shown in Figure 3. Brands A and B were more preferably evaluated by the sensory test panel than the other brands of F, G, and H. Brand H was evaluated as the worst among the eight tested brands. Various distances between each of two groups show the similar trend with the difference of aroma quality evaluated by the sensory panel. Furthermore, these scattergrams suggested to us that the difficulty in the discrimination among these brands in sensory tests might be equal to the difficulty in the classification of these samples on the basis of GC profiles.

In the stepwise discriminant analysis, one peak was selected on the basis of analysis of variance and it was entered into a computed function at each step as shown in Table 4. These functions were computed in order to maximize the variation between brands and to minimize the variation in each brand. Consequently the selection order might be considered as one of the standards used to assess the importance of peaks. Namely, peaks that greatly contribute to differentiate the quality of soy sauce aroma. One interesting result in the selection is that aroma compounds which belong to quite different compound groups were selected up to step 7. Such selection shows that peaks with much information for differentiation were preferentially entered into the discriminant functions. However, further investigation on aroma compounds is needed in order to connect these compounds with various microorganisms and conditions in soy sauce making.

Principal component analysis

At first a matrix of correlation coefficients among 39 peaks was calculated in order to extract PCs from the GC data. In the correlation matrix, several groups consisting of highly correlated peaks such as peaks 2, 3, and 4 are observed as shown in Table 5. An interesting result deduced from the matrix is that peak quantities of aliphatic and cyclic compounds negatively correlated with each other. This inverse suggests that these two groups might be derived from competitive conditions such as species of microorganisms or process temperature in soy sauce making. Then PCs were extracted from the 39 peaks. Eigen values surpassed 1.0 up to the eighth PC and the cumulative proportion attained 81% on the basis of extracted 8 PCs as shown in Figure 4. This result means that 39 peaks in the original GC data were contracted into 8 PCs with 19% loss of the information. Factor-loading on the first and second PCs were plotted in order to investigate the meanings contained in

Table 3–Number of samples classified into the eight brands. Each sample is classified on the basis of discriminant functions and GC data

	Brand							
Brand	A	B	C	D	E	F	G	H
A	20	0	0	0	0	0	0	0
B	1	19	0	0	0	0	0	0
C	0	0	20	0	0	0	0	0
D	0	0	0	20	0	0	0	0
E	0	0	0	0	20	0	0	0
F	0	0	0	0	0	20	C	0
G	0	0	0	0	0	0	2C	0
H	0	0	0	0	0	0	0	20

Table 4–Selection of peaks by stepwise discriminant analysis

Step no.	Peak entered	F-Value to enter
1	11; trans-2-Hexen-1-ol	120.91
2	33; 4-Ethyl guaiacol	41.68
3	19; γ-Butyrolactone	40.64
4	31; 2-Acetylpyrrole	33.87
5	36; iso-Varelic acid	27.17
6	8; Ethyl lactate	22.15
7	10; 2-Ethyl-6-methyl pyrazine	18.27
8	39; 2,6-Dimethoxy phenol	23.86

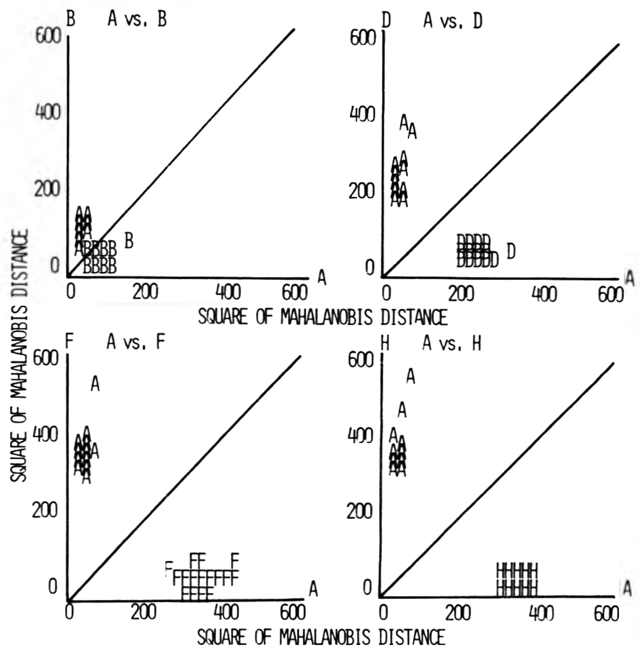

Fig. 3–Scattergrams of brand A vs. each of brand B, D, F, and H. Mahalanobis distance was calculated on the basis of GC data.

Table 5—A matrix of correlation coefficients among 11 major peaks[a]

Peak number	Peak number										
	2	3	4	8	18	19	20	22	30	31	33
2	1.000	0.726**	0.846**	0.251**	−0.487**	−0.188**	−0.348**	−0.145	−0.346**	−0.343**	−0.622**
3		1.000	0.520**	0.505**	−0.394**	−0.386**	−0.104	−0.431**	−0.600**	0.014	−0.603**
4			1.000	0.078	−0.487**	−0.038	−0.523**	−0.082	−0.051	−0.356**	−0.659**
8				1.000	−0.220*	−0.355**	0.083	−0.448**	−0.674**	−0.161	−0.271**
18					1.000	−0.329**	0.567**	−0.283**	0.103	0.441**	0.200*
19						1.000	−0.402**	0.629**	0.344**	−0.105	0.244**
20							1.000	−0.387**	−0.441**	0.437**	0.125
22								1.000	0.550**	−0.303**	0.297**
30									1.000	−0.055	0.173
31										1.000	−0.249**
33											1.000

[a] Peaks: 2, iso-butyl alcohol; 3, n-butyl alcohol; 4, iso-amyl alcohol; 8, ethyl lactate; 18, γ-valerolactone; 19, γ-butyrolactone; 20, furfuryl alcohol; 22, methionol (3-methylthio-1-propanol); 30, β-phenethyl alcohol; 31, 2-acetylpyrrole; 33, 4-ethyl guaiacol

* $P < 0.05$;

** $P < 0.01$

Table 6—Relation between the principal components (Z_i) and sensory score analyzed by multiple regression analysis

Z_i	Correlation (r) Z_i vs. Y	Contributing proportion (%)	F-Value
1	0.741**	54.96	506.8**
2	0.300**	9.02	83.2**
3	−0.239**	5.71	52.5**
4	−0.013	0.02	0.2
5	0.046	0.02	1.9
6	0.140	1.97	18.2**
7	−0.336**	11.30	104.2**
8	−0.067	.45	4.1**

** $P < 0.01$, $F(8, 151; 0.01) = 2.64$

each PC as shown in Figure 5. Peaks located near the circumference of 1.0 highly correlate to the first or second PC. Judging from the factor loading of each peak on the first PC, we deduced the meaning contained in the first PC as the difference in mash fermentation that is to say, difference in yeast species during mash period, Yokotsuka et al. (1967). Regarding the other seven PCs, however, we could not recognize reasonable meanings in each of them so far. The first and seventh PCs were plotted against the sensory score in order to study the relationship between the aroma quality and extracted PCs as shown in Figure 6. Then the relationships between the eight PCs and sensory score were analyzed by multiple regression analysis as shown in Table 6, and the resulting multiple regression model showed a highly significant relationship between the PCs and aroma quality as below.

$$Y = 0.392Z_1 + 0.173Z_2 - 0.199Z_3 - 0.12Z_4 + 0.046Z_5 + 0.183Z_6 - 0.477Z_7 - 0.104Z_8 + 4.497$$

Multiple correlation coefficient: 0.915

Coefficient of multiple determination: 0.836

F-value: 96.38**; $P < 0.01$, $F(8.151; 0.01) = 2.64$

The equation could explain 84% of variation in the total variation contained in it. The second, third, and seventh PCs showed relatively high contributions to the aroma quality and, on the contrary, the fourth and fifth PCs did not show significant relationship between the sensory score in spite of their large eigen values. Therefore, it seemed that these two PCs represented factors which gave no effects for the aroma quality and the multiple regression model consisting of six PCs except for these two PCs was calculated as below.

$$Y = 0.392Z_1 + 0.176Z_2 - 0.200Z_3 + 0.179Z_6 - 0.475Z_7 - 0.118Z_8 + 4.497$$

Multiple correlation coefficient: 0.913

Coefficient of multiple determination: 0.833

F-Value: 128.09** $P < 0.01$, $F(6, 153; 0.01) = 2.94$

Aroma patterns consisting of six PCs are described for each of eight brands in Figure 7. The fourth and fifth PCs were excluded from the patterns because of their lack of significance to the aroma quality. The middle line in each pattern denotes the mean value of the PCs and the inner and outer lines show the standard deviation from the mean value. Therefore, the distance between the middle line and

Text continued on page 1731

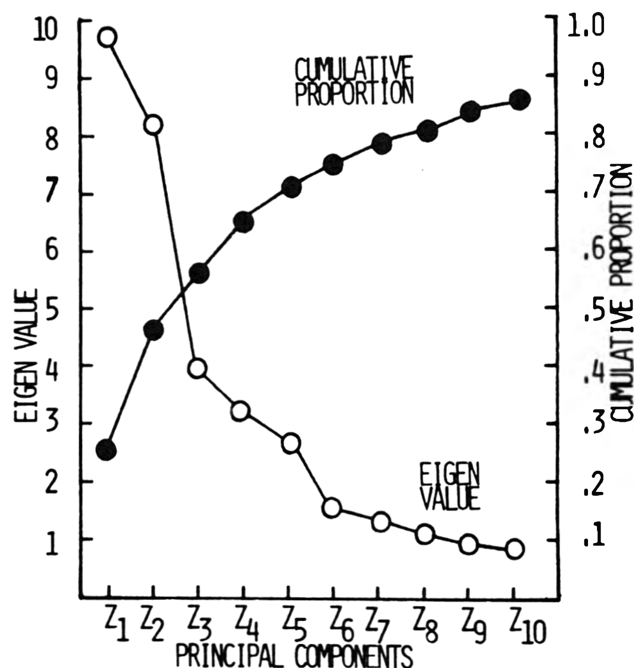

Fig. 4—Eigen values and cumulative proportion in total variance on the basis of 39 GC peaks.

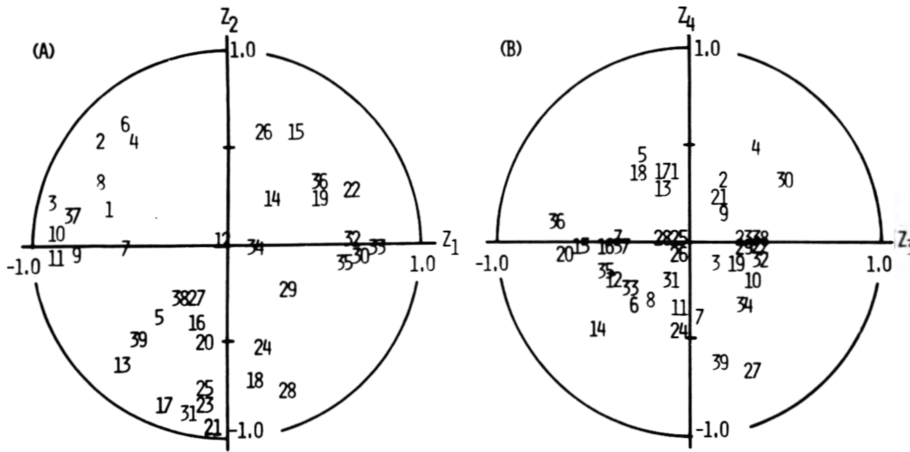

Fig. 5—Relationships between factor loading for the first and second factors in (A), and for the third and fourth factors in (B).

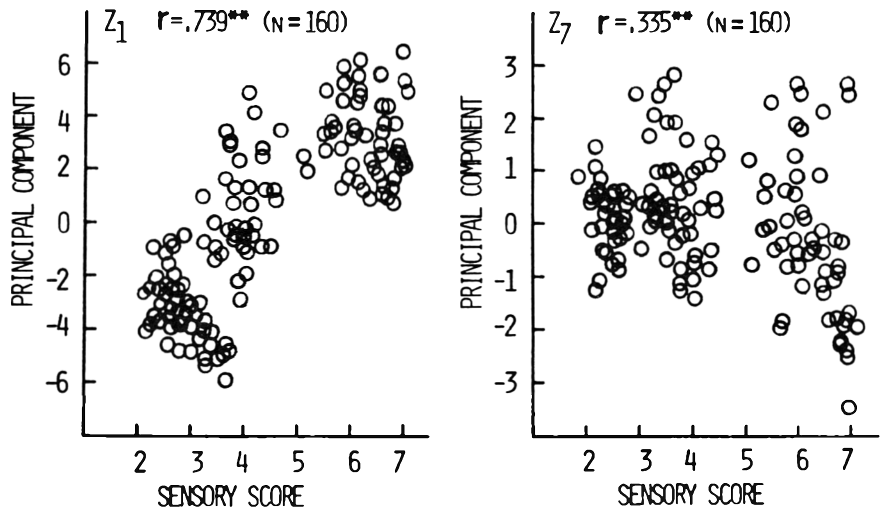

*Fig. 6—The sensory score vs the first principal component (Z_1) and seventh principal component (Z_7). (**$P < 0.01$)*

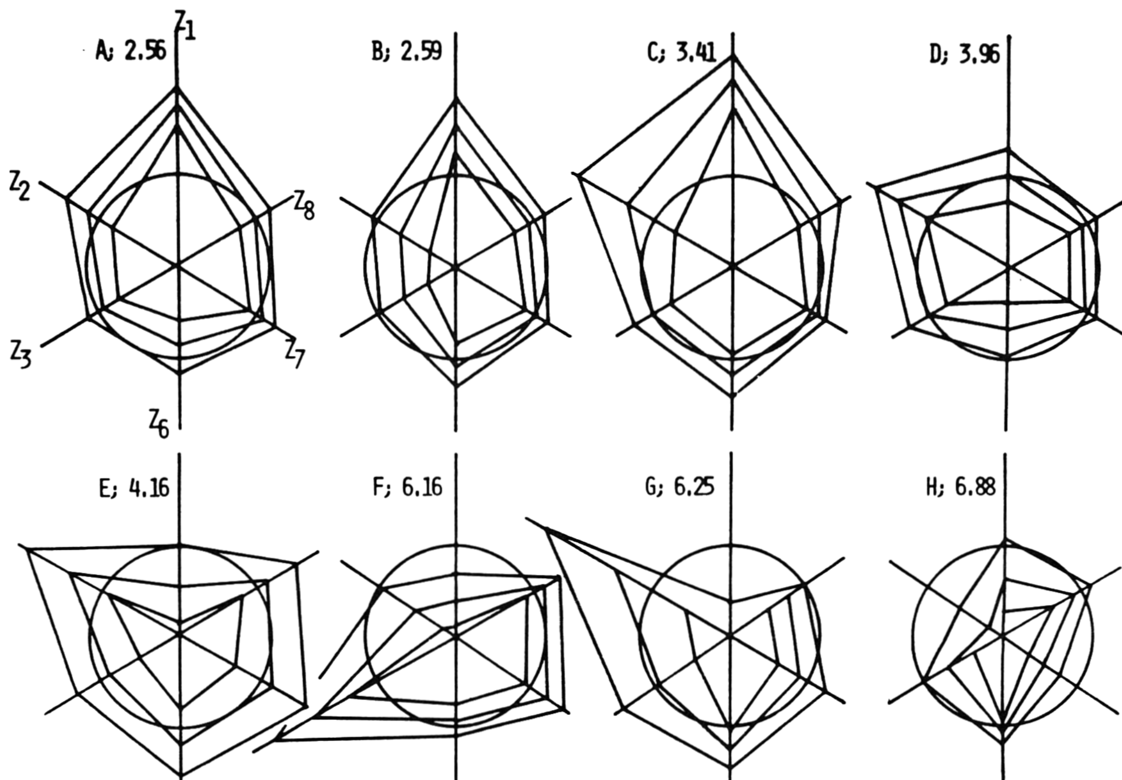

Fig. 7—Aroma patterns consist of six principal components (Z_i) except for the fourth and fifth components and average sensory score for each of eight brands.

EFFECTS OF DEHYDRATION AND STORAGE CONDITIONS ON THE PANCREATIC ALPHA AMYLASE SUSCEPTIBILITY OF VARIOUS STARCHES

K. KAYISU and L. F. HOOD

ABSTRACT

Corn, waxy maize, potato and tapioca starches were cooked, subjected to different dehydration (oven-, freeze- and drum drying) and storage (frozen and refrigerated) conditions, and evaluated for pancreatic alpha amylase susceptibility. Dehydration reduced the enzyme susceptibility of all the starches. Oven drying had a greater effect than freeze- or drum drying. Frozen and refrigerated storage affected the susceptibility of corn, potato and tapioca starches but not waxy maize starch. Refrigerated storage had a greater effect than frozen storage. No differences were observed between constant and variable temperature frozen storage or between quick and slow freezing. The enzyme susceptibility did not change during 6 wk of storage.

INTRODUCTION

THE ENZYME susceptibility of starches has been a subject of several studies (Sandstedt, 1955; Leach and Schoch, 1961; Walker and Hope, 1963; Rogols and Meites, 1968; Evers and McDermott, 1970; Evers et al., 1971; Gallant et al., 1973; Rasper et al., 1974; Hood and Arneson, 1976). These studies have shown that starches vary in their resistance to the action of amylolytic enzymes. These differences were found not only in the degree of hydrolysis but also in the mode of attack on the starch granule and in the products of hydrolysis. Generally, it is accepted that these differences are due to variations in molecular association within the starch granule. However, Guilbot and Mercier (1962) considered the molecular association as a secondary factor. They believed that the differences in amylase susceptibility of starches were primarily due to the presence of a system of external and internal layers that present different degrees of resistance to enzyme penetration.

Rasper et al. (1974) have shown that cereal starches are less resistant to enzymatic degradation than starches extracted from roots, tubers, and fruits. Wheat starch was found to be the most susceptible while large granule yam and plantain starches were found to be the most resistant to all enzymes tested. Cassava starch had a relatively high enzyme susceptibility compared to other noncereal starches. Leach and Schoch (1961) showed that potato starch was much more resistant to enzyme action than corn starch. Waxy starches were somewhat more susceptible to bacterial alpha amylase enzyme attack than the corresponding nonwaxy varieties. However, Walker and Hope (1963) showed that after a 2-hr digestion with salivary alpha amylase, waxy maize was less hydrolyzed than maize starch, while potato starch was hydrolyzed very little. Rogols and Meites (1968) found that starches with high amylopectin content tended to be digested at higher rates. They suggested that amylase activity would depend not only upon the carbohydrate fraction but upon the presence of trace levels of other materials such as protein.

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Chemical modification and processing treatments also affect the enzyme susceptibility of starches. The in vitro digestibility of gelatinized hydroxypropyl starches by pancreatin decreased exponentially with increasing degree of substitution (Leagwater and Luten, 1971). Similar conclusions were reached by Yoshida et al. (1973). Hood and Arneson (1976) studied the effect of various processing and storage conditions on the degree of hydrolysis of hydroxypropyl distarch phosphate and unmodified tapioca starch. They found that modification increased the enzyme susceptibility of ungelatinized starch but decreased the enzyme susceptibility of gelatinized starch. Gelatinization at pH 3.5 increased the degree of hydrolysis of unmodified starch about 4% more than gelatinization at pH 7.0. However, this pH effect was not evident for gelatinized modified starch.

In the present study we have examined the effect of some food processing treatments and storage conditions on the enzyme susceptibility of unmodified corn, waxy maize, potato, and tapioca starches. The hydrolysis conditions were chosen so as to simulate the conditions in vivo during digestion.

EXPERIMENTAL

Materials

Tapioca starch was obtained from Stein, Hall and Company, Inc., New York, NY. Corn, waxy maize, and potato starches were obtained from A.E. Staley Manufacturing Co., Decatur, IL.

Distilled, deionized water was used in all experiments.

Cooking

Starch slurries were cooked in a Brabender Visco-amylograph, Model VA-VE, fitted with a 700 cm g head (C.W. Brabender Instruments, Inc., South Hackensack, NJ). This instrument was used in order to obtain the same degree of gelatinization for each starch. Cooking conditions were selected to simulate the effects of food processing procedures on the starch granules. Each starch was cooked to slightly below its peak viscosity in order to gelatinize the granules and minimize granule rupture (Fig. 1). Starch concentration in the slurries varied from 5–8%. Slurries were heated in the Brabender bowl at 1.5°C/min. The bowl was rotated at 75 rpm. At a predetermined time (arrows, Fig. 1), the heating was stopped and the starch paste removed from the bowl. The cooked strach was subjected to various dehydration and storage conditions.

Dehydration

Cooked pastes were dehydrated by three methods: oven drying, drum drying and freeze drying. For oven drying, pastes were placed in petri dishes (about 20 g/dish) and dried at 106°C in a forced-air oven until they reached a constant weight (about 24 hr).

Starch pastes were diluted to 2% (W/V) with water, frozen in a −18°C freezer, and freeze dried. The freeze dryer operated at a pressure of about 2 μm Hg and a shelf temperature of 50°C.

Prior to drum drying, the starch pastes were diluted to 4% (W/V) with water. A laboratory model atmospheric drum drier with two 6-in. diam × 7 5/8 in. length chrome-plated drums (Blaw Knox Co., Buffalo, NY) was used. The drum speed was 3 rpm. The interdrum space was very small and the steam pressure was about 30 psi. The temperature inside the drums was estimated at 120°C. The contact time between the starch slurry and the drums was about 10 sec.

All dehydrated samples were ground with a mortar and pestle, and kept in screw-capped glass jars until analysis. Moisture contents were determined by drying to constant weight in a 100°C oven.

Storage

Cooked starch pastes were diluted to 2% (W/V) with NaCl solu-

Table 1—Effect of drying on alpha amylase susceptibility of cooked starches[a,b]

Starch	Undried	Oven dried	Freeze dried	Drum dried
Corn	76.0 ± 0.0 (100)	63.3 ± 1.9 (83)	68.8 ± 1.4 (91)	71.9 ± 0.8 (95)
Waxy maize	75.4 ± 2.0 (100)	67.4 ± 1.2 (89)	71.7 ± 1.3 (95)	ND[c]
Potato	82.6 ± 1.0 (100)	69.9 ± 2.1 (85)	76.1 ± 1.6 (92)	ND
Tapioca	74.7 ± 1.0 (100)	49.5 ± 1.8 (66)	71.0 ± 0.0 (95)	67.4 ± 1.3 (90)

[a] Mean % hydrolysis ± SD, n = 3. Corrected for moisture content of starch.
[b] Values in ( ) are an index of enzyme susceptibility based on 100 for the same starch, undried.
[c] Not determined

Table 2—Effect of freezing rate and storage conditions on alpha amylase susceptibility of cooked corn starch[a,b]

Storage conditions	Storage time		
	1 Wk	3 Wk	6 Wk
Refrigerated	69.5 ± 0.8 (91)	69.1 ± 2.3 (90)	67.2 ± 0.7 (88)
Quick frozen	71.9 ± 0.0 (95)	71.4 ± 1.6 (94)	70.5 ± 0.C (93)
Constant temp freezer	69.5 ± 0.8 (91)	72.4 ± 0.8 (95)	71.9 ± 1.4 (95)
Variable temp freezer	70.9 ± 1.6 (93)	73.7 ± 0.7 (97)	70.5 ± 1.4 (93)

[a] Mean % hydrolysis ± SD, n = 3. Corrected for moisture content of starch.
[b] Values in ( ) are an index of enzyme susceptibility based on 100 for the control (undried, Table 1).

Table 3—Effect of freezing rate and storage conditions on alpha amylase susceptibility of cooked waxy maize starch[a,b]

Storage conditions	Storage time		
	1 Wk	3 Wk	6 Wk
Refrigerated	74.5 ± 2.0 (99)	75.8 ± 0.8 (101)	74.4 ± 1.6 (99)
Quick frozen	75.8 ± 0.8 (101)	76.8 ± 1.4 (102)	74.9 ± 1.6 (99)
Constant temp freezer	76.3 ± 0.8 (101)	76.8 ± 1.4 (102)	75.4 ± 1.4 (100)
Variable temp freezer	75.4 ± 1.4 (100)	76.3 ± 0.8 (101)	74.4 ± 0.8 (99)

[a] Mean % hydrolysis ± SD, n = 3. Corrected for moisture content of starch.
[b] Values in ( ) are an index of enzyme susceptibility based on 100 for the control (undried, Table 1).

tion (0.016M for tapioca, corn, and waxy maize starch; 0.02M for potato starch). The different molarities were used to compensate for the differences in slurry concentrations. The NaCl enhances alpha amylase activity and the diluent was prepared so as to obtain a final concentration of 0.01M NaCl in the starch-enzyme mixture. Aliquots of 5 ml were taken from the 2% starch suspension and placed in 26 ml ethanol-washed polyethylene vials. Three drops of toluene were added to each vial to prevent microbial growth, and the vials were capped and stored under one of the following conditions: (1) in a 3°C refrigerator; (2) quick frozen by dipping the vials in liquid nitrogen for 15 min and stored in −18°C constant temperature freezer; (3) in a constant temperature −18°C freezer; and (4) in an automatic defrost freezer. The defrost cycle occurred four times per day. The freezer temperature was −18°C but reached about −9°C during the defrost cycle (Hood and Seifried, 1974).

Hydrolysis

Twice crystallized hog pancreatic alpha amylase in a 0.5 saturated NaCl solution containing 3 mM $CaCl_2$ (ICN Pharmaceuticals, Inc., Cleveland, OH) was used. The preparation had a reported activity of 935 units/mg.

Frozen and refrigerated vials were tempered to room temperature for about 1 hr. Dehydrated starches (100 mg) were weighed into 26 ml polyethylene vials and 5 ml of 0.012M NaCl added. The concentration of NaCl in the starch-enzyme mixture was then equivalent to that used for the undried (refrigerated and frozen) samples.

One ml of 0.1M phosphate buffer, pH 7.0, and an appropriate amount of enzyme were added to each vial. Hydrolyzates were incubated in a 37°C shaker water bath for 1 hr. The enzyme was inactivated by placing the vials in a boiling water bath for 10 min. Vials were cooled to room temperature and the contents quantitatively transferred to 15 ml centrifuge tubes. Two ml of 0.35M $ZnSO_4$ and 2 ml of 0.5N NaOH were added to each tube and the volume adjusted to 12.5 ml with water. The tubes were shaken vigorously and centrifuged at 1500 × G for 5 min. Supernatants were filtered through Whatman #1 and analyzed for reducing sugar content by the alkaline ferricyanide method (Friedemann et al., 1962). A maltose standard curve was prepared. Percent hydrolysis was calculated as:

$$\frac{\text{mg maltose equivalents}}{\text{mg starch (dry wt basis)}} \times 100$$

Seventy units of enzyme per 100 mg starch was used with corn, waxy maize, and potato starches, while 23 units per 100 mg starch was used with tapioca starch. These levels were selected because they yielded high but not maximum levels of hydrolysis on the respective cooked starch. With this approach, reductions in enzyme susceptibility caused by dehydration and storage treatments could be detected.

RESULTS & DISCUSSION

Effect of dehydration

Drying reduced the alpha amylase susceptibility of all the starches evaluated (Table 1). Hydrolysis indexes for the different drying methods relative to the undried starch are shown in parenthesis. Calculation of these susceptibility indexes facilitates comparisons among starches and drying methods. The decrease in enzyme susceptibility may be partially explained by the retrogradation which takes place during drying. Retrogradation is known to decrease enzyme susceptibility of starch and is thought to occur primarily among the linear amylose polymers (Greenwood, 1970; Hellendoorn, 1971). The alpha amylase susceptibility indexes of oven dried corn, potato, and tapioca starches decreased 17−34%. However, waxy maize starch, which is almost exclusively composed of amylopectin, showed a decrease of only 11%.

Oven drying had a greater effect on the enzyme susceptibilities of the individual starches than freeze- or drum drying. Enzyme susceptibility indexes of freeze- and drum-dried starches decreased only 5−10%. Interestingly, freeze-dried corn starch was less susceptible to alpha amylase than drum-dried corn starch, whereas the reverse was true for tapioca starch. This difference between starches cannot be explained. The relatively high susceptibility of freeze-dried starch, as compared to oven dried, may be due to the open spongy structure that remains after sublimation of the large ice crystals. Drum-dried starches were relatively high in enzyme susceptibility as compared to oven-dried starches. This may be due to the rapid dehydration rate attained with the drum dryer because of the combination of the high temperature and the thin layer of starch paste on the drum. In this case, dehydration takes place so quickly that the linear starch polymers do not have enough time to align themselves and retrograde (Huddersfield and Grimsby, 1971). Hellendoorn et al. (1970) observed a slight improve-

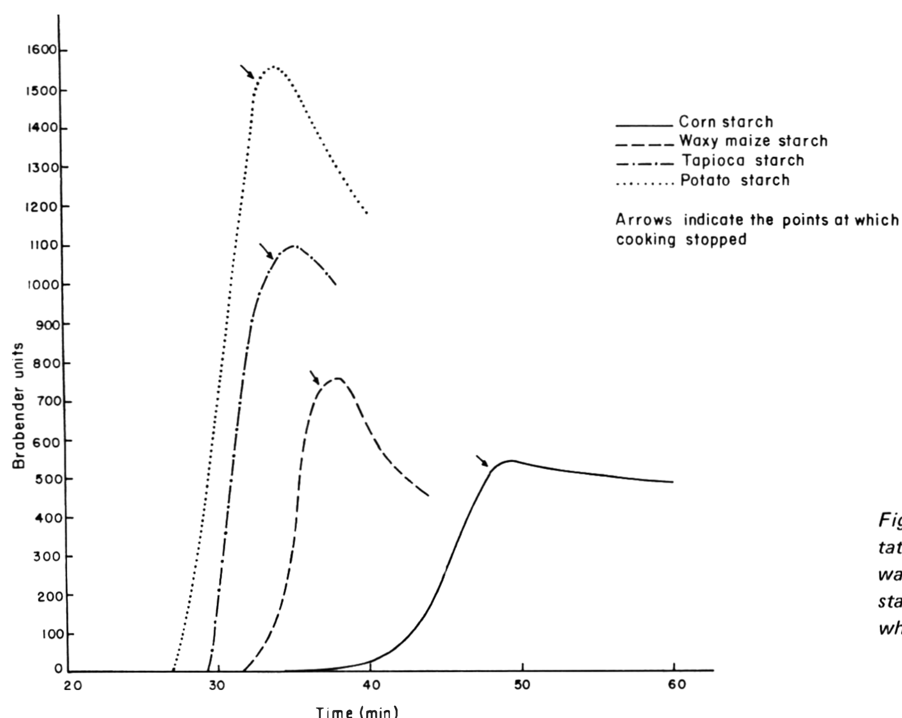

Fig. 1—Brabender amylograms of 5% potato (····), 8% tapioca (-·-·-), 7% waxy maize (---), and 8% corn (——) starch pastes. Arrows indicate points at which cooking was stopped.

ment in digestibility of potato starch when drum drying was used.

Effect of frozen and refrigerated storage

The effects of freezing rate and storage conditions are shown in Tables 2–5. In general, frozen and refrigerated storage reduced the alpha amylase susceptibility of the amylose-containing starches. As in the case of dried starches, this decrease in enzyme susceptibility probably resulted from the retrogradation process. This is supported by the fact that neither refrigeration nor frozen storage affected the enzyme susceptibility of waxy maize starch (Table 3). In the case of corn, potato, and tapioca, refrigeration reduced the enzyme susceptibility slightly more than freezing. This may be attributed to greater retrogradation at +3°C than at −18°C. The index of enzyme susceptibility was about 90 for all amylose-containing starches after 1 wk of refrigerated storage. This did not change for corn or tapioca starch after longer storage but did continue to decrease for potato starch. Frozen storage time beyond 1 wk did not have a consistent effect.

No noticeable differences were observed in enzyme susceptibility between different rates of freezing (i.e. quick freezing vs slow freezing) or between variable and constant temperature frozen storage. It has been shown that subjecting starch gels to cyclic freezing and thawing markedly increased the degree of syneresis (Schoch, 1969; Hood and Seifried, 1974). This is a manifestation of retrogradation. The variable temperature freezer heat shocks the product and some thawing occurs each time it goes through a defrost cycle. Apparently, temperature fluctuation during frozen storage did not have a detectable effect on enzyme susceptibility.

The results of these experiments indicate that conventional food processing procedures and storage conditions affect the alpha amylase susceptibility of several common starches. The physiological and nutritional implications of changes in enzyme susceptibility can only be determined in vivo. Unfortunately, digestibility studies with animals or humans often produce inconclusive results. In vitro methods, such as those described here, can provide useful information on the relative digestibility of food starches.

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Table 4—Effect of freezing rate and storage conditions on alpha amylase susceptibility of cooked potato starch[a,b]

Storage conditions	Storage time 1 Wk	3 Wk	6 Wk
Refrigerated	71.6 ± 1.4 (87)	68.2 ± 1.7 (83)	64.3 ± 2.9 (78)
Quick frozen	76.0 ± 1.4 (92)	78.9 ± 1.4 (96)	77.0 ± 0.8 (93)
Constant temp freezer	75.0 ± 1.6 (91)	77.0 ± 0.8 (93)	76.0 ± 1.4 (92)
Variable temp freezer	75.0 ± 2.2 (91)	76.3 ± 1.6 (93)	74.5 ± 1.4 (90)

[a] Mean % hydrolysis ± SD, n = 3. Corrected for moisture content of starch.
[b] Values in ( ) are an index of enzyme susceptibility based on 100 for the control (undried, Table 1).

Table 5—Effect of freezing rate and storage conditions on alpha amylase susceptibility of cooked tapioca starch[a,b]

Storage conditions	Storage time 1 Wk	3 Wk	6 Wk
Refrigerated	66.0 ± 2.1 (88)	66.0 ± 2.1 (88)	ND[c]
Quick frozen	72.1 ± 2.1 (97)	70.7 ± 0.8 (95)	74.4 ± 1.6 (100)
Constant temp freezer	ND	71.6 ± 0.8 (96)	67.9 ± 1.6 (91)
Variable temp freezer	74.4 ± 2.1 (100)	74.0 ± 0.0 (99)	70.9 ± 1.5 (95)

[a] Mean % hydrolysis ± SD, n = 3. Corrected for moisture content of starch.
[b] Values in ( ) are an index of enzyme susceptibility based on 100 for the control (undried, Table 1).
[c] Not determined

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This work was supported by USPHS Grant 5R01 FD 00607.

COMPUTER ANALYSIS OF SOY SAUCE AROMA . . . From page 1727

the other two expresses the consistency of aroma quality in each brand. PC patterns of brand A and B show not only preferable flavor patterns but also greater consistency in the quality of aroma. On the contrary, patterns of E, F, and G suggested inconsistency of aroma and dissimilarity with those of brands A and B. Thus we can describe aroma patterns as consisting of contracted GC profiles without loss of significant information from the original 39 peaks. Comparison of these aroma patterns can easily reveal similarity and difference in the aroma quality of soy sauce. The pattern similarity or dissimilarity among them suggests that soy sauce aroma is ascribed to an integrated response of many flavor compounds and it also suggests to us the importance to the harmonious balance of each aroma compound for good soy sauce.

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Ms received 2/15/79; revised 4/28/79; accepted 5/5/79.

The authors thank Dr. A. Nobuhara, Central Research Laboratories of Kikkoman Shoyu Co., Ltd., for his kind suggestions. The authors also thank Mr. M. Kojima and Mr. K. Uematsu, Kikkoman Shoyu Co., Ltd., and Mr. M. Ogawa, Nippon Univac Co., Ltd., for their kind and valuable advice for multivariate analyses.

FIBER COMPONENTS–QUANTITATION AND RELATIONSHIP TO CAKE QUALITY

MELISSA SHAFER JELTEMA and MARY E. ZABIK

ABSTRACT

Cereal and legume brans were fractionated to measure individual dietary fiber components. Wheat brans were similar in dietary fiber content, showing values of approximately 0.8% pectin, 26% water insoluble hemicellulose, 8% cellulose, and 4% lignin. The dietary fiber content of soy, oat, and corn brans varied widely. Oat bran was lowest in most fiber components. Soy hulls was the only bran containing a substantial quantity of pectin. Corn bran contained the largest quantity of water-insoluble hemicellulose. Fiber components could be used to partially predict the following parameters of cake quality: tenderness, volume, viscosity, cell size, cell wall thickness, and grain. Hemicelluloses appeared to have a large effect on cake quality.

INTRODUCTION

RECENT CONCERNS over the need for dietary fiber in the human diet have led to research into methods of incorporating fiber into the diet. Work with the cake system has shown that fiber in the form of soft red wheat bran can be successfully incorporated in levels up to 30% based on the weight of the flour (Springsteen et al., 1977). Several objective and sensory attributes were significantly altered; however, no attempt was made to determine which dietary fiber components were responsible for the change in cake parameters noted.

Work has been done with individual fiber components using physically separated water-soluble and insoluble hemicelluloses of the wheat endosperm (Baldi et al., 1965; Donelson and Wilson, 1960), and the addition of cellulose and pectin-coated cellulose to white layer cakes (Zabik et al., 1977).

This research was conducted to examine the differences in the fiber constituents of various cereal and legume brans and to relate the quantities of dietary fiber components to the differences seen in cake quality by the addition of these brans to white layer cakes.

EXPERIMENTAL

Materials

The same cereal and legume brans and control flour were analyzed as were previously incorporated into white layer cakes (Shafer and Zabik, 1978). These included two soft red wheat brans, two hard red wheat brans, two soft white wheat brans, corn bran, oat bran and soy hulls.

Cake parameters

The values used for evaluating the quality of the white layer cakes were the same as those quoted by Shafer and Zabik (1978). These included objective measurements for viscosity, volume, and tenderness and sensory evaluation for cell size, cell wall thickness and grain.

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0022-1147/79/0006-1732$02.25/0
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Fiber component analyses

Cellulose, pectin, lignin, and water soluble and insoluble hemicelluloses along with their sugar component composition were analyzed as described by Jeltema and Zabik (1979).

Statistical analyses

Dietary fiber components of the brans and flour were statistically analyzed using the same orthogonal comparisons as previously described by Shafer and Zabik (1978). In addition, statistical programs LS and LSSTEP were run on the CDC 6500 to obtain correlation coefficients and prediction equations for cake parameters using fiber components as the independent variables.

RESULTS & DISCUSSION

Fiber quantitation

The quantities of the various fiber components in bran and flour samples are shown in Tables 1 and 2. The control flour (Table 2) contained the lowest quantities of most fiber constituents. Physical separation methods have shown the water-soluble pentosans content of wheat endosperm after enzymatic starch digestion to be between 0.4 and 0.6%. Fifty to sixty-eight percent of this was carbohydrate in nature (Lineback et al., 1977). The current study showed 0.41% water-soluble carbohydrates (Table 2).

Physical separation methods have shown that the endosperm contains 2–3% water-insoluble hemicelluloses (Neukom et al., 1962). Southgate's fractionation scheme listed 2.8% water-insoluble hemicelluloses (Southgate, 1977) while distillation of furfural components gave values of 1.8 and 2.4% hemicellulose (Fraser et al., 1956; Fraser and Holmes, 1957). These compare to 2.7% obtained in the current study. Cellulose and lignin values obtained in this study (2 and 0.5%) were higher than those listed by the previously mentioned studies (0.6–0.8%; 0.03%). Cellulose values of the control flour obtained in this study are probably high since most of the hemicellulosic material remained in the cellulose extraction.

The water-insoluble hemicellulose contents of the bran samples in the current study ranged from 24–30%. Distillation of furfural components and Southgate's fractionation scheme (Southgate, 1977) showed results of 26 and 35% respectively. Data by McConnel and Eastwood (1974) using Southgate's method listed 25–28% water-insoluble hemicelluloses. Data of Adams cited by Hlynka (1964) listed 30% water-insoluble hemicelluloses.

Cellulose contents of the wheat brans in the current study ranged from 6–9% (Tables 1 and 2). Rasper (1978) treated an acid detergent fiber residue with 72% sulfuric acid and obtained values of 8.6–12.4% cellulose. Southgate (1977) found 8.6% cellulose by his fractionation scheme. McConnel and Eastwood (1974) found 9–11% cellulose. Adams found higher values in the range of 25% cellulose (Hlynka, 1964). The lignin values of this study (3.6–5.4) were again higher for the wheat bran samples than previously found (1.7–3.4%) by Rasper (1978) and Southgate (1977). However, McConnel and Eastwood (1974) found 4.6–5.8% lignin using Southgate's method. Adam's values showed 5.6% lignin.

The corn bran of this study was found to contain 45.6% water-insoluble hemicelluloses, 15% cellulose and 2% lignin. This compares to 47–67% hemicellulose, 14–23% cellu-

Table 1—Dietary fiber components of wheat bran[a]

Source	Water-soluble hemicellulose (%)	Pectin[b] (%)	Water-insoluble hemicellulose[c] (%)	Cellulose (%)	Lignin[d] (%)
Hard Red					
Commanche	0.46 ± 0.27	0.75 ± 0.39	30.34 ± 5.99	9.17 ± 0.96	4.35 ± 0.21
Shawnee	0.53 ± 0.46	0.71 ± 0.39	25.41 ± 2.41	8.58 ± 0.62	4.72 ± 0.68
Soft Red					
Oasis	0.52 ± 0.21	0.57 ± 0.28	24.90 ± 3.54	7.79 ± 0.42	5.12 ± 1.25
Arthur	0.50 ± 0.26	0.90 ± 0.25	27.07 ± 2.72	8.44 ± 1.09	4.19 ± 0.14
Soft White					
Ionia	0.45 ± 0.27	1.68 ± 0.65	24.20 ± 2.49	5.98 ± 2.22	3.90 ± 0.47
Yorkstar	0.54 ± 0.32	0.77 ± 0.48	24.29 ± 2.48	6.82 ± 1.16	3.65 ± 0.48

[a] 4 replications
[b] The orthogonal comparison between Ionia and Yorkstar was significant at $P < 0.01$.
[c] The following orthogonal comparisons were significant at $P < 0.001$: hard versus soft, Commanche vs Shawnee.
[d] 3 replications

Table 2—Dietary fiber components of various plant materials[a]

Source	Water-soluble hemicellulose (%)	Pectin (%)	Water-insoluble hemicellulose (%)	Cellulose (%)	Lignin (%)
Control flour[d]	0.41 ± 0.19	0.44 ± 0.57	2.75 ± 1.18	2.04 ± 0.52[b]	0.53 ± 0.25[c]
Commercial wheat bran[e]	0.44 ± 0.38	0.61 ± 0.54	28.49 ± 4.38	8.40 ± 1.19	5.35 ± 1.78
Corn bran[f]	0.30 ± 0.21	0.56 ± 0.60	45.62 ± 4.38	14.99 ± 1.49	2.02 ± 0.07
Oat bran[f]	0.93 ± 0.56	1.11 ± 0.21	8.20 ± 1.24	2.92 ± 0.86	3.36 ± 0.11
Soy hulls[f]	0.89 ± 0.25	6.92 ± 0.89	17.57 ± 3.31	36.19 ± 4.69	3.10 ± 0.33

[a] 4 replications
[b] 3 replications
[c] 2 replications
[d] All orthogonal comparisons between control flour and rest significant at $P < 0.001$.
[e] Water-insoluble hemicellulose orthogonal comparison between commercial vs other flours significant at $P < 0.01$.
[f] All orthogonal comparisons of corn vs oat vs soy except for lignin were significant at $P < 0.001$.

Table 3—Composition of the hemicellulosic material in wheat brans[a]

Source	Water-soluble hemicellulose		Water-insoluble hemicelluloses		
	Pentoses (%)	Uronic acids (%)	Pentoses (%)	Hexoses (%)	Uronic acids (%)
Hard Red					
Commanche	0.22 ± 0.14	0.26 ± 0.15	23.87 ± 4.28	4.17 ± 0.90	2.30 ± 0.60
Shawnee	0.30 ± 0.25	0.23 ± 0.21	20.18 ± 2.23	3.49 ± 0.48	1.74 ± 0.08
Soft Red					
Oasis	0.31 ± 0.12	0.19 ± 0.09	19.90 ± 3.87	3.21 ± 0.52	1.79 ± 0.23
Arthur	0.25 ± 0.10	0.25 ± 0.23	21.45 ± 1.80	3.56 ± 0.34	2.06 ± 0.35
Soft White					
Ionia	0.35 ± 0.17	0.08 ± 0.13	19.73 ± 2.50	2.85 ± 0.20	1.62 ± 0.21
Yorkstar	0.35 ± 0.20	0.20 ± 0.13	19.53 ± 2.48	3.03 ± 0.81	1.68 ± 0.18

[a] Means and standard deviations are based on four replications.

Table 4—Composition of the hemicellulosic material of various plant materials[a]

Source	Water-soluble hemicellulose		Water-insoluble hemicelluloses		
	Pentoses (%)	Uronic acids (%)	Pentoses (%)	Hexoses (%)	Uronic acids (%)
Control flour[b]	0.20 ± 0.11	0.20 ± 0.10	2.18 ± 0.38	0.48 ± 0.25	0.06 ± 0.13
Commercial wheat bran	0.22 ± 0.22	0.23 ± 0.17	22.72 ± 4.20	3.55 ± 0.38	2.22 ± 0.77
Corn bran[c]	0.18 ± 0.09	0.12 ± 0.12	34.38 ± 4.24	7.61 ± 2.63	3.70 ± 1.37
Oat bran[c]	0.40 ± 0.20	0.53 ± 0.38	7.12 ± 1.37	0.71 ± 0.11	0.37 ± 0.11
Soy hulls[c]	0.37 ± 0.10	0.52 ± 0.16	14.61 ± 3.64	1.51 ± 0.24	1.45 ± 0.26

[a] Means and standard deviations are based on four replications.
[b] The orthogonal comparison for pentoses in the water-insoluble fraction comparing control versus rest was significant at $P < 0.001$.
[c] The following orthogonal comparison of corn vs oat vs soy were significant: Uronic acids in both water-soluble and insoluble fractions ($P < 0.01$); pentoses and hexoses in water-insoluble fraction ($P < 0.001$).

lose, and 0.2–0.7% lignin (Schaller, 1977; Rasper, 1978).

Oat bran was found to contain 8% water-insoluble hemicellulose, 3% cellulose and 3% lignin. Oat hulls have previously been reported to consist of 35% cellulose and 6.7% lignin (Rasper, 1978). Soybean hulls contained 36% cellulose and 3% lignin. This compares with 41% cellulose and 3% lignin (Rasper, 1978).

Statistical analyses on the wheat brans showed significant differences between pectin and water-insoluble hemicellulose content (Table 1). The differences seen in pectin were between the two white wheat brans due to a much higher pectin content found for Ionia. Insoluble hemicelluloses showed significant differences between the hard versus soft and the two hard red brans.

More significant differences in fiber component proportions appeared in the comparison of the control versus all the other types; the wheat versus the nonwheat brans. The control versus the rest and wheat brans versus nonwheat brans differed in pectin, insoluble hemicellulose, cellulose, and lignin content. The three nonwheat brans differed in all fiber component contents except lignin. Corn bran contained the greatest amount of water-insoluble hemicellulose, while soy hulls contained the greatest quantity of cellulose. Oat bran was low in both of these.

The hemicelluloses were also measured as hexoses, pentoses, and uronic acids (Tables 3 and 4). The sugar classes of the hemicelluloses in wheat bran showed no significant differences (Table 3). Analysis of variance of the other cereal brans showed significant differences in the contents of the three sugar classes. These differences occurred in comparisons of the control versus any bran and among the nonwheat brans. When the proportion of hexoses or uronic acids to pentose content were compared, corn and oat brans were slightly lower in uronic acids and slightly higher in hexose content than the wheat brans. Soy hulls contained a greater proportion of both uronic acids and hexoses than the wheat brans. Legumes have previously been found to contain more uronic acids than cereals (Pigman and Horton, 1970). Ratios of uronic acids to pentose content found for corn bran (11) are similar to those found by Seckinger et al. (1960). The minor constituents of wheat brans are also similar to those found by Southgate (1977). He found the following proportions: 17% uronic acids and 27% hexose to pentose content. The water insoluble hemicelluloses of wheat flour have been found to contain approximately 41% hexoses (Cole, 1967) when fractionated on DEAE-cellulose columns. Southgate listed 80% hexose, 11% pentose and 9% uronic acids.

The uronic acid content of water soluble hemicelluloses has not been studied. The values found by this study were higher than those found in most hemicellulose fractions, showing values as high as the pentose content. It is probable that some of this was water soluble pectic materials.

Correlation of fiber components to cake quality

Several significant simple correlations were evident between fiber components and cake parameters. Table 5 shows those correlations which were significant at $P < 0.01$. Those cake parameters involved were tenderness, volume, cell size, cell wall thickness and grain. Viscosity correlated at $P < 0.1$ while sensory tenderness, moistness, softness, objective moisture and uniformity although showing significant differences among cakes showed no significant simple correlations.

Previous research has shown that water insoluble hemicelluloses improve cake volume and internal characteristics (Baldi et al., 1965; Donelson and Wilson, 1960). This was found to be the case here, where insoluble hemicelluloses were positively correlated with increased volume, increased tenderness, and thinner cell walls. Water-soluble hemicelluloses have been found to decrease cake volume (Donelson and Wilson, 1960). Again this was found to be true. Soluble hemicelluloses were correlated with decreased tenderness, decreased volume, larger cells and thicker cell walls.

Table 5—Simple correlations[a] of fiber components with cake parameters

Component	Cake Parameters: Tenderness	Volume	Cell size	Cell thickness[b]	Grain
Water-soluble hemicellulose (WSHEM)	–0.50	–0.51	–0.64	–0.71	–0.40
Water-insoluble hemicellulose (HEM)	0.57	0.53		0.50	
Pectin (PECT)			–0.44	–0.39	
Lignin (LIG)	0.58				
WSHEM Sq	–0.56	–0.45	–0.66	–0.73	–0.47
HEM Sq	0.37	0.64		0.43	
PECT Sq			–0.42	–0.36	
LIG Sq	0.59			0.41	
Cellulose (CEL) Sq			–0.37		
CEL X HEM		0.42			0.41
CEL X PECT			–0.40	–0.34	
CEL X WSHEM			–0.39		
HEM X LIG	0.79		0.37	0.63	
HEM X WSHEM	0.55				
WSHEM X LIG		–0.49	–0.39		
PECT X LIG			–0.43	–0.34	
PECT X WSHEM			–0.47	–0.43	

[a] Significant at $P < 0.01$

[b] A positive correlation denotes thinner cell walls.

Pectin correlated with larger cells and thicker cell walls. The lower levels of pectin found in the brans did not show decreased volume or increased tenderness as did the high levels of the pectin coated variables of the cellulose study (Zabik et al., 1977). However, since only one variable (soy) contained a substantial amount of pectin, and the other pectin values were not considered accurate, these correlations should not be considered conclusive.

Cellulose by itself showed no significant correlations while square of the cellulose component correlated only with larger cells. The study incorporating cellulose into cakes also showed few significant effects. Brys and Zabik (1976) showed few significant differences until substitution levels greater than 40%. The correlations which were significant for the component squared tended to be the same as those exhibited by the component itself.

Several interactions were seen between fiber components. In most cases the interactions were between components which showed the same correlations. Several interactions also appeared where one component had shown a correlation and the other had not. These were seen between CELXHEM, WSHEMXLIG, and PECTXLIG. In these cases the correlation followed that seen by the hemicellulose or pectin component. In only one case was there an interaction between components which had shown the opposite simple correlations. This was HEMXWSHEM for tenderness. In this case the correlation followed that of the water-insoluble hemicellulose.

These simple correlations cannot be thought of as indicating the absolute effect of these substances on the parameter measured, because of the possible interactions between component levels. For instance, lignin showed a correlation with increased tenderness. This effect seems unlikely because lignin is often associated with vegetable toughness. However, lignin content may be correlated with

Table 6—Prediction equations for cake parameters

Parameter	Equation	R^2
Tenderness	Y = −1.8990 WSHEM + 1.7685 WSHEMSQ − 0.0016 HEMPECT − 0.0020 HEM LIG + 1.9649	0.77
Volume	Y = 0.0004 HEM SQ − 0.1390 LIG WSHEM + 10.1433	0.49
Viscosity[a]	Y = −59.42 CEL − 200.57 LIG + 48.20 HEM + 4853.86 WSHEM − 1773.43	0.17
Cell size	Y = −3.1268 WSHEM + 10.0681	0.41
Cell wall thickness	Y = 0.0325 CEL + 0.3535 LIG − 0.0234 HEM − 5.2528 WSHEM + 10.7395	0.67
Grain	Y = −6.2064 WSHEM − 0.0023 HEMSQ + 0.3225 HEM WSHEM + 16.9894	0.68

[a] This equation does not include the oat data.

the content of some other component. In fact, there is a significant correlation ($P < 0.01$) with the water-insoluble hemicelluloses which show the same effect. Lignin content has been found to follow hemicellulose and cellulose contents (Gaillard, 1962).

Batter viscosity has been implicated as an indicator of final cake quality. Studies have shown that more viscous batters result in cakes which have finer texture with more evenly distributed air cells (Collins and Sunderline, 1940).

In the present study, viscosity showed no correlation with volume, tenderness, or cell size. Significant correlations did exist, however, between moistness and cell uniformity. While objective moisture showed a negative correlation, sensory moisture and cell uniformity showed positive correlations.

Tenderness was positively correlated with cell size and cell wall thickness. Objective moisture was negatively correlated with cell uniformity, sensory moisture and softness. Uniformity showed a positive correlation with sensory moistness and softness, while uniformity was positively correlated with cell size.

Prediction equations

Prediction equations were calculated using fiber components as the independent variables. The following parameters could be predicted by fiber content: volume, tenderness, viscosity, grain, cell size, and thickness of cell walls (Table 6). Viscosity was significant at $P < 0.1$, while the other were significant at $P < 0.0005$.

These equations start with one component and perform partial correlations of the other components with the component chosen.

How well the equation predicts the parameter measured is shown by the R^2 value. This tells how much of the variation in quality can be predicted by the equation. Therefore, 77% of the variation in tenderness can be predicted by this equation. Between 40 and 60% of the variation in other cake parameters was predicted by the fiber components. These prediction values are good since they indicate that over 50% of the variation in cake quality can be predicted by fiber components alone. Viscosity was the exception. Only 17% of the variation could be explained. Pectin and hemicelluloses are known to have a high water-binding capacity (Eastwood, 1973; Jelaca and Hlynka, 1971) and hemicelluloses have been shown to increase viscosity (Baldi et al., 1965). Also, high levels of cellulose (less than 20% of the flour) increase viscosity (Brys and Zabik, 1967). The high levels of cellulose and pectin in the cellulose study both caused an increase in viscosity. However, although most of these parameters figured into the prediction equation, prediction was still quite poor. It must be noted that all of the brans increased viscosity, even oats which are low in most fiber components. Therefore, something other than fiber is causing the large effect seen. Protein and starch values were also put into an equation along with total fiber, but did not help the predictability.

Water-soluble or insoluble hemicelluloses always figured into the prediction equations, indicating that these are important indicators of cake quality. Water-soluble hemicellulose components were of major importance to tenderness and cell size while water-insoluble hemicellulose was the most important factor to volume. Taking this factor out would have reduced predictability from 0.49 to 0.24. Both hemicellulose fractions were important to grain appearing both singly and in interactions. Pectin, cellulose, and lignin most often occurred as interactions with one of the hemicelluloses indicating that these are of less importance.

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Ms received 3/29/79; revised 5/26/79; accepted 6/5/79.

Michigan Agricultural Experiment Station Journal Article No. 8950.

Yersinia enterocolitica: A REVIEW OF THE PSYCHROTROPHIC WATER AND FOODBORNE PATHOGEN

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ABSTRACT

Infection in humans may be caused by *Yersinia enterocolitica*. Several foodborne outbreaks implicating this microorganism have been documented. *Y. enterocolitica* was shown to be the causitive agent of a foodborne disease outbreak in school children in upstate New York. Gastroenteritis and pseudoappendicitis were the predominant symptoms in this outbreak. It is hypothesized that the organism may be transmitted directly or indirectly to foods and water through fecal contamination or by the urine of a carrier animal. Swine have been shown to be the species most consistently harboring serotypes of *Y. enterocolitica* implicated in enteritis. The etiology of *Y. enterocolitica* diseases and infection and the most current isolation methodology is also reviewed. The distinguishing characteristics of this microorganism are its psychrotrophic growth capabilities, motility at 25°C, nonmotility at 37°C, negative phenylalanine deaminase, and positive urease activity. Controversy exists regarding classification of the species: whether the species belongs within the genus *Yersinia* and if the species should be further subdivided according to invasiveness and other biochemical distinctions are considered in this review.

INTRODUCTION

Yersinia enterocolitica (Schleifstein and Coleman, 1939: Frederiksen, 1964) has been increasingly implicated in outbreaks of food and water-borne enteritis and other diseases (Asakawa et al., 1973; Black et al., 1978; CDC, 1977; Highsmith et al., 1977; Keet, 1974; Nilehn, 1969a; Toma and Lafleur, 1974). Aside from being one additional foodborne pathogen to consider, *Y. enterocolitica* has the distinction of surviving and multiplying at refrigeration temperatures. The psychrotrophic nature of this microorganism presents the food industry with a unique problem in maintaining food safety.

Y. enterocolitica has been the object of intensive study in the United States and Europe over the past 15 years. Some of the earliest work done involved the clinical isolation of this microorganism. Schleifstein and Coleman (1939) accurately described many of the important clinical and laboratory features of this bacterium nearly 40 years ago. During the 1960's, many isolations of *Y. enterocolitica* from clinical and environmental sources were reported by European laboratories. Characterization of *Y. enterocolitica* through biochemical descriptions and growth requirements now appear in the literature. Increasing scientific evidence of biochemical heterogeneity suggests the further subdivision of the species. The isolation and characterization methodology for *Y. enterocolitica* has improved considerably and consequently the organism has been reported more frequently by clinical and food microbiologists. The etiology of yersinial infection and the role of the organism in gastroenteritis and pseudoappendicitis has also become increasingly well documented. The purpose of this review is to place this information together in a manner useful to food science interests.

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0022-1147/79/0006-1736$02.25/0
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Table 1—Distinguishing Biochemical Characteristics of Yersinia enterocolitica[a]

Adonitol	–[b]
Amygdalin	–
Arabinose	d[c]
Arabitol	–
Arbutin	+[b]
β-galactosidase	+
Cellobiose	+
Dextrin	d
Galactose	+
Glucose	+
Gas from Glucose	–
H_2S Production	–
Inulin	–
KCN (Growth in)	d
Lactose	–
Melibiose	–
Nitrate reduction	+
Oxidase	–
Rhamnose	–
Salicin	–
Sorbitol	+
Sorbose	+
Starch	d
Sucrose	+
d-Tartrate	–
Urea hydrolysis	+
Xylose	d

[a] Mollaret and Thal (1974); Delorme et al. (1974)
[b] – = no utilization or production; + = utilization or production.
[c] d = variable utilization or production.

TAXONOMICAL ASPECTS

Yersinia enterocolitica is a gram-negative, nonsporeforming facultatively anaerobic rod. It is classified in the family of *Enterobacteriaceae* by virtue of its common antigens, and its biochemical and core DNA relatedness (Edwards and Ewing, 1972; Brenner et al., 1976). Frederiksen (1964) proposed the classification of the microorganism into the genus *Yersinia* by virtue of superficial similarities.

The vegetative cells in a young culture are typically ovoid to rod-shaped and later develop into the characteristic rod-shaped morphology. They are generally motile at temperatures below 30°C and are nonmotile at 37°C (Nilehn, 1969b; Toma, 1973). Indole is differentially produced; the methyl red test is positive; the Voges-Proskauer test is negative at 37°C and is generally positive at 22–30°C for this species. *Y. enterocolitica* does not usually utilize citrate as a carbon source, except for certain strains (Bottone et al., 1974; Chester et al., 1977). Some authors categorize these atypical strains as the "environmental" strains and suggest a separate species for them. In Bergey's 8th edition, the temperature growth range was listed as –2 to 45°C with optimum growth at 30–37°C (Mollaret and Thal, 1974). Very good growth was also observed at temperatures of 18°C (Mollaret et al., 1964), and 40°C is the upper limit for growth of many strains.

Table 1 lists the distinguishing biochemical characteristics of *Y. enterocolitica* (Mollaret and Thal, 1974; Delorme et al., 1974). Reactions of *Y. enterocolitica* in Triple Sugar Iron (TSI) agar after overnight incubation at 25°C are as follows: acid (yellow) slant and acid butt, no gas, and no blackening (H_2S production) in the butt (Feeley et al., 1976; Toma, 1973). Strains the ferment sucrose may cause the slants to rapidly revert to alkaline after 1 day of incubation, and give alkaline slant and acid butt reactions. *Y. enterocolitica* is O-nitrophenyl-β-D-galactopyranoside (ONPG) positive for β-galactosidase, but typically the organism does not ferment lactose (Chester et al., 1977; Schiemann and Toma, 1978). Another key biochemical identification marker is the negative-phenylalanine deaminase reaction (Toma, 1973; Delorme et al., 1974).

Y. enterocolitica may be characterized immunologically on the basis of two cell-surface antigens, the O, or somatic antigen, and the H, or flagellar antigen (Wauters, 1970; Wauters et al., 1971; Wauters et al., 1972). However, many strains isolated from foods are nontypable (Schiemann and Toma, 1978; Highsmith et al., 1977). The serological reactions, when they do occur, are similar in principle to those found with *Salmonella* (Carter, 1974). The O antigens are complex lipopolysaccharides that are part of the endotoxin structure of *Y. enterocolitica*. The immunological reactions depend on the kinds of sugars and the manner of linkage. Further serological subdivisions are based on the antigenic specificities of the flagellar H antigens. The various serotypes of *Y. enterocolitica* are determined through reactions of these antigens with antisera. Cross reactions of antisera with the endotoxin of *Brucella* have been observed (Hurvell et al., 1971).

Some scientists suggest that *Yersinia pestis* and *Yersinia pseudotuberculosis* are more closely related to each other than they are to *Y. enterocolitica*, which might more properly be placed into another genus (Brubaker, 1972; Brenner et al., 1976). Indeed other researchers also consider that there is a need for reclassifying atypical strains of *Y. enterocolitica* (Chester et al., 1977; Knapp and Thal, 1973). The above mentioned biochemical reactions of *Y. enterocolitica* are most frequently encountered, yet large and significant variations exist among strains. "Atypical" strains are sucrose-negative, rhamnose-positive (Weaver and Jordan, 1973; Knapp and Thal, 1973; Bottone et al., 1974), citrate positive (Chester et al., 1977), lactose positive (Schiemann and Toma, 1978), raffinose-positive and melibiose-negative (Bottone et al., 1974; Chester et al., 1977). All the inconsistencies in these biochemical reactions lend to the confusion in classification that abounds in the literature.

Biotyping of *Y. enterocolitica* separates strains into 5 biochemically differentiated categories (Nilehn, 1969a, Wauters, 1970). Both the specific biotypes and serotypes have significance in incidence, virulence, and in distribution throughout the world. The Wauter's biotype scheme is most widely used and is helpful in tracking the source of epidemic strains. Table 2 demonstrates this scheme, while Table 3 shows the Nilehn scheme.

The terms "typical" and "atypical" as found in the literature on *Y. enterocolitica*, serve only to confuse the situation. "Atypical" strains along with strains of biotype 1 are commonly found in many foods such as raw oysters (Toma, 1973), raw milk (Schiemann and Toma, 1978), and raw meats (Inoue and Kurose, 1975). These "environmental" strains have also been isolated from clinical sources (Bottone et al., 1974), as have the "typical" clinical strains, belonging to biotypes 2, 3 and 4. The "environmental" strains, however, seem to have a different foci of disease (Bottone, 1978; Aldova et al., 1977). The pathogenic potential of the environmental strains is less severe causing self-limiting enteritis without mesenteric lymphadenitis or

Table 2—Biotype scheme for Yersinia enterocolitica[a]

	Biotypes				
Tests[b]	1	2	3	4	5
Lecithinase	+[c]	–[c]	–	–	–
Indole	+	+	–	–	–
Lactose (O/F) medium	+	+	+	–	–
Xylose (48 hours)	+	+	+	–	–
Nitrate	+	+	+	+	–
Trehalose	+	+	+	+	–
Ornithine decarboxylase	+	+	+	+	–
β-galactosidase	+	+	+	+	–

[a] Wauters (1970)
[b] All test cultures are incubated for 48 hr at 25°C except indole which is incubated at 29°C.
[c] – = no utilization or production; + = utilization or production.

Table 3—Biotype scheme for Yersinia enterocolitica[a]

	Biotypes				
	1	2	3	4	5
Tests at 36°C					
Salicin	+[b]	–[b]	–	–	–
Esculin	+	–	–	–	–
Indole	+	+	–	–	–
Nitrate	+	+	+	+	–
Trehalose	+	+	+	+	–
Sorbitol	+	+	+	+	d[c]
Sorbose	+	+	+	+	d
Tests at 25°C					
Lactose (O/F)	+	+	+	–	–
Xylose	+	+	+	–	–
Ornithine decarboxylase	+	+	+	+	–
Voges-Proskauer	+	+	d	+	–
β-galactosidase	+	+	+	+	–
Sucrose	+	+	+	+	d

[a] Nilehn (1969a)
[b] – = no utilization or production; + = utilization or production.
[c] d = variable utilization or production.

terminal ileitus (Bottone, 1978). Moore and Brubaker (1975) reported that "only strains of biotype 5, which differ markedly from the strains of the other biotypes, clustered separately in the similarity matrix...," and therefore may merit classification as a separate species.

INCIDENCE OF Yersinia enterocolitica IN FOOD

ONE HUNDRED EIGHTY-EIGHT school children and one teacher, from the same school in Japan, suffered from an illness resembling bacterial food poisoning, between January 31 and February 26, 1972 (Asakawa et al., 1973). This was possibly the first report of a community outbreak of infection due to *Y. enterocolitica* in the world. All strains isolated from the patients belonged to the O antigen, serotype 3 (0:3). The source and transmission of the infection was not determined.

The only known outbreak of illness from *Y. enterocolitica* for which foodborne transmission was proven occurred in September, 1976, among 220 school children in Oneida County, New York. Thirty-six children were hospitalized and 16 had appendectomies before the bacterial basis of the illness was determined. *Y. enterocolitica* was isolated from both the ill children and from the incriminated chocolate milk which they had consumed. Serotype 0:8 was isolated from the clinical specimens of the infected school children and an unopened carton of the suspect chocolate milk. It is postulated that the adulteration occurred during processing. Chocolate syrup was added to a large open vat of pasteur-

ized milk and the resulting chocolate milk was not repasteurized prior to dispensing into milk cartons (CDC, 1977; Black et al., 1978).

In April, 1975. an outbreak of febrile gastroenteritis occurred in two Montreal elementary schools. In one school, 57 children and one adult developed enteritis symptoms and in another school 80 children also developed these same symptoms. Raw milk which was consumed contained *Y. enterocolitica* serotype 0:6,30. *Y. enterocolitica* serotype 0:5,27 was isolated from the ill children. The milk was not epidemiologically incriminated because the *Y. enterocolitica* isolated from the ill children and the milk were not of the same serotype. It was however, difficult to explain the difference in serotypes since *Y. enterocolitica* was the only pathogenic agent identified. The evidence strongly pointed toward a foodborne infection (Health and Welfare Canada, 1976).

Another outbreak of enteritis attributed to *Y. enterocolitica* occurred in a Japanese junior high school on July 14, 1972. In this outbreak, a total of 198 pupils suffered from acute abdominal pains for a duration of up to 8 days. The predominant serotype isolated belonged to the 0:3 group. Although the infection route and source were not determined, the outbreak was presumed to be from a point source contaminated with the bacteria since all the patients became ill within 3 days of each other (Zen-Yoji et al., 1973).

Y. enterocolitica has been isolated from water sources on several occasions. In Novermber, 1972, an elderly man became ill during a hunting/camping trip. *Y. enterocolitica* was recovered in pure culture from his blood and surgically drained shoulder. The reporting physician (Keet, 1974) thought it probable that the infection source was from the mountain stream water used for drinking, because all food eaten during the patients hunting trip was canned. Serotype 0:8 was recovered from both the patient and the mountain streams. It is theorized that infected animals could have contaminated the area with their droppings. With a subsequent rain or thaw, the bacterium could have found its way into and contaminated a stream that was used for drinking water.

Lassen (1972) collected 50 samples of drinking water in Europe 10 of which contained *Y. enterocolitica*. These samples were taken from either private wells or from waterworks where chlorine was not added. The serotypes most frequently involved in illness were not found in the randomly selected samples. It was proposed that pathological serotypes might also be conveyed through drinking water.

In another report (Highsmith et al., 1977) recovered *Y. enterocolitica* from well water which was implicated in a water-borne outbreak of gastrointestinal illness. Most of the isolates were of Nilehn's biotype 1 and were serologically nontypable. Highsmith et al., (1977) reported growth of 4 isolates in sterile distilled water without added nutrients at 4, 25 and 37°C. This finding raises questions about the safety of non-chlorinated water when held at lower temperatures. There has been little documentation to substantiate this possible health hazard.

Many documented reports of clinically isolated *Y. enterocolitica* from humans exist (Kohl et al., 1975; Lafleur and Martineau, 1973; Toma, 1973; Zen-Yoji and Maruyama, 1972). More than 5,800 isolates of *Y. enterocolitica* were on deposit at the Pasteur Institute as of January, 1977 (Bottone, 1977). The distribution of pathogenic serotypes show 0:3 and 0:9, biotype 4 (Nilehn's) predominant in Europe (Aldova et al., 1977: Vandepitte et al., 1973), Japan (Zen-Yoji and Maruyama, 1972), and Canada (Toma and Lafleur, 1974), while in the United States biotypes 2 and 3 (Nilehn's) serotype 0:8 is the most prevalent pathogen (Quan et al., 1974; Toma and Lafleur, 1974; Weaver and Jordan, 1973). Explanations for the difference in the prevailing serotypes and biotypes in adjacent Canadian and United States areas are still lacking.

Yersinial outbreaks follow specific distributions. Seasonal variations show that the lowest frequency is found in spring, with increases in summer and fall, and the highest frequency in October and November (Zen-Yoji et al., 1974; Winblad, 1973; Wauters and Janssens, 1976). The precise peak in infection depends on the geographical location and climate of the reporting station. The incidence of infection has predominated in persons between birth to 15 years of age (Vandepitte et al.. 1973; Rusu et al., 1973), as well as in debilitated individuals (Keet, 1974). Disease attributable to *Y. enterocolitica* can no longer be considered as a rarity, rather it has become a definite health problem. Between 1966 and 1975 the Center for Disease Control (CDC) in Atlanta, GA, received 74 isolates of *Y. enterocolitica* relating to bacterial disease (Kohl et al., 1975). Some experts in the field suggest that bacterial enteritis caused by this organism has surpassed the incidence of *Shigella* and is only second to *Salmonella* in Canada (Lafleur, 1973; Toma and Lafleur, 1974; Pai and Mors, 1978).

Y. enterocolitica is a zoonotic organism with the major route of transmission to food possibly being through the contamination of foods with feces, urine or insects (Sonnenwirth and Weaver, 1970; Keet, 1974). Evidence exists that mammals may serve some role in the contamination of foods. Rats inhabiting a Japanese slaughterhouse carried *Y. enterocolitica* at the rate of 35% (Zen-Yoji et al., 1974). An interfamilial outbreak of *Y. enterocolitica* enteritis suggested that a bitch and its litter of sick puppies were the source of an infection (Gutman et al., 1973). *Y. enterocolitica* has been isolated from cats (Toma, 1973), chinchillas, hares, swine, and dogs in European countries (Mollaret et al., 1964), and from monkeys and deer in Japan (Otsuki et al., 1973).

Y. enterocolitica has been recovered from food sources such as beef, lamb, chicken and brown trout in different areas of the world (Hanna et al., 1976; Inoue and Kurose, 1975; Kapperud and Jonsson, 1976; Leistner et al., 1975). Swine seems to be the major reservoir for human infections caused by *Y. enterocolitica* (Toma and Deidrick, 1975; Leistner et al., 1975: Zen-Yoji et al., 1974; Essevald and Goudzwaard, 1973; Tsubokura et al., 1973). Narucka and Westendoorp (1977) isolated *Y. enterocolitica* most frequently from the tonsils of pigs, compared to other portions sampled. Serotype 0:3, one of the chief human pathogenic serotypes in most of the world, as well as other clinical serotypes, have frequently been isolated from swine (Wauters et al., 1976). Wauters and Janssens (1976) also isolated 0:3 serotypes from pig throat swabs. In a survey of Japanese foods by Asakawa et al., (in press), two potentially pathogenic 0:3 serotype strains and one 0:5 serotype strain were recovered from 300 pork samples studied. The 0:3 strain was also recovered from 2 of 50 chopping blocks and 2 of 571 butcher feces samples examined. Swine are the only recognized animal species consumed by humans which recurrently have been shown to harbor pathogenic *Y. enterocolitica* serotypes (Pederson, in press). One perplexing and contradictory note has recently been sounded by Lee et al., (1977): none of the *Y. enterocolitica* cultures isolated from pork (or from other food and water samples) proved invasive by the HeLa cell test for virulence. Since this report, several *Y. enterocolitica* strains isolated from pork have proven to be HeLa cell invasive (Lee, personal communication). Most of the *Y. enterocolitica* strains isolated from pork and other foods have been classified into the environmental strain category (Leistner et al., 1975; Inoue and Nagao, 1976). Environmental *Y. enterocolitica* strains have been recovered from chicken in Germany, Ja-

pan, and the U.S. (Leistner et al., 1975; Inoue and Nagao, 1976; Mehlman et al., 1978), from turkey meat samples in the United States (Guthertz et al., 1976), from oysters in Canada and the United States (Lee et al., 1977; Toma, 1973), and from vegetable samples tested in France (Louiseau-Marolleau and Alonsa, 1976). If food and water supplies only infrequently carry HeLa cell invasive *Y. enterocolitica*, then alternative explanations that resolve the transmission question of the pathogenic variety of the bacterium are needed.

In addition, *Y. enterocolitica* has been isolated from raw milk (Aldova et al., 1975; Mollaret et al., in press) and pasteurized milk (Schiemann, 1978; Hughes, 1979; Schiemann and Toma, 1978) in various locations throughout the world. Aside from the isolates from the two large outbreaks previously mentioned, there is no evidence that the strains isolated from milk were pathogenic to humans.

PATHOGENICITY

THE DEVELOPMENT of yersinial enteritis begins with contamination of the food or water by the pathogen. The organism must proliferate under favorable growth conditions and must be present in sufficient numbers to cause infectivity, and finally, the food or water must be ingested. Other factors affecting the etiology of *Y. enterocolitica* caused disease are the infectivity of the particular strain, the resistance of the consumer and the incubation temperature at which the culture was grown (Lee et al., 1977; Carter and Collins, 1974; Abramovitch and Butas, 1973). The infectious nature of the organism seems to be related to incubation temperatures, that is, a culture grown at 25°C in synthetic media is more virulent than one incubated at 37°C (Nilehn, 1973).

Y. enterocolitica has been implicated in certain acute human diseases. *Y. enterocolitica* has been implicated as the causative agent of enteritis, pseudoappendecitis (Black et al., 1978; Winblad, 1973; Nilehn, 1969b), mesenteric lymphadenitis (Bronstein et al., 1971), terminal ileitis (Mayer and Greenstein, 1976), and arthritis (Winblad, 1975). It has been isolated from the cerebrospinal fluid, blood, urine, and eyes of infected patients (Sonnenwirth, 1969). In one study (Nilehn et al., 1968) it was reported that *Y. enterocolitica* accounted for 3.8% of the appendectomies performed because of the similarities in *Y. enterocolitica* enteritis and appendicitis (Asakawa, 1973; Jepsen et al., 1976; Zen-Yoji et al., 1973). Indeed appendectomies were performed on 16 children after they contracted *Y. enterocolitica* enteritis in the New York chocolate milk outbreak (Black et al., 1978). The organism has been isolated in large numbers of clinical syndromes including peritonitis, abscesses of the colon, spleen and neck, and cholecystitis (Sonnenwirth and Weaver, 1970). Septicemia due to the organism does occur, but it is the rarest form of *Y. enterocolitica* infection and carries the highest mortality rate attributable to the species (Abramovitch and Butas, 1973; Keet, 1974).

Gastroenteritis in humans is the major expression of the pathogenic strains. Frequently reported symptoms of *Y. enterocolitica* enteritis include: diarrhea, fever, vomiting, abdominal pain, nausea and headaches (Kohl et al., 1975; CDC, 1977; Asakawa et al., 1973; Zen-Yoji et al., 1973; Lafleur and Martineau, 1973; Delorme et al., 1974; Health and Welfare Can., 1976; Nilehn, 1969a; Nilehn et al., 1968). Although relatively rare, fatality due to *Y. enterocolitica* does occur, but the patient's recovery is generally complete within 1 to 2 days.

In a human volunteer study, one individual took an oral dose of 3.5×10^9 cells of *Y. enterocolitica* (Szita et al., 1973). The results were similar to those reported in other food and water-borne bacterial infections; the organism caused enterocolitis, diarrhea and a fever. The acute symptoms ceased after 2 days of discomfort. Tenderness in the stomach and liver region lasted 4 wk.

Tests on the pathogenicity of the species for mice and other laboratory animals have been studied. Mollaret and Guillon (1965) found all their strains of *Y. enterocolitica* were devoid of any experimental pathogenicity, whatever the dose, animal species, or inoculation route. However, Quan et al. (1974) suggested that long maintenance on artificial media may be associated with loss of virulence, and this could explain the discrepancy between their findings and that of Mollaret and Guillon's. Quan et al. (1974), found that LD_{50} levels for 3 cultures injected into white mice ranged from 1.6×10^4 to 54.0×10^4 organisms. In another study on pathogenicity, Carter (1974) inoculated mice intravenously with low numbers of *Y. enterocolitica* and produced a laboratory infection that was similar to the naturally occurring human infection. The mice developed a systemic, pyogenic infection involving the liver, spleen and lungs. Carter and Collins (1974) found greater virulence of cultures grown below 26°C when compared to organisms grown at 37°C. This increase in virulence was confirmed by Lee et al., (1977) who reported an increase of invasiveness in HeLa cells when *Y. enterocolitica* was grown at 22°C compared to 36°C. These findings merit the interest of food microbiologists concerned with refrigerated foods adulterated with *Y. enterocolitica*; however, extrapolation of these findings to refrigerated foods is ill-advised until experimental evidence is produced.

Feeley et al. (in press) tested *Y. enterocolitica* strains isolated from the New York chocolate milk outbreak for tissue invasiveness. Some of these strains were shown to have the ability to cause conjunctivitis in the quinea pig eye, giving positive Sereny tests. The ability to cause conjunctivitis was detected only in cultures grown at 25°C and not at 36°C. Zink et al. (1978), by using the Sereny test on one of the chocolate milk outbreak *Y. enterocolitica* strains, also found that invasiveness (positive Sereny test) correlated with the presence of a 41 Mdal plasmid.

Une (1976) speculated that in addition to the ability of *Y. enterocolitica* to invade epithelial cells in the human gut, toxin production by the microorganism may be significant in its pathogenicity. Pai and Mors recently (1978) confirmed this speculation by showing the production of a heat stable enterotoxin by the microorganism in a synthetic medium. They demonstrated enterotoxin activity "only when organisms were grown at 30°C or below, with the highest activity at 26°C." The production of enterotoxin, HeLa cells invasivity, Sereny testing, or any other method for detecting *Y. enterocolitica* virulence has proven to be inconsistent when tested against each other. Therefore, some alternative, objective plan needs to be developed to predict human pathogenicity of *Y. enterocolitica* isolates.

As already mentioned, the "environmental" strains of *Y. enterocolitica* have been isolated from a wide variety of foods and water. Consumption of these strains might be considered similar to eating nonpathogenic strains of other bacteria. However, "environmental" strains of *Y. enterocolitica* have caused confirmed cases of human infection, and therefore if isolated from a food source should also be tested for virulence. Clinical strains must also be tested for virulence if isolated from food or water.

GROWTH POTENTIAL AND CONTROL OF Yersinia enterocolitica IN FOODS

RELATIVELY FEW studies have been done on the growth potential of *Y. enterocolitica* in food. *Y. enterocolitica* has been shown to grow in pork at 4–5°C with 10^8 cells per gram obtained in less than 2 wk from a starting inoculum of 300–3000 cells per gram (Leistner et al., 1975; Asakawa et

al., in press). Similar results were obtained by Hanna et al. (1977a) in experiments with raw pork stored at 7°C. *Y. enterocolitica* was grown in cooked beef starting with an inoculum of about 300 cells per gram, which increased to about 10^{10} cells per gram within 10 days. Growth of *Y. enterocolitica* in raw beef was not as great as in the cooked product (Hanna et al., 1977a). A combination of 4 *Y. enterocolitica* strains (2 clinical and 2 environmental strains) inoculated into sterile whole milk at a level of 100 per ml, grew to over 10^7 cells per ml after 20 days incubation at 0–2°C (Stern, unpublished data).

Freezing conditions may have an adverse effect on the survival of *Y. enterocolitica*. Hanna et al. (1977b) described the effects of frozen storage on *Y. enterocolitica* in inoculated beef. Cell numbers per gram fell from approximately $10^{6.5}$ to 10^4 within 28 days storage at –23°C. Lower numbers of approximately 10^4 cells per gram decreased to nondetectable levels within 28 days of storage at the same temperature. Asakawa et al. (in press), showed that about $10^{3.5}$ *Y. enterocolitica* per gram of sliced ham stored at –10°C fell to less than 50 cells per gram within 7 days of storage. Leistner et al. (1975), in contrast, showed that $10^{2.5}$ *Y. enterocolitica* per gram of frozen chicken broilers stored at –18°C decreased only slightly in numbers over a 90-day storage period.

An internal temperature in beef roasts of 60°C was adequate to inactivate approximately $10^{6.5}$ cells per gram, while 51°C left some survivors (Hanna et al., 1977b). The heat resistance of 2 *Y. enterocolitica* and *Y. enterocolitica*-like strains in skim milk has been determined (Hanna et al., 1977c). Among these cultures, 3–10 min heating at 55°C caused large reductions in counts. At 60°C, no survivors remained after 1–3 min. These thermal inactivation levels appear similar to those found with other *Enterobacteriaceae*.

The effect of pH on the growth of *Y. enterocolitica* was studied in brain heart infusion broth (BHIB) at pH levels ranging from 5–9 (Hanna et al., 1977b). The most rapid growth occurred between pH 7 and 8, while growth of *Y. enterocolitica* ATCC 23715 ceased at a pH of 5.0 within 24 hr. Growth of two clincial (CDC A2611 and CDC A2635) and two environmental (IP 867 and IP 955) strains at a pH of 4.6, at 25°C within 24 hr in BHIB was observed (Stern, unpublished data). The recovery of *Y. enterocolitica* in tartar sauce in Czechoslovakia may further suggest some resistance of *Y. enterocolitica* to acidic environment and seems to warrant further investigation (Aldova et al., 1975). The effects of the commonly used antimicrobial agents in foods on *Y. enterocolitica* have not been reported. Inhibition of four *Y. enterocolitica* strains in BHIB was observed at 5–7% NaCl at 25°C (Stern, unpublished results). This salt concentration in BHIB correpsonded to a water activity of 0.945.

Recently, El-Zawahry and Rowley (1979) reported on the radiation resistance of *Y. enterocolitica*. They found that *Y. enterocolitica* was among the most radiation sensitive bacteria. It was concluded that the microorganism should not be a health hazard in a low-dose irradiation process.

Y. enterocolitica has the ability to grow at refrigeration temperatures and possesses a potential health hazard. At temperatures of 4–7°C small numbers of the microorganism have the capability to develop into large numbers of viable cells. Fresh meats may carry the pathogen or the meat may be contaminated by the handlers through the transfer of intestinal materials to the individual cuts. The bacterium may be transferred by carriers that appear to be healthy animals (Inoue and Kurose, 1975). Home or delicatessen foods adulterated with *Y. enterocolitica* might serve as vectors of disease, especially if left at refrigeration temperatures for long periods of time.

RECOVERY AND ISOLATION METHODOLOGY

THE METHODS used for the isolation of *Y. enterocolitica* from food, water or clinical specimens are still being developed and improved. The groundwork for isolation includes the satisfactory collection and handling of a sample, which determines the adequacy of any microbiological investigation. When testing for the presence of *Y. enterocolitica* in any specimen, refrigeration temperatures should be employed until the specimen is ready for laboratory analysis. Greenwood et al. (1975) pointed out that even when using conventional media and procedures, the organism is difficult to isolate.

Authors have presented various methods used to isolate *Y. enterocolitica*. Primary isolation procedures include the use of cold enrichment temperatures (Feeley et al., 1976; Wauters, 1973a; Nilehn, 1969b; Nilehn and Sjostrom, 1967). Various enrichment broths have been suggested to help in the isolation of *Y. enterocolitica*, and typically, the methods used are successful only for certain strains, serotypes or biotypes. There may also be differences in efficacy of different lots of the same medium. Wauters (1973a) found satisfactory results when Rappaport's *Salmonella* medium was used in comparison to selenite enrichment medium. Toma (1973) reported that the use of selenite F enrichment broth for isolating *Y. enterocolitica* from feces specimens proved satisfactory. Several investigators (Inoue and Kurose, 1975; Feeley et al., 1976; Tsubokura et al., 1973; Leistner et al., 1975) suggested enrichment in saline or 1/15M disodium phosphate buffer at pH 7.6 for 21 days at 4°C. Asakawa et al. (1973) used an enrichment culture of phosphate buffer solution at pH 7.6 for 10 days or more at 8°C before plating onto 4 separate types of differential media. Schieman and Toma (1978) reported that the largest number of isolations of *Y. enterocolitica* from raw milk samples were obtained by inoculating the milk into Butterfield's phosphate buffer and incubating at 4°C for 14 days. This buffer was then transferred into modified Rappaport's broth which was incubated at 23°C for 5 days. Because all the clinically important serotypes and biotypes are not recoverable by any single enrichment method, two or more methods are suggested for isolation of *Y. enterocolitica* from a food system.

The clinical 0:3 and 0:9 strains may grow in selenite-cystine broth with 40 mg/l novobiocin or in Rappaport's broth (RMC) containing magnesium chloride, malachite green and carbenicillin (Wauters, 1973b). In an inoculated food test, the RMC broth recovered about 100 cells of the 0:3 serotype per gram of fresh oysters in 1 of 3 tests and from ground pork in 5 of 6 tests (Lee, in press). A 0:8 strain was completely inhibited by RMC broth in this same study. Phosphate buffered saline, pH 7.6, cold enrichment was successful in recovering the 0:3 strain in only 2 of a total of 9 tests and the 0:8 strain in 4 of 9 tests (Lee, in press).

Aulisio et al. (1979), reported on the use of alkalai for the rapid recovery of *Y. enterocolitica* from foods. Enrichment broths contained food inoculated with 10–1,000 cells per gram. After 2 days at 25°C, a combination of enrichment broth and KOH was streaked onto MacConkey agar. The plates were incubated 48 hr at 26°C and the resulting colonies were characterized biochemically. This recovery method increases sensitivity 10–1,000 times. Lee et al. (1979), reported the rapid recovery of *Y. enterocolitica* by the use of selenite enrichment broths. Sodium selenite, malachite green, and carbenicillin were filter sterilized into pH 7.5 phosphate buffer. Food suspensions were inoculated into the selenite enrichment broths, incubated 2 and 3 days at 22°C, and streaked onto various differential plating media. Recovery of *Y. enterocolitica* 0:3 strains was good, while high levels of indigenous bacteria in some food sam-

ples blocked recovery of a sensitive 0:8 strain.

The various differential and/or selective enteric plating media used with nonuniform success for the isolation of *Y. enterocolitica* include deoxycholate citrate agar (Szita et al., 1973; Wauters, 1973a) also known as Leifson agar (1935 modification); bismuth sulfite agar (Hanna et al., 1977d); lysine-sucrose-urea agar (Nilehn, 1969b; Lee 1977a; Asakawa, 1973; Toma, 1973; Wauters, 1973a); MacConkey's agar (Asakawa 1973; Feeley et al., 1976); and others. Lee (1977b) reported that "MacConkey agar modified with Tween 80, and deoxyribonuclease agar modified with Tween 80 plus sorbitol were used in differentiating *Y. enterocolitica* colonies from other lactose-negative bacterial colonies." Again, the efficacy of one plating medium over another has yet to be proven for all *Y. enterocolitica* strains and therefore it is best to employ 2 or 3 media for recovery work (Feeley et al., 1976).

Lactose, one of the common carbohydrate sources in enteric media, is usually not fermented by *Y. enterocolitica*. However, lactose-positive colonies similar in size and morphology to known *Y. enterocolitica* colonies should also be picked and cultured along with lactose-negative, Gram-negative colonies. These lactose-positive colonies may be members of the "environmental" group of *Y. enterocolitica* and the pathological significance of lactose-positive strains is not clear. Once isolated, a culture from the specimen in question should first be positively identified as belonging to the *Enterobacteriaceae*. Procedures for identification are presented in Edward and Ewing's *Identification of Enterobacteriaceae* (1972) and by Vanderzant and Nickelson (1969). Gram-negative, oxidase-negative organisms showing growth on MacConkey agar and fermentation of Hugh-Leifson carbohydrates are categorized as members of the *Enterobacteriaceae*. Once this classification has been established, final identification of the genus and species is made via motility and biochemical tests. Motility should be observed at room temperature and a lack thereof at 37°C. Among the most significant biochemical tests used in identification are TSI reactions, negative phenylalanine deaminase, and urea hydrolysis. Other biochemical tests necessary to identify include: lysine decarboxylase (−), mannitol (+), and sorbitol (+). After isolation, a suspected culture of *Y. enterocolitica* might also be screened for identification by the use of a miniaturized multitest system, such as API strips. Guthertz and Okoluk (1978) reported that API strips were employed to identify 96.1% of *Enterobacteriaceae* isolates to the species level. We suggest that the multitest system should only be used as a screening procedure. Further serotyping and/or biotyping of sample isolates should also be reported.

CONCLUSION

Yersinia enterocolitica has the unique distinction of being the first known psychrotrophic water and foodborne enteropathogen. Accordingly, increased research should be directed toward this microorganism to establish its significance as a water and foodborne pathogen. Microbiologists who isolate bacteria from foods that are Gram-negative rods, urease positive, phenylalanine deaminase negative, with characteristic *Y. enterocolitica* TSI reactions, and that exhibit motility only at room temperature should be suspicious of the presence of *Y. enterocolitica*. If isolates are positively identified, virulence testing should be considered.

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Ms received 3/10/79; revised 5/19/79; accepted 5/25/79.

EFFECT OF IRRADIATION TEMPERATURE AND DRYING ON SURVIVAL OF HIGHLY RADIATION-RESISTANT BACTERIA IN COMPLEX MENSTRUA

M. A. BRUNS and R. B. MAXCY

ABSTRACT

The influence of temperature and water availability on the shape of the sigmoidal radiation inactivation curves of highly resistant bacteria was determined. The resistance of *Micrococcus radiodurans* and *Moraxella* sp. to gamma radiation was much higher in ground beef at subfreezing temperatures (−30 ± 10°C) than at ambient temperatures (20–37°C). Irradiation in the frozen state lengthened the shoulder (phase of resistance prior to simple exponential death rate) of the inactivation curve. An even greater extension of the shoulder and protective effect resulted after lyophilization of *Moraxella* sp. Radiation sensitivity in the dry state was not temperature dependent. Lyophilized samples that had subsequently absorbed water exhibited greater sensitivity to radiation with a shorter shoulder on the inactivation curve. These results indicated that the effectiveness of irradiation in inactivating radiation-resistant bacteria in foods is highly dependent on the microenvironment.

INTRODUCTION

Micrococcus radiodurans, Moraxella Acinetobacter (M-A) and other vegetative bacteria which are highly resistant to gamma radiation have been isolated from foods and a broad spectrum of environmental sources (Anderson et al., 1956; Davis et al., 1963; Ito and Iizuka, 1971; Lewis, 1971; Tiwari and Maxcy, 1972; Krabbenhoft, 1965; Gulistani, 1977). Some of these bacteria have radiation resistance greater than that of bacterial spores (Duggan et al., 1963a; Matsuyama et al., 1964; Welch and Maxcy, 1975).

This resistance is particularly pronounced in frozen beef, where a *Moraxella* sp. was shown to be more resistant than *Clostridium botulinum* spores (Bruns and Maxcy, 1978). Determination of radiation resistance in frozen meat is appropriate, because the currently proposed method for radappertizing meat involves irradiation at −30°C (Maxcy and Rowley, 1978). Radiation pasteurization, on the other hand, employs temperatures above freezing.

Comparative radiation inactivation curves have shown that the frozen state offers a protective effect against radiation damage for a number of vegetative bacteria (Matsuyama et al., 1964; Anellis et al., 1973). Therefore, highly radiation-resistant bacteria should have slower inactivation rates in meat at −30°C than at the temperatures used for radiation pasteurization. However, the only data available on comparative resistance of *M. radiodurans* under frozen and nonfrozen conditions in a food system suggested that freezing does not afford significant protection during irradiation (Duggan et al., 1963b).

More extensive radiation studies on *M. radiodurans* in beef had not been undertaken, because *M. radiodurans* was not considered to pose a problem in a radappertization procedure (Anellis et al., 1973). *M. radiodurans* has no public health significance. It is relatively heat sensitive (Duggan et al., 1963c). Thus, it would not be likely to survive the heat treatment given meat in the course of a radappertization process. The heat treatment is necessary for enzyme inactivation.

Comparative radiation studies of highly radiation-resistant bacteria in beef, however, are in order because certain *Moraxella* sp. are sufficiently heat resistant to survive the heat treatment for enzyme inactivation (Maxcy and Rowley, 1978). Furthermore, hemolytic activity was found among several micrococci isolated from irradiated chicken in this laboratory, thus raising the question of public health significance for these bacteria (Welch and Maxcy, 1979).

There is a lack of understanding of the relationship between radiation resistance of highly resistant bacteria and the microenvironment during irradiation. Therefore, this work was directed to obtain a better understanding of the effect of irradiation temperature and water availability on survival of highly radiation-resistant bacteria in a food system.

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0022-1147/79/0006-1743$02.25/0
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MATERIALS & METHODS

Cultures

Isolates 4 and 7 were chosen as highly radiation-resistant representatives of the M-A isolated from irradiated beef in a study by Welch and Maxcy (1975). The two isolates were classified as members of Group M-5 of the bacteria that resemble *Moraxella* sp. (Tatum et al., 1974). These cultures were compared to *M. radiodurans* ATCC 13939.

Cultures were propagated on Plate Count Agar (PCA; Difco) slants by incubating for 24 hr at 32°C. Between transfers, cultures were stored at 2°C. Cultures to be irradiated were grown in m-Plate Count Broth (PCB; Difco) at 32°C in a reciprocating shaker bath. Radiation resistance was determined with 48-hr cultures of Isolates 4 and 7 and 32-hr cultures of *M. radiodurans*.

Radiation inactivation curves

Ground beef patties were inoculated with approximately 10^7 bacteria per gram, irradiated, and sampled as described by Bruns and Maxcy (1978). All CFU added to the meat were recoverable within experimental error. Dose rate provided by the Cobalt-60 source was approximately 7 Krad per min.

For irradiation at subfreezing temperatures, patties were held near dry ice with appropriate insulation so that the temperature was maintained at −30 ± 10°C as determined with a thermocouple and recorder. During ambient temperature irradiation, the temperature of the patties (initially at 20°C) rose steadily until a temperature of 34–37°C was reached. Patty temperatures did not exceed 37°C.

Radiation resistance of Isolate 4 also was determined at ambient and subfreezing temperatures in the broth in which it had been grown. All frozen samples were thawed in a 32°C water bath and plated immediately.

Inactivation curves in the dry state in air

Portions of 48-hr cultures of Isolate 4 were lyophilized with a Virtis Model No. 10-145 MR-BA lyophilizer. Radiation inactivation curves were determined at ambient and subfreezing temperatures with the lyophilized samples, which were rehydrated with PCB.

To study the effect of exposure to a humid environment on radiation resistance of lyophilized Isolate 4, the stoppers on some of the samples were removed, and the vials were held over distilled water in a desiccator for 1 hr at 25°C. The stoppers were then replaced. Control samples were held stoppered for 1 hr at 25°C.

Plate counts

Dilutions and pour plate counts in PCA were performed according to the procedures outlined by Speck (1976). For inactivation studies comparing lyophilized samples, parallel plate counts were

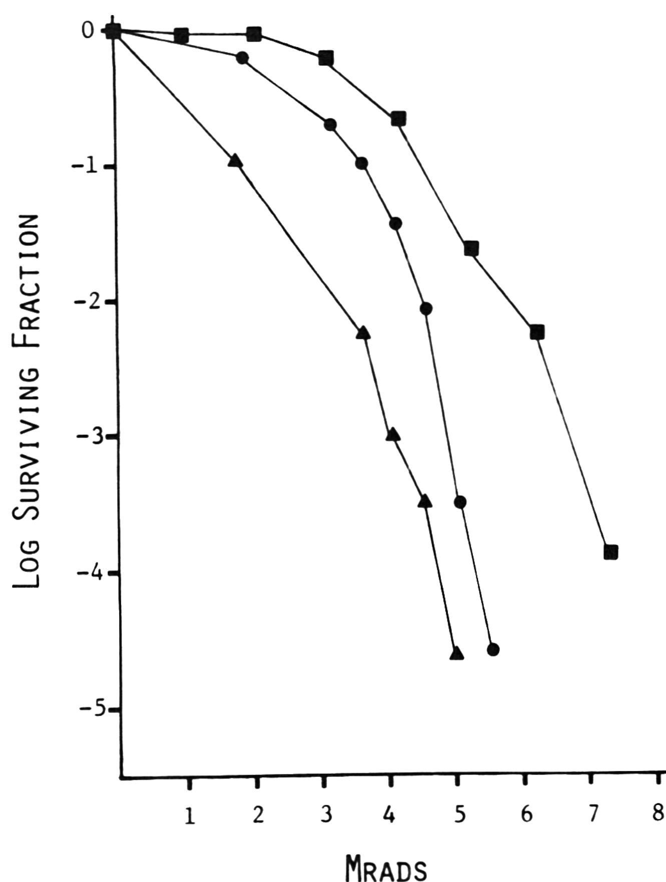

Fig. 1—Comparative radiation resistance of M. radiodurans (■), Isolate 7 (●), and Isolate 4 (▲) in ground beef at −30 ± 10°C.

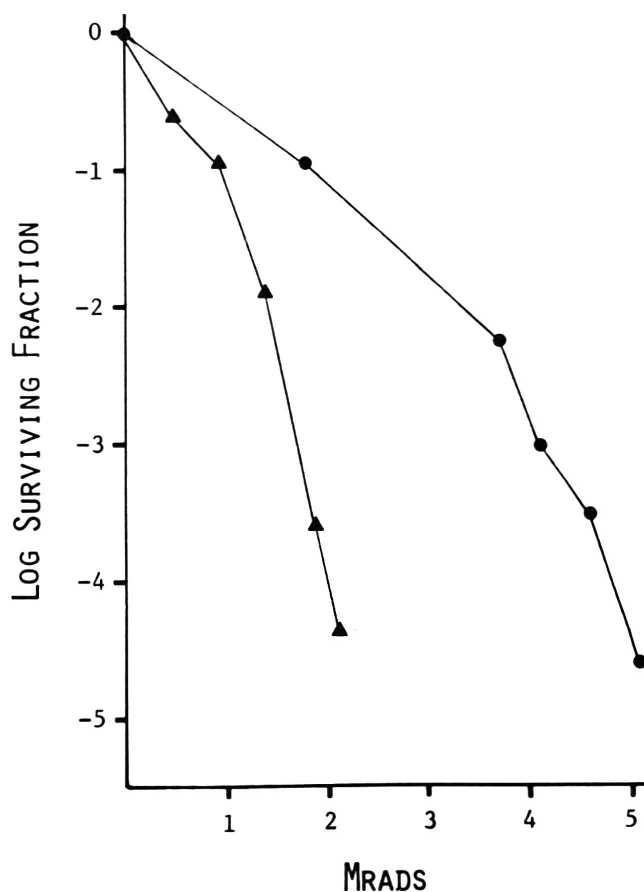

Fig. 2—Comparative radiation resistance of Isolate 4 in ground beef at −30 ± 10°C (●) and ambient temperature (▲).

made on PCA, and PCA with 0.5% NaCl in order to evaluate injury (Bruns and Maxcy, 1978). Plates were incubated at 32°C until there was no further increase in the number of colonies (up to 7 days incubation for samples receiving doses above 2 Mrad).

All data represent values averaged from at least two trials.

RESULTS

Comparative radiation inactivation curves in frozen ground beef

Radiation inactivation curves are given in Figure 1 for Isolates 4 and 7 and *M. radiodurans* in frozen beef (−30°C). Isolate 7 and *M. radiodurans* had more extensive shoulders (phase of resistance prior to simple exponential death rate) than that of Isolate 4. Percent survival over the 4.5 Mrad range was 0.05%, 2%, and 10% for Isolate 4, Isolate 7, and *M. radiodurans*, respectively. This range is the approximate maximum for 12D destruction of *C. botulinum* spores in proposed systems for radappertization of frozen meats (D.B. Rowley, Personal communications).

Comparative radiation resistance at subfreezing and ambient temperatures

The radiation inactivation curves of Isolate 4 in ground beef at −30°C and ambient temperatures (20−37°C) are shown in Figure 2. The resistance of *M. radiodurans* in beef at these temperatures is indicated in Figure 3. Also shown in Figure 3 is the inactivation curve of *M. radiodurans* (R_1 culture) irradiated in a beef purée at 5°C. This curve was replotted from the data of Duggan et al. (1963a). A similar protective effect due to freezing was also seen with Isolate 4 in PCB (Fig. 4).

Though the transition point is not distinct, the biphasic nature of the inactivation curves is apparent. The frozen state resulted in an extension of the shoulders, and the exponential inactivation was temperature-dependent. The slope of the exponential portion of the curve for *M. radiodurans* in beef at −30°C was approximately one-half that obtained when suspended in beef purée at 5°C.

Effect of the dry state on resistance of Isolate 4 in air

When more water was physically removed from cultures of Isolate 4 by lyophilization, they demonstrated even greater resistance (Fig. 4). After 6 Mrad, the viable populations had only been reduced to approximately 10% of the original number. Resistance of Isolate 4 in the dry state was not temperature-dependent.

Effect of exposure to humid atmosphere on the resistance of Isolate 4

Another approach to substantiate the contention that water availability was critical in determining resistance was to reverse the effect of lyophilization by exposing the dried samples to a moist environment for 1 hr. After exposure, the lyophilized cultures of Isolate 4 were difficult to rehydrate, thus indicating a physical change. Cells having been exposed to rehydration were considerably more susceptible to radiation (Fig. 5).

DISCUSSION

THE MICROENVIRONMENT markedly influences the resistance of certain bacteria to gamma radiation. Previous observations on the effect of freezing (Duggan et al., 1963b) and on drying (Christensen and Holm, 1964) on the radiation resistance of *M. radiodurans* were not extended to actual food systems. Duggan et al. (1963b) used a mentruum of a 1:1 mixture of beef and saline and concluded

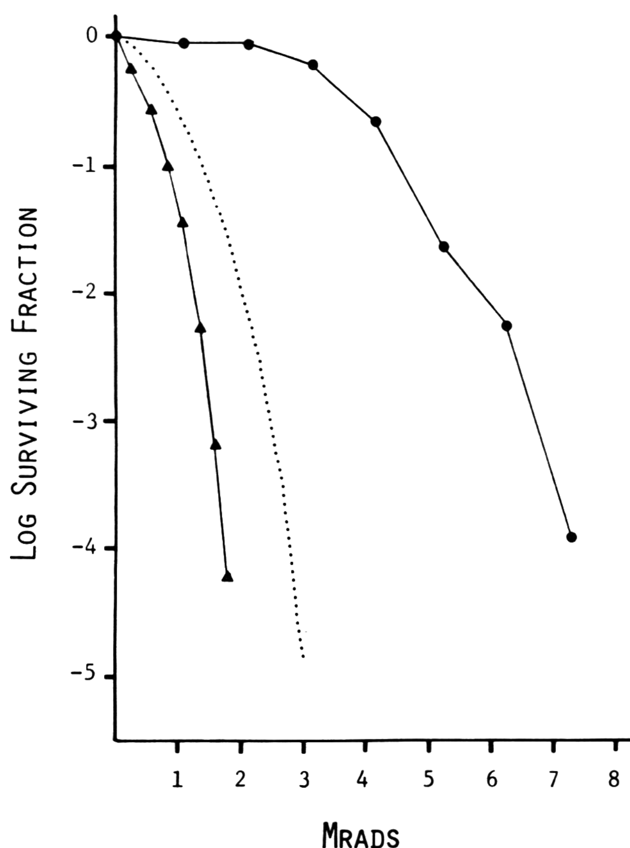

Fig. 3—Comparative radiation resistance of M. radiodurans in ground beef at −30 ± 10°C (●) and ambient temperature (▲). Also shown is curve (. . . . .) showing resistance of M. radiodurans in beef purée at 5°C (replotted from results of Duggan et al. 1963a).

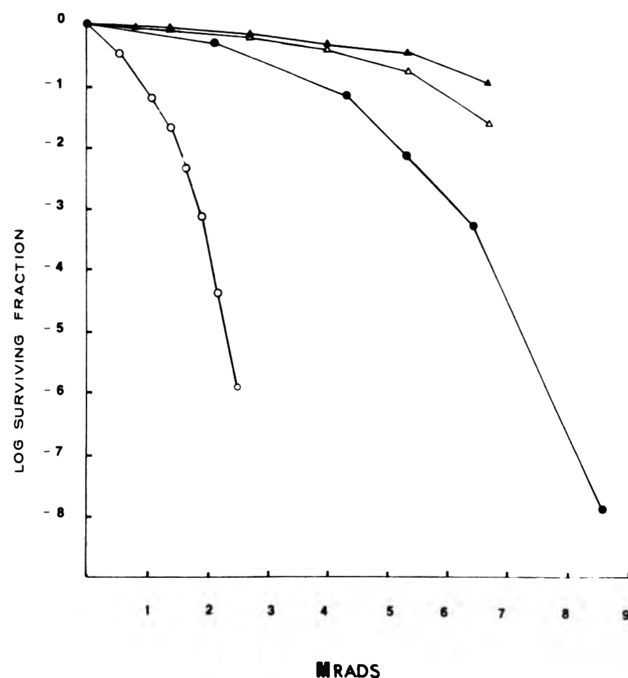

Fig. 4—Comparative radiation resistance of Isolate 4 in PCB at ambient temperature (○) and PCB at −30 ± 10°C (●) and of Isolate 4 lyophilized in PCB before irradiation at ambient temperature (△) and −30 ± 10°C (▲).

that the frozen state was not protective for *M. radiodurans*. Their results, however, were based on single-dose level comparisons.

It is apparent (Fig. 2, 3) that the difference in the degree of inactivation in the frozen and nonfrozen states varies with radiation dose. This variation arises from the sigmoidal characteristic of the inactivation curves of the highly radiation-resistant bacteria. For example, 4.0 Mrad is within the shoulder range of the inactivation curve for *M. radiodurans* in the frozen state. A comparable dose at ambient temperature, however, results in inactivation well into the exponential phase.

Comparative radiation effectiveness can also be expressed in terms of dose ratios (Table 1). Comparison of different phases of the inactivation curves provide different ratios. Matsuyama et al. (1964) also observed variation in dose ratios when the sigmoidal inactivation curves of *Streptococcus faecium* and *Alcaligenes* sp. were determined at −79°C and at room temperature. Thus, it appears that the reduction of water availability due to freezing markedly influences the radiation resistance of bacteria.

Most of the alteration in radiation resistance associated with temperature reduction was attributable to decreased water availability, since comparable reductions in temperature of lyophilized bacteria had little effect on the radiation resistance. The increased resistance both in the frozen and dried states was due primarily to an extension of the shoulder of the inactivation curve. These results extend the observations of Christensen and Holm (1964) who showed that irradiated *M. radiodurans* exhibited a pronounced shoulder after air drying.

Radiation damage can result in either killing or injury. Injury of Isolate 4 is indicated by lowered recovery on PCA with 0.5% NaCl (Bruns and Maxcy, 1978). Parallel platings on PCA and PCA with 0.5% NaCl of lyophilized samples after irradiation showed little difference in recovery (Fig. 5). Thus, significant injury was not detected in this medium after irradiation in the dry state. Irradiated samples that had been exposed to a moist atmosphere subsequent to lyophilization also showed similar recoveries on PCA and PCA with 0.5% NaCl. Thus, the increase in sensitivity of Isolate 4 due to exposure was due to kill rather than injury.

The effects of freezing and drying on the kinetics of inactivation of the highly resistant bacteria used in our studies were not the same as those obtained by freezing the more radiation-sensitive bacteria studied by Matsuyama et al. (1964). Even though the inactivation curves of *S. faecium* and *Alcaligenes* sp. were sigmoidal, the main effect of the frozen state was a decrease in the slope of the exponential portions. The main effect of the frozen state on the resistance of *M. radiodurans* and Isolate 4, on the other hand, was the extension of the shoulders of the inactivation curves. Therefore, it is necessary to evaluate extended inactivation curves in the specific food system and at the temperature to be used, because of the variability of the inactivation patterns with changes in the microenvironment.

In our study the resistance of *M. radiodurans* and two M-A was evaluated in a specific food system, beef, and at the specific temperature proposed for radappertization. The results indicate some of these bacteria would likely survive an irradiation dose of 4.5 Mrad given frozen meat.

It has been assumed that highly radiation-resistant bac-

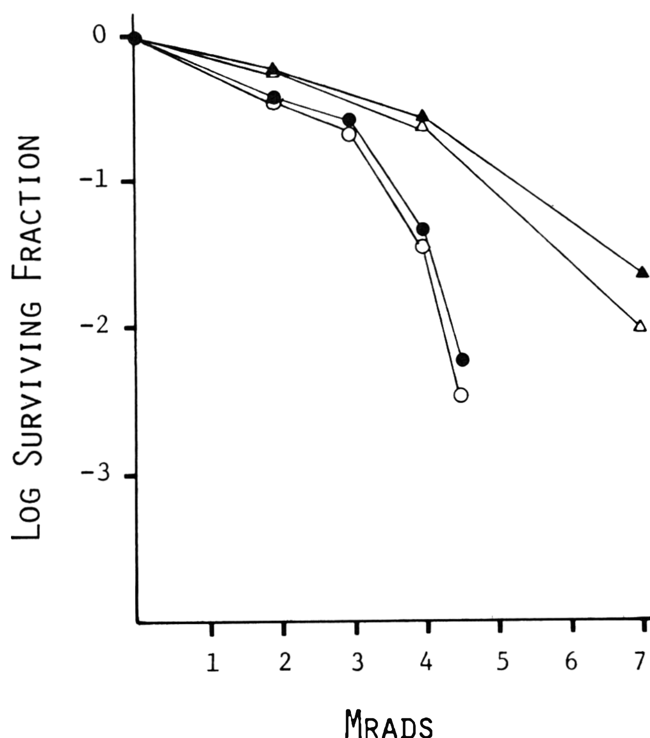

Fig. 5—Radiation inactivation curves of Isolate 4 lyophilized in PCB and subsequently exposed to a moist atmosphere for 1 hr at 25°C (○, ●) and Isolate 4 which was lyophilized in PCB but kept dry (Δ, ▲). Closed symbols indicate recovery on PCA. Open symbols indicate recovery on PCA with 0.5% NaCl. Irradiation was at ambient temperature.

teria are destroyed by an enzyme inactivation heat treatment, because of earlier reports on the heat sensitivity of *M. radiodurans*. However, radiation-resistant M-A have been shown to be more heat resistant than *M. radiodurans*. The interaction of sequential heat and radiation would likely eliminate these bacteria in a radappertization process. Furthermore, these bacteria are extremely sensitive to the post-irradiation microenvironment (Bruns and Maxcy, 1978) and may not grow in meats (Snyder and Maxcy, 1979). The nature of the response of these bacteria to the complex microenvironment of foods warrants further study.

Table 1—Protective effect of the frozen state during irradiation on survival of M. radiodurans in ground beef

Log cycles of reduction	Dose (Mrad)[a] needed to achieve reduction at: ambient temp	−30 ± 10°C	Dose ratio[b] (Protective factor due to freezing)
−0.25	0.3	3.2[c]	12.8
−0.50	0.5	3.8[c]	8.4
−1.00	0.8	4.5	5.6
−2.00	1.2	5.8	4.8
−3.00	1.6	6.7	4.2
−4.0	1.8	7.4	4.1

[a] Data from Figure 3
[b] Dose needed at −30 ± 10°C/dose needed at ambient temp
[c] Shoulder range of inactivation curve at −30 ± 10°C

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Published as Paper No. 5681, Journal Series, Nebraska Agricultural Experiment Station, Lincoln, Nebraska. Research was conducted on Project Number 16-023.

This work was supported in part by Contract DAAK 60-78-C-0039 from the United States Army, Natick Research & Development Command.

PURIFICATION AND SOME PROPERTIES OF LIPASE FROM Streptococcus faecalis

HARISH CHANDER, B. RANGANATHAN and JASJIT SINGH

ABSTRACT

Streptococcus faecalis possesses a glycerol ester hydrolase (lipase). The enzyme was purified from cell-free extract by ammonium sulphate precipitation, Sephadex G-25 filtration, acetone fractionation, Sephadex G-75 filtration, ion exchange chromatography on DEAE cellulose, to 140-fold with 30% recovery. The isoelectric point of enzyme was found to be 3.6. Its molecular weight was 20,900. With regards to amino acid content, glutamic acid was present in maximum amounts as compared to other amino acids, while cysteine was present in minimum amounts. Michaelis constant of lipase was 5.0 × 10^{-3} M at pH 7.5 at 40°C. Of the simple triglycerides, tributyrin was hydrolyzed most easily by the enzyme, as compared to tricaproin, tricaprylin and triolein. The relative specificity of purified lipase for natural triglycerides was in the following order: butter oil > olive oil > linseed oil > coconut oil. Maximum enzyme activity with butter oil as substrate, was observed at pH 7.5 at 40°C. The enzyme was stable for 1 month at −18°C and was completely inactivated in 10 min at 90°C. The enzyme was stable at pH levels ranging from 6.0–8.0. Addition of bile salts (0.2%) stimulated enzyme activities.

INTRODUCTION

MICROBIAL LIPASES play an important role in fat hydrolysis contributing to both desirable and undesirable flavors in foods. Considerable work on the properties of crude or partially purified lipase has been reported by several workers (Khan et al., 1967; Umemoto et al., 1968). Lipases have been obtained in a highly purified state from a variety of microorganisms (Jensen, 1974; Mencher and Alford, 1967; Oi et al., 1969; Lu and Liska, 1969; Vadhera, 1974). However, purified lipases from streptococci have not been thoroughly investigated except for very few reports (Carini, 1969; Umemoto, 1969). The present paper deals with the purification and some properties of lipase from *S. faecalis*.

EXPERIMENTAL

Culture

Streptococcus faecalis used in the present study was isolated from *khoa*, a concentrated Indian milk product and maintained in chalk litmus milk.

Cultivation of microorganisms

The organism was cultivated as a stationary culture in a nonsynthetic medium; peptone (Oxoid), 2% yeast extract (Oxoid), 0.3% glucose (BDH), 1% NaCl (BDH), 0.5% tomato juice (10.0%), pH 7.5.

Lipase activity

The method of Oi et al. (1969) was used with some modifications. Reaction mixture contained 5.0 ml of 5% butter oil emulsified in 7% gum acacia, 5.0 ml of 0.02M Tris-HCl buffer (pH 7.5), 2.0 ml of 0.2M $CaCl_2$ sol., 1.0 ml enzyme and 2.0 ml glass distilled water. Except for determination of Michaelis constant, where tributyrin was used, butter oil as substrate was taken. Lipase unit: 1 unit of lipase activity was defined as that which produces 1 μmole of free fatty acids from the substrate by 1 ml enzyme sol at 40°C in 3 hr.

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0022-1147/79/0006-1747$02.25/0
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Protein content

The enzyme was examined for protein content by the method of Lowry et al. (1951) using crystalline serum albumin as standard.

Purification procedures

The cell-free extract from *S. faecalis* prepared as previously described by Chander and Ranganathan (1975) was precipitated at 60% saturation with ammonium sulphate. After centrifugation at 9000 × G for 10 min, the resulting precipitate was dissolved in 10 ml Tris HCl buffer (pH 7.5) and examined for specific activity. The enzyme was desalted using Sephadex G-25 in a Pyrex glass column (2.5 × 31 cm) and equilibrated with 0.02M Tris-HCl buffer (pH 7.5). Thirty fractions of 5 ml each were collected at a flow rate of 1 ml per min following the procedure of Majumdar et al. (1970).

Cold acetone (−10°C) was added dropwise to the partially purified enzyme solution kept at 0°C to a final concentration of 60% V/V, which resulted in precipitation of enzyme.

Sephadex G-75 (Pharmacia, Sweden) was packed in a column (2.5 × 50 cm) and the flow rate was adjusted to 1.0 ml/min. The partially purified enzyme was eluted with 0.02M Tris-HCl buffer (pH 7.5).

DEAE cellulose (W and Bolstan, England) was packed in a column (2.5 × 25 cm) and fractions were collected at a flow rate of 20 ml/hr. Protein was eluted by a linear gradient of NaCl obtained by using 100 ml of 1.0M NaCl in 0.02M Tris-HCl buffer (pH 7.5) in the reservoir and 100 ml of the same buffer in 0.1M NaCl in the mixing chamber. The active fractions were pooled and dialyzed at 4°C against 0.02M Tris-HCl buffer (pH 7.5) for 30 hr with one change of buffer.

Homogeneity of lipase was determined by sodium dodecyl sulphate polyacrylamide electrophoresis (Weber and Osborn, 1969) by applying 30 μg of enzyme in 15% sucrose for each gel disc. Electrophoresis was performed at a constant current of 8 mA per gel with a positive electrode (anode) in the lower chamber.

Isoelectric focussing of lipase. The method of Wrigley (1968) was adopted with slight modifications. Three hundred μg of purified lipase in 8M urea containing 2 mM dithiothreitol was mixed with 1.0 ml of working gel solution. A constant current of 1–2 mA per gel was applied and the experiment was run for 6 hr at 4°C. The run was completed when there was a reduction in current to 1.5 mA. Four gels without any sample were also run to measure the pH gradient formed by the carrier ampholyte during the run. For measurement of pH of the discs, destained gel pieces of 5 mm size were cut and macerated in 2 ml water after vigorously shaking in a Vortex mixer. The pH of each gel was measured with a Radiometer pH meter using the expanded scale.

Purified lipase was subjected to a gel filtration technique as described by Andrews (1964) for molecular weight determination in a glass column (2.5 × 75 cm) packed with Sephadex G-100. The markers used were bovine serum albumin (mol wt 69,000, Fluka Switzerland), β-lactoglobulin (mol wt 36,000 Sigma, U.S.A.), Ovalbumin (mol wt 45,000, Sigma, U.S.A.) and α-chymotrypsinogen (mol wt 22,500, Sigma, U.S.A.). Protein content was determined by Lowry et al. (1951) using crystalline bovine serum albumin as the standard.

Amino acid analysis was conducted by hydrolyzing 1 mg lipase in 2 ml 6N HCl at 110°C for a 22-hr period under vacuum below 100 microns in a thick-walled test tube. At the end of hydrolysis, the hydrolysate was evaporated to dryness in a rotatory Buchi type vacuum evaporator. The residue was dissolved in distilled water and evaporated until complete removal of acid was achieved. The acid-free residue was dissolved in 0.1N sodium citrate (pH 2.0) and filtered. The analysis of the sample was performed on a Technicon TSM Automatic amino acid analyzer.

Text continued on page 1748

RESULTS & DISCUSSION

Purification of enzyme

The purification steps and enzyme yields are summarized in Table 1. At the end of purification, the recovery of enzyme was 30% with 140-fold purification. Figure 1 presents a typical elution profile for protein and lipase activity. Total enzyme activity was concentrated between elution volumes of 30 and 65 ml. Electrophoretic studies of purified lipase showed a single band moving towards the anode, thereby confirming the homogeneity of the lipase preparation.

Isoelectric focussing of purified lipase revealed one band of protein at the anode. The isoelectric point of the enzyme was found to be 3.6.

The elution volumes of standard proteins like bovine serum albumin, ovalbumin, β-lactoglobulin and α-chymotrypsinogen were 110, 170, 202 and 271 ml respectively, while the volume of purified *S. faecalis* lipase was 275 ml.

Table 1—Purification procedure for lipase from S. faecalis

Fraction	Total lipase activity[a]	Total protein (mg)	Specific activity[b]	Recovery %	Purification fold
1. Crude enzyme (cell free extract)	4000	16000	0.25	100	1
2. Precipitation at 50% ammonium sulphate saturation	3400	1100	3.1	85	12.4
3. Sephadex G-25	2800	800	3.5	70	14.0
4. Acetone precipitation	2320	400	5.8	58	23.2
5. Sephadex G-75	2000	114	17.5	50	70
6. Chromatography on DEAE cellulose	1200	34	35.0	30	140

[a] Expressed as μmoles free fatty acids released/ml enzyme solution.
[b] Specific activity as μmoles of free fatty acids released per mg of protein.

Table 2—Amino acid analysis of purified Streptococcus faecalis lipase

Amino acids	Amino acid g/100g of protein
Lysine	6.47
Histidine	2.12
Arginine	3.73
Asparic acid	7.64
Threonine	3.68
Serine	4.43
Glutamic acid	11.88
Proline	7.15
Glycine	7.40
Alanine	7.86
Cysteine	1.08
Valine	5.31
Methionine	3.92
Isoleucine	6.74
Leucine	8.30
Tyrosine	5.21
Phenylalanine	7.14

Table 3—Substrate specificity of purified lipase

Lipid source	Lipase activity[a]	Relative activity (%)
Tributyrin	7.8	100
Tricaproin	6.5	83
Tricaprylin	5.6	64
Triolein	4.5	58
Butter oil	5.0	64
Olive oil	4.5	58
Coconut oil	3.1	40
Linseed oil	4.0	51

[a] μmoles of free fatty acids liberated from the substrate/ml of enzyme solution

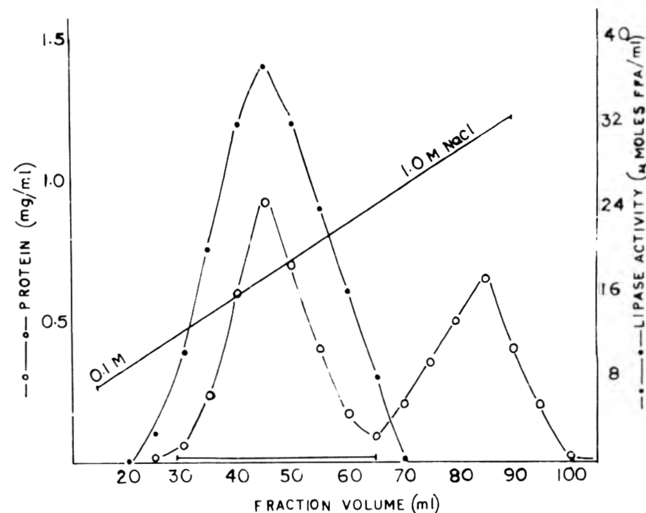

Fig. 1—DEAE cellulose chromatography: Ion exchange chromatography of lipase on DEAE cellulose column (2.5 × 50 cm). After the initial buffer (0.02M Tris-HCl, pH 7.5), protein was eluted with 0.1–1.0M NaCl gradient in the same buffer, 5 ml fractions were collected with a flow rate of 20 ml/hr.

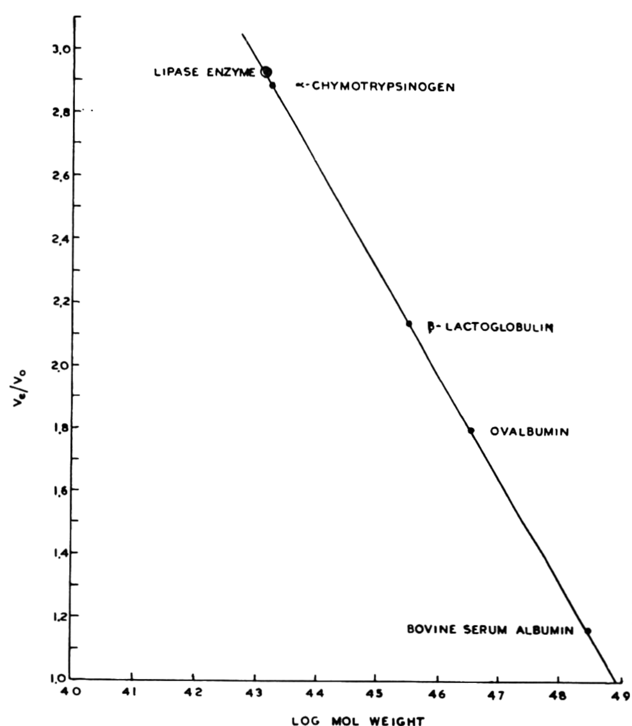

Fig. 2—Ve/Vo vs log molecular weight plot by Sephadex G-100. Sephadex G-100 was equilibrated with 0.02M Tris-HCl buffer (pH 7.5) and packed in a column 2.5 × 75 cm. The purified enzyme 5 mg protein was dissolved in 0.5 ml of the same buffer and applied to the column.

The void volume of standard dextran was 94 ml. From the known molecular weight of standard proteins, the molecular weight of purified lipase was calculated as 20,900 (Fig. 2). The molecular weight of *S. faecalis* lipase was comparable to that obtained with *Micrococcus, Pseudomonas* (Lawrence et al., 1967). According to Pollock (1962), exoenzymes are usually small proteins and the results of the present investigation support this view.

Amino acid composition of *S. faecalis* is presented in Table 2. Glutamic acid was present in maximum amounts as compared to other amino acids, while cysteine was present in minimum amounts amongst other amino acids. *Geotrichum candidum* lipase in comparison to *S. faecalis* lipase showed the absence of sulphur-containing amino acids (Tsujisaka et al., 1973).

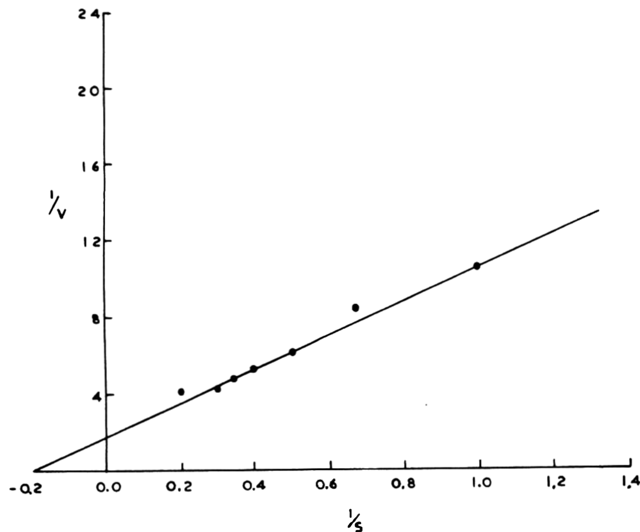

Fig. 3—Double reciprocal plot of Lineweaver and Burk for the determination of Km and Vm values. Reaction mixture for lipase activity contained tributyrin concentration ranging from 1–5 mM, 0.02M Tris-HCl buffer (pH 7.5), 0.02M $CaCl_2$ solution and enzyme solution. Km (5.0×10^{-3} M) was calculated from these data.

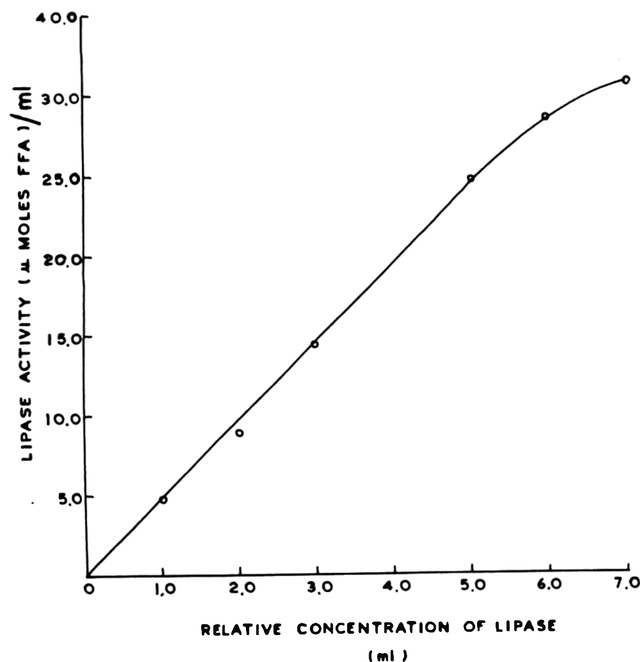

Fig. 4—Effect of enzyme concentration on lipase activity. Reaction mixture contained 5% butter oil emulsion, 0.02M Tris-HCl buffer (pH 7.5), 0.2M $CaCl_2$ solution and lipase in different concentrations.

Properties of purified S. faecalis lipase

The relation between substrate concentration and lipase activity was established by determining the reaction rate at various concentrations ranging from 1–5 mM. Tributyrin was used as substrate. The enzyme activity increased linearly up to 2.5 mM of the substrate (Fig. 3). Michaelis constant of purified lipase was 5.0×10^{-3} M at pH 7.5 and at 40°C by the double reciprocal method of Lineweaver and Burk (1934).

A linear relationship was noted between the activity of *S. faecalis* lipase and enzyme concentration up to 5 ml of enzyme solution (Fig. 4).

Synthetic triglycerides were hydrolyzed by the purified lipase in the following order: tributyrin > tricaproin > tricaprylin > triolein with relative activities of 100, 83, 64 and 58% respectively. The relative specificity of purified lipase for natural triglycerides was as follows: butter oil > olive oil > linseed oil > coconut oil. The relative activity by the enzyme on the above substrates were 64, 58, 51 and 40%, respectively (Table 3). Amongst synthetic triglycerides, *S. faecalis* lipase hydrolyzed tributyrin more readily than other simple triglycerides and in this respect, *S. faecalis* lipase resembles several other microbial lipases like *Lactobacillus casei, L. plantarum, L. helveticus, L. acidophilus* and *Streptococcus diacetilactis* lipases (Sato et al., 1967; Umemoto et al., 1968; Umemoto, 1969). However, *S. faecalis* lipase hydrolyzed butter oil to a lesser degree than tributyrin. Since natural oils are not degraded more readily, it may be inferred that *S. faecalis* lipase has less preference for lipids with long chain unsaturated fatty acids. The apparent specificity of *S. faecalis* lipase for short chain unsaturated fatty acids in the triglycerides moiety, may be due to the ease with which substrates form emulsions at 37°C. Further, the similar rates of hydrolysis may not be achieved, presumably due to marked differences in the molecular weights as also the different physical states between synthetic and natural triglycerides.

The optimum pH for enzyme activity was 7.5 at 40°C and any variation in pH level and temperature of the reaction mixture inhibited enzyme activity (Fig. 5, 6). A similar

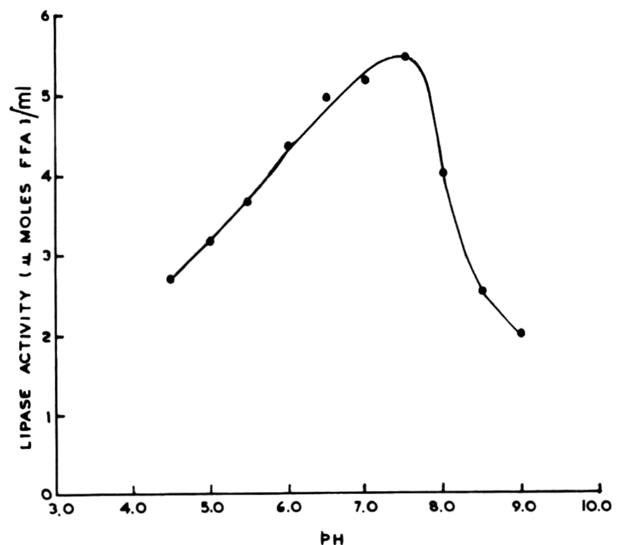

Fig. 5—Effect of pH on the activity of purified S. faecalis lipase. Except for the pH changes, the assay conditions were those described in the legend to Fig. 4.

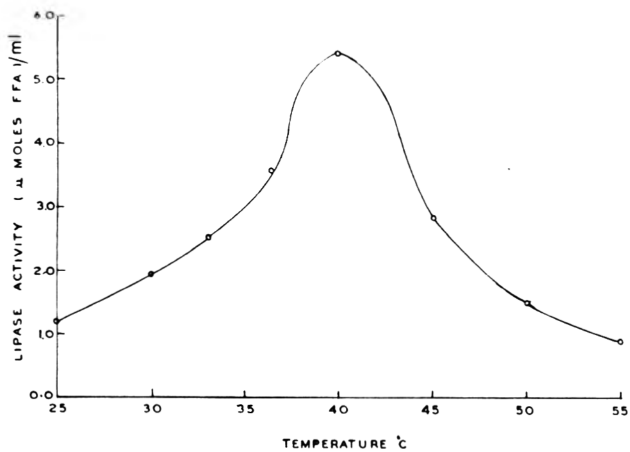

Fig. 6—Effect of temperature on the activity of purified S. faecalis lipase. Except for the temperature changes indicated, the assay conditions were those described in the legend to Fig. 4.

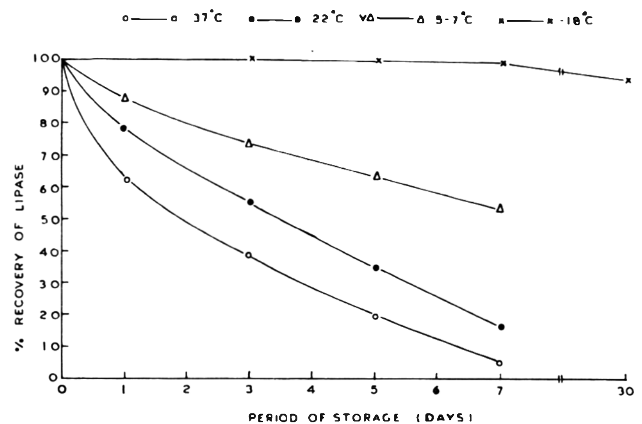

Fig. 7—Effect of temperature and period of storage on the activity of purified S. faecalis lipase. Except for different incubation temperatures and period of storage, the assay conditions were those described in the legend to Fig. 4.

pH and temperature optimum for lipase activity has been reported amongst lactic acid bacteria (Umemoto et al., 1968). It may be presumed that emulsified fat globules may be in a more dispersed state in the temperature ranges mentioned above and the optimum temperature for lipase activity usually falls within this range. Since lipolysis occurs at the interface between fat globules and aqueous phase, the stability of fat emulsion is reported to vary with different reaction temperatures (Sumner, 1954). The optimum reaction temperature for lipase action may, therefore, be considered an important factor in controlling the rate of this reaction. According to Khan et al. (1967) the physical state of the substrate may not be conducive to interaction with the active site of enzyme, when the reaction temperature is less than the optimum.

Maximum loss of activity of enzyme (95%) was observed at 37°C after 7 days of storage, while at 22°C, 82% of enzyme activity was lost (Fig. 7). No loss in lipase activity was noted at −18°C up to 7 days. This finding is in agreement with that of Troller and Bozeman (1970) who reported no loss in activity of *Staphylococcus aureus* lipase under frozen storage condition for 4 wk.

No loss in enzyme activity occurred at 40°C for 10 min, while at 60°C the enzyme activity was reduced to 40% (Fig. 8). The enzyme was completely inactivated at 90°C in 10 min. According to Driessen and Stadhouders (1971), *Alcaligenes viscolactis* lipase exhibited 92% loss of its activity at 84°C for 10 sec and was completely inactivated at 90°C for 10 sec. *S. faecalis* lipase differed markedly from *Achromobacter lipolyticum* lipase in that the latter was more heat stable and was inactivated at 99°C within 30 min (Khan et al., 1967). Although the mechanism of thermostability of lipase is not clearly understood, Manning et al. (1961) attributed the above property to the structure of the enzyme itself. According to the above workers, the enzyme exists either as a randomly coiled structure or it is in a more rigid condition. It is possible that purified *S. faecalis* lipase may have a similar structure in view of its thermostability.

Samples of purified lipase were preincubated for 1 hr at

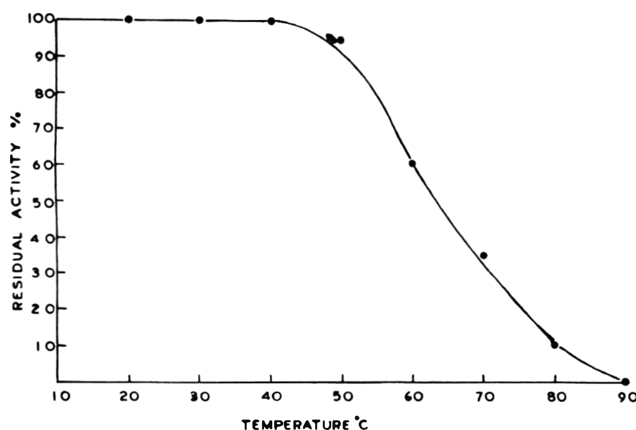

Fig. 8—Effect of heat treatment on the stability of purified S. faecalis lipase. The enzyme preparation was subjected to various heat treatments for 10 min and then assayed as described in the legend to Fig. 4.

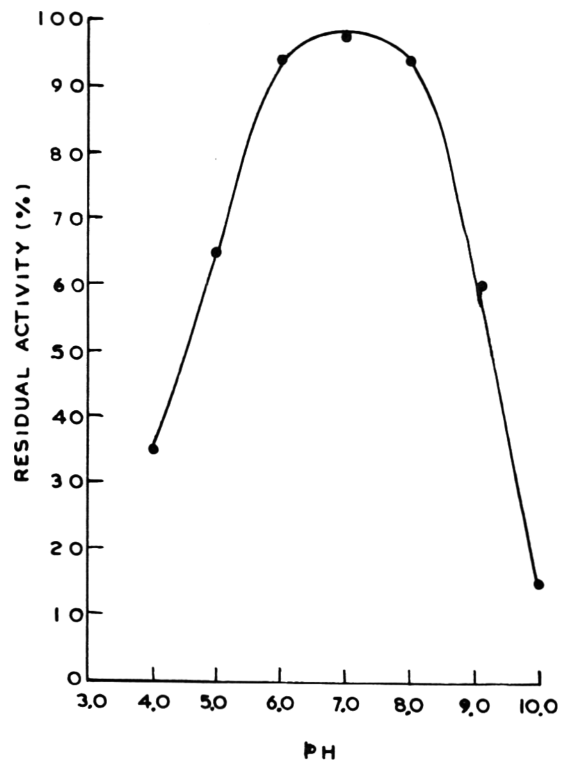

Fig. 9—Effect of pH on the stability of purified S. faecalis lipase. The enzyme preparation was held at indicated pH for 1 hr. The sample was then assayed as described in the legend to Fig. 4.

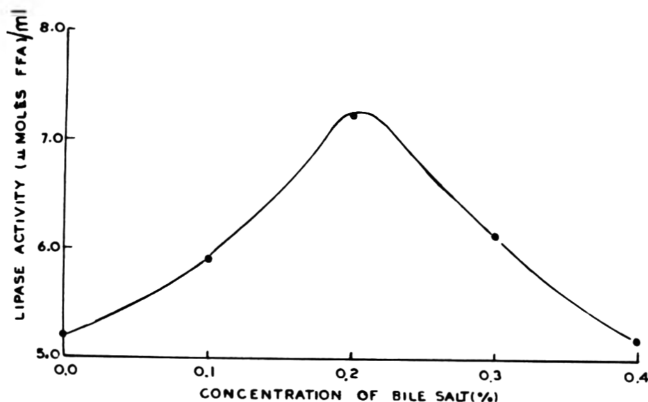

Fig. 10–Effect of bile salt concentrations on the activity of S. faecalis lipase. Except for addition of different salt concentrations, the conditions were the same as described in the legend to Fig. 4.

37°C at pH levels ranging from 3.0–10.0. Purified lipase maintained the activity at pH levels between 6.0–8.0 (Fig. 9). These results are in partial agreement with the findings of Mencher and Alford (1967) on *Pseudomonas fragi* which showed pH stability between 6.0–9.0.

Addition of 0.2% sodium taurocholate stimulated enzyme activity to the extent of 39% as compared to control. However, at higher concentration of Bile salts (0.4%), no stimulation in enzyme activity was observed (Fig. 10). Enhanced enzyme activity by bile salts has also been reported for lipases obtained from *Chromobacterium* (Sugiura et al., 1974). Wills (1965) suggested that the stimulatory action to bile salt on the activity of mammalian lipases may be due to its surface active property which promotes the rate of hydrolysis by increasing the interfacial area of fat-aqueous pahse. It is possible that the stimulatory action of bile salt on *S. faecalis* lipase may be due to better alignment of the enzyme to the substrate molecule, thereby accelerating the hydrolysis of triglycerides.

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Ms received 3/1/79; accepted 6/8/79.

EFFECTS OF ACETYLATED MONOGLYCERIDE COATINGS ON PECAN KERNEL SHELF-LIFE

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ABSTRACT

Pecan kernels, coated with hot dip applications of acetylated monoglycerides and acetylated monoglycerides containing legally acceptable quantities of antioxidants, were evaluated over 24 wk of accelerated storage for changes in color and peroxidation of the indigenous oils. Both types of coatings showed significant effects on the degree of color changes occurring as determined from Hunter ΔE values. However, it appears that these differences were due to a masking of the red-brown coloration that developed rather than a retardation of the color development. The coatings did not have a significant effect on the amounts of anthocyanidins and phlobaphenes formed when compared to untreated kernels. Peroxidation of the oils in the kernels was retarded by both types of coatings, but differences were not significant until after the oils were oxidized to levels that would have been detectable subjectively. Differences among varieties in susceptibility to color changes and peroxidation of the kernels appeared to be more influential to storage stability than the application of monoglyceride coatings.

INTRODUCTION

THE FRESH QUALITY of pecan kernels is usually maintained at processing plants by refrigeration of both shelled and inshell pecans from time of harvest until distributed to food processors and retailers. At temperatures of −2°C and below, kernel quality can be maintained for up to 3 yr (Heaton, 1974). When pecans are used as condiments by food processors, refrigeration is usually continued to assure the use of high quality ingredients in their products. However, at the retail level this protection is usually not provided for shelled kernels and maintenance of quality is dependent on the material in which they are packaged. Often, this material offers more display appeal than preservation characteristics. Consequently, substandard kernels are often offered for sale at the retail level. Williams et al. (1973) estimated that more than 50% of pecan kernels offered for sale by retailers did not meet minimum USDA requirements because of discoloration, rancidity and staleness.

Quality in pecan kernels is characterized by a light color, crisp texture and freedom from staleness and rancidity. Initial coloration of fresh pecan kernels varies among varieties (Heaton et al., 1975). Senter et al. (1978) reported color change to be a function of leucoathocyanidin oxidation in the testa of pecan kernels. While some coloration is desirable to produce the typical hue that is associated with a mature pecan kernel, exposure to adverse conditions during harvest, storage and marketing causes excessive darkening of the testas (Woodroof and Heaton, 1961, 1967). Crispness relates to the moisture content of the kernels and is optimum at 3.5–4.0% for texture and storage stability. Because deviation from this level results in decreased shelf-life and substandard quality, the optimum level must be maintained during processing and storage. Rancidity is primarily associated with oxidation of the unsaturated fatty acids that predominate the lipid composition of pecan oils (Senter and Horvat, 1976). Although it does occur in the oils of stored pecans (Forbus and Senter, 1976), hydrolytic rancidity is not a significant contributor to off-flavor as in other foods with high lipid contents for the fatty acid complement of pecans does not contain the short-chain acids usually associated with this type of deterioration (Senter and Horvat, 1978).

Numerous studies have been conducted to develop processes that will maintain the chemical integrity of stored pecan kernels and other nutmeats without refrigeration. Packaging materials that can contain controlled atmospheres and exclude oxygen and sunlight (Cavaletto and Yamamoto, 1971; Heaton and Shewfelt, 1976; USDA, 1977) and coatings that provide a barrier to atmospheric oxygen have been developed. These coatings have generally been formulations of sucrose base syrups (Godkin et al., 1951); oils and antioxidants (Swarthout et al., 1958); confectioners glaze (Harris et al., 1972); formulations of sugars, dextrin alcohols and sucrose fatty acid esters (Kobayashi and Hisamatsu, 1977); and formulations of acetylated monoglycerides and acetylated monoglycerides containing antioxidants (Shea, 1965; Luce, 1967). Reasonable success in the retardation of oxidative rancidity was reported with the application of these compounds, especially those using the acetylated monoglycerides alone or as the base compound.

Acetylated monoglycerides form flexible films on products when heated and applied as a dip or spray, and are noted for their enhancement of product appearance, compatability with foods with high lipid contents, and relative impermeability to moisture, oxygen and other atmospheric constituents (Newman, 1962). In highly accelerated storage studies, Shea (1965) and Luce (1967) reported that the normal shelf-life of pecans and other nutmeats, as determined by the retardation of oxidative rancidity could be extended for up to 1 yr by acetylated monoglyceride coatings. Additionally, the application of these coatings cleansed the kernels and reduced the microbial count on the processed kernels.

In this study, objective evaluations were made in changes in the quality of pecan kernels coated with acetylated monoglycerides and stored for 24 wk at an elevated temperature. Special emphasis was placed on observing the ability of these coatings to (a) retard discoloration of the kernels, one of the important factors in kernel quality, and (b) influence the stability of the indigenous oils to oxidation.

EXPERIMENTAL

PECANS from the 1976 crop were used. The Eastern Schley and Halbert varieties were grown at Brownwood, TX, and the seedlings in Georgia. Nuts were obtained in 22.7-kg lots and were representative of samplings from various trees in the two locales. Samples were obtained shortly after harvest, packaged in-shell in polyethylene bags and stored at −33°C. In July 1977, the nuts were taken from storage, equilibrated to ambient temperature, and then cracked and shelled mechanically with pilot plant equipment. About 6-kg of perfect halves were selected from each variety. The remainder of the yield within each variety was selectively sorted or manually broken to obtain 6-kg of pieces of quarter-section size. The two samples within each variety were then subdivided into 2-kg subsamples and (1) left untreated (controls), (2) coated with acetylated monoglycerides, or (3) coated with acetylated monoglycerides and incorporated antioxidants.

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Coatings

Acetylated monoglycerides (Myvocet 7-00, Eastman Chemical Products) with a melting range of 30–40°C and containing 68% acetylation, were used as coatings in this study. Preliminary analyses showed that individual kernels at ambient temperature dipped into 85°C Myvocet for 5 sec increased 1.8% in weight. Submersions at this temperature, followed by shaking to remove the excess monoglycerides, resulted in a coating that was free from excessive deposits in the crevices of the kernels and provided a continuous film that was not readily visible.

For the coating procedure, two 9-kg quantities of the monoglycerides were melted and tempered to 85°C in a steam kettle. To one lot, 72.6g of Tenox-20 antioxidant (Eastman Chemical Products) was added and thoroughly mixed. Based on estimates of the amount of acetylated monoglycerides that adhered to the dipped nuts in the preliminary tests and on the average quantities of lipids present in the kernels (73% as determined by the procedures of Folch et al., 1957), it was concluded that the antioxidants in the kernels were present at less than 0.02% of the total fat present, in compliance with FDA limitations.

In the dipping procedure, kernels were placed in the basket of a squirrel-cage spinning apparatus and submerged in the tempered coating for 5 sec, then removed and spun to aid draining of any excess coating. The kernels were then spread on absorbent paper to drain and left at ambient temperature for several hours until the coatings solidified. They were then placed in open aluminum trays to a depth not to exceed 5 cm and stored in the dark at 30°C and 50% relative humidity.

Quality evaluations

The effectiveness of the coatings in retarding kernel discoloration and rancidification was determined by analyzing about 75g of kernel halves and pieces every 3 wk for 24 wk for (1) increases in phlobaphene and anthocyanidin formations, (2) changes in Hunterlab color meter values, and (3) increases in peroxide formation in the oils.

Increases in the absorbance of methanol-HCl extracts of the kernels at 450 and 550 nm were indicative of the amounts of phlobaphene and anthocyanidin formed, respectively, by the oxidation of indigenous leucoanthocyanidins (Senter et al., 1978). Extracts were prepared by soaking about 20g of kernels for 1 hr at ambient temperature in a 1:10 (W/V) ratio of 0.1% HCl in methanol. After filtering through filter paper, the kernel/solvent ratio was readjusted and the absorbance of the solution was determined with a Beckman Acta III spectrophotometer.

Sixty halves and about 55g of pieces were randomly selected from the samples for color measurements with a Hunterlab D25D measuring unit equipped with a 2-in. measuring head. Values were

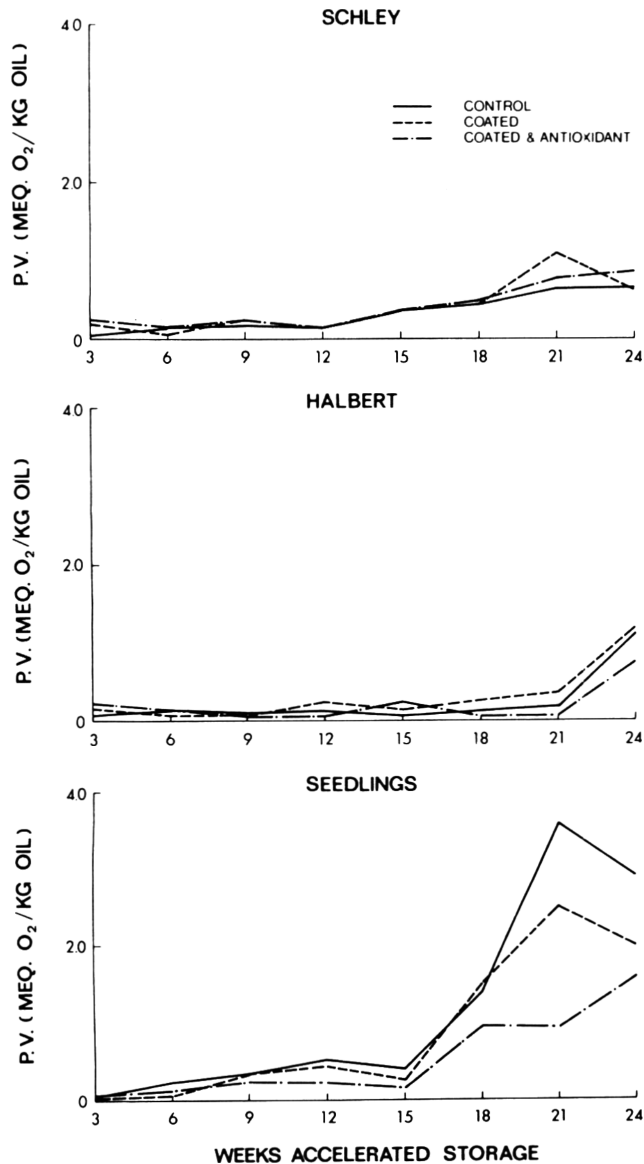

Fig. 1—Effects of acetylated monoglycerides and acetylated monoglycerides + Tenox-20 coatings on the development of peroxides (PV) in pecan kernels stored at 30°C and 50% relative humidity for 24 wk.

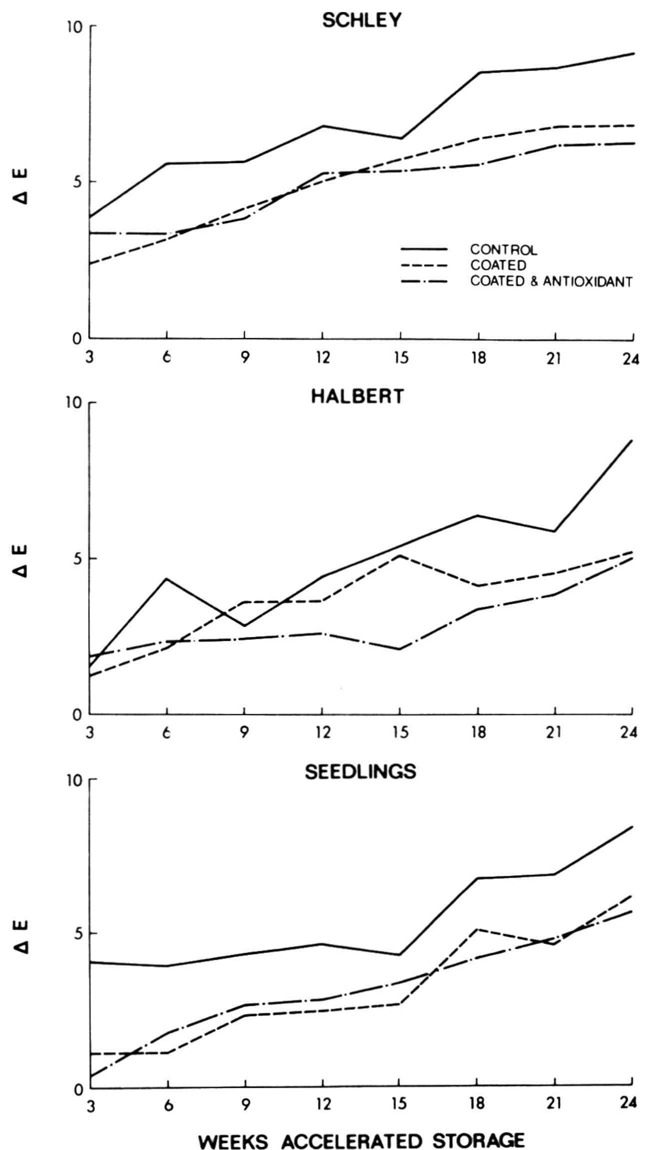

Fig. 2—Effects of acetylated monoglycerides and acetylated monoglycerides + Tenox-20 coatings on the total color change (Hunter ΔE values) in pecan halves stored at 30°C and 50% relative humidity for 24 wk.

determined on three subsamples of 20 halves and about 18g of pieces. The halves were arranged in a 2-in. diam Plexiglass holder so that the flat side of the half faced up; the pieces were arranged randomly. The L (lightness), a (red) and b (yellow) values for each subsample were determined from four positions (the sample cell was rotated 90° between readings). From these values, means were calculated and total color change (ΔE) during storage was computed from the Hunter-Schofield equation (Schofield, 1943):

$$\Delta E = [(\Delta L)^2 + (\Delta a)^2 + (\Delta b)^2]^{1/2}$$

where ΔE represents total color change during storage and ΔL, Δa and Δb represent changes during storage of each value.

Peroxide values, expressed as milliequivalents of O_2 per kilogram oil, were determined by the procedures of Holloway (1966) on 75 mg (±2 mg) of cold pressed oil prepared as reported in a previous communication (Senter et al., 1978). Increased peroxide values are indicative of decreased quality in pecan oils.

Analysis of variance was performed on the entire data set by the statistical analysis system of Barr and Goodnight. Means within each variable were grouped and separated by Duncan's multiple range and multiple F test, and the regression of each variable as a function of time was determined.

RESULTS & DISCUSSION

THE MEAN VALUES of quality factors determined on pecan kernels over 24 wk of accelerated storage are given in Tables 1 and 2 by variety, form and treatment. Variables differing significantly among treatments and by varieties are presented in Figures 1 and 2.

Pigmentation

Differences in the amounts of pigmentation produced during storage were not significant ($P > 0.05$) for treatments or for forms within varieties, but differences between varieties were significant at $P < 0.01$. The data for treatments and forms within varieties were then pooled for regression analyses and separation by Duncan's multiple range test. Significantly higher levels ($P < 0.05$) of both phlobaphenes and anthocyanidins were produced in Seedling and Halbert pecans than in Schley pecans. Differences between Halbert and Seedling pecans were not significant at $P < 0.05$.

Peroxide values

Analysis of variance of peroxide values showed that the degree of rancidification among varieties varied significantly ($P < 0.01$) over the 24 wk of storage. Higher levels of peroxides were formed in the Seedling kernels (both halves and pieces) than in kernels from the Halberts and Schleys. Peroxide values did not vary significantly at the 5% level among the latter two varieties. Although overall mean values for peroxidation were not significantly different ($P > 0.05$) by treatments within varieties (Tables 1 and 2), the interactions of treatment and time were significant ($P < 0.05$) at 18 wk in the Seedling halves, at 21 wk in the Halbert halves (Fig. 1) and at 18 wk in the Halbert pieces, and these differences became progressively greater with time. After these time periods, it appears that the application of acetylated monoglyceride and antioxidant coatings

Table 1—Mean values of quality factors of coated and uncoated pecan kernel halves during 24 wk of accelerated storage

Pecan varieties and treatments[a]	Anthocyanidin absorbance A_{550}	Phlobaphene absorbance A_{450}	Peroxide values meq O_2/kg oil	Hunter values L	a	b	ΔE
Schley							
1	0.13b	0.40b	0.34b	31.42b	10.22b	13.52b	5.72b
2	0.10b	0.31b	0.22b	31.67b	9.48c	13.46b	3.87c
3	0.10b	0.31b	0.30b	31.37b	9.34c	13.11b	3.85c
Halbert							
1	0.19b	0.61b	0.10b	29.45b	9.77b	12.11b	3.85b
2	0.14b	0.49c	0.12b	29.35b	8.86c	11.83b	2.82c
3	0.14b	0.51c	0.06b	29.14b	8.69c	11.71b	2.10c
Seedling							
1	0.18b	0.62b	0.85b	29.78b	9.35b	12.19b	4.34b
2	0.17b	0.61b	0.88b	29.60b	9.11bc	12.16b	2.88c
3	0.18b	0.63b	0.56b	29.41b	8.90c	12.16b	2.86c

[a] 1 = Untreated, 2 = acetylated monoglyceride coating, 3 = acetylated monoglyceride coating + antioxidant. Values within a column followed by the same letter or letters do not differ significantly at $P < 0.05$.

Table 2—Mean values of quality factors of coated and uncoated pecan kernel pieces during 24 wk of accelerated storage

Pecan varieties and treatments[a]	Anthocyanidin absorbance A_{550}	Phlobaphene absorbance A_{450}	Peroxide values meq O_2 kg oil	Hunter values L	a	b	ΔE
Schley							
1	0.11b	0.31b	0.48b	36.99b	7.87b	13.00b	3.52b
2	0.10b	0.24b	0.37b	36.69b	6.77c	12.71b	3.01bc
3	0.10b	0.27b	0.31b	36.15b	7.03c	12.63b	2.47c
Halbert							
1	0.18b	0.54b	0.06c	34.67b	7.63b	11.80b	3.40b
2	0.20b	0.57b	0.16bc	33.35c	7.01c	11.24c	3.10bc
3	0.21b	0.58b	0.18b	33.29c	7.18c	11.19c	2.68c
Seedling							
1	0.19b	0.59b	0.82b	35.16b	7.50b	12.17b	4.08bc
2	0.18b	0.52b	0.69b	34.25bc	6.89c	11.57c	2.97c
3	0.15b	0.49b	0.62b	33.84c	6.87c	11.48c	4.42b

[a] 1 = Untreated, 2 = acetylated monoglyceride coating, 3 = acetylated monoglyceride coating + antioxidant. Values within a column followed by the same letter or letters do not differ significantly at $P < 0.05$.

retarded the development of rancidification. When the effects of coatings became apparent, peroxide values were approaching 1.0 gm O_2/kg of oil, indicating that the oils in the kernels had reached a stage of deterioration that would be readily detected subjectively and the kernels would be classified as substandard.

Hunter color values

Greater variance due to treatments was found in the Hunter color values than in the preceding factors that were measured. Values for total color changes (ΔE) in both halves and pieces were significantly lower ($P < 0.01$) initially and throughout the storage period in coated kernels than in uncoated kernels (Fig. 2). These conditions were also true for the a values (red color) in the varieties. However, the L (lightness) and b (yellow) values did not vary significantly ($P > 0.05$) among treatments either initially or during storage, although significant changes ($P < 0.01$) with time were observed in these factors. Therefore, it appears that since ΔE is a function of ΔL, a and b values, and the initial differences in the a values appeared to be present throughout the storage period, differences in ΔE and a values by treatments can possibly be explained as a masking effect of the red-brown coloration by the coatings. This is further substantiated by the lack of significant differences among treatments in the analyses for pigment (anthocyanidin and phlobaphene) formation in the stored kernels.

Differences among varieties in susceptibility to color changes were observed in the Hunter measurements as in the values for pigmentation and peroxidation. Kernels from the Schley variety showed higher degrees of color change then did kernels from the Halbert and Seedling varieties. Mean values for the Halbert and Seedling varieties were not significantly different in respect to any of the Hunter values measured.

CONCLUSION

ALTHOUGH COATING pecan kernels with acetylated monoglycerides did influence quality over extended storage periods, the variations found among varieties in susceptibility to kernel deterioration in storage appears to have greater influence on stability than does the addition of protective coatings.

Differences in the storage quality of pecan kernels appears to be influenced more by their indigenous chemical properties and the packaging materials in which they are stored than by other factors previously studied. Therefore, it appears advisable to emphasize research in these areas. Many chemical properties of nut kernels have not been elucidated. A concentrated effort should therefore be implemented to evaluate these properties within varieties, determine difference that can be related to storage stability and provide indices for breeding programs to develop pecan varieties with desirable kernel characteristics including storage stability.

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Ms received 4/20/79; revised 6/13/79; accepted 7/1/79.

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GROWTH OF Clostridium perfringens STRAINS ON α-GALACTOSIDES

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ABSTRACT

Five strains of *Clostridium perfringens* type A varied greatly in their ability to utilize and grow on the α-galactosides raffinose, stachyose, and melibiose when these sugars were added (1%) to a casein-digest medium. Stachyose and raffinose were utilized rapidly by NCTC strain 8798, and at moderate rate by NCTC strains 8238 and 10240. Strain KA3 utilized raffinose at moderate rates, but stachyose not at all. Strain FD-1 utilized raffinose only to a slight extent, and stachyose not at all. NCTC strains 8238 and 10240 sporulated heavily during growth on raffinose and stachyose. The implications of these findings on previous work linking *C. perfringens* to legume-induced human flatulence is discussed.

INTRODUCTION

THE POTENTIAL IMPORTANCE of many leguminous seeds in human nutrition is undermined to a considerable extent by the flatulence commonly associated with their consumption (Calloway et al., 1971; Steggerda et al., 1966; Murphy et al., 1972). At least part of the gas formed has been attributed to the relatively high content of α-galactosides, particularly raffinose and stachyose, present in beans and related seeds (Calloway et al., 1966; Rackis et al., 1970; Taeufel et al., 1965). These α-galactosides are not significantly hydrolyzed by mammalian digestive enzymes (Taeufel et al., 1965), and are presumably fermented by the intestinal flora (Calloway et al., 1966; Richards and Steggerda, 1966; Richards et al., 1968; Taeufel et al., 1967) with the release of large amounts of H_2 and CO_2, gases commonly associated with flatulence (Calloway et al., 1966; Richards et al., 1968; Gumbmann and Williams, 1971).

Although little is known about the microorganisms involved, some work implicates anaerobic spore formers (*Clostridium*). Richards et al. (1968) demonstrated large increases in spore-forming anaerobes in the ileum and colon of a dog which had been fed soybean homogenates 6 hr previously. They also reported that pure cultures of *C. perfringens*, commonly present in the intestinal tract, produced gas when exposed to bean homogenates, the volume and composition of which were consistent with the results obtained when intestinal isolates were grown on the same material. They proposed that "the organisms responsible are the same as or closely related to the gram-positive *C. perfringens* type" (Richards et al., 1968).

There are few studies concerning the ability of *C. perfringens* to ferment α-galactosides, and, to our knowledge, there is no evidence that *C. perfringens* can ferment stachyose. *C. perfringens* ATCC 3624 formed no gas from stachyose after 6 hr growth in a defined medium (Rockland et al., 1969). The purpose of this investigation was to ascertain the ability of various *C. perfringens* strains to ferment some common α-galactosides.

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MATERIALS & METHODS

Strains

Strain KA3 was obtained from the Center for Disease Control, Atlanta. Strains FD-1, and NCTC strains 8798, 8238, and 10240 were obtained from S.M. Harmon, Food & Drug Administration, Washington, DC. Strain 8-6 was obtained from G. Gould, Unilever Research, England. A spore stock of strain 8-6 was prepared on D medium (Sacks and Thompson, 1978) supplemented with 100 μg/ml caffeine. Spore studies of all other strains were prepared on CPS medium (Sacks and Thompson, 1977) as previously described. All spore stocks were stored at 5°C.

Media

Spore stocks were prepared on D medium (Sacks and Thompson, 1978) and on CPS medium (Sacks and Thompson 1977). Inocula were out-grown from spore stocks in Fluid Thioglycolate medium (Difco) and transferred to a Casitone (a pancreatic digest of casein) medium containing 6% Casitone (Difco); 0.2% K_2HPO_4; 0.1% yeast extract; 2.0% raffinose; and 0.02% sodium thioglycolate (added just before autoclaving). After 16 hr growth on this medium, these raffinose adapted cultures were used to inoculate a medium of identical composition, but containing 1% raffinose, stachyose or melibiose, rather than 2% raffinose. When glucose was employed, it was autoclaved separately as a 10% solution and added aseptically to the freshly autoclaved basal medium.

Inocula

Spore stocks (see **Strains**) were allowed to outgrow in fresh Fluid Thioglycolate medium for 4–7 hr, as previously described (Sacks and Thompson, 1978). On attaining appropriate cell density (80–150 Klett units, #66 filter) the cultures were diluted 1:50 in freshly boiled, sterile 0.1% peptone and 0.4 ml used to inoculate 16 mm culture tubes containing 13 ml of freshly autoclaved Casitone medium with 2% raffinose. After 16 hr at 37°C, 0.4 ml of these "adapted" cultures were used to inoculate 13 ml of fresh Casitone medium containing 1% of the desired sugar in 16 mm culture tubes.

Turbidimetry

Growth was monitored by periodic readings in a Klett-Summerson colorimeter (#66 filter), with a water standard.

Viable spore counts

Viable spore counts were determined as described previously (Sacks and Thompson, 1977).

Carbohydrate analysis

A Waters Associates (Milford, MA) high pressure liquid chromatography (HPLC) system consisting of a model 6000A solvent delivery system, a U6K sample injector, a R401 differential refractometer and a 30 cm × 4 mm i.d. reverse phase μBondapak Carbohydrate column was used for these analyses. A 30 mm × 2 mm i.d. stainless steel precolumn packed with Corasil AX (Waters Associates) was used to reduce contamination of the analytical column. Data were recorded on an Esterline Angus (Indianapolis, IN) Speed Servo II strip chart recorder and peaks were integrated using a Chromatopac-E1A data processor (Waters Associates).

Culture samples (1.3 ml) were centrifuged 3 min in a microfuge. The supernate was shaken for 2–3 min with about 0.2g of mixed resins to reduce the amount of noncarbohydrate material in the sample. The resin mix consisted of 6 parts Duolite C-20 (H^+ form), 1 part Duolite A-4 (OH^- form), and 1 part Duolite S-37. Duolite resins were obtained from Diamond Shamrock Chemical Co., Redwood City, CA. The resins were mixed with water, ground with a Tekmar homogenizer and dried under a stream of nitrogen for 2 hr. After shaking with the resin mix the sample was centrifuged for 3 min in a clinical centrifuge and the supernatant was filtered through a 0.22 μm Millex disposable filter (Millipore Corp., Bedford, MA). Glass distilled acetonitrile (Burdick and Jackson Laboratories Inc.,

Table 1—Utilization of stachyose by C. perfringens in Casitone medium

Strain	Stachyose % remaining			pH	Gas cc/ml (est)	Spores: Heat-resistant viable		Spores: Microscopy % (est)
	5 hr	11 hr	24 hr	24 hr	24 hr	5 hr	24 hr	11 hr
NCTC 8798	28	4	0	5.2	>3.7	7×10^5	1×10^3	0
NCTC 8238	89	46	0	5.3	2.7[a]	1.4×10^6	8×10^7	80
NCTC 10240	67	46	39	5.3	1.6[b]	7.1×10^4	2.2×10^6	80
KA3	104	96	101	7.0	0.2	4.7×10^4	5.9×10^4	0
FD-1	104	99	105	7.1	0.3	3.2×10^5	3.9×10^6	5

[a] 46% H_2; 47% CO_2
[b] 45% H_2; 44% CO_2

Table 2—Utilization of raffinose by C. perfringens in Casitone medium

Strain	Raffinose % remaining			pH	Gas cc/ml (est)	Spores: Heat-resistant viable		Spores: Microscopy % (est)
	5 hr	11 hr	24 hr	24 hr	24 hr	5 hr	24 hr	11 hr
NCTC 8798	38	0	0	5.2	>3.7	3.9×10^5	1×10^3	0
NCTC 8238	89	64	20	5.2	2.9[a]	1.1×10^6	8×10^7	50
NCTC 10240	89	54	0	5.3	0.7[b]	1.2×10^4	2.6×10^6	90
KA3	75	37	25	5.2	3.2[c]	8×10^5	1.6×10^7	0
FD-1	96	87	87	7.0	0.4[d]	4×10^5	1.2×10^7	5

[a] 42% H_2; no CO_2 analysis
[b] 35% H_2; no CO_2 analysis
[c] 39% H_2; 55% CO_2
[d] 55% H_2; no CO_2 analysis

Table 3—Utilization of glucose by C. perfringens in Casitone medium

Strain	Glucose % remaining			pH	Gas cc/ml (est)	Spores: Heat-resistant viable		Spores: Microscopy % (est)
	5 hr	11 hr	24 hr	24 hr	24 hr	5 hr	24 hr	11 hr
NCTC 8798	15	0	0	5.6	4.0[a]	5.0×10^5	10^3	0
NCTC 8238	58	43	4	5.1	4.4[b]	1.5×10^6	3.7×10^6	3
NCTC 10240	18	21	8	4.9	1.1[c]	2.0×10^4	1.2×10^4	0
KA3	0	0	–	5.6	2.6[d]	2.5×10^5	3.7×10^4	0
FD-1	0	0	–	5.4	5.6[e]	1.6×10^5	3.2×10^6	5

[a] 41% H_2; 45% CO_2
[b] 43% H_2; 45% CO_2
[c] 28% H_2; no CO_2 analysis
[d] 37% H_2; 43% CO_2
[e] 40% H_2; 46% CO_2

Muskegon, MI) and glass distilled water were individually filtered through FHUP and HAWG membrane filters respectively (Millipore Corp.) and combined in the ratio 65/35 and used as the eluting solvent at a flow rate of 2 ml/min. Analyses were done by injecting 10 μl of treated filtered sample onto the column. Solutions of melibiose, raffinose and stachyose (obtained from Sigma Chemical Co.) were prepared and used as standards. Solutions of sterile media containing these sugars gave the same response factors as the standards, showing no interference in the analysis by the media. Elution times for melibiose, raffinose, and stachyose were typically 4.0, 4.5, and 6.3 min.

Glucose concentrations were determined by gas-liquid chromatography of the trimethylsilyl derivatives. Aliquots were lyophilized to dryness and silylated at 60°C for 1 hr with Tri-Sil reagent (Pierce Chemical Co). Analyses were done on a Hewlett Packard #5830A with dual flame ionization detectors and helium carrier gas-flow of 25 cc/min. One-μl aliquots were injected onto a 5 ft × 1/8 in. stainless steel column packed with 3% OV-17 on 100/120 mesh Variport 30. After a 2 min hold at 130°C, the oven temperature was increased at 10°/min until 310°C was reached. Injector temperature was 260°C and detector temperature 300°C. Glucose eluted as both the α- and β-anomers at 7.1 and 8.0 min and was quantitated by using peak areas from solutions containing known amounts of glucose.

Gas analysis

Gas evolved during fermentation in a sealed syringe was accommodated by plunger movement (cf. Richards et al., 1968). An excess of freshly inoculated Casitone medium was drawn into a sterile 20 ml plastic syringe (Burron Medical Products, Bethleham, PA), and all air and fluid in excess of 6 ml (4 ml in the case of glucose cultures) were expelled. The syringe tips were capped with snug-fitting rubber sleeve-type serum bottle caps, and placed in a horizontal position in enamel trays in a 37°C incubator. Sticking was not a serious problem with these syringes. After 24 hr, gas volume was estimated, and gas withdrawn through the septum for analysis.

Hydrogen and carbon dioxide analysis

Varian series 1520 gas chromatograph equipped with dual thermal conductivity cells was used to analyze for these gases. Argon was the carrier gas. Integrated peak areas were obtained from a

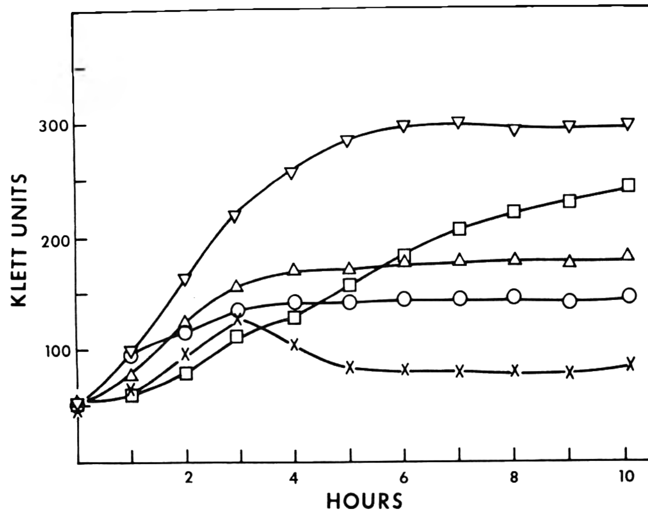

Fig. 1—Growth of C. perfringens strains in a Casitone-yeast extract medium with 1% stachyose. Strains: KA3, ○; FD-1, △; NCTC 8238, □; NCTC 8798, ▽ ; NCTC 10240, X.

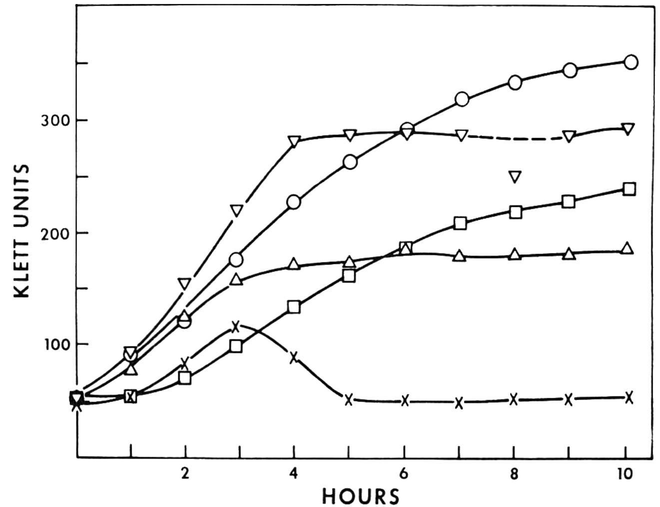

Fig. 2—Growth of C. perfringens strains in a Casitone-yeast extract medium with 1% raffinose. Strains: KA3, ○; FD-1, △; NCTC 8238, □; NCTC 8798, ▽ ; NCTC 10240, X.

Hewlett Packard 18850A GC terminal. The instrument oven was operated isothermally at 40°C for hydrogen analysis and 200°C for carbon dioxide analysis. Separation was effected with molecular sieve 5A (45-60 mesh obtained from Hewlett Packard). A 12 ft × 1/8 in. stainless steel column packed with this material was used for hydrogen analysis, and a 28 in. × 1/8 in. stainless steel column of this material was used for carbon dioxide analysis. Carrier gas flow was 13 cc/min for the 12 ft column and 15 cc/min for the 28 in. column.

A flask fitted with a septum was continuously purged with either hydrogen or carbon dioxide. From this flask relatively pure samples of the gas (less than 1% air in flask) could be sampled and chromatographed for the determination of response factors. All hydrogen samples were diluted tenfold with air prior to the chromatography of 0.5 ml. All samples for carbon dioxide analysis were analyzed directly without dilution by injecting 0.25 ml. Standards were chromatographed frequently throughout a series and their integrated peak areas were used for quantitation.

Methane analysis

A Hewlett Packard model 1530A GC equipped with dual flame ionization detectors was used for this analysis. The initial oven temperature of 50°C was held for 3 min and then programmed to 200°C at 20°C/min. Helium carrier gas flow rate was 38 ml/min. A 3 ft × 1/8 in. SS column packed with activated alumina (100-200 mesh obtained from Bio-Rad) gave the desired separation. Relatively pure samples of natural gas (approx. 90% methane) could be obtained as described above for use as a standard. Comparison of the integrated areas obtained from chromatographing 0.25 ml of sample or standard was used for quantitation.

RESULTS

TABLES 1, 2 AND 3 show sugar utilization, terminal pH, gas evolution and spore formation for various strains of *C. perfringens* growing on Casitone medium supplemented with 1% stachyose, raffinose, and glucose, in parallel culture tubes. Table 1 shows that NCTC strain 8798 utilizes stachyose rapidly. reducing stachyose content by 96% within 11 hr. NCTC strain 8238 reduced stachyose content more slowly, but by 24 hr, no stachyose was detectable. Gas evolution was relatively high in both cases, and terminal pH markedly reduced (initial pH was 7.0). Terminal pH is a useful index of sugar fermentation; in the presence of adequate iron, *C. perfringens* ferments glucose primarily to acetic and butyric acids (Pappenheimer and Shaskan, 1944; Moore et al., 1966), with release of CO_2 and H_2 in approximately equimolar amounts. Gas chromatographic analysis of collected gas showed H_2 and CO_2 to be major components, regardless of the sugar metabolized; no significant CH_4 was detected. Strain NCTC 10240 utilizes stachyose slowly, but the low pH at 24 hr indicates considerable sugar fermentation; gas evolution was appreciable, but less than that observed with strains 8798 and 8238. The high sporulation exhibited by NCTC 10240 and NCTC 8238 is probably related to reduced rates of sugar utilization by these strains (Hsu and Ordal, 1969); it is also possible that sporulation of NCTC 10240 is accompanied by an aberrant fermentation pattern, characterized by more acid and less gas. Strains KA3 and FD-1 do not utilize stachyose to a significant extent; virtually all of the added stachyose remained after 24 hr, pH remained high, and only insignificant amounts of gas were collected.

The growth curve for the above experiment (Fig. 1) shows rapid and extensive growth of NCTC strain 8798, consistent with the stachyose analyses shown in Table 1. NCTC 8238 grows more slowly, also consistent with the rate of stachyose disappearance revealed in Table 1. NCTC 10240 grows well for several hours, then flocculates severely, reflected in the growth curves as declining Klett values. The heavy sporulation of strains 8238 and 10240, and slow utilization of stachyose are probably related to the reduced growth rates observed. Strain FD-1 grows at moderate rates for several hours, then ceases to grow. Strain KA3 follows a similar pattern. The limited growth of these two strains is probably supported by small amounts of sugars present in the yeast extract or Casitone.

Raffinose is utilized at rates similar to the rate of stachyose utilization by NCTC strains 8798, 8238 and 10240 (Table 2; Fig. 2). Terminal pH and gas evolution are consistent with this observation, although gas evolution by NCTC 10240 again is low, with a reduced H_2 content. The growth curve of NCTC 10240 again showed the peculiar pattern observed in the stachyose cultures. Strain KA3, which showed no stachyose utilization, does utilize raffinose; at 24 hr, 75% of the raffinose is gone, terminal pH reduced, and considerable gas formed. The growth curve for KA3 shows abundant multiplication has occurred. Strain FD-1 shows little raffinose utilization, and the growth curve is similar to that of the stachyose culture.

Glucose containing media were run as controls (Table 3; Fg. 3). Figure 3 shows that growth rates on glucose were distinctly higher for strains KA3, FD-1, and 8798 than on

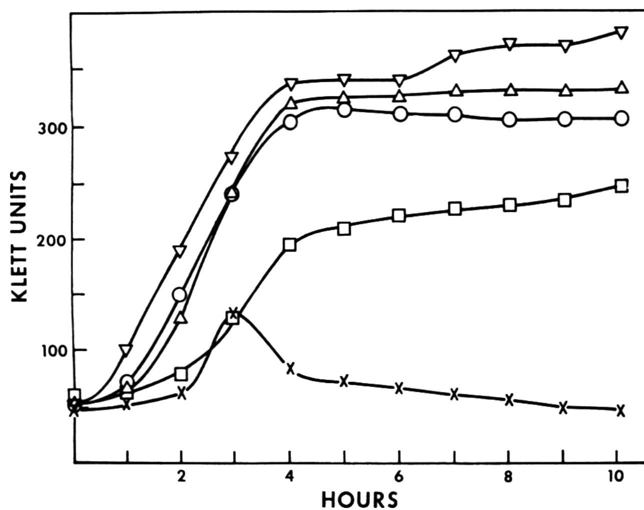

Fig. 3—Growth of C. perfringens strains in a Casitone-yeast extract medium with 1% glucose. Strains: KA3, ○; FD-1, △; NCTC 8238, □; NCTC 8798, ▽ ; NCTC 10240, X.

the α-galactosides, and glucose disappears rapidly from the medium with all strains except NCTC 8238, where appreciable sporulation was detected as early as 5 hr. Strains 10240 and 8238 show the distinctive growth curve characteristics noted in connection with the α-galactoside containing cultures, but terminal pH and analyses of residual glucose indicate extensive glucose utilization by 24 hr. Gas evolution for NCTC 10240 is again low with reduced H_2 content.

Melibiose utilization is shown in the results of a similar experiment summarized in Table 4. It is quite evident that melibiose is utilized very slowly and incompletely by all strains; only strain KA3 grows well and forms appreciable gas on melibiose. All the strains, however, do show some utilization of melibiose, as evidenced by sugar analyses, and terminal pH.

Results showing very similar characteristics for melibiose and raffinose were obtained using a very different medium, a completely defined medium (Sacks and Thompson, 1978), thus showing that the results do reflect sugar utilization rather than other medium characteristics.

DISCUSSION

PREVIOUS WORK (Cristofaro et al., 1974; Rackis et al., 1970; Taeufel et al., 1965; Taeufel et al., 1967) has implicated the α-galactoside content of bean with the flatulence associated with their ingestion. These sugars, undigested in the mammalian alimentary tract, enter the distal portion of the intestine and are presumably fermented by *C. perfringens* and/or related microorganisms (Richards et al., 1968).

The present work demonstrates for the first time that some strains of *C. perfringens* can utilize stachyose and that some strains (e.g. NCTC 8798) metabolize raffinose and stachyose much more rapidly than others, while some strains (e.g. FD-1) cannot utilize either sugar. Strain KA3 ferments raffinose, but cannot metabolize stachyose. Although capacity to ferment raffinose is widely distributed in the *C. perfringens* group, strains have been reported that are unable to metabolize this sugar (Harmon and Kautter, 1978).

This demonstration that ability to ferment the low molecular weight α-galactosides is distributed among strains of *C. perfringens* to different extents has some implications with regard to the microbial origins of flatulence postulated above. If the α-galactosides are involved in the occurrence of flatulence, the *C. perfringens* group must be considered as playing a role in the intestinal fermentation. All the strains used in the present work belong to type A, the type generally found in the human gut (Collee, 1974). The evidence associating the genus *Clostridium* with flatulence is strong (Richards and Steggerda, 1966; Richards et al., 1968); a single report (Richards et al., 1968) asserting that pure cultures of *C. perfringens* produced gas of expected volume and composition from bean homogenates implicated this particular species. The *C. perfringens* population in the intestine is relatively low, compared to that of other intestinal clostridia (Finegold et al., 1977; Mitsuoka and Ohno, 1976). It seems possible that one of the more numerous clostridia might play an even more important role in flatulence.

The present work shows that *C. perfringens* at least may possess the necessary ability to degrade α-galactosides. Flatulence studies on humans have been characterized by considerable variability, between individuals, (Steggerda and Dimmick, 1966; Wagner et al., 1977) and within a single individual (Calloway and Murphy, 1968). The *C. perfringens* population is also highly variable, fluctuating in an individual (Mitsuoka and Ohno, 1976) and between individuals, both in number and serotype (Akama and Otani, 1970; Mitsuoka and Ohno, 1976; Yamagishi et al., 1976).

A related, and seemingly neglected factor in considering the microbial origins of flatulence, is the possibility for rapid increase in microorganisms capable of attacking α-galactosides after these substances enter the small intestine. *C. perfringens* is capable of extremely rapid growth; genera-

Table 4—Utilization of melibiose by C. perfringens

Strain	Sugar	Residual sugar % remaining		pH	Total gas cc/ml	Klett units	Time of max Klett
		6 hr	24 hr	24 hr	24 hr	(max)	hr
KA3	Glucose	0	0	5.32	>3.7	365	12
	Melibiose	45	33	6.20	1.5	260	11
FD-1	Glucose	24	0	5.22	>3.7	400	12
	Melibiose	67	57	6.61	0.3	180	4
NCTC 8238	Glucose	32	24	5.22	>3.7	220	11
	Melibiose	67	38	6.28	0.3	155	12
NCTC 8798	Glucose	0	0	5.51	>3.7	365	11
	Melibiose	57	39	6.61	0	190	3
NCTC 10240	Glucose	0	0	4.80	2.5	192+	4
	Melibiose	–	60	6.18	0.3	105	5

+ Flocculating

tion times of 10 min or even less have been reported (Duncan, 1976; Schroder and Busta, 1971; Willardsen et al., 1978). Richards et al. (1968) demonstrated very large increases in the anaerobic spore population in the ileum and colon of dogs 6 hr after ingestion of soybean homogenates. Thus, a normally low *C. perfringens* population does not preclude the possibility that this organism might rapidly increase in numbers to the point where its fermentation products could be significant. However, *C. perfringens* is a fastidious organism with complex growth requirements (Duncan, 1976; Fuchs and Bonde, 1957; Ting and Fung, 1972) which vary from strain to strain (Fuchs and Bonde, 1957; Ting and Fung, 1972). Thus, the presence of particular strains, the food intake of the host, and his physiology may all contribute to the occurrence of flatulence following the consumption of leguminous seeds.

Ability to ferment stachyose is certainly not restricted to *Clostridium* species; a number of lactobacilli have been shown to ferment stachyose, raffinose, and melibiose (Mital et al., 1974) and it is likely the complex flora of the intestine contains a number of microorganisms capable of attacking the α-galactosides. *C. perfringens*, however, may be unique in its capacity for unusually rapid growth. Thus, after ingestion of α-galactoside containing foods, appropriate strains of *C. perfringens* might grow much more rapidly than competing microorganisms. A sustained supply of α-galactoside containing foods might subsequently favor the replacement of *C. perfringens* by slower growing α-galactoside fermentors with perhaps different metabolic products. Continued bean consumption by rats results in greatly diminished hydrogen evolution (Gumbmann and Williams, 1971). Heterolactic fermentation by lactobacilli is accompanied by CO_2 evolution, with no H_2. Rats fed guar gum [linear chains of (1→4)-β-D-mannopyranosyl units with α-D-galactopyranosyl units attached by (1→6) linkages] for 8–10 days showed large increases in counts of *Clostridium* and *Lactobacillus* in their faeces (Münzer and Harmuth-Hoehne, 1978). Some *Bacteroides* strains have also been shown to ferment guar gum (Salyers et al., 1977). Another explanation for reduced H_2 might revolve about CH_4 formation.

Sporulation of *C. perfringens* after, or during, growth following ingestion of flatulent foods, is another factor which has escaped attention. *C. perfringens* can sporulate in the intestine (Duncan, 1976; Simonds, 1915) and some strains liberate an enterotoxin in the course of sporulation (Duncan, 1976; Genigeorgis, 1975); ingestion of food containing large numbers of *C. perfringens* vegetative cells can result in a type of food poisoning, symptoms of which occur concomitant with sporulation in the intestine (Duncan, 1976). Raffinose and melibiose are known to enhance sporulation of *C. perfringens* (Nakamura and Nishida, 1974; Ellner, 1956), and the work of Richards et al. (1968) indicates that sporulation as well as growth occurs following ingestion of bean homogenates by dogs. Flatulence in humans has been reported to be accompanied sometimes by headache, dizziness, slight retinal edema, and reduced ability to concentrate (Askevold, 1956). The possible effects of liberation of small amounts of *C. perfringens* enterotoxin during a flatulence episode should be considered. In the experiments reported here, NCTC strains 8238 and 10240 apparently sporulate concomitant with growth on α-galactosides.

Previous work has shown that extraction of the low molecular weight α-galactosides from beans reduces but does not eliminate intestinally produced gas (Calloway et al., 1971; Olson et al., 1975). It seems likely that other polysaccharides, also undigested by human digestive tract enzymes, enter the distal intestine, and are similarly fermented, though not necessarily by the same microorganisms (cf. Kurtzman and Halbrook, 1970; Olson et al., 1975).

The relatively poor growth and gas production on melibiose by all strains of *C. perfringens* tested suggests a difference in the way these organisms attack this sugar. Perhaps a different transport mechanism is required for cell entry, or a different mode of hydrolytic attack is employed.

The extremely rapid utilization of stachyose by NCTC 8798 is noteworthy. Equally rapid stachyose fermentation was displayed by a mutant derivative (Cassier and Ryter, 1971) of the above strain, designated 8-6. Active α-galactosidase preparations have potential value in industrial applications (Yamano, 1971) and reducing the flatulence potential of leguminous seeds (Cristofaro et al., 1974). The possibility that NCTC strain 8798 might represent a promising source of α-galactosidase has been considered and is being investigated.

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DIETARY CAFFEINE, GLUCOSE TOLERANCE AND INSULIN SENSITIVITY IN MICE

P. E. ARAUJO and J. MAYER

ABSTRACT

Mice consumed diets which contained caffeine and predominated in either corn starch, sucrose or hydrogenated vegetable oil. When compared by means of an oral glucose tolerance test to mice which had eaten the same diets without caffeine, it was found that they had a more rapid return of blood glucose to resting values. In vivo measurements of glucose uptake by diaphragm and epididymal fat pad revealed differences in this adaptation to caffeine. The adaptive mechanism appeared to vary with type of diet. When mice were given caffeine chronically in their drinking water there was evidence of adaptation occurring in the ability of muscle to increase glycogen stores in the presence of caffeine in vitro. No differences were found in other tissue glycogen levels.

INTRODUCTION

THE ROLE of the methyl xanthines, such as caffeine, in the regulation of cyclic 3′,5′-adenosine monophosphate levels has led to an increased awareness of the importance of these dietary compounds in the control of the metabolic apparatus (Sutherland, 1972). Studies have previously reported direct effects on the pancreatic islets and peripheral changes (Kuftinec and Mayer, 1964; Mayer, 1966; Jankelson et al., 1967). With current emphasis on the ultimate metabolic effects of the source of dietary calories, it was hypothesized that an interaction between diet and caffeine could be observed. It was impossible to decide a priori between the two effects of cyclic AMP–first its catabolic action on glycogen and lipid stores, and second its enhanced release of insulin from the pancreas. It was the purpose of this study to determine which of the known effects of the ingestion of caffeine (Jankelson et al., 1967; Wachman et al., 1970) would predominate.

MATERIALS & METHODS

ADULT MALE albino mice (20–25g) of the Charles River COBS strain were maintained on purified diets (Table 1) differing in caloric source. A high fat diet (60% hydrogenated vegetable oil), a starch diet (77% corn starch), and a sucrose diet (77% sucrose) were available ad libitum. All diets were isocaloric for protein. Half the mice on each regimen received 0.13 mg of caffeine per calorie of diet; the controls received the diet without the additive. The mice were found to average approximately 4 kcal per day or 0.5 mg caffeine per day.

All tests described were performed after the mice has been on their respective diets for 4 wk. Before each test the mice were fasted 18 hr. For the measurement of glucose tolerance, 0.1 mg/g of body weight of glucose in solution (15g/100 ml) was administered via stomach tube. Blood samples were taken from the suborbital sinus at 0, 1, 2, and 4 hr, cleared by $Zn(OH)_2$ precipitation and glucose was measured by the glucose oxidase method.

To determine the effect of the dietary caffeine on the ability of

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0022-1147/79/0006-1761$02.25/0
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Table 1–Diets

Ingredients (g/kg)	101	203	204	701	703	704
Casein	275	145	145	275	145	145
Vitamin mix[a]	25	25	25	25	25	25
Corn oil	50	29	29	50	29	29
Hydrogenated vegetable oil	600	–	–	600	–	–
Corn starch	–	772	–	–	772	–
Sucrose	–	–	772	–	–	772
Salts (Hegsted IV)	50	29	29	50	29	29
Caffeine	–	–	–	0.91	0.52	0.52

[a] Vitamin mix = biotin – 16 mg, folic acid – 400 mg, pyridoxine – 800 mg, thiamin HCl – 800 mg, riboflavin – 1600 mg, α-tocopherol (500 IU/g) 7000 mg, menadione – 800 mg, A/D mix (A acetate = 5×10^5 USP, D = 5×10^4 USP/g) – 1300 mg, nicotinic acid – 4000 mg, pantothenic acid – 800 mg, cyanocobolamine – 30 mg, choline – 120 g in 2 kg vitamin-free casein.

the metabolic machinery to maintain the glycogen and lipid stores of the body, the uptake of uniformly labeled glucose (0.1 μC) into liver glycogen and epididymal fat pad lipid was measured in vivo with and without the stimulation of exogenous insulin (Rafaelson et al., 1965; Stauffacher and Renold, 1969). Since the small amounts of insulin (0.001 μU/g of body weight) remain in the peritoneum into which it and the glucose are injected, only direct effects on glucose uptake and metabolism are measured and no systemic response to insulin interferes. One hour post injection, the animals were sacrificed and glycogen was extracted from the liver by NaOH digestion and ethanol precipitation. The fat pad lipid was extracted by chloroform:methanol (2:1) according to the method of Fain (Fain et al., 1963). Radioactivity of both compounds was determined by liquid scintillation counting.

In a second trial caffeine was administered in the drinking water of mice consuming laboratory chow. The animals receiving caffeine were drinking tap water ad libitum which contained 5.68 mg/100 ml. This chronic ingestion of caffeine continued for about 10 weeks. The mice drank about 8 ml of tap water per day or approximately 0.45 mg per day.

At the end of this period the mice were challenged with a glucose tolerance test as described above except half of the animals received 0.43 mg/100 ml caffeine in their glucose load. In vitro measurements of tissue metabolism were performed using standard manometric techniques. In the first series of experiments, liver slices, kidney slices, hemidiaphragm and minced fat pads were incubated in a Krebbs phosphate buffer with added glucose for 60 min. At the end of this incubation the contents of a side arm containing either insulin or insulin plus caffeine were emptied into the flask and oxygen consumption was observed for an additional 60 min.

In a second series of experiments in vitro, the tissues were incubated in the buffer plus caffeine for 30 min at the end of which time the contents of the side arm containing either the same medium or the medium plus insulin and caffeine were added to the flask. Oxygen consumption was maintained for 45 min after the side arm was emptied. Upon completion of the incubation in both sets of experiments the tissues were analyzed for glycogen by the anthrone method and for protein by the Biuret technique. Tissues from another set of animals were analyzed for cholesterol content using an automated method.

RESULTS

FIGURE 1 presents the data from the glucose tolerance tests. While the decrease in glucose tolerance with the feeding of a high fat carbohydrate-free diet was expected,

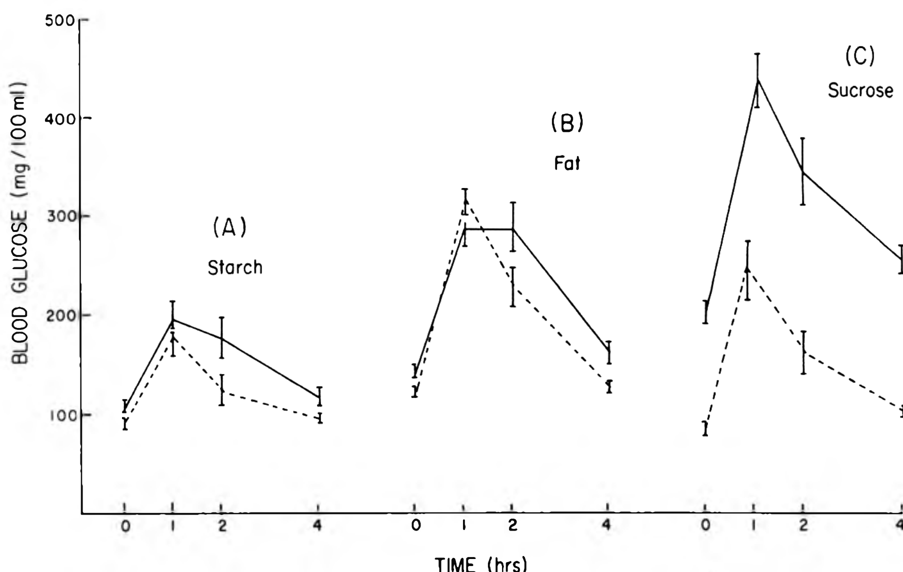

Fig. 1—Glucose tolerance curves: Solid lines represent curve for blood glucose values from animals which were consuming the basal diet and dotted lines the curve for animals consuming the basal diet plus caffeine. Bars indicate SEM. There were 8 mice in each group: (A) Mice fed the starch diet; (B) Mice fed the fat diet; and (C) Mice fed the sucrose diet.

there was an even greater response by the sucrose fed mice. In each case the mice on the diets which included caffeine exhibited a greater ability to remove glucose from the blood than the control animals on the diet alone. The change is least in the mice on the fat diet and starch diet and the greatest with sucrose feeding. Figure 2 contains data from the second set of glucose tolerance tests which were performed. The laboratory chow diet fed to these mice resembles the starch diet in composition and the glucose tolerance curve of the control animals is similar to that found in the preceding experiment. When the glucose challenge contains caffeine, however, the response is a more rapid return of blood glucose to original values.

The data in Table 2 are from the in vivo test of glucose metabolism and insulin action. Since pool sizes of precursors were not determined in these animals under the various conditions of the experiment, the ratio of uptakes by diaphragm and adipose tissue was calculated. A high ratio could be indicative of greatly increased diaphragm uptake or of decreased adipose tissue utilization of glucose. The relatively slow removal of glucose from the blood would suggest the latter situation and rapid removal of the former. Inspection of the data in Table 2 reveals an inconsistent pattern. Fat feeding depresses the response to insulin by the adipose tissue but feeding the fat-plus-caffeine diet restores the insulin response as indicated by both the D/At ratios and the number of counts per milligram of dry weight for that tissue.

On the other hand, the starch fed mice show a clear response to insulin which is depressed when the diet includes caffeine. The data from the number of counts appear to indicate that lipogenesis is proceeding at a rapid rate in the absence of insulin and is exaggerated in response to that hormone. The metabolic pattern in the animals eating the sucrose diet shows no effect of the caffeine treatment.

The data presented in Tables 3 and 4 resulted from the in vitro measurements of metabolic activity. Since changes in metabolic pattern were more reproducible and of greater interest the data are comparisons of rates between samples of tissues from the same mouse. Percent stimulation was determined by the equation: $\% = [(+SA)-(-SA)/(-SA)] \times 100$ where the (−SA) is the amount of tissue glycogen accumulation or Q_{O_2} after addition of the control side arm's contents and (+SA) is the observation in the experimental situation. In the first test (Table 3) the liver of the chronic caffeine drinkers showed a lessened Q_{O_2} response to insulin if caffeine was similtaneously presented when compared to the water drinking animals. Lack of tissue differences in diaphragm and fat pad as measured by either Q_{O_2} or glycogen stores, decreases the utility of these observations. To ascertain the effect of a chronic level of caffeine in body fluids the data in Table 4 were obtained. In this situation the diaphragm of the chronic caffeine drinkers is able to respond to an insulin and glucose challenge by maintaining a higher glycogen level. The glycogen level occurs after preincubation with caffeine.

A measure of changed lipid metabolism was attempted by determination of tissue cholesterol levels as presented in Table 5. In no case was there a statistically significant difference between controls and chronic caffeine ingestors.

DISCUSSION

THE CHANGED ABILITY of the mice to respond to an oral glucose load can only be related to a basic metabolic adaptation to chronic caffeine ingestion. Reports of de-

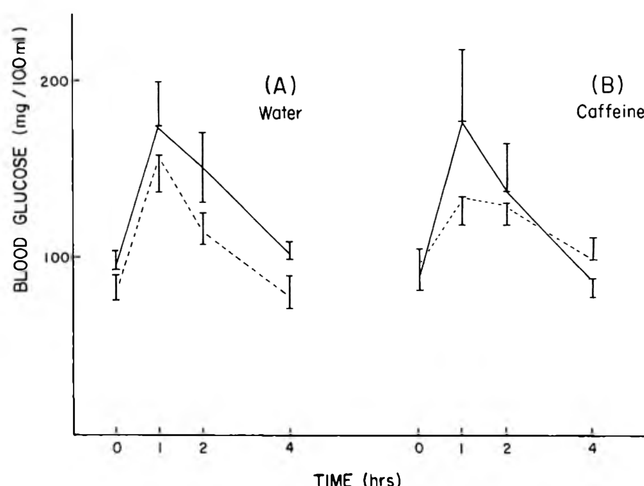

Fig. 2—Glucose tolerance curves: (A) Control mice consuming tap water; and (B) Experimental mice consuming caffeine solution. Solid lines are blood glucose levels after an acute challenge with an oral glucose solution. Dashed lines are blood glucose levels after an oral glucose solution which contained caffeine. Bars indicate SEM. There were 8 mice in each group.

Table 2—Glucose metabolism – in vivo

	cpm/mg Dry wt				Diaphragm/Adipose tissue		
	Diaphragm		Adipose tissue		Ratio		% shift
Diet	Glucose	Glucose + Insulin	Glucose	Glucose + Insulin	Glucose	Glucose + Insulin	+ insulin
Fat	28 ± 9	465 ± 52	6 ± 1	28 ± 5	4.7 ± 0.5	20.9 ± 4.8	445
Fat + caffeine	30 ± 5	605 ± 67	11 ± 6	92 ± 26	6.0 ± 1.5	12.3 ± 3.4	205
Starch	32 ± 8	530 ± 85	16 ± 4	72 ± 20	2.2 ± 0.4	11.0 ± 2.9	500
Starch + caffeine	22 ± 6	380 ± 72	83 ± 58	244 ± 86	1.2 ± 0.6	2.5 ± 0.6	208
Sucrose	22 ± 6	353 ± 70	13 ± 3	52 ± 13	2.2 ± 0.6	13.3 ± 3.6	605
Sucrose + caffeine	17 ± 4	646 ± 53	14 ± 5	98 ± 26	2.0 ± 0.8	10.5 ± 2.3	525

Table 3—Percent caffeine stimulation insulin action

	Tissue glycogen			Fat
Pretreatment	Liver	Kidney	Diaphragm	pad
Water	−15[a]	− 3	+12	−12
Caffeine	− 9	−12	+11	− 6
	Tissue Q_{O_2}			
Water	+33	−13	+10	−60
Caffeine	−32[b]	+18	+ 4	−24

[a] Percent stimulation = [(+SA)−(−SA)/(−SA)] × 100 where −SA = side arm content of buffer only and +SA = side arm contents of buffer plus insulin and caffeine

[b] Significant difference in rate between tissue from water and caffeine drinking group p ⩽ 0.05 as determined by the nonparametric Mann-Whitney test.

Table 4—Percent caffeine stimulation of glycogenolysis

	Tissue glycogen			Fat
Pretreatment	Liver	Kidney	Diaphragm	pad
Water	−12[a]	+68	−14	− 7
Caffeine	− 7	−21[b]	+78[b]	+ 7
	Tissue Q_{O_2}			
Water	+20	+16	− 6	+318
Caffeine	−17[b]	− 2	−18	− 48[b]

[a] Percent stimulation = [(+SA)−(−SA)/(−SA)] × 100 where −SA = side arm contents of buffer plus caffeine and +SA = side arm contents of buffer plus insulin and caffeine

[b] Significant difference is rate between tissue from water and caffeine drinking groups p ⩽ 0.05 as determined by the nonparametric Mann-Whitney test.

creased clearance after acute ingestion of a caffeine solution can be explained as representing caffeine effects on cyclic AMP levels via a blocking action on degradation of the latter compounds by the enzyme phosphodiesterase (Sutherland, 1972). Since all food during the preceding 4 wk contained caffeine it can be hypothesized that a homeostatic equilibrium had occurred which allowed for the presence of the caffeine and an adjustment of the metabolic apparatus had occurred. The new equilibrium could be achieved by increasing phosphodiesterase levels or by decreasing cyclic AMP production or by other possible mechanisms too numerous to speculate upon.

Care must be taken in proposing a single mechanism for the observed effect on glucose tolerance curves in view of the in vivo data. These data show a significant difference in response to caffeine's action in relation to type of diet fed and sensitivity to insulin. Caffeine increases the fat tissue response to insulin except in the sucrose consuming mice. Enhanced lipogenesis in both the presence and absence of insulin in the fat pads from mice fed the starch diet may be related to effects observed after fasting and refeeding of a high starch diet (Pffeifer and Debro, 1966; Szepesi and Berdanier, 1971). It can be postulated that high caffeine levels mimic the stress of a fast especially by an increase in lipolysis. In the test situation, refeeding would be simulated by a decreasing caffeine level and a slowing of lipolytic stress. It must be noted that only trace amounts of glucose are injected into the peritonea of the mice which have just undergone an 18 hr fast.

Sucrose feeding seems to decrease the effects of caffeine on the tissues investigated. This result appears to conflict with the great change in the glucose tolerance curves. The discrepancy may be due to the reported shift of lipogenesis from adipose tissue to liver in fructose fed animals (Chevalier et al., 1972). Moreover, the reported effects of fructose (Curry et al., 1972) and caffeine (Levey et al., 1972; Chatles et al., 1973) on the release of insulin from the pancreas may lead to a state in which the animals are less dependent upon that hormone for metabolic homeostasis. Both compounds—fructose and caffeine—apparently would increase the amount of insulin released in response to a stimulus and thereby set into play mechanisms to maintain an equilibrium.

Thus, the chronic feeding of a diet containing caffeine causes a variety of adaptations. Effects reported after acute caffeine ingestion may not be applicable to the chronic situation. To differentiate among facilitated insulin release (Chatles et al., 1973), increased steroid levels (Sutherland, 1972), metabolic adaptation and any possible combination of these circumstances, the second series of tests were performed. The only clear response observed in vitro was a greater glycogen level in the diaphragm of the chronic caffeine drinkers. In view of the findings in vivo this result can only be interpreted as indicating that the fat pad has been maximally stimulated and cannot further increase its capacity. That is, the chow diet most resembles the starch diet; hence the fat pad is at a high level of anabolism while the

Table 5—Cholesterol in tissues

Tissue	No. of mice	Pretreatment	mg cholesterol g dry wt
Liver	7	water	5.4 ± 0.6[a]
	7	water + caffeine	5.9 ± 0.9
Kidney	6	water	8.1 ± 1.3
	7	water + caffeine	5.7 ± 0.8
Intestine	6	water	3.3 ± 0.5
	7	water + caffeine	3.2 ± 0.8
Serum	4	water	62.0 ± 11 mg/100 ml
	6	water + caffeine	59.0 ± 3 mg/100 ml

[a] Mean ± SEM

diaphragm in these animals appears to have a decreased rate.

In view of the concern that caffeine drinking may have a link with heart disease, a measurement of cholesterol levels was made. These data indicated no difference in tissue cholesterol levels between the two groups of mice. Evidently the higher lipogenic activity of the adipose tissue as measured in vivo for mice consuming the starch diet with caffeine does not include a higher amount of cholesterolgenesis and storage and no further investigation of this metabolite appeared warranted.

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Ms received 11/16/78; revised 5/4/79; accepted 5/10/79.

Florida Agricultural Experiment Stations Journal Series No. 1300.

We thank Cecilia Fiscus, Timothy Patton and Paul Gustafson for their assistance.

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The authors are indebted to P.A. Thompson and C.R. Sell for valuable technical assistance.

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PHOSPHORUS AND MAGNESIUM BALANCE OF ADOLESCENT FEMALES FED TWO LEVELS OF ZINC

J. L. GREGER, J. HUFFMAN, R. P. ABERNATHY, O. A. BENNETT and S. E. RESNECK

ABSTRACT

Utilization of phosphorus and magnesium by 11 girls (12.5–14.2 yr of age) was measured during a 30-day period in which two different levels of zinc (11.5 and 14.7 mg daily) were fed. The former level is similar to that consumed usually by adolescent females; the latter level is similar to the Recommended Dietary Allowance for zinc. Apparent retention of phosphorus was reduced significantly ($p < 0.02$) when subjects were fed the higher level of zinc. Both urinary and fecal phosphorus levels were greater, but not significantly greater, when subjects consumed the higher level of zinc. The variations in dietary zinc levels had no effect on magnesium utilization.

INTRODUCTION

SEVERAL INVESTIGATORS have reported finding Americans who were in poor nutritional status in regard to zinc (Greger, 1977; Hambidge et al., 1972, 1976; Henkin et al., 1974; Pories et al., 1967; Sandstead, 1973). Before programs to increase dietary zinc levels are implemented, the effect of varying dietary zinc levels on the use of other nutrients needs to be studied thoroughly. In animal studies and in vitro preparations, zinc has been demonstrated to be antagonistic to the absorption of other minerals (Magee and Matrone, 1960; Murthy et al., 1974; Van Campen and Scaife, 1967). During severe dietary zinc deficiency, animals have been found to lose excessive amounts of nitrogen in the urine (Hsu and Anthony, 1975). Chu and Cox (1972) observed decreased amounts of phosphorus containing compounds in the tissues of animals fed high levels of zinc. However, the effect of moderate alterations in dietary zinc levels on the utilization of other nutrients by human subjects has received limited attention (Greger et al., 1978a, b, d; Hess et al., 1977; Keltz et al., 1978; Meiners et al., 1977; Tamura et al., 1978).

The purpose of this study was to determine if moderate alterations in dietary zinc levels resulted in changes in the utilization of phosphorus and magnesium by adolescent females.

METHODS

Subjects

Eleven girls, between 12.5 and 14.2 yr of age, agreed to participate in a 30-day metabolic study. Parental approval was also obtained. All subjects were given a routine physical examination by a physician with urine analysis prior to their participation in the study. All procedures used in this investigation were approved by Purdue University's committee on the use of human subjects.

The subjects' mean height was 158 ± 7 (SD) cm; their mean weight was 52.5 ± 13.6 kg. Six of the subjects had already experienced menarche.

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0022-1147/79/0006-1765$02.25/0
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Experimental design

The subjects were split randomly into two groups. During period 1, the first 14 days of the study, group A was fed 11.3 ± 0.8 mg zinc daily (Diet Z11.5) and group B was fed 14.5 ± 0.8 mg zinc daily (Diet Z14.7). During period 2, the last 16 days of the study, group A was fed 14.8 ± 1.1 mg zinc daily (Diet Z14.7) and group B was fed 11.6 ± 1.1 mg zinc daily (Diet Z11.5). The higher levels of dietary zinc were achieved by adding a zinc sulfate solution (donated by Mericon Industries, Inc., Peoria, IL) to the lemonade served at lunch to subjects.

Experimental diet

A 6-day cycle menu containing foodstuffs typical of adolescent food patterns was served throughout the study. The diet was calculated by computer using U.S. Department of Agriculture food composition tables (Watt and Merrill, 1963) and nutrition information supplied by companies to contain 100% of the Recommended Dietary Allowances (RDA) (Food and Nutrition Board, 1974) for 11–14 yr old girls of energy, protein, vitamins A, B_6, and C, thiamin, riboflavin, niacin, and iron. More details on the diets were given in a previous paper (Greger et al., 1978d).

When similar menus were fed previously, the level of magnesium was found to be low (Greger et al., 1978b). Hence, the girls were given 25 mg of magnesium in the form of magnesium gluconate at each meal. The diet was determined by analysis to contain 1049 ± 50 mg calcium, 906 ± 92 mg phosphorus, 271 ± 22 mg magnesium daily in period 1 and to contain 1058 ± 112 mg calcium, 946 ± 130 mg phosphorus, and 281 ± 26 mg magnesium daily in period 2.

Analyses

Fecal markers of brilliant blue were given to subjects on days 5 and 14 during period 1 and on days 19 and 28 during period 2. Fecal composites for the two periods were prepared accordingly. Acidified urine samples were pooled for each period also. Food was composited on a daily basis.

Food and fecal samples were ashed as described by Osis et al. (1972). The magnesium content of the ashed samples and of urine samples diluted with 0.5% strontium chloride were determined by atomic absorption spectrophotometry. The recovery of magnesium added to three food samples that were processed in this manner ranged from 96–99%. Phosphorus content of ashed fecal and food samples and diluted urine samples were determined spectrophotometrically by a modification of the Fiske and Subbarow procedure (Lindberg and Ernster, 1956). The recovery of phosphorus added to three food samples that were processed in this manner ranged from 93–96%.

Fecal and food composites were analyzed for dry matter content. Duplicate aliquots of the composites were dried in a vacuum oven at 70°C for at least 6 hr and dry matter content of samples were calculated.

All statistical analyses were done by computer utilizing the Statistical Package for the Social Science program (Nie et al., 1975). Paired "t" were used to evaluate differences between treatments (Steel and Torrie, 1960).

RESULTS & DISCUSSION

THE RETENTION of phosphorus by ten of the eleven subjects was less ($p < 0.02$) when the subjects were fed Diet Z14.7 rather than Z11.5 (Table 1). In a previous study with adolescents, we observed no significant effect of dietary zinc levels on phosphorus retention (Greger et al., 1978b). However, in the previous study, the subjects did not serve as their own controls which caused some loss of sensitivity.

The mechanism by which zinc affected phosphorus utilization is unclear. Subjects lost slightly, but not signifi-

Table 1—Phosphorus excretion and retention of adolescent females fed two levels of zinc

Subject no.	Diet Z11.5			Diet Z14.7		
	U[a]	F[b]	R[c]	U	F	R
	(mg/day)					
1	500	508	– 62	580	506	–180
2	329	419	198	430	796	–320
3	535	385	26	583	317	6
4	270	597	79	429	434	43
5	346	660	– 60	430	860	–384
6	377	419	110	408	598	– 60
7	436	462	8	371	600	– 25
8	435	502	29	461	781	–296
9	295	530	81	410	514	22
10	396	402	108	335	590	21
11	382	727	–203	413	674	–141
Mean	393	510	23	441	606	–120
SD[d]	84	111	110	77	164	155

[a] Urinary losses
[b] Fecal losses
[c] Apparent retention = dietary intake – fecal losses – urinary losses
[d] Standard deviation

Table 2—Magnesium excretion and retention of adolescent females fed two levels of zinc

Subject no.	Diet Z11.5			Diet Z14.7		
	U[a]	F[b]	R[c]	U	F	R
	(mg/day)					
1	116	151	11	147	145	–21
2	115	136	30	124	213	–66
3	135	115	31	111	87	73
4	68	172	41	110	114	7
5	129	145	7	147	169	–45
6	110	151	10	113	150	18
7	125	145	1	74	167	40
8	120	152	– 1	94	178	9
9	128	100	43	137	121	23
10	95	183	– 7	96	194	– 9
11	108	163	0	139	151	– 9
Mean	114	147	15	117	157	2
SD[d]	19	24	18	24	34	38

[a] Urinary losses
[b] Fecal losses
[c] Apparent retention = dietary intake – fecal losses – urinary losses
[d] Standard deviation

cantly, more phosphorus in both their urine and feces when fed Diet Z14.7 rather than Diet Z11.5.

Fecal phosphorus losses in this study tended to be somewhat higher than fecal phosphorus levels reported in other studies in which subjects were fed between 800–1000 mg phosphorus daily (Greger et al., 1978b; Leverton et al., 1962; Spencer et al., 1978). There are several possible explanations. The diet contained slightly more calcium than phosphorus. Spencer et al. (1978) observed somewhat increased fecal phosphorus losses as dietary calcium levels were increased. The diet contained the level of nitrogen suggested in the RDA (Food and Nutrition Board, 1974). This is a lower level of protein than adolescent females generally consume (Greger et al., 1978c). Absorption of several minerals is reduced when dietary protein levels are lowered (Schwartz et al., 1973; Walker and Linkswiler, 1972; Van Campen and House, 1974).

The magnesium intake of the subjects in this study was about 90% of the Recommended Dietary allowance for magnesium (1974) and was about 50 mg daily greater than estimated usual intake of adolescent females (Marhefka, 1978). However, two of the girls were in negative magnesium balance when fed Diet Z11.5 and five of the girls were in negative magnesium balance when fed Diet Z14.7 (Table 2). The differences in magnesium excretion and retention due to dietary zinc levels were not statistically significant. Previously, alterations in dietary zinc levels were also not demonstrated to affect magnesium balance significantly (Greger et al., 1978b).

The dry matter content of fecal samples when subjects were fed Diet Z11.5 was 20 ± 4g daily and when subjects were fed Diet Z14.7 was 21 ± 4g daily. While the dietary treatments did not affect the dry matter content of the feces, the dry matter content of fecal samples was correlated to their magnesium content ($r = 0.619$, $p < 0.005$), but not their phosphorus content.

The practical significance of alterations in dietary zinc levels on phosphorus utilization by Americans is unclear. The two levels of zinc fed in this study were representative of the level suggested in the RDA (Diet Z14.7) and of the level consumed by adolescent females (Diet Z11.5) (Greger et al., 1978c). The dietary phosphorus level in this study was about 0.9g daily. Marston and Friend (1966) on the basis of retail weight of food sold in the U.S.A. suggested that Americans consumed 1.5g phosphorus daily. Perhaps if the subjects had consumed a higher level of phosphorus, the majority (7 out of 11) of the subjects would not have been in negative balance in regard to phosphorus when fed Diet Z14.7. Even so, this interaction deserves further study.

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METHIONINE STABILITY IN METHIONINE-FORTIFIED MODEL FOOD SYSTEMS AS INFLUENCED BY METHOD OF METHIONINE INCORPORATION AND BY LIPID OXIDATION

M. C. TUFTE and J. J. WARTHESEN

ABSTRACT

Model systems were used to evaluate the influences of method of methionine incorporation and lipid oxidation on the stability of added methionine during heating or storage. High pressure liquid chromatography was used to separate and quantify the dansyl derivatives of methionine, methionine sulfoxide and methionine sulfone. In a system undergoing nonenzymatic browning, the loss of added methionine was greater when the methionine was added in water solution than when it was incorporated by dry blending. In systems undergoing lipid oxidation, the initial peroxide value was directly related to the loss of free methionine and the formation of methionine sulfoxide. The conversion of methionine to methionine sulfoxide due to oxidizing lipid was reduced by the presence of protein.

INTRODUCTION

METHIONINE is the limiting amino acid in many foods such as potatoes (Kies and Fox, 1972), oats (Kies et al., 1975), peas, beans (Russell et al., 1946) and other legumes (Anonymous, 1973). When these foods are the sole source of protein in the diet, the lack of adequate methionine can result in an amino acid imbalance which reduces the protein quality of the food. Fortification of certain protein sources with methionine has been shown in many instances to improve either the protein efficiency ratio in rats or nitrogen balance in humans. Improvements in protein quality with methionine fortification have been shown in diets based on several varieties of beans and peas, soybeans, soy milks, various soybean-based blended foods and in diets based on cowpeas, oats, textured vegetable protein and potatoes (Russell et al., 1946; Kon et al., 1971; Bookwalter et al., 1975; Onayemi and Potter, 1976; Kies et al., 1975; Kies and Fox, 1971).

Added methionine may be inactivated or destroyed during processing or storage, resulting in less nutritionally available methionine. A major mechanism likely to cause losses of free methionine involves the reaction of the amino group with a carbonyl compound in Maillard browning. Horn et al. (1968) demonstrated loss of nutritional availability when methionine was reacted with glucose. O'Keefe and Warthesen (1978) suggested Maillard browning as the main mechanism of free methionine destruction in glucose-containing model systems. A second mechanism of methionine loss in foods is alteration of the sulfur atom. Tannenbaum et al. (1969) reported that methionine residues in casein were oxidized to methionine sulfoxide in the presence of oxidizing lipid while Cuq et al. (1973) found the same effect in the presence of hydrogen peroxide. It is likely the free methionine that has been involved in Maillard browning is nutritionally unavailable while there is uncertainty as to the availability of methionine sulfoxide (Miller and Samuel, 1970; Gjoen and Njaa, 1977; Cuq et al., 1978).

While there are several possible reactions that can lead to methionine destruction, the factors influencing the stability of free methionine in methionine-fortified foods have not been fully examined. The purpose of this research was to investigate the influence of the method of methionine incorporation and the presence of oxidizing lipid on free methionine stability in fortified systems.

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0022-1147/79/0006-1767$02.25/0
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MATERIALS & METHODS

THE RESEARCH was conducted in two parts with Part I comprising two experiments and Part II comprising three experiments. In Part I, the influence of various methods of free methionine incorporation on free methionine stability was evaluated. In Part II the influence of oxidizing lipid on free methionine stability was examined.

Preparation and treatment of model systems

Model systems conducive to Maillard browning were prepared in order to evaluate the influence of method of methionine incorporation on free methionine stability. In the first experiment, two different methods of model system preparation were compared. The ingredients were either blended in the dry state or mixed as a slurry. A 400-g model system composed of 55.5% microcrystalline cellulose (MCC, FMC Corp.), 40.0% isolated soy protein (Promine-D, Central Soya Co., Inc.), 4.0% D-glucose (Mallinckrodt Inc.), and 0.5% DL-methionine (Sigma Chemical Co.), was dry blended for 20 min using a Kitchen Aid Model K45 mixer. Half of the sample was then removed, and the remaining half (200g) was slurried with 2000 ml of deionized water and blended for an additional 5 min. Then both halves were placed in freeze dryer trays. The samples were frozen at −20°C and were then freeze dried. After drying, the samples were ground and placed in plastic weighing dishes. To impart water activities of 0.68 and 0.75, the samples were placed in desiccators containing saturated salt solutions of cupric chloride and sodium chloride, respectively (Rockland, 1960). The desiccators were held at room temperature and the samples were allowed to equilibrate for 5 days. Ten-gram samples were then placed in foil pouches which were evacuated and heat sealed. Unheated control samples were held at room temperature while the heated samples were placed in an oven at 94°C for 2 hr. Two heated and two control samples were then analyzed in duplicate for free methionine.

While the first experiment compared dry blending to slurrying, a second experiment compared three other methods of methionine incorporation into a model system. These were: (1) methionine added directly by dry blending, (2) methionine added in water solution, and (3) methionine and glucose added together in solution. Model systems of the following composition were used: 39.5% MCC, 35.0% soy protein, 5.0% D-glucose, 20.0% deionized water and 0.5% DL-methionine. Model system 1 was prepared by dry blending all of the ingredients except the water for 5 min. Then the water was added and the system was mixed for an additional 15 min. For model system 2, the methionine was dissolved in the water while the remaining ingredients were dry blended for 5 min. Then the methionine solution was added to the dry blend of MCC, protein and glucose, and the entire mixture was blended for an additional 15 min. For model system 3, the methionine and glucose were both dissolved in the water while the MCC and protein were dry blended for 5 min. Then the solution of methionine and glucose was added to the dry ingredients and the entire system was mixed for an additional 15 min. Six-gram samples of each system were then placed in foil pouches which were evacuated and heat sealed. Unheated controls were held at room temperature while six pouches of each system were heated in a water bath at 80°C for 3 hr. Six

heated and six control samples were then analyzed in duplicate for free methionine.

Part II of the research consisted of three experiments which investigated the influence of oxidizing lipid on free methionine stability. In the first experiment, four model systems were used to evaluate the influence of initial peroxide value of an oil on oxidation and/or destruction of added methionine. The first system was a no oil control in which 85.0% MCC (containing 0.5% DL-methionine) was blended with 15.0% deionized water. The remaining model systems were prepared by blending 65.0% methionine-fortified MCC with 15.0% deionized water, and then blending in 20.0% stripped corn oil (Eastman Kodak Co). Oils with three different peroxide values were used, and the peroxide value was varied by bubbling air through the oil until different degrees of oxidation were reached. The peroxide values of the oils were determined by AOAC Method 28.023 (1975). The peroxide value of the oil in the model systems was determined by using the model system directly in the peroxide test. Seventy-five milliliters of the acetic acid/chloroform mixture were added to approximately 8g of sample. The AOAC procedure was then followed as with the oil samples. After blending, 10g of each system were placed in foil pouches which were heat sealed. These pouches were held at room temperature and on days 0, 8, 13, 20 and 28, two samples of each of the systems were analyzed for methionine, methionine sulfoxide, methionine sulfone and peroxide value in duplicate.

The second experiment investigated the influence of oxidizing lipid and protein on the stability of free methionine. Two model systems, one with and one without soy protein, were used to assess the influence of protein on the oxidation of free methionine. The composition of the systems were: 85.0 or 65.0% MCC (fortified with 0.5% methionine), 0.0 or 20.0% soy protein, 15.0% deionized water and 20.0% oxidized corn oil. The peroxide value of the oil used was initially 155.4 meq/kg. Each system was blended for 20 min. Ten-gram samples of each system were placed in foil pouches which were heat sealed. Control samples were held at room temperature while the heated samples were placed in an 80°C oven for 3 hr. The following day six samples of each system were analyzed in duplicate for methionine, methionine sulfoxide and methionine sulfone. Because protein interfered with the peroxide determination, model systems containing soy protein were not analyzed for peroxide value.

The third experiment evaluated the stability of free methionine in a full-fat soy flour. Full-fat soy flour was purchased at a retail outlet and was analyzed for percent fat and protein using AOAC Methods 14.019 and 14.026, respectively (1975). The flour was fortified with 0.5% DL-methionine by dry blending. Ten-gram samples were placed in foil pouches which were heat sealed. These pouches were then stored at −20, 21, 37 and 55°C for 112 days. Two samples from each of the treatments were removed and analyzed in duplicate for methionine, methionine sulfoxide and methionine sulfone after 51 and 112 days of storage.

Methionine extraction

The sample extraction procedure used was a modification of the procedure of O'Keefe and Warthesen (1978). Approximately 2g of each sample were weighed into a 50 ml beaker. Thirty milliliters of 12% trichloroacetic acid (TCA) were then added, and the sample plus TCA was stirred by magnetic stirrer for 20 min. The slurry was filtered through a 10–20 μm fritted glass filter (Ace Glass Inc.) using an aspirator. The beaker and filter plus sample were then washed twice with 20 ml of TCA. The sample extract was quantitatively transferred to a 200-ml volumetric flask, and the pH was adjusted to 9.0 with 5N NaOH. Water was added to bring the extract to volume.

Reaction with dansyl chloride

The reaction with 1-N,N'dimethylaminonaphthalene sulfonyl chloride (dansyl chloride, Sigma Chemical Co.) was adapted from the procedure of Bayer et al. (1976) for amino acids. One-half milliliter of sample extract, 0.5 ml of pH 9.0 borate buffer (0.1M) and 0.5 ml of 10 mM dansyl chloride in acetonitrile were reacted for 40 min at 40°C to form the dansyl derivatives of methionine, methionine sulfoxide and methionine sulfone. The samples were then filtered through 0.2 μm membrane filters (Gelman Metricel TCM-200). Separation and quantification of methionine and related compounds were accomplished by high pressure liquid chromatography (HPLC).

HPLC of methionine and related compounds

HPLC was accomplished using reverse phase chromatography on a Waters Associates High Pressure Liquid Chromatograph (model U6K injector, 6000A solvent delivery system, 440 absorbance detector). The data were recorded and integrated by a Hewlett-Packard 3380A recorder-integrator.

Two different HPLC systems were used for the analysis. In both systems the detector monitored absorbance at 254 nm. For methionine measurement only, a μBondapak C_{18} column (30 cm, 3.9 mm i.d., Waters Associates) was used with a mobile phase composed of acetonitrile (Mallinckrodt distilled in glass, nanograde) and 0.01M phosphate buffer pH 7.0 (23/77, v/v). A flow rate of 2.0 ml/min was used. To measure methionine, methionine sulfoxide and methionine sulfone, an Altex LiChrosorb RP-8 column (25 cm, 3.2 mm i.d.) was used in conjunction with a Model 660 solvent programmer (Waters Associates). The programming conditions used were 1.2 ml/min; pump A: 90/10 (v/v), pH 7.0 (0.01M) phosphate buffer/acetonitrile; pump B: 50/50 (v/v), pH 7.0 (0.01M) phosphate buffer/acetonitrile. The program was run from 20% B to 50% B over 4 min via programming curve #10. A 2-min reverse program was used to return the column to initial conditions in between sample injections. The acetonitrile used with the programmer was spectral grade, distilled in glass (Burdick and Jackson Laboratories, Inc.). All sample injections were 10 μl.

For the experiments in Part I, where no lipid was present, methionine sulfoxide or sulfone would not be expected to form (O'Keefe and Warthesen, 1978). To ensure that this was true, representative samples from these experiments were analyzed for both of these derivatives as well as for methionine. If no methionine sulfoxide or sulfone was found in the representative samples, then all of the samples were analyzed for methionine only using the μBondapak C_{18} column. This method of analysis was more rapid than the programmed analysis (5 min per sample as opposed to approximately 12 min per sample for the programmed analysis). All samples from the experiments in Part II were analyzed for methionine, methionine sulfoxide and methionine sulfone using the programmed analysis.

DL-methionine when reacted with dansyl chloride gave a peak which co-chromatographed with standard dansyl methionine (Sigma Chemical Co.). Dansyl methionine sulfoxide and sulfone standards were prepared by reaction with dansyl chloride. Dansyl derivatives of methionine and related compounds were quantified by comparing peak areas from sample data to those of external standards prepared each day by reacting water solutions of 0.1 mg/ml DL-methionine, DL-methionine sulfoxide (Sigma Chemical Co.) or DL-methionine sulfone (Sigma Chemical Co.) in the same manner as sample extracts.

All treated samples in any one experiment were heated at one time and analyzed the following day to ensure equal treatment. In all experiments, untreated samples were used as controls, and the loss of free methionine due to treatments was represented as a percent of the methionine in the untreated control. This accounts for errors in fortification level (due to weighing and blending) as well as inefficiencies in the extraction procedure. All results were calculated on a dry basis, and moisture determinations were made using Method 44-15A of the American Association of Cereal Chemists (AACC 1975). Fischer's F test and the Student's t test were used for statistical analysis of the data (Snedecor and Cochran 1967).

RESULTS & DISCUSSION

HIGH PERFORMANCE liquid chromatography was chosen for determining free methionine because it is fairly rapid, sensitive, and is capable of quantifying methionine sulfoxide and methionine sulfone (O'Keefe and Warthesen, 1978). Twelve percent trichloroacetic acid was chosen as the extraction solvent because it gave good recoveries of added methionine and precipitated the proteins, thus improving filtration through the fritted glass filters. Methionine extraction efficiency from model systems was 90.4 ± 4.0% (mean ± standard deviation). Extraction efficiencies of methionine sulfoxide and methionine sulfone were similar to the recovery of methionine, as determined via fortification and extraction studies.

Experiments in Part I examined the influence of several methods of methionine incorporation on free methionine stability in systems conducive to Maillard browning. The first experiment investigated the difference between slur-

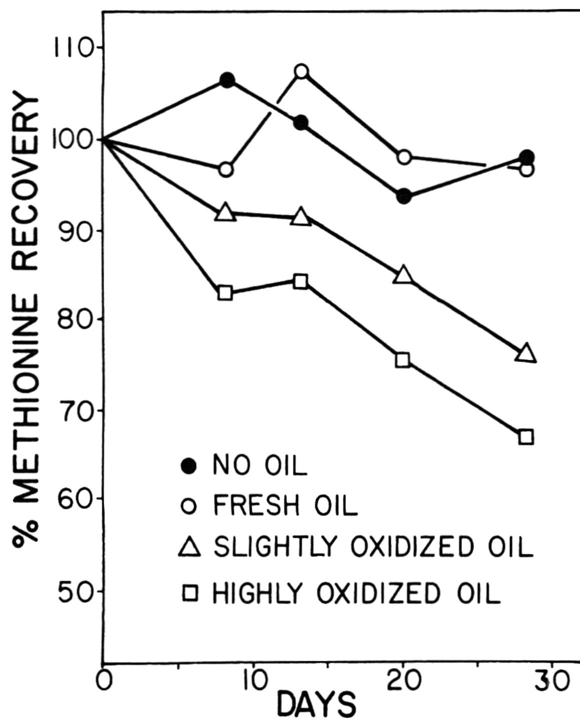

Fig. 1–Methionine recovery (as % of control samples) during room temperature storage.

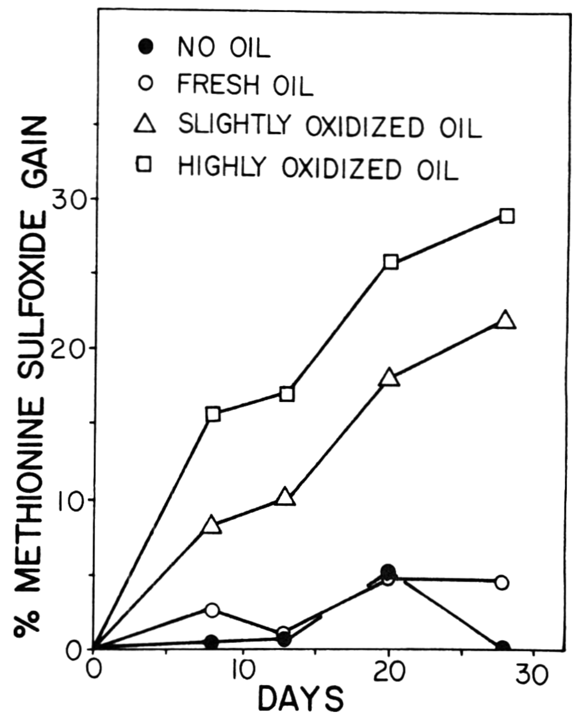

Fig. 2–Methionine sulfoxide gain (as % of original methionine) during room temperature storage.

rying and dry blending a model system (Table 1). At a given water activity, the slurry blended and dry blended samples had the same moisture content which was 9.6% at water activity 0.68 and 11.2% at 0.75. The slurry blending resulted in extensive methionine loss upon heating, while the dry blend had only slight methionine loss. The difference between the two blending techniques was highly significant ($p = 0.005$). There was not, however, a significant difference in methionine loss between the two water activities tested. The results indicate that slurrying as a method of methionine incorporation can lead to greater losses than dry blending.

The second experiment in Part I compared addition of methionine in the dry state with addition of methionine in solution and addition of methionine and glucose together in solution. All systems contained 21.3 ± 0.4% moisture and no methionine sulfoxide or methionine sulfone was detected in any of the representative samples.

After heating at 80°C for 3 hr, 71.6% (±2.9) of the methionine was recovered from the dry blended system. Only 50.2% (±4.4) of the methionine was recovered from the system where methionine was added in solution and 50.4% (±3.7) was recovered when methionine and glucose were added together in solution. Thus, addition of methionine in solution, or methionine and glucose together in solution, resulted in significantly more free methionine loss than did dry blending ($p = 0.001$). There was not a significant difference between the two solution systems, however. This result was somewhat surprising, as greater interaction of methionine and glucose might be expected when both were added together in solution. These results suggest that loss of free methionine in systems susceptible to Maillard browning is greater when methionine has been added in solution.

In both experiments in Part I, the loss of methionine during heating was less in dry blended systems than when the system was slurried or when the methionine was added in solution. There are several possible reasons for the difference in methionine loss even though the final moisture contents during heating were the same for comparable samples. The slurry or solution might result in a better dispersion of the individual glucose and methionine molecules. This increased dispersion would allow more interaction at the molecular level, even after the dispersing water was removed (in the case of the slurry). With dry blending, the methionine molecules exist in fairly large crystals with fewer exposed amino groups. This may result in localized areas of browning, but less overall browning and subsequent methionine loss.

O'Keefe and Warthesen (1978) suggested Maillard browning as the most likely mechanism of free methionine loss when heated in the presence of glucose. Many factors

Table 1–Effect of slurry or dry blending on free methionine stability at two different water activities after heating at 94°C for 2 hr

Water activity	Methionine recovery as % of control[a,b]	
	Slurry blend	Dry blend
0.68	24.4 (± 4.4)	98.1 (± 4.3)
0.75	37.4 (±11.3)	84.3 (±13.2)

[a] Each value represents the average of two replicates analyzed in duplicate.
[b] Values in parentheses are one standard deviation.

Table 2–Peroxide values of model systems during room temperature storage

Type of oil added to model system	Peroxide value (meq/kg)				
	Day 0	Day 8	Day 13	Day 20	Day 28
Fresh oil	2.9	2.3	2.2	1.4	1.9
Slightly oxidized oil	23.6	103.5	105.9	100.7	116.2
Highly oxidized oil	58.2	142.7	91.3	105.4	92.9

Table 3–Methionine (M) recovery and methionine sulfoxide (MX) gain as influenced by protein and oxidizing lipid[a,b,c]

System	% of control		
	M recovery	MX gain	Total recovery
No protein	79.4 (±4.0)	21.9 (±1.8)	101.3 (±2.9)
Protein	97.5 (±2.5)	8.8 (±7.6)	106.3 (±7.6)

[a] Heated at 80°C for 3 hr
[b] Each value represents the average of six replicates analyzed in duplicate.
[c] Values in parentheses are one standard deviation.

such as water activity, water content, type of reducing compound, pH and temperature can influence the rate of browning (Eichner and Karel, 1972; Spark, 1969; Reynolds, 1963). In systems susceptible to browning, it is likely that factors such as these will influence stability of free methionine in the same way that they influence Maillard browning. Apart from this is the observation that when all other factors are held constant, the method of methionine incorporation can significantly influence free methionine stability. It appears that solubilizing the added methionine will promote losses of the added amino acid. This factor should be considered in any amino acid fortification system or when studying methionine stability in model systems.

Part II of this study examined the influence of oxidizing lipid on the stability of free methionine. The first experiment was designed to determine whether higher initial peroxide values might result in greater loss of methionine. To better observe the interaction of lipid and methionine, protein was not incorporated into the model systems. Table 2 shows the changes in peroxide values that occurred in the model systems during storage at room temperature. The peroxide value of the model system made with the highly oxidized oil increased initially and then decreased, probably indicating peroxide decomposition with storage.

Figure 1 shows the retention of free methionine over time as a percent of the methionine originally present in each of the systems. There was no significant difference between the no oil and fresh oil systems, where methionine loss was negligible. However, the three systems containing oil were significantly different from one another after 20 days of storage ($p = 0.05$) with the most oxidized system giving the greatest methionine loss. The differences due to initial peroxide level were most significant by day 28 ($p = 0.001$). At the same time that methionine was being lost, methionine sulfoxide was being formed, as shown in Figure 2. The percent methionine sulfoxide gain is based on the moles of methionine initially present in the control samples. There was no significant difference between the levels of methionine sulfoxide detected in the no oil control and the fresh oil system. Significant differences were observed between each of the oil systems ($p = 0.05$), with the greatest formation of methionine sulfoxide occurring in the highly oxidized oil system. No methionine sulfone was detected in any of the samples.

The results suggest that in the presence of oxidized lipid, a major mechanism of methionine loss was by conversion to methionine sulfoxide. There is the possibility, however, that some of the lost methionine was not converted to methionine sulfoxide, but rather reacted in a Maillard browning reaction with carbonyls formed as lipid oxidation products. If all of the methionine lost was converted to methionine sulfoxide, the total recovery of methionine plus methionine sulfoxide should equal 100% of the methionine present in the control samples. If, on the other hand, some of the methionine or methionine sulfoxide was lost by reaction with carbonyls formed from lipid oxidation, the total recovery of methionine plus methionine sulfoxide would be less. In nearly every case, the total recovery was approximately 100%, suggesting that at room temperature in the presence of oxidizing lipid, methionine is quantitatively converted to methionine sulfoxide. It also appears that the higher the initial peroxide value of the oil, the greater the subsequent methionine oxidation.

The second experiment in Part II examined the influence of protein on free methionine stability in the presence of oxidizing lipid (Table 3). No methionine sulfone was detected in any of the samples. The system without protein lost considerably more methionine upon heating than did the protein system. This difference was highly significant ($p = 0.005$). The no protein system also resulted in significantly more methionine sulfoxide production ($p = 0.005$). The methionine sulfoxide level was low in the system with protein, and the measurement was more variable at these low levels. The total recovery of methionine plus methionine sulfoxide was close to 100% of the amount of methionine initially present, indicating that nearly all of the methionine lost was converted to methionine sulfoxide. Even though the temperature in this experiment was higher than room temperature, it appears that methionine loss via Maillard browning reactions involving products of lipid oxidation was negligible.

There are several possible mechanisms whereby the protein might protect the methionine from oxidation. The presence of protein may have altered the lipid oxidation reaction directly, resulting in less lipid oxidation and consequently less methionine oxidation. This would be possible if the protein acted as an antioxidant, quenching free radicals. Schaich and Karel (1975) demonstrated that lipid radicals arising during oxidation are transferred to protein, thus forming protein radicals which eventually cause the protein to polymerize. This polymerization would eliminate free radicals, thus reducing the extent of lipid oxidation. It is also possible that the sulfur atoms of the side chains of some of the methionine residues in the protein were oxidized, rather than the free methionine sulfur atoms, resulting in less free methionine oxidation. Tannenbaum et al. (1969) demonstrated loss of methionine residues in casein, and suggested that the methionine residues acted as peroxide decomposers with concomitant conversion of methionine to methionine sulfoxide. A further possibility is that the added protein altered the water activity of the system, such that the rate of lipid oxidation was reduced. Water activity and water content can significantly affect lipid oxidation (Karel et al., 1975), and if the protein binds water differently than MCC, then a change in water activity could result in less lipid oxidation and hence less methionine oxidation. Regardless of the mechanism, it is apparent that the presence of protein can significantly affect the oxidation of free methionine in the presence of oxidizing lipid.

The last experiment in Part II examined the stability of free methionine in full-fat soy flour stored for 112 days at four different temperatures ranging from −20 to 55°C. A full-fat soy flour was chosen because it is a food source which has fortification potential and provides a realistic comparison to the model systems. The fat content of the flour was 12.1% and the protein content was 40.2%. There were no significant differences in methionine recoveries at the four temperatures studied, and the methionine appeared to be stable under these conditions. Methionine recoveries ranged from 100.2–107.0%. No conversion to methionine sulfoxide or sulfone was observed.

There are several possible factors contributing to the stability of methionine in the full-fat soy flour. Extensive oxidation of the lipid may not have occurred, due either to lack of conditions favorable to oxidation or to the presence of natural antioxidants in soy (such as tocopherols). If the lipid present did not oxidize, it is unlikely that the methio-

nine would oxidize. It is also possible that the protein present in the flour exerted a protective effect on methionine as observed in the model systems containing soy protein. In any case, free methionine appears to be stable in full-fat soy flour for nearly 4 months at temperatures as high as 55°C. These results suggest that from a storage stability standpoint, fortification of soy flour with free methionine is a feasible method of improving soy protein quality.

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Presented at the 38th Annual Meeting of the Institute of Food Technologists, Dallas, TX, June 4—7, 1978.

Paper No. 10,756, Scientific Journal Series, Minnesota Agricultural Experiment Station, St. Paul, MN 55108

This research was supported in part by the USDA under the PL-89-106 Grant Program.

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Purdue University Experiment Station journal paper 7077. Supported in part by Cooperative State Research Service, U.S. Department of Agriculture grant #516-15-101 and by the University of Wisconsin, College of Agricultural and Life Sciences.

X–RAY FLUORESCENCE ANALYSIS OF SULFUR AND PHOSPHORUS WITH A FUNDAMENTAL MATRIX CORRECTION CALCULATION

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ABSTRACT

A comparison of X-ray and chemical analyses of phosphorus and sulfur standards of KH_2PO_4– and $(NH_4)_2SO_4$–cellulose was performed to determine the range of applicability of a numerical matrix correction technique. Over the range 0.1–1.0%, the X-ray technique was accurate to within 8% for phosphorus and 17% for sulfur. At 0.03%, the X-ray values are low by 17% or more. At 3.0% and 10.0%, particle size effects caused the values to be low by more than 17%. X-ray analysis of pure sulfur and $(NH_4)_2SO_4$ was accurate to within 13% and indicates the validity of the mathematical technique. Application was made to the analysis of wheat and standard reference plant materials.

INTRODUCTION

A NUMBER of important agricultural trace elements can be determined with energy dispersive X-ray spectrometers (Giauque et al., 1973; Pippen et al., 1975). The advantage of the energy dispersive systems compared to wet chemical methods is that about 13 elements can be observed in two separate machine runs on dried agricultural products without destroying the sample. This feature permits a rapid qualitative or quantitative assessment of the sample minerals without any prior knowledge of the minerals or concentration ranges present.

Since most X-ray spectrometers use an air path which absorbs the X-rays from light elements, they are limited to analysis of elements heavier than chlorine or calcium. A vacuum path spectrometer permits detection of the important minerals sulfur and phosphorus making X-ray fluorescence (XRF) a candidate for the rapid analysis of these two elements. However, the routine utilization of the technique is limited by two problems: particle size effects and calibration curves.

Claisse (1970) has pointed out that heterogeneity, particle size and surface roughness can affect the number of low energy X-rays escaping from a sample. These effects require fusion techniques and polishing for metalurgical and geological samples. For agricultural materials, however, the light element nature of the matrix may permit one to overcome these problems with grinding alone. The effect of particle size on the XRF determination of phosphorus in freeze-dried meat diluted with cellulose has been demonstrated by Isherwood and King (1976).

The ability to measure solid material directly is slowed if a different calibration curve is required for each sample type. Reed (1973) has demonstrated that a large variety of plant samples containing 0.1–0.4% sulfur can be treated with the same calibration curve. Reuter (1975) has shown that no calibration curves are necessary for heavy element determination in plant material when changes in matrix absorption are treated mathematically.

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With model agricultural materials of cellulose plus a salt, and actual plant materials (National Bureau of Standards reference samples), we have explored the extension of applicability of the mathematical matrix correction procedure (Reuter, 1975) to the X-ray determination of the lighter elements phosphorus and sulfur, and the use of grinding to minimize particle size effects.

EXPERIMENTAL

A SALT PLUS CELLULOSE was chosen as a model agricultural substance for this study. Cellulose was used as a diluent because of the similarity in its composition and matrix correction to plant material. The range of concentrations chosen corresponded to the concentrations of phosphorus and sulfur typically encountered in the analysis of foodstuffs and agricultural products. Readily water-soluble inorganics [$(NH_4)_2SO_4$ and KH_2PO_4] were used as sources of the sulfur and phosphorus to minimize the analytical chemical error by eliminating the procedures necessary to convert organic sulfur and phosphorus to sulfate and phosphate ions, respectively, the forms in which the elements were determined chemically.

Preparation of standards

Phosphorus and sulfur standards were prepared by mixing known amounts of KH_2PO_4 (NBS 186–1) and $(NH_4)_2SO_4$ (reagent grade, recrystallized), respectively, with Whatman Chromatographic grade cellulose, 200 mesh, (W. & R. Balston, Ltd., England) to make concentrations of 0.03, 0.1, 0.3, 1.0 and 3.0% phosphorus or sulfur. The dry mixtures were wet with redistilled water, air dried to insure homogeneity, and ground into a fine powder with an agate mortar and pestle. Further grinding was performed with a Spex mixer mill (Spex Industries, Inc., Metuchan, NJ) in a Tool Steel container. One-half to five grams of sample was covered with 95% ethanol and milled with stainless steel balls for 10 min. After air drying and regrinding in an agate mortar, 300 mg of powder were pressed at 16,000 psi into a 3.18 cm diameter wafer. The wafers were self supporting and could be easily stored until measurement. Similar wafers were made from standard reference materials (S.R.M.) of the National Bureau of Standards (NBS): Trace Elements in Spinach S.R.M. 1570, Tomato Leaves S.R.M. 1573, and Pine Needles S.R.M. 1575 (dried in air in an oven at 85°C for 2 hr), and Orchard Leaves S.R.M. 1571 (dried in air in an oven at 85°C for 4 hr). The S.R.M. samples, as received, were already finely ground (comminuted to pass a 40–60 mesh sieve).

XRF spectrometry

The X-ray measurements were performed with a Finnigan Model 80 energy-dispersive X-ray fluorescence spectrometer (Finnigan Corp., Sunnyvale, CA). Sulfur and phosphorus atoms were excited with the spectrum from an unfiltered rhodium X-ray tube operated at 20 kV and 0.1 ma. Fluorescent X-rays were collimated and detected in a solid state lithium-drifted silicon detector with a resolution of 165 ev, FWHM at 5.9 Kev (Mn $K\alpha$), 1000 counts/sec. Samples were analyzed for 1000 sec in a vacuum.

Machine and elemental X-ray parameters were obtained from one time calibration with thin film standards (Reuter and Raynolds, 1974). The concentrations of phosphorus and sulfur in the 300 mg wafers were determined mathematically from the X-ray signal, X-ray production probabilities determined from the thin film standards, and fundamental X-ray constants with a Fortran computer program described in detail by Reuter (1975). This program involves an iterative numerical technique that directly computes the amount of absorption of each of the analyte lines by the light element (C, H, O, N) constituents of the sample matrix. In plant materials this matrix is assumed to be chiefly cellulose, a carbohydrate polymer $(C_6H_{10}O_5)_n$. An allowance is made in the program to include other light elements such as N, Na, and Mg in the matrix formula if they

Table 1—Comparison of X-ray fluorescence spectrometry and chemical analyses for phosphorus in KH_2PO_4—cellulose standards

Standard (%)[a]	XRF n[b]	XRF Avg (%)	XRF Range	Chemical n[b]	Chemical Avg (%)	Chemical Range	XRF—chemical Dif (%)
0.03	(2)	0.024	0.019–0.028	(3)	0.029	0.026–0.032	–17.2
0.10	(2)	0.089	0.082–0.096	(3)	0.096	0.095–0.098	– 7.3
0.30	(2)	0.282	0.270–0.293	(3)	0.281	0.276–0.289	+ 0.4
1.0	(2)	0.919	0.895–0.942	(3)	0.993	0.970–1.019	– 7.5
3.0	(2)	2.34	2.24 –2.43	(3)	2.90	2.82 –2.95	–19.3

[a] Prepared phosphorus concentration (weight basis), approximate
[b] n = number of determinations entering into the average value.

Table 2—Comparison of X-ray fluorescence spectrometry and chemical analyses for sulfur in $(NH_4)_2SO_4$—cellulose standards

Standard (%)[a]	XRF n[b]	XRF Avg (%)	XRF Range	Chemical n[b]	Chemical Avg (%)	Chemical Range	XRF—chemical Dif (%)
0.03	(2)	0.021	0.021–0.021	(4)	0.032	0.030–0.032	–34.4
0.10	(2)	0.085	0.084–0.086	(3)	0.102	0.101–0.102	–16.7
0.29	(2)	0.258	0.251–0.265	(3)	0.297	0.294–0.299	–13.1
1.0	(1)	0.964	–	(4)	1.02	1.01 –1.02	– 3.7
3.0	(2)	2.44	2.42 –2.46	(6)	2.99	2.97 –3.01	–18.4

[a] Prepared sulfur concentration (weight basis), approximate
[b] n = number of determinations entering into the average value.

occur in high concentrations. However, it was shown (Reuter, 1975) that 15% N caused less than 1% change in the matrix correction factor for sulfur and phosphorus. The program also corrects for overlapping peaks (e.g., potassium Kβ – calcium Kα), and interelement absorption effects of the measured elements.

Chemical methods

Published methods were used with slight modifications to accommodate the samples and amounts analyzed.

Phosphorus was determined colorimetrically (Allen, 1940). Inorganic phosphorus in the KH_2PO_4—cellulose mixtures was determined directly on filtered, aqueous solutions of the samples. For the determination of total phosphorus in the NBS standard reference plant materials, the dried sample was digested with 60% perchloric acid (Mallinckrodt, A.R.) and the digest used for the development of color.

Sulfur was determined as sulfate titrimetrically (Fritz and Yamamura, 1955). Inorganic sulfur in the $(NH_4)_2SO_4$—cellulose mixtures was determined on filtered, aqueous solutions of the samples treated before filtration with Dowex 50W–X8 ('Baker Analyzed' reagent) (20–50 mesh, regenerated-acid form) to remove cations which interfere in the titration. For the determination of total sulfur in the NBS standard reference plant materials, the dried sample was decomposed by the oxygen flask combustion technique (Corliss, 1962). The sulfate in the absorbing solution was titrated after first removing phosphate interference with silver oxide (Colson, 1963) and cations with Dowex 50W–X8 (20–50 mesh, regenerated-acid form).

RESULTS & DISCUSSION

TABLES 1 AND 2 show the results of the phosphorus and sulfur determinations, respectively, on the salt-cellulose standards. All the X-ray values are lower than the prepared values, and all except one are also lower than the chemical values. The reason for this negative bias in the X-ray results is not known at this time, and considering the concentration level may not be significant. Over the range 0.1–1.0%, the X-ray values agree with the chemical values by 8% or less for phosphorus, and by 17% or less for sulfur. This is reasonable considering that the original thin fim X-ray calibration standards are accurate to ± 10% (Reuter, 1975). Below this range (at 0.03%), both the phosphorus and sulfur values are low by 17% or more. The low results for

Table 3—Effect of grinding on sulfur and phosphorus concentrations by X-ray fluorescence spectrometry

Sample	Element	XRF, % Wiley Mill	XRF, % Ball Mill	Chemical (%)
$(NH_4)_2SO_4$—cellulose[a]	S	5.43	7.39	10.2
Wheat germ	S	0.244	0.283	0.300
Whole wheat	P	0.129	0.259	0.280

[a] 10.0% sulfur, prepared concentration (weight basis), approximate

phosphorus and sulfur at this level can be explained by the fact that 0.03% is close to the detection limit for these elements. This same trend has also been observed by Reed (1973) with sulfur concentrations less than 0.03%. At 3.0% phosphorus and sulfur, the X-ray values are low by more than 17%.

In order to determine the contribution of the mathematical model to the error at 3.0% and higher, finely ground pure sulfur and $(NH_4)_2SO_4$ were analyzed for sulfur by the X-ray technique. The sulfur concentrations found by XRF were 89.8 ± 2.6% for pure sulfur (theoretical, 100.0%) and 27.4 ± 0.2% for $(NH_4)_2SO_4$ (theoretical, 24.3%). The measurements for pure sulfur and for $(NH_4)_2SO_4$ were within 10% and 13% of theoretical, respectively. Over this range, the matrix correction factor increased from about 23 for the $(NH_4)_2SO_4$—cellulose standards to 102 for the pure sulfur. Even though particle size effects were probably not entirely eliminated at these high concentrations, such a good agreement over this large concentration range gives credence to the mathematical technique.

The contribution of particle size to the error was studied by comparing the effect of grinding on the phosphorus and sulfur results (Table 3) in several different materials ground in a Wiley Mill (20 mesh) and a ball mill. A mixture of $(NH_4)_2SO_4$ and cellulose (10% sulfur) showed an increase from 5.43 to 7.39% sulfur when the mixture was ground with a Wiley Mill and ball mill, respectively. This indicates

Table 4–Phosphorus and sulfur in NBS standard reference plant materials (dry-weight basis)

Sample	Elements	XRF (%)[a]	Chemical (%)[b]	NBS (%)[c]
S.R.M. 1570	P	0.59 ± 0.09	0.54 ± 0.005	0.55 ± 0.02
(Trace elements in Spinach)	S	0.45 ± 0.04	0.46 ± 0.02	—
S.R.M. 1571	P	0.29 ± 0.02	0.20 ± 0.002	0.21 ± 0.01
(Orchard Leaves)	S	0.22 ± 0.01	0.18 ± 0.01	0.19
S.R.M. 1573	P	0.48 ± 0.05	0.34 ± 0.005	0.34 ± 0.02
(Tomato Leaves)	S	0.60 ± 0.04	0.63 ± 0.06	—
S.R.M. 1575	P	0.13[d]	0.12 ± 0.003	0.12 ± 0.02
(Pine Needles)	S	0.11[d]	0.13 ± 0.01	—

[a] Mean and standard deviation of four to six determinations for each element

[b] Mean and standard deviation of seven determinations for each element

[c] Certified values for phosphorus, noncertified for sulfur

[d] Single determination

that dissimilar mixtures cannot be ground fine enough to overcome particle size effects. This is corroborated by Claisse (1970). This result probably explains the divergence of the XRF values for phosphorus and sulfur at 3.0%. Below 3.0% the material appeared homogeneous. At 3.0%, however, which is about 13% total salt concentration, the salt-cellulose mixture was observed to have a salty texture and to take much longer to dry than the lower concentration mixtures. This indicates that the 3.0% mixture was no longer uniform. We conclude, therefore, that the divergence of the X-ray and chemical values at 3.0% phosphorus and sulfur is the result of particle size effects from insufficient grinding. Initially, the XRF value for the 1.0% sulfur cellulose standard was less than half of the prepared value. Rewetting and regrinding (agate mortar) the sample brought the sulfur value up to 0.964% (Table 2). Purely agricultural material such as wheat germ and whole wheat show visible heterogeneity after grinding with a Wiley Mill (20 mesh). After being ball-milled these materials yield X-ray values within 8% of the chemical values (Table 3). Fellers and Mossman (1976) have shown that the majority of the phosphorus in whole wheat resides in the bran. Wiley milling whole wheat leaves readily visible bran flakes while ball milling produces a homogeneous looking powder. One problem with the ball mill, however, is that it left a large iron contaminant in the sample, due to the stainless steel construction of the grinding chamber, that made measurement of iron and the adjacent trace minerals difficult. This contamination can be avoided by using a ceramic or plastic milling container.

Table 4 shows the application of the XRF technique to the determination of phosphorus and sulfur in NBS standard reference plant materials. The chemical and NBS values are given for comparison. The results show that the XRF technique measured the phosphorus and sulfur with a precision range of ± 5% to ± 16%, and with an accuracy adequate for routine analyses at these low concentrations.

In conclusion we feel that the calibration curves can be eliminated in the XRF determination of phosphorus and sulfur in agricultural material over the concentration range 0.1–1.0% by using a numerical matrix correction procedure. In addition the method can be extended to materials of higher concentration if particle size effects can be minimized by grinding the material adequately. Use of XRF techniques results in a great savings of time over the chemical methods used.

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Ms received 12/31/78; revised 5/18/79; accepted 5/27/79.

Presented at the 35th Annual Meeting of the Institute of Food Technologists, Chicago, Ill., June 8–12, 1975.

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A Research Note

PALATABILITY OF PRERIGOR AND MECHANICALLY PROCESSED NITRITE-FREE HAMS

M. D. JUDGE and J. J. CIOCH

ABSTRACT

The effects of mechanical treatment (massaging) and prerigor infusion of a nitrite-free salt solution on the palatability of nonheat-processed hams were determined. Massaging in the prerigor state after salt infusion resulted in reduced thiobarbituric acid (TBA) reaction values in muscle tissue at the end of a 14-day storage period at 3°C as compared to salt infusion alone either pre- or postrigor. Extended frozen storage (13 wk at −40°C) resulted in TBA values in adipose tissue in all treatment groups of a magnitude normally associated with rancid flavors. A meat tenderizing action by the massaging treatment was noted from Warner-Bratzler shear tests performed on several muscles of dry roasted hams. Taste panel data also identified massaging treatment effects on tenderness but not on juiciness or flavor. The taste tests established that all treatment combinations produced nitrite-free hams that were generally acceptable in most sensory attributes. Panel members associated the flavor and texture with that of uncured roasted pork.

INTRODUCTION

DOUBTS about the safety with which nitrite salts may be used in processed meats have stimulated scientific, regulatory agency, and consumer interest in nitrite-free processed products. Aside from the protection against botulism afforded by nitrite ions, several palatability factors (cured meat color, "cured" flavor and stability against lipid autoxidation) are known to be influenced by this additive.

Processes designed to improve the palatability of cured pork have been developed recently with some application by the meat industry. The mechanical treatment of boneless hams known as tumbling or massaging improves the uniformity and degree of tenderness and water-binding capacity of the finished product (Theno et al., 1977; Krause et al., 1978). Likewise, some palatability characteristics of pork, particularly color and texture, are improved when the curing salts are introduced prerigor rather than postrigor (Kastner, 1977). The effects of such innovations in the absence of the nitrite ion in the curing process are of interest.

This research was performed to evaluate the palatability that nitrite-free nonheat-processed pork possesses when manufactured with advanced technologies. The study did not address the question of microbiological hazards, if any, that such products may represent.

EXPERIMENTAL

Experimental design

Experiment I. Forty hams weighing approximately 8−11 kg each after skinning were randomly assigned to one of four treatment groups consisting of 10 hams each. The treatments constituted a 2 × 2 factorial design as follows:

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0022-1147/79/0006-1775 $02.25/0
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Treatment 1–Prerigor salt infusion and mechanical treatment;
Treatment 2–Prerigor salt infusion;
Treatment 3–Postrigor salt infusion and mechanical treatment;
Treatment 4–Postrigor salt infusion.

Experiment II. Twenty pairs of hams weighing approximately 7−9 kg each after skinning were used in this experiment. One ham of each pair (random selection of right or left side) was assigned to either Treatment 1 or 2 described above. The mate to each selected ham was used as its control and was processed as Treatment 4 above. Four groups of 10 hams each were subsequently formed as follows:

Treatment 1
Treatment 4 (Paired control for Treatment 1)
Treatment 2
Treatment 4 (Paired control for Treatment 2)

Salt infusion

The salts infused into the hams were those typically used in commercial meat cures with the omission of nitrite salt. A solution was prepared using the following ingredients:

Water	29.5 kg
Sodium chloride	3.6 kg
Brown sugar	2.3 kg
Sodium tripolyphosphate	284 g
Sodium erythorbate	168 g

The phosphate and erythorbate were included even in the absence of nitrite because Tims and Watts (1958) demonstrated that these additives act synergistically to prevent lipid autoxidation in meat.

The hams were stitch pumped to 118 and 110% of original weight in Experiments I and II, respectively. For Treatments 1 and 2 the infusion occurred within approximately 1 hr postmortem and for Treatments 3 and 4 it was accomplished at approximately 18 hr postmortem.

Mechanical treatment and storage

Immediately after salt infusion all hams were made boneless and those to receive mechanical treatment were placed intact in a Knud Simonsen (Ontario, Canada) laboratory model meat massager. The massaging was continuous for approximately 18 hr at 3 rpm in a room temperature of approximately 3°C. All hams were placed in polyvinyl chloride bags which were then vacuumized and sealed. They were stored at approximately 3°C until 7 days postmortem at which time they were opened for sample collection and resealed. They were then stored for an additional 13 wk at −40°C (Experiment I) or 7 days at 3°C (Experiment II).

TBA analyses

The oxidation products of unsaturated fatty acids were quantitatively estimated with the thiobarbituric acid (TBA) test of Tarladgis et al. (1960). In Experiment I, samples of subcutaneous adipose tissue were obtained immediately following salt infusion and at 7 days and 14 wk postmortem. In Experiment II, samples of the gluteus medium muscles were obtained prior to prerigor processing and at 7 and 14 days postmortem. All analyses were performed in triplicate.

pH

In Experiment II, the pH values of minced samples of the gluteus medium muscles were determined with a Beckman pH meter at 14 days postmortem.

Cooking and palatability evaluation

All hams were cooked one time only at the end of the respective storage periods by dry roasting in electric institutional-size baking ovens. The oven temperature was approximately 150°C and the

Table 1—Influence of ham processing methods on adipose tissue TBA numbers, consumer panel scores and cooked meat shear resistance (Experiment I)

	Treatment				Significance	
	1 Prerigor salt and massage	2 Prerigor salt	3 Postrigor salt and massage	4 Postrigor salt	Massage	Rigor
TBA[a]						
After salting	0.91	0.49	0.46	0.60		
7 days postmortem	1.08	0.82	0.86	0.71		
14 wk postmortem	2.73	3.25	3.39	3.23		
Consumer scores[b]	7.61	7.38	7.26	7.33		
Shear (kg)[c]						
Light semitendinosus	1.04	1.63	1.30	1.60	**	
Dark semitendinosus	1.17	1.87	1.35	1.91	**	
Semimembranosus	1.38	1.59	1.48	1.83	*	
Biceps femoris	1.12	1.66	1.50	2.21	**	*
Avg	1.18	1.69	1.41	1.89	**	

[a] Mg malonaldehyde/1,000g sample; n=10/treatment group.
[b] 1=dislike extremely, 5=neither like nor dislike, 9=like extremely.
[c] n=4/treatment group
** $P < 0.01$
* $P < 0.05$

hams were roasted to an internal temperature of 77°C (including post cooking temperature rise) with a glass thermometer in place.

Six randomly selected hams from each treatment group in Experiment I were used on the menu of a public cafeteria operated by the Department of Restaurant, Hotel and Institutional Management. Servings were prepared immediately after roasting of hams from a single treatment group on any given day and were displayed on a hot table with other meat items. They were identified as roasted leg of pork. When consumers voluntarily selected the item, a score sheet was handed to them on which they were asked to record their overall impression of the meat serving by checking the appropriate position on a line labeled at the extremes with the phrases "dislike extremely" and "like extremely." The position of the mark was later converted to a numerical value based on a 1–9 scale. A total of 224 meat servings were evaluated.

An experienced but untrained laboratory taste panel consisting of a minimum of 5 persons evaluated the tenderness, juiciness and flavor of samples of the semimembranosus muscles of the hams in Experiment II. Each palatability factor was scored on a 9-point hedonic scale. The samples were served warm within 30 min of removal of the hams from the ovens.

The light semitendinosus, dark semitendinosus, semimembranosus and intermediate biceps femoris muscles of four randomly selected cooked hams from each treatment group in Experiment I and all hams in Experiment II were subjected to Warner-Bratzler shear tests. A minimum of 10 cores 1.25 cm in diameter were prepared and sheared from each tested muscle after the hams had been refrigerated overnight.

Data analyses

Data were analyzed using a 2 × 2 factorial analysis of variance (Experiment I) and pair difference analysis (Experiment II) from the Statistical Package for the Social Sciences (Nie et al., 1970).

RESULTS & DISCUSSION

Experiment I

There were no significant treatment differences in the degree to which the lipids of the subcutaneous adipose tissue of the hams in Experiment I underwent autoxidation (Table 1). However, the magnitude of the TBA values after extended storage at −40°C suggests some flavor deterioration although the precise relationship between TBA values and sensory perception of rancid flavor is obscure (Pearson et al., 1977). The TBA values for the frozen hams are in line with those observed by Olsen and Rust (1973) in the fat component of dry-cured hams that were judged by taste panelists to be slightly inferior. The results of the present study discourage the practice of holding nitrite-free uncooked salted products in a frozen state even at temperatures substantially below those normally used for frozen storage.

In spite of the rancidity which developed in the adipose tissue of the hams, the meat served in the cafeteria was scored well up on the hedonic scale by the consumers (Table 1). However, the meat servings did not include subcutaneous fat or large deposits of intermuscular fat. Another likely reason for their acceptability is the process design which included a single heat treatment just prior to consumption and did not include storage in a precooked state. The consumer scores for the prerigor salt-massage treatment combination tended to be higher than those of the other treatment groups although the differences lacked statistical significance.

A tenderizing action by the mechanical processing (massaging) and, to a minor degree, by the prerigor salt infusion is apparent from Warner-Bratzler shear values (Table 1). The consequences of the meat massaging process include the application of frictional energy to the product, the disruption of cellular structures and the extraction of salt soluble proteins to form an exudate (Theno et al., 1977). Consequently the observed tenderizing action may have resulted from physical damage to the connective tissues, sarcolemmae, or other structures; or it may have stemmed from the solubilization and improved water-binding capacity of the myofibrillar proteins after having been exposed to the easily diffusing salt. In any case the changes occurred in both pre- and postrigor massaged hams (i.e., there were no significant interactions for the massaging and rigor related salt treatments on shear values).

The tendency for prerigor processed hams to be more tender than those processed postrigor (Table 1) contrasts with the results of studies on uncured hot processed pork, but is in agreement with several reports on hot processing of cured pork (Kastner, 1977). It is possible that the presence of the salt prior to the completion of rigor mortis caused increased myofibrillar protein hydration as compared to postrigor salting, an action which improves the water-holding capacity (Hamm, 1977) and consequently the tenderness of the meat.

Experiment II

The data generated in Experiment II extend the above observations on the relative importance of prerigor salt infusion and massaging on the palatability and stability of nitrite-free hams. The paired control hams, having been

Table 2–Influence of ham processing methods on muscle tissue TBA numbers and pH and on cooked meat panel scores and shear resistance (Experiment II)[a]

	Paired treatments			Paired treatments	
	1 Prerigor salt and massage		4 Postrigor salt	2 Prerigor salt	4 Postrigor salt
TBA[b,c]					
Prerigor	0.17			0.21	
7 days postmortem	0.22		0.29	0.40	0.24
14 days postmortem	0.20	*	0.50	0.31	0.38
pH					
14 days postmortem	5.761		5.716	5.821	5.785
Panel scores[d]					
Tenderness	6.99	**	6.38	6.96	7.34
Juiciness	5.66		5.79	6.03	6.29
Flavor	6.84		6.63	6.77	6.67
Shear (kg)					
Light semitendinosus	1.27		1.47	1.62	1.56
Dark semitendinosus	1.43	**	1.73	1.57	1.71
Semimembranosus	1.83		1.97	1.79	1.90
Biceps femoris	1.50	*	1.74	1.62	1.79
Avg	1.52	**	1.73	1.65	1.74

[a] n=10/treatment group.
[b] Mg malonaldehyde/1,000g sample
[c] Gluteus medius
[d] Semimembranosus; 1=dislike extremely, 5=neither like nor dislike, 9=like extremely.
** $P < 0.01$
* $P < 0.05$

pumped with salt solution postrigor, provide a common basis for comparison of Treatments 1 and 2.

The combination of prerigor salting and massaging afforded a protective effect against development of oxidative rancidity during unfrozen storage (Table 2). This was not evident in the muscle tissue of hams treated by prerigor salting alone, however. The explanation for such a protective effect may relate to differences in glycolytic enzyme activity in view of the reports of Honikel and Hamm (1978) and Owen and Lawrie (1975). The former authors reported that one of the effects of prerigor sodium chloride addition to minced muscle at temperatures of −3 to 24°C is a reduction of total lactate production by inactivation of glycolytic enzymes. In the present study the massaging treatment may have partially simulated the effects of mincing by allowing rapid diffusion of salt through damaged membranes. The research of Owen and Lawrie (1975) showed that high ultimate pH reduces lipid oxidation in pork as measured by peroxide values. Although the pH differences in the present study were not marked nor statistically significant, in both paired treatments, the gluteus medius muscles of prerigor salted hams had slightly higher pH values than those salted postrigor even after 14 days of storage (Table 2).

Data on meat tenderness again show a stronger tenderizing action by the prerigor salt and massage treatment than by prerigor salt infusion alone. The taste panel confirmed the differences detected by shear tests as caused by prerigor salting and massaging (Table 2). The taste panel data also give some impression of the degree of acceptability of the products and reveal that the tenderness and flavor of all samples were rated reasonably high. Juiciness scores were somewhat marginal and probably reiterate the absence of any adhering subcutaneous or intermuscular adipose tissue on the samples received by the panel. Based on prior meat tasting experience, the taste panelists revealed that nitrite-free processed hams were quite palatable but their flavor and texture did not resemble that of hams cured with nitrite. Panel members generally described the flavor and texture as that of uncured roasted pork.

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Ms received 3/10/79; accepted 7/1/79.

Journal Paper No. 7537 of the Purdue Agricultural Experiment Station.

The authors acknowledge with thanks the technical assistance of Loretta Gong, Glen May, James McCollum and David Hayes.

A Research Note

FATTY ACID STABILITY OF GULF OF MEXICO BROWN SHRIMP (Penaeus aztecus) HELD ON ICE AND IN FROZEN STORAGE

NESTOR R. BOTTINO, MARTHA L. LILLY and GUNNAR FINNE

ABSTRACT

The fatty acid composition of iced, frozen and frozen-glazed brown shrimp (*Penaeus aztecus*) was determined as a function of storage time. The iced shrimp were analyzed every third day for 18 days while both frozen sets were analyzed at regular intervals for 183 days. No significant changes in neither the iced, frozen nor the frozen-glazed shrimp could be detected.

INTRODUCTION

MANY STUDIES have been reported on the storage stability of shrimp held on ice. Most of these studies have concentrated on the combined action of tissue enzymes and microbial activity (Nair and Bose, 1964; Cobb and Vanderzant, 1971; Flick and Lovell, 1972). On the basis of data derived from these studies, specific assays have either been used or proposed as quality indices of shrimp held on ice. The potential spoilage of shrimp held in frozen storage, on the other hand, has been less investigated. Although there are abundant studies in the literature on the shelf preservation of fish lipids, this is not the case with lipids of shrimp. Bullard and Collins (1978) reported recently on the stability of Alaskan pink shrimp (*Pandalus borealis*) kept in carbon dioxide refrigerated sea water and in ice, but the only lipids analyzed were carotenoids. This scarcity of information prompted the present studies on the stability of shrimp fatty acids during both iced and frozen storage.

MATERIALS & METHODS

BROWN SHRIMP (*Penaeus aztecus*) were obtained directly from fishing boats in Aransas Pass, TX, and immediately packed in ice and brought to the Seafood Laboratory at the Texas A&M University Experiment Station and Extension Center, in Corpus Christi, TX. The shrimp were beheaded, thoroughly washed, sampled and divided into three groups. Group one shrimp were stored in an ice chest well mixed with twice their weight of ice. The ice chests were held in a walk-in refrigeration unit. The shrimp were sampled, drained, and re-iced every third day for a period of 18 days. Group two was packed in five 5-lb cardboard boxes and frozen at −20°C. Group three was packed and frozen as group two, but the boxes from this group were removed from the freezers after 6 hr, opened, the void spaces filled with water (glazing) and the boxes placed back in the freezer. The latter treatment is the standard way for frozen green headless shrimp production in Texas. The primary purpose of glazing is to prevent dehydration and toughening of shrimp meat during frozen storage. However, an additional benefit of glazing may be protection from oxidation. Both groups of frozen shrimp were sampled at 4, 14, 37, 92 and 183 days. Shrimp tails (shells on) belonging to 2–4 animals were thoroughly mixed and a 5-g aliquot was extracted for lipids with a chloroform:methanol (2:1, v/v) mixture. These mixtures were purged with oxygen-free nitrogen, sealed and sent to College Station for analysis. The total lipids were converted to fatty acid methyl esters, purified, identified and quantitated by duplicate analyses by gas-liquid chromatography as previously described (Bottino, 1975). Quantitative results with National Heart Institute Fatty Acid Standard D agreed with the stated composition data with the relative error of less than 5% for major components (>10% of total mixture) and less than 33% for minor components (<10% of total mixture).

Differences between fatty acid compositions were quantitatively determined by calculating the distance or D value between sets of data by the following formula

$$D_{jh} = [\sum_{i=1}^{n} (P_{ij} - P_{ik})^2]^{1/2}$$

where D_{jh} is the degree of difference between the *j*th and *h*th species and P_{ij} is the percentage of the total fatty acids in the *j*th species.

Table 1—Fatty acids of ice-stored brown shrimp, Penaeus aztecus

Fatty acid[a]	Storage time (days)[b] 0	3	6	9	12	15	18
	Weight %						
<14:0	T						
14:0	1.5	1.9	2.5	2.3	1.8	2.7	2.3
15:0	0.6	0.9	2.4	1.3	1.2	0.9	2.0
16:0	18.4	17.5	18.8	17.9	15.3	16.7	17.9
17:0	1.7	1.2	1.3	1.6	1.9	1.1	1.8
18:0	7.7	7.4	7.4	7.6	7.8	6.2	7.6
13:1	1.0	2.0	3.8	0.4	0.3	0.9	0.1
15:1	0.8	1.0		1.3	1.2	1.6	
16:1 ω7[c]	8.4	8.5	8.3	8.5	8.4	7.6	8.6
17:1	1.0	0.7	0.6	1.3	2.2	0.1	1.0
18:1 ω9[c]	16.0	14.6	15.6	16.0	15.2	14.1	16.6
20:1 ω9	1.7	1.3	1.2	2.0	2.0	2.0	2.2
16:2	0.5	0.8	0.2	0.6		0.2	
18:2 ω6	1.4	1.0	0.9	2.0	2.4	0.5	2.4
22:2 ω3?	0.5	0.3	0.4	0.7	0.5	0.5	0.4
18:3 ω3	T			0.3	0.1		0.1
20:3 ω3	0.2	0.2	0.2	0.2	0.1	0.2	0.2
18:4 ω3	0.3	T	0.1	0.2	0.2	0.2	0.2
20:4 ω6	5.6	6.2	6.9	6.4	7.0	8.7	6.4
20:4 ω3	0.3	0.3	0.3	0.1	0.1	0.3	0.5
22:4 ω6	1.4	1.5	1.1	0.7	0.9	1.0	0.8
22:4 ω3	1.0	1.3	0.6	0.4	0.7	0.6	0.5
20:5 ω3	18.5	19.5	17.5	17.6	19.4	21.3	17.6
22:5 ω3	1.9	1.8	1.4	1.3	1.6	1.7	1.5
22:6 ω3	9.5	10.1	8.5	8.8	9.7	10.9	8.7
Unknowns	0.1			0.5			0.4

[a] Chain-length: number of double bonds; ω = number of carbons from methyl end to first double bond counting from that end.
[b] Each sample composed of 2–4 individuals analyzed collectively.
[c] It may contain other isomers.

RESULTS & DISCUSSION

THE CONCEPT of distance or D value is a useful tool for determining whether there are significant differences between fatty acid compositions (Bottino, 1974). In our experience, the distance between the results of two gas-

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0022-1147/79/0006-1778$02.25/0
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Table 2–Fatty acids of frozen brown shrimp, Penaeus aztecus

Fatty acid[a]	Storage time (days)[b]					
	0	4	14	37	92	183
	Weight %					
<14:0	T	0.1	0.4	0.1	0.6	
14:0	1.5	1.7	2.3	1.6	1.7	1.1
15:0	0.6	1.8	2.8	0.7	1.5	0.6
16:0	18.4	15.6	16.3	14.6	16.1	15.3
17:0	1.7	1.2	1.7	1.9	2.5	1.5
18:0	7.7	6.7	7.2	7.7	7.8	8.3
13:1	1.0	2.9	0.7			
14:1					1.0	0.1
15:1	0.8			1.2		0.2
16:1 ω7[c]	8.4	7.4	8.2	7.5	7.6	7.0
17:1	1.0	0.8	1.4	1.7	1.2	1.0
18:1 ω9[c]	16.0	15.1	15.7	16.7	15.8	16.6
20:1	1.7	2.1	2.0	1.7	2.1	2.0
16:2	0.5			0.8		
18:2 ω6	1.4	2.1	1.1	2.3	2.4	2.6
22:2 ω3?	0.5	0.7	0.5	0.4	0.5	0.2
18:3 ω3	T	0.2		T	0.2	0.3
20:3 ω3	0.2	0.2	0.1	0.1	0.2	0.3
18:4 ω3	0.3	0.3	0.1	0.1	0.2	0.3
20:4 ω6	5.6	6.9	6.5	7.0	6.5	8.7
20:4 ω3	0.3	0.3	0.3	0.2	0.1	0.3
22:4 ω6	1.4	1.0	1.3	1.1	1.0	1.2
22:4 ω3	1.0	0.6	1.0	0.1	0.5	0.6
20:5 ω3	18.5	21.2	18.2	20.0	19.4	20.9
22:5 ω3	1.9	1.5	1.9	1.7	1.5	1.5
22:6 ω3	9.5	9.3	10.3	10.3	9.3	8.5
Unknowns	0.1	0.3		0.5	0.3	0.9

[a] Chain-length: number of double bonds; ω = number of carbons from methyl end to first double bond counting from that end.
[b] Each sample composed of 2–4 individuals analyzed collectively.
[c] It may contain other isomers.

Table 3–Fatty acids of frozen-glazed brown shrimp, Penaeus aztecus

Fatty acid[a]	Storage time (days)[b]					
	0	4	14	37	92	183
	Weight %					
<14:0	T	1.6	1.1	1.3	2.1	
14:0	1.5	2.3	1.5	2.5	2.1	1.1
15:0	0.6	2.4	0.8	1.2	1.2	0.9
16:0	18.4	17.8	16.1	13.3	17.7	15.7
17:0	1.7	1.7	1.8	1.7	2.0	2.0
18:0	7.7	8.4	7.2	7.2	8.4	8.1
13:1	1.0					0.1
15:1	0.8		0.8	1.6		0.3
16:1 ω7[c]	8.4	8.4	7.6	7.2	8.1	7.8
17:1	1.0	1.4	0.7	2.8	0.7	1.5
18:1 ω9[c]	16.0	16.3	14.9	14.3	14.9	16.8
20:1 ω9	1.7	2.1	2.2	2.6	1.6	2.0
16:2	0.5					
18:2 ω6	1.4	2.3	1.5	3.2	1.3	2.2
22:2 ω3?	0.5	0.3	0.6	1.5	0.7	0.6
18:3 ω3	T	0.3	T	0.6		0.2
20:3 ω3	0.2	0.2	0.2	0.6	0.2	0.2
22:3 ω6					0.4	0.6
18:4 ω3	0.3	0.1	0.2	0.6	0.1	0.3
20:4 ω6	5.6	6.5	7.3	6.8	6.7	7.9
20:4 ω3	0.3	0.3	0.5	0.8	0.3	0.3
22:4 ω6	1.4	0.1	1.0	1.6	1.4	1.0
22:4 ω3	1.0	0.4	0.9	1.0	0.5	0.9
20:5 ω3	18.5	18.7	20.3	17.7	18.2	18.1
22:5 ω3	1.9	1.3	1.4	1.4	1.8	1.3
22:6 ω3	9.5	7.4	10.5	8.5	7.9	9.6
Unknowns	0.1		0.9		0.2	0.5

[a] Chain-length: degree of unsaturation; ω = number of carbons from methyl end to first double bond counting from that end.
[b] Each sample composed of 2–4 individuals analyzed collectively.
[c] It may contain other isomers.

chromatographic analyses of the same sample of marine fatty acids is about 2.2 ± 1.8 (average ± standard deviation). The distance between the fatty acids of two biologically related samples, for example shrimp and its food, is about 10–15. Finally, the distance between two widely different fatty acid compositions is about 25–30 (Bottino, 1974).

In the present case, the distance D = 3.0 between the fatty acid compositions at 0 and 18 days of storage in ice (Table 1) is as small as the distance commonly found between two consecutive analyses of the same sample. The D values for the frozen and frozen-glazed samples between 0 and 183 days of storage (Tables 2 and 3) were 5.7 and 4.2, respectively. Again, these values are as small as D values found between repeated analyses of the same sample.

In conclusion, the results in Tables 1 through 3 indicate that oxidative deterioration of shrimp fatty acids did not occur to a measurable extent after 18 days of storage in ice or 183 days of storage in frozen conditions, with or without glazing.

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Ms received 5/19/79; revised 7/27/79; accepted 8/1/79.

Supported in part by the National Marine Fisheries Service, the Texas Agricultural Experiment Station, and the Sea Grant Program, Texas A&M Univ.

A Research Note

A LIQUID SCINTILLATION TECHNIQUE FOR MICROBIAL VITAMIN ANALYSES

M. N. VOIGT and R. R. EITENMILLER

ABSTRACT

A radiometric procedure for the assay of the B-vitamins was investigated. The method, based on the release of $^{14}CO_2$ from C-14 labeled glucose, gave proportional responses to vitamin concentration for thiamin, pyridoxine, and pantothenic acid. This procedure was not significantly faster than measuring increases in absorbance due to growth when bacteria and yeast were used as test organisms. However, for the protozoan assay of thiamin with *Ochromonas danica*, the analysis time was decreased from 4–5 days to 20 hours.

INTRODUCTION

MICROBIOLOGICAL METHODS for vitamin assay are based on the observation that certain microorganisms can reproduce only in the presence of specific vitamins. When aliquots of the sample containing the vitamin are added to the medium and the latter inoculated with the test organism, growth is correlated to the level of vitamin present. This growth can be measured either photometrically or the metabolic products can be measured (acids or CO_2). Over a certain concentration range, the measured response is directly proportional to the amount of vitamin present, and, within this range, the sample solution and reference solution can be compared accurately.

Most of the isotopic methods developed for vitamin analysis are directed at determining clinical vitamin status and not at analysis of natural materials. These methods generally utilize isotopically labeled vitamins, which are relatively expensive, and a competitive protein binding procedure. Such methods have been developed for riboflavin (Fazekas et al., 1974), biotin (Dakshinamurti et al., 1974; Shimada et al., 1972, 1969), folic acid (Herbert, 1972), and vitamin B_{12} (Mortensen, 1972). These assays usually require less than 2 hr and are not affected by antibiotics and antimetabolites (Herbert, 1972). Reinken (1972) developed an isotopic assay for vitamin B_6 using tyrosine decarboxylase and ^{14}C-tyrosine and applied this procedure to clinical samples. Carlucci and Bowers (1972) developed a $^{14}CO_2$ uptake method for measuring thiamin, biotin, and vitamin B_{12} using labeled carbonate and the diatom, *Cyclotella nana*. This technique decreased the normal assay time of 4–5 days in half. DeBlanc et al. (1972) developed a rapid and quantitative method for evaluating bacterial growth by measuring the $^{14}CO_2$ resulting from ^{14}C-glucose metabolism.

This study was designed to apply the procedure of De Blanc et al. (1972) to the analysis of B-vitamins. Therefore, the objective was to examine the feasibility of measuring the release of $^{14}CO_2$ from ^{14}C-glucose to decrease the time required to complete biological vitamin assay procedures.

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0022-1147/79/0006-1780$02.25/0
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MATERIALS & METHODS

THE FOLLOWING VITAMINS and corresponding test organisms were used: thiamin–*Lactobacillus viridescens* and *Ochromonas danica*; pyridoxine–*Saccharomyces uvarum*; and pantothenate–*Lactobacillus plantarum*. The parameters varied in the experiments were: incubation time (0.5–20 hr), glucose level in the basal media (media prepared as described in the literature or prepared omitting the glucose) and inocula size (diluted or not diluted). The vitamin analyses were conducted as previously described (Voigt et al., 1978a, b). Microbial growth was monitored spectrophotometrically at 620 nm and by the radioactivity of $^{14}CO_2$ released from the media. Culture dilutions, when used, were 1 ml culture to 99 ml saline for the Lactobacilli, 1 ml culture to 6.5 ml distilled H_2O for *O. danica*, and 1 ml culture to 1 ml H_2O for *S. uvarum*.

The isotopic procedure was based upon the incubation of the microbiological vitamin assays to which ^{14}C-glucose had been added (Bachrach and Bachrach, 1974; DeBlanc et al., 1972; Hill, 1972). The technique consisted of adding 0.2 μCi of D-(U-^{14}C)-glucose with a specific activity of 180 μCi/mmole (New England Nuclear, Boston) to the vitamin mixtures. Polyethylene liquid scintillation vials were used as assay vessels for the radiometric vitamin assays that employed bacteria and yeast, while 25 ml Erlenmeyer flasks were used for the assays that employed *O. danica*. After inoculation, the assay vessels were immediately sealed with rubber stoppers. Rolled paper cylinders (3 MM Whatman, 1 × 3 cm) coated with 2-phenylethylamine were suspended over the reaction solutions by wire inserted into rubber stoppers. Following incubation, 1 ml of 1N HCl was injected into the reaction vessels to ensure complete release of $^{14}CO_2$ during an additional 15 min incubation. The filter paper $^{14}CO_2$ traps were then transferred to scintillation vials containing 10 ml of a toluene scintillation cocktail (Beckman, Fullerton, CA) and counted in a Beckman Model LS-100 C Liquid Scintillation Counter. Control samples consisted of uninoculated assay media.

RESULTS & DISCUSSION

THE EFFECTS of inoculum size and incubation time on release of $^{14}CO_2$ and absorbance of growth of *L. viridescens* in thiamin assays was studied. In glucose-free media, diluted inocula yielded a lower CPM measurement after 14 hr incubation than did the assays which received nondiluted inocula after 0.5 hr incubation. After 14 hr incubation, the vitamin assays which received the larger inoculum did not elicit $^{14}CO_2$ release or absorbance responses that were proportional to the levels of thiamin present. The thiamin assays prepared with glucose and receiving nondiluted inoculum of *L. viridescens* developed proportional radioactivity and absorbance responses to thiamin dosage after 10 hr incubation. Since the radiometric technique required a minimum of 10 hr incubation to give a quantitive response, it was evaluated to be unuseful–since the procedure could not be completed within a normal 8 hr workshift. Figure 1 illustrates a comparison of the radiometric assays for thiamin using *O. danica* and *L. viridescens* as the test organisms. Diluted inocula were used and basal media contained glucose. The *L. viridescens* assays for thaimin were terminated after 16.5 hr, while the assays using *O. danica* were terminated after 20 hr. The incubation time of 20 hr for the radiometric technique using *O. danica* was usefully shorter than the 4–5 days required for measurement of absorbance.

For the pyridoxine assay employing *S. uvarum*, no proportional responses in either radioactivity or absorbance to

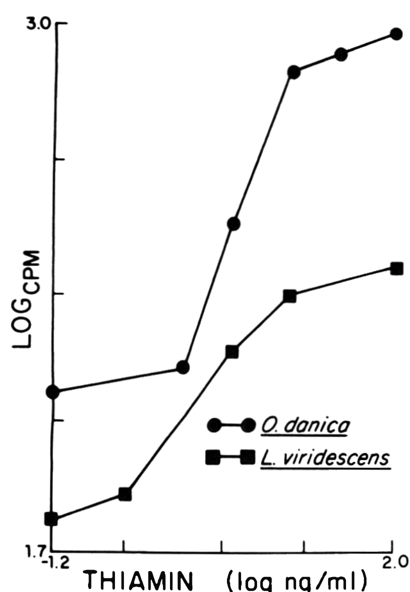

Fig. 1—Comparison of the $^{14}CO_2$ release (CPM) during the O. danica and L. viridescens thiamin assays. Four drops of inoculum preparations were used. The incubation times were 20 hr for O. danica and 16.5 hr for L. viridescens. Both media included glucose.

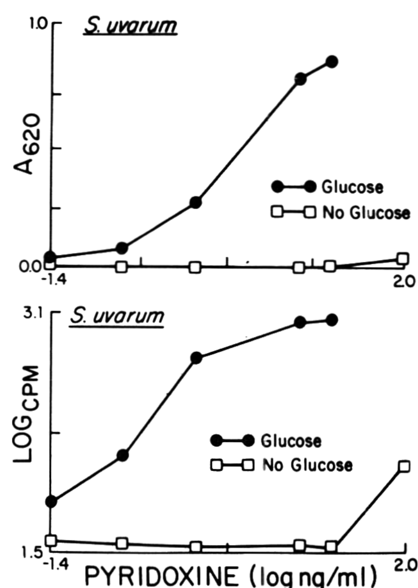

Fig. 2—Effect of the assay medium glucose content on the absorbance and the $^{14}CO_2$ release (CPM) of the S. uvarum pyridoxine assay. Four drops of inoculum preparation were used. Assay incubation time was 16 hr. Medium without glucose was prepared from the Difco Pyridoxine Y Medium formula omitting glucose. Top: Absorbance at 620 nm (A_{620}) data; Bottom: $^{14}CO_2$ release (Log CPM) data.

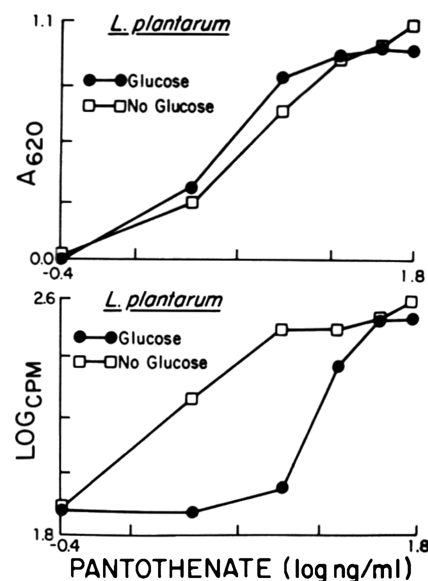

Fig. 3—Effect of the assay medium glucose content on the absorbance and $^{14}CO_2$ release (CPM) of the L. plantarum pantothenate assay. Four drops of inoculum preparation were used. Assay incubation time was 11.5 hr. Medium without glucose was prepared from the Difco Pantothenate Assay Medium USP formula omitting glucose. Top: Absorbance at 620 nm (A_{620}) data; Bottom: $^{14}CO_2$ (Log CPM) data.

pyridoxine dosage were evident in glucose-free media (Fig. 2). When glucose was added to the medium, dosage related increases in CPM and absorbance were obtained (Fig. 2). However, as with the thiamin assays using *L. viridescens*, the incubation time required to complete the radiometric *S. uvarum* assays for pyridoxine was too long to permit the analyses to be completed within an 8 hr workshift. Both the radiometric and absorbance techniques would require an overnight incubation.

Figure 3 illustrates the effects of the presence and absence of glucose in basal media on the growth and radioactivity responses of the *L. plantarum* assay for pantothenate. The absorbance of the growth slightly decreased in absence of glucose in the media. However, the absence of glucose in the media increased the response isotopically measured. Proportional responses of CPM to pantothenate dosage were evident after 7 hr of incubation, which was not usefully shorter than the overnight incubation used when monitoring absorbance.

Except for folate, protozoan methods have been developed for quantitating all of the B-vitamins (Voigt et al., 1978a). Since the protozoa possess more mammalian-like responses than bacterial or yeast to the various forms of the vitamins that occur in natural materials, they are the preferable test organisms to use in determining vitamin contents in foods (Voigt and Eitenmiller, 1978). The problem is that they require 4–6 days to complete vs 1 day for the bacteria and yeast. The radiometric procedure described can shorten the assay time for the protozoan methods to 1 day. For the radiometric procedure to be economically worthwhile, an inexpensive monitoring method is necessary. The use of a radiometric gas flow analysis system, such as the Bactec 460 (Johnson Laboratories, Inc., Cockeysville, MD) would avoid the costs of scintillation cocktail and vials. Future studies need to be completed using the protozoan, *Tetrahymena*, food samples and gas-flow analysis.

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Ms received 10/5/78; revised 7/27/79; accepted 8/1/79.

A Research Note

SPINNING OF PROTEIN ISOLATES WITH UREA AS SOLVENT

LARS GILLBERG

ABSTRACT

A new method for the spinning of biological materials is described in which urea is used as solvent and deionized water as coagulation medium. Rapeseed and soybean protein isolates and a mixture thereof were spun. The higher the temperature of the spinning bath, the faster the fibers coagulated. The influence of the pH of precipitation when preparing the protein isolates and the influence of the electrolyte composition of the spinning bath on the strength of the fibers are discussed. Starch was spun with the technique outlined using ethanol as spinning bath.

INTRODUCTION

THIS RESEARCH NOTE describes a method for the spinning of biological materials in which urea is used as solvent and water for the coagulation of the fibers. Although it has been known for a long time that urea is a good solvent for proteins, no study treating the spinning of biological materials dissolved in urea seems to have been published previously.

MATERIALS & METHODS

Preparation of the rapeseed protein isolates (RPI)

Previously heat-treated and defatted rapeseeds were extracted at pH 11.1. The undissolved material was separated and the dissolved proteins precipitated in one or two steps by the addition of carboxymethylcellulose (CMC) or sodium hexametaphosphate (HMP) and an acid as is shown schematically in Figure 1. The process is described in detail by Gillberg (1978). By this technique the polyacids and all of the proteins extracted from the meal, plus the polyacids added, are recovered in the protein isolates (Lönnerdal et al., 1977; Åman and Gillberg, 1977; Gillberg, 1978; Gillberg and Törnell, 1976).

The isolates, which were prepared on a large scale, were neutralized to pH 7 and then spray dried. The neutralization was carried out as the major proportion of the isolates was to be used for other purposes than the one described in this research note. Before use in the present study the protein isolates were suspended in deionized water and the pH of the suspensions was adjusted to the pH where the isolates had a minimum in nitrogen solubility. The isolates were then recovered by centrifugation and lyophilized.

Preparation of the soybean protein isolates (SPI)

Four hundred twenty grams of defatted non-heat treated soybean meal (Sojafluff 200W, Central Soya) were dispersed in 3,600 ml deionized water at room temperature, and 0.2M of aqueous sodium hydroxide was added until the pH of the dispersion was 10. The dispersion was continuously agitated for 60 min during which time its pH was kept constant by the addition of small amounts of sodium hydroxide solution. A total amount of 600 ml of sodium hydroxide solution was added. Undissolved material was separated by centrifugation. The extract was then divided into three parts, from each of which a protein isolate was prepared. SPI(4.8) and SPI(4.5) were prepared by adding hydrochloric acid to the meal extract, under agitation, until the pH of the mixture was 4.8 and 4.5, respectively. SPI(CMC) was prepared by adding 26 ml of a 1%-aqueous solution of CMC (Cellugel 3,000 Special, SCA, Sundsvall, Sweden) per 100 ml of the soybean meal extract and then, under agitation, adding hydrochloric acid until the pH of the mixture was 3.8. The isolates were recovered by centrifugation and lyophilized.

The (S+R)PI isolate was prepared by mixing equal amounts of lyophilized SPI(CMC) and RPI(D) isolates.

Preparation of the spinning dopes

The spinning dopes were prepared by mixing 10g of protein isolate with the following amounts of urea (grams) and water (ml): RPI(A) 26, 26; RPI(C) 26, 26; RPI(D) 35, 35; SPI(4.8) 30, 30; SPI(4.5) 35, 35; SPI(CMC) 46, 46; and (S+R)PI 42, 42. The mixtures were stored with occasional stirring for 2–16 hr after which periods the protein isolates were completely dissolved. After degassing the solutions by centrifugation for 1 min at 3,100 × G, clear spinning dopes were obtained.

Method of spinning

The various spinning dopes were transformed to a tube of polymethacrylate and pressed through a spinneret containing 60 holes, each with a diameter of 0.1 mm, out into the spinning baths. The filaments spun were collected on two reelers, with adjustable speeds, between which the filaments could be stretched.

Deionized water with temperatues in the range 20–70°C, with no or small amounts of NaCl or $CaCl_2$, was used as coagulation medium.

RESULTS & DISCUSSION

THE AMOUNT OF UREA and water needed for the preparation of spinning dopes with comparable rheological properties varied with the type of protein isolate. The higher the carbohydrate content of the isolates the more urea and water were needed for the preparation of spinning dopes with comparable rheological properties.

From visual observations it was observed that the jets of spinning dope coagulated within a distance of 15 cm from the spinneret and that the velocity of the coagulation process increased with the temperature of the spinning bath.

For all protein isolates, except the SPI(4.8) isolate, it was found that the lower the ionic strength of the spinning bath the stronger were the filaments. When the SPI(4.8) isolate was spun stronger filaments were formed in 0.01M $CaCl_2$ than in deionized water as spinning bath.

The filaments spun could be stretched to about twice their original length without breaking.

Due to the high dielectric constant of urea, the intensity of all secondary attraction forces (hydrogen bonding, electrostatic- and dispersion forces) are lower in urea solutions than in water and other liquids with a lower dielectric constant. When the spinning dopes came in contact with the spinning bath the urea was released into the spinning bath and the protein isolates precipitated probably due to the increasing intensity of the secondary attraction forces between the molecules of the protein isolates.

The urea content of the spinning bath had little effect on the velocity of the coagulation of the fibers. A separate experiment showed that the fine jets of spinning dope coagulated, although not as rapidly as in deionized water, even when the spinning bath contained 10% urea by weight.

The pH of precipitation used when preparing the protein isolate to be spun, plays an important role for the recovery of the protein isolate as fibers. When using deionized water

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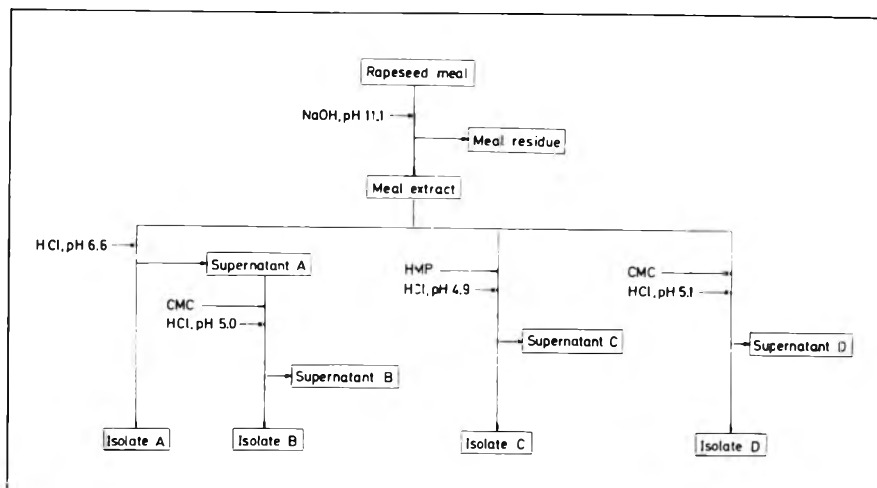

Fig. 1—Scheme for the preparation of the rapeseed protein isolates.

as spinning bath, highest recovery will be obtained when the protein isolate is prepared at a pH of precipitation where the protein isolate has its lowest solubility in deionized water (its iso-electric point). The further the pH of precipitation is from the iso-electric point of the protein isolate, the more the fibers formed will tend to dissolve in the spinning bath. To exemplify this it can be mentioned that when the RPI(C) and RPI(D) isolates neutralized to pH 7 were dissolved in urea solutions and spun into deionized water, the fibers formed soon disintegrated in the spinning bath.

Salt present in the spinning bath will tend to screen fixed charges and thereby weaken electrostatic interactions between the molecules of the filaments. This fact leads to the result that when a protein isolate, prepared by precipitation to its iso-electric point is spun, the strongest filaments will be obtained in a spinning bath with the lowest possible ionic strength. This was found to be true for all protein isolates spun at their iso-electric points.

However, when a protein isolate prepared with a pH of precipitation different from its iso-electric point is spun the presence of a low concentration of salt in the spinning bath may have a beneficial effect on the strength of the fibers formed. Selective ion binding may neutralize the excess charge of the protein isolate, thereby decreasing the number of sites with repulsive forces between the molecules of the protein isolate, which leads to a decreased solubility of the protein isolate (Gillberg, 1978) and thereby stronger fibers. When a protein isolate prepared using a pH of precipitation below its iso-electric point is spun, the strongest filaments will be obtained in a spinning bath containing a low concentration of a salt the anions of which have a considerably stronger tendency than its cations to bind to the molecules of the protein isolate. Similarly, when a protein isolate prepared at a pH of precipitation above its iso-electric point is spun, the strongest filaments will be obtained in a spinning bath containing a low concentration of a salt the cations of which have a considerably stronger tendency to bind to the protein isolate than its anions. This explains why stronger filaments were formed in 0.01M $CaCl_2$ than in deionized water when the SPI(4.8) isolate was spun.

Filaments produced from the SPI(4.5) were stored in deionized water without any sign of deterioration. In this respect they differed from filaments spun from a soybean protein isolate using the spinning technique described by Boyer (1953). Such filaments decomposed in deionized water but were stable in physiological and 4.7% saline.

Attempts were made to use the Boyer technique for the conversion of the rapeseed protein isolates to fibers. It was, however, impossible to completely dissolve the rapeseed protein isolates entirely in alkali. When attempts were made to dissolve 10g of the respective rapeseed protein isolates in 55–100 ml aqueous sodium hydroxide, gels were formed. By heating such gels to temperatures in the range 30–70°C, most of the gels were broken. Upon centrifugation of such broken gels a sediment was always obtained above which there was a clear solution. The fibers formed when such clear solutions were pressed through spinnerets into acid baths were very weak and broke upon a gentle touch with a glass-rod.

No attempts were made to spin other protein isolates or mixtures of protein isolates with urea as solvent than those described in this research note. However, it seems most likely that all protein isolates, mixtures of protein isolates, and other materials dissolvable in urea and precipitable in a medium in which urea is solvable successfully can be spun with the technique outlined (e.g. carbohydrates and nucleic acids). Thus, filaments of starch were formed when starch dissolved in urea and water was spun in 85% ethanol.

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Ms received 12/6/78; revised 5/18/79; accepted 5/27/79.

The study was carried out at the Dept. of Chemical Technology, The Lund Institute of Technology, Lund, Sweden.

Financial support from the Swedish Board for Technical Development is gratefully acknowledged.

A Research Note
FERMENTATION AND RELATIVE NUTRITIVE VALUE OF RICE MEAL AND CHIPS

PENKWAN TONGNUAL and MARION L. FIELDS

ABSTRACT

A natural lactic acid fermentation of rice meals produced a significant ($P < 0.05$) increase in isoleucine, lysine, relative nutritive value and riboflavin content of the meals. Niacin and thiamin decreased significantly ($P < 0.05$) during fermentation. Although the levels of isoleucine, lysine and riboflavin decreased during making of rice chips, the chips still contained more of these nutrients than the control chips made from unfermented meal. Chips made from fermented rice meal and control rice meal were generally acceptable, thus showing that acceptable foods could be made from fermented rice meal.

INTRODUCTION

IN STUDIES comparing the effect of germination and fermentation upon cereals, Hamad and Fields (1979) reported that the % relative nutritive value (RNV) increased significantly ($P < 0.05$) in naturally fermented wheat, barley and rice and highly significantly ($P < 0.01$) in millet and maize. Fermented rice also increased significantly ($P < 0.01$) in lysine.

Hamad (1978) confirmed that these cereal fermentations were dominated by lactic acid bacteria as Frazier had reported (1967). Hamad isolated *Lactobacillus fermentum, Lactobacillus cellobiosus* and *Pediococcus acidilactici* from fermenting wheat and corn meals.

According to Van Veen and Steinkraus (1970), organoleptic properties of fermented foods were generally improved over the raw ingredients. In studies conducted in Ecuador, fermented rice increased in soluble nitrogen and riboflavin.

The hull is the outer structure of the rice kernel. When the outer layers are removed, proteins, fats and vitamins in the remaining kernel decrease, whereas the carbohydrate increases (Marz and Beachell, 1969). The hulls contain thiamin, riboflavin and niacin, essential nutrients for cereal eaters. Cellulose and hemicellulose are part of the crude fiber found in hull. Additional fiber would be a valuable component in fabricated foods such as rice chips.

Therefore, this research was instituted to determine the nutritive value of fermented rice meal. Separate batches of rice meals were prepared with and without hulls. Rice chips were prepared from these meals to determine acceptability and nutrient quality.

MATERIALS & METHODS

Sample preparation

Rice (*Oryza sativa*) with hulls was purchased from Central Foods Stores at the University of Missouri, Columbia. Rice was milled in a laboratory food grinder to remove the hulls to produce rough rice or brown rice, as described by Greddles (1951). Other rice was used with the hulls still attached to the grain. Rice samples were ground in a Thomas Wiley laboratory mill (Model 4, Arthur Thomas Co.,

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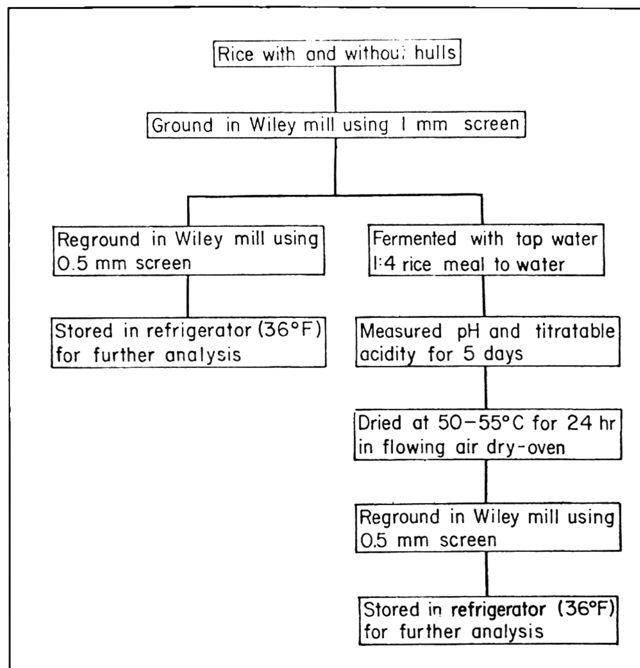

Fig. 1—Preparation procedure for making rice meals.

Philadelphia, PA) using a 1 mm screen after being dehulled. Rice samples were divided into two portions. One portion was reground using 0.5 mm screen and stored at 2°C until it was analyzed. This portion was designated as the control sample. The other was allowed to undergo natural lactic fermentation. This was done by adding tap water (1:4, w/v) at room temperature (about 25°C), as illustrated in the flow chart, Figure 1. Titratable acidity and pH were measured daily. After 5 days, the fermented rice was dried at 50–55°C for 24 hr in a flowing air drying oven (Freas Model 835, Precision Scientific, Chicago, IL). Dried samples were ground in the Thomas Wiley laboratory mill using a 0.5 mm screen and stored (2°C) for further analysis. The fermentations were replicated four times.

pH and titratable acidity determinations

pH was measured by using a Beckman Zeromatic pH meter. Titratable acidity was determined by titrating the samples with 0.1N NaOH to an endpoint of pH 8.2. The acid in the fermented rice sample was expressed as lactic acid.

Determination of relative nutritive value

The procedure was outlined by Wang and Fields (1978).

Vitamin determination

Preparation of samples for riboflavin and niacin were according to Association of Vitamin Chemists (1966). Preparation of thiamin samples followed the method of Sarett and Cheldelin (1944). Procedures for preparation of stock cultures, standard solutions and assay tubes were recommended by Difco (1977). Vitamin assays were replicated four times.

Amino acid determination

Preparation of samples for assays followed the method described by Ford (1964) whereas the procedures for isoleucine and lysine analyses were Difco (1977).

Preparation of rice chips and sensory evaluation

Both the preparation of the rice chips and the sensory evaluation of the chips are given by Tongnual (1978).

Table 1—Means[a] for selected nutrients in rice meals and chips

	Rice Meals			
	Rice with hulls		Rice without hulls	
Nutrients	Control	Fermented	Control	Fermented
Isoleucine mg/g N	1.22c	8.28a	1.05c	6.65b
Lysine mg/g N	2.96c	14.80a	2.81c	9.24b
Niacin mcg/g	65.98a	44.40b	63.08a	41.60b
Riboflavin mcg/g	0.87b	1.47a	0.80b	1.35a
Thiamin mcg/g	0.60a	0.35b	0.51a	0.35b
RNV (%)	66.22d	80.50b	71.43c	83.84a

	Rice Chips			
	Rice with hulls		Rice without hulls	
Nutrients	Control	Fermented	Control	Fermented
Isoleucine mg/g N	0.57b	7.10a	0.85b	6.46a
Lysine mg/g N	2.29a	6.85b	2.71a	7.72b
Riboflavin mcg/g	0.85c	1.30a	0.79c	1.13b
RNV (%)	52.00a	62.90b	56.04c	65.12bc

[a] N = 4. Values calculated on dry basis. Means followed by the same letter are not significantly different (P < 0.05).

Statistical analyses

Data were analyzed by analysis of variance (Snedecor and Cochran, 1967) and by Duncan's new multiple range test (Duncan, 1955).

RESULTS & DISCUSSION

pH and titratable acidity

At the end of the fifth day, the pH of rice with and without hulls was 3.8 and the titratable acidities were 0.56% and 0.51%, respectively.

Fermented rice meals

Fermented rice meals with and without hulls increased significantly ($P < 0.05$) in isoleucine, lysine, and % RNV but decreased significantly ($P < 0.05$) in niacin and thiamin (Table 1). Riboflavin, however, was the only vitamin to increase significantly ($P < 0.05$) after fermentation regardless of whether or not the rice meal contained hulls.

Rice chips

The only values which were not significantly different from the controls were the RNV of chips made without hulls (Table 1). The fermentation process produced a more nutritious meal and the nutrients in the chips made from fermented meal were higher than the nutrients in chips made from nonfermented rice.

The mean scores for rice chips made from fermented rice with hulls (3.80), without hulls (3.89) and control rice without hulls (3.59) were in the "like" category while the mean scores of rice chips made from control rice with hulls (3.23) were in the "neither like nor dislike" category. The mean scores for rice chips made from fermented rice with and without hulls were significantly ($P < 0.05$) higher than those of the control chips. This is in agreement with Van Veen and Steinkraus (1970) who reported that organoleptic acceptability of fermented foods was generally higher than that of the cooked raw materials. Some consumers commented that the chips were good but lacked flavor. The hedonic rating might have been higher if the chips had been seasoned. Development of products and sensory evaluation need further study but the data in this study indicate that nutritious foods can be made from fermented rice.

Cellulose and hemicellulose are part of the crude fiber found in the hull. With the recent emphasis on increasing the crude fiber in the diet of consumers, chips made with hulls could be advertized as such. The consumer could have a snack item that provided increased isoleucine, lysine, riboflavin as well as the additional crude fiber, thus providing a more nutritious snack than snacks made from nonfermented rice.

The data presented here suggest that rice chips might be an acceptable item in the United States and certainly in rice-eating populations. Also, it might be possible for other food items to be produced from fermented rice meal.

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Ms received 12/30/78; revised 7/4/79; accepted 7/10/79.

Contribution from the Missouri Agricultural Experiment Station Journal Series No. 8195.

A Research Note

INHIBITION OF POLYGALACTURONASE SOFTENING OF CUCUMBER PICKLES BY CALCIUM CHLORIDE

R. W. BUESCHER, J. M. HUDSON and J. R. ADAMS

ABSTRACT

Cucumbers were fermented and held for 30 days in equilibrated solutions containing low NaCl (11–13° salometer) and high NaCl (18.5–20.5° salometer) with and without $CaCl_2$ and/or polygalacturonase (PG). $CaCl_2$ and PG treatments did not interfere with fermentation. Separately, $CaCl_2$ enhanced firmness of pickles while PG was effective in causing excessive softening. When $CaCl_2$ was present in PG containing solutions, softening by PG was inhibited.

INTRODUCTION

EXCESSIVE SOFTENING of cucumbers during fermentation and brine storage causes severe losses annually to the pickle industry. Breakdown of pectic substances by polygalacturonase (PG) has been shown to be a major contributor to softening (Bell et al., 1950; Demain and Phaff, 1957; Fabian and Johnson, 1938). Bell and Etchells (1961) demonstrated that PG mediated softening was directly related to NaCl level. In 6% NaCl, softening was twice as severe as it was in pickles held in 20% NaCl. However, prevention of softening by high salt levels is not possible since low salt levels are necessary for rapid and complete fermentation (Etchells et al., 1964). In addition, if methods were available for inhibiting enzymatic softening, utilization of low levels of salt for storage would allow for substantial reductions of salt in waste water (McFeeters et al., 1978).

Several plants produce substances which inhibit PG activity (Bell and Etchells, 1958; Bell et al., 1965a, b) but none is available or accepted for commercial use. Fleming et al. (1978) observed that calcium in fermentation brine was beneficial to the firmness of sliced and small whole pickles. Calcium is used extensively by the food industry to enhance firmness of several processed products, but information is lacking on its effects on firmness in the presence of active PG. Bateman and Lumdsen (1965) suggested that calcium pectate was resistant to PG and that its formation accounted for enhanced disease resistance during maturation of red kidney beans. In the study presented herein we observed the efficacy of $CaCl_2$ on retarding softening of cucumbers during fermentation and storage in two levels of NaCl and in the presence of high PG activity. Our data indicate that softening is greatly retarded when $CaCl_2$ is present in cucumber pickling brine.

MATERIALS & METHODS

Source

Pickling cucumbers were obtained at the time of delivery to Atkins Pickle Company (Atkins, AR). Fruits (4.5–5.5 cm diameter) were sorted for uniformity and freedom from defects.

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0022-1147/79/0006-1786$02.25/0
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Brining treatments

Brine solutions (1.5L) containing 4.5% NaCl, 4.5% NaCl with 0.1M $CaCl_2$, 9.0% NaCl, and 9.0% NaCl with 0.1M $CaCl_2$ were added to 3.8L glass containers. The tap water source used to prepare brines contained less than 100 ppm calcium. Each solution also contained 0.1% K-sorbate to inhibit yeast and mold growth. From prior experiments we observed that this level of K-sorbate did not interfer with fermentation or pickle quality but was effective in preventing loss of acidity. Brine pH was adjusted to 3.1 with acetic acid, cucumbers (9–10) were then packed to fill the jars and held submerged. Pack-out ratio was about 60% cucumber to 40% brine volume. After 24 hr, solution pH was raised to 5.0 with NaOH and then inoculated with about 10^8 cells of *Lactobacillus plantarum* (Etchells et al., 1964; 1973). To determine the effect of $CaCl_2$ on PG mediated softening, 150 mg of PG (ICN Nutritional Biochemicals pectinase) was added to jars containing high and low NaCl levels alone and some containing $CaCl_2$. This level of PG provided an activity about 1000 times greater in the brines than what is considered to be strong and would need to be removed from contact with cucumbers (Bell and Etchells, 1955). Each of the eight treatments was duplicated.

Assays

Titratable acidity (expressed as % lactic acid), pH, and optical density of brine were determined as described by Etchells et al. (1964). Salt levels were estimated by determining degrees salometer. PG activity in brines was determined as described by Bell and Etchells (1955). Firmness was evaluated with a U.C. Fruit Firmness Tester (Western Industrial Supply, Inc.) equipped with an Ametek LKG-14 gage and a 5/16-inch tip. Pickles were cut in half and evaluated for soft centers, bloating, and uniformity of color.

RESULTS & DISCUSSION

THE PRESENCE of $CaCl_2$ and/or PG in brine did not interfer with fermentation as observed by rates of acid production and changes in optical density (data not shown). The greatest difference in rate of fermentation occurred between the two levels of NaCl with the high salt having the slowest rate. Fermentation in the low salt treatment was essentially completed after 12 days while in the higher salt level, fermentation was completed in about 17 days. It should be noted that the high salt level in this investigation would actually be considered low by industry standards, especially for storage of pickles. Addition of $CaCl_2$ increased salometer readings by 1.5–2.0° and reduced pH of the brine, but did not affect the level of titratable acidity (Table 1). PG activity was not observed in treatments unless it had been added, then activity was consistently high and appeared stable.

Both $CaCl_2$ and PG dramatically altered pickle firmness, regardless of NaCl level. $CaCl_2$ enhanced firmness of pickles by 1.7 and 2.0 kg (3.7 and 4.4 lb) in low and high NaCl levels, respectively. PG caused substantial reductions in firmness. Firmness was reduced by PG most in low NaCl conditions which supports previous results by Bell and Etchells (1961). With $CaCl_2$ present in brines containing PG, softening was inhibited. In low NaCl conditions, firmness of pickles from the $CaCl_2$ + PG treatment was not significantly different from firmness of the control and $CaCl_2$ treatments while in high NaCl, firmness of $CaCl_2$ + PG treated pickles was equal to those treated with $CaCl_2$ alone and was significantly greater than the control.

Since softening did not occur in brines containing $CaCl_2$ even though PG activities were very high, the method to

Table 1—Characteristics of spent brines and pickles 30 days after initiating fermentation as influenced by NaCl level, $CaCl_2$ and PG

Treatment of fermentation brine		Characteristics of spent brine				Pickle firmness[b] (kg)
		Degrees salometer	pH	Acidity (% lactic)	PG units[a]	
Low salt	Control	11.3	3.40	1.21	0	7.9c[c]
	$CaCl_2$	13.0	3.18	1.11	0	9.6ab
	PG	11.0	3.43	1.15	>280	2.3e
	$CaCl_2$ + PG	13.0	3.23	1.07	264	8.9bc
High salt	Control	18.5	3.23	1.02	0	8.0c
	$CaCl_2$	20.3	3.08	1.00	0	10.0a
	PG	18.8	3.15	1.00	225	5.8d
	$CaCl_2$ + PG	20.5	3.05	1.04	>280	10.0a

[a] Units as defined by Bell and Etchells (1955)
[b] Average of 18 pickles per treatment
[c] Mean separation by L.S.D. test; values with same letters are not significantly different.

predict softening from PG activity as described by Bell and Etchells (1955) would not be meaningful in brines containing $CaCl_2$. $CaCl_2$ did not appear to inhibit PG activity although it was removed by dialysis before assaying for PG and PG might be reversibly inhibited. In contrast, Ca^{++} might be binding with demethylated pectins to form Ca-pectates which are then resistant to degradation by PG (Bateman and Lumdsen, 1965). Regardless of the mode of action, $CaCl_2$ was highly effective in preventing softening in the presence of exceptionally high PG activity.

While soft centers were not a problem even when PG was present, it was noted that $CaCl_2$ treatments improved internal firmness. External color was not affected and bloating was not a problem in any treatment. Internal color was better in all pickles held in low NaCl than in high NaCl conditions since in low NaCl they were mostly uniform in olive color while in high NaCl, considerable amount of white tissue was apparent.

Utilization of $CaCl_2$ in brines to retard PG mediated softening would be economical, easily adapted to present systems and appears to provide the potential for reducing levels of NaCl in pickling operations.

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Ms received 5/26/79; revised 7/27/79; accepted 8/1/79.

A Research Note

pH AND BUFFERING CAPACITY OF CANNED GREEN PEPPERCORNS

JOHN J. POWERS and KATHLEEN SHINHOLSER

ABSTRACT

The pH, total acidity, and buffering capacity of canned green peppercorns against increases and decreases in pH were determined. The mean pH of nonacidified, canned green peppercorns was 6.51. The total acidity was 0.048%, calculated as citric acid. The buffering capacity between the original pH of the peppercorns and pH 8.35 was 0.188 milliequivalents. To lower the pH to 4.4, from 219–247 mg citric acid per 100g fill-in weight of peppercorns is required. Canned green peppercorns acidified to pH 4.0 retained much of the pungency and pepperiness of fresh green peppercorns.

INTRODUCTION

INCREASED INTEREST in gourmet cookery is leading to increased importation of canned green peppercorns for dishes *au poivre vert*. Peppercorns are a low-acid fruit; consequently, they are acidified for canning so as to permit boiling-water processes to be used. Prior to May 15, 1979, acidification to below pH 4.6 exempted them from the thermal-processing regulations for low-acid foods (Code of Federal Regulations, 1975); but on that date, Good Manufacturing Practices were also instituted for acidified foods (Federal Register, 1979). The new regulations, in effect, require that sound thermal processes be established and adhered to in the canning of acidified foods. Since data on the pH of peppercorns are limited, this note reports on the pH and buffering capacity of Madagascan peppercorns and provides estimates of the amount of citric acid needed to bring the pH to different levels.

EXPERIMENTAL

FIFTY 200-g cans of peppercorns were processed in a commercial cannery in Tamatave, People's Republic of Malagasy, and airfreighted to Athens, GA. Although some of the cans were crushed as a result of abuse during the 6 wk in transit, and the product had received only a 13-min process in boiling water, none of the cans showed any signs of microbial growth. Part of the shipment consisted of peppercorns which had received various acidification treatments. Details will not be given as to these treatments since the purpose of this note is to report on the normal pH of green peppercorns and the amount of acid needed to lower the pH to various levels.

Canning

The peppercorns were stripped from the cluster and winnowed on small screens by hand to remove chaff. Aside from removing foreign matter, the women carrying on the stripping and screening operations also graded the raw peppercorns by removing corns not of the proper maturity for canning as green peppercorns. The graded peppercorns were then washed in a ripple washer. In the particular cannery where the peppercorns were packed, the peppercorns are next given a preliminary cook in a citric acid solution to soften the corns, remove surface coatings, and to bring about some reduction in pH. For the nonacidified peppercorns analyzed here, the preliminary cook was in plain water.

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0022-1147/79/0006-1788 $02.25/0
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The softened peppercorns were removed from the boiling water with sieves and immediately filled into 200-g cans. Each can was check weighed, which is also a standard practice in this cannery for its commercial packs. The cans then were filled with 2% brine at 95°C, immediately sealed, and conveyed to a continuous spiral water cooker operated at 99–100°C. The temperature of the contents as the cans entered the waterbath was 66–68°C. The residence time in the waterbath was 13 min; the center temperature of the contents of the cans was 91–93°C upon exiting from the cooker. From the continuous cooker, the cans went directly to a continuous water cooler.

Analyses

The drained weight of six cans was determined so we would know the respective proportions of peppercorns and packing brine. The pH of the peppercorns and packing brine was determined separately. The pH meter was standardized against buffers at pH 4.0 and 7.0, and the pH meter was checked frequently to be sure it was still properly standardized. To determine total acidity and buffering capacity, 25g of peppercorns were made into a slurry with a small amount of water. Pureed peppercorns by themselves were so thick that pH equilibrium upon the addition of each increment of base was extremely slow even with vigorous stirring. The peppercorn slurry was titrated to pH 10 with a 0.094N NaOH solution so as to be able to observe the form of the titration curve beyond the neutralization point. Total acidity was calculated as citric acid, assuming neutralization occurred at pH 8.35. The pH and buffering capacity of the packing brine were determined as above except 25 ml were measured out volumetrically. To determine the amount of citric acid needed to lower the pH to various levels, 25g of peppercorn slurry and 25 ml of packing brine were separately titrated with 0.068N citric acid solution.

Based upon the average drained weight of peppercorns and the volume of packing brine, calculations were then made as to how many milligrams of citric acid are needed per 100g peppercorns to reduce the pH to different levels.

RESULTS

THE MEAN pH of the peppercorns was 6.51. Since the product had been canned in 2% brine, and if peppercorns are like some other products, the pH of which is reduced about 0.1 unit upon the addition of salt (Pray and Powers, 1966; Lopez and Schoenemann, 1971; Powers, 1976), then the mean pH of the peppercorns as the raw product might be as high as 6.6. The pH of the raw product probably varies by season and maturity as Sane et al. (1950) pointed out for pimiento peppers, which was reaffirmed and extended by Flora et al. (1978). The pH and the standard deviations of the means for peppercorn slurry and the packing brine are listed in Table 1. If our data are representative, then the pH of nonacidified canned peppercorns may be expected to range from 6.19–6.83.

One peculiarity noted in making pH measurements was that the slurry became higher in pH the longer it stood. The pH values given in Table 1 are for determinations made within 15 min of pureeing the peppercorns. By chance, some samples were not discarded right away and the pH was determined again later. The new pH values were higher than the original ones. There were not enough cans left to study the matter thoroughly, but it was observed that in the course of 3 hr, the pH ultimately rose by about 0.4 units, progressively with elapsed time. One possibility might be that pureeing brought components into contact with each other so that they could react. Another possibility is

Table 1—pH and buffering capacity of canned peppercorns and the packing brine

	Peppercorns			Packing brine		
	No. of determinations	Mean	Standard deviation	No. of determinations	Mean	Standard deviation
pH	27	6.51	0.160	19	6.47	0.116
Milliequivalents to raise pH to 8.35	14	0.188	0.023	10	0.229	0.047
Milliequivalents to lower pH to 4.4	12	0.560	0.109	9	0.505	0.064
Grams peppercorn per 200-g can	6	112.8	4.70	–	–	–
Grams packing brine per 200-g can	–	–	–	6	110.9	8.19
% drained weight		50.4%				

that adsorption onto starch or other particles was involved, but the binding force must have been quite weak because the total acidity values did not differ according to the length of time the slurry stood prior to titration. A similar phenomenon was not observed for the packing brine. Frequent standardization of the pH meter ruled out "drifting" of the instrument as the casue of the increase in pH observed.

The buffering capacity (van Slyke, 1922) of the brine seemed to be slightly greater than that of the peppercorns themselves. The buffering capacity was not determined from the mean pH and titration value but by averaging the buffering capacity of each sample. The buffering capacity was less than reported for pimientos (Sane et al., 1950) and tomatoes (Powers and Godwin, 1978). This perhaps is to be expected since the pH is considerably higher than that of tomatoes or pimientos.

Calculated as citric acid, the acidity of the peppercorns was 0.048%.

Table 1 gives the milliequivalents required to lower the pH to 4.4. For a so-called 200-g can, 0.16g of anhydrous citric acid would be needed to lower 112.8g of peppercorns to pH 4.4. Enough acid has to be added, however, so that at equilibrium the pH of the packing brine will be at 4.4 too. For 110.9 ml of brine, this would require 0.143g citric acid. For the contents of a can, 0.305g of citric acid is thus required. The fill-in brine therefore has to contain 0.305g per 110.9 ml or 0.275g anhydrous citric acid per 100 ml of packing brine. Cans of 850-g capacity may have a drained weight as high as 65%. Upon the same basis as above, the concentration of citric acid in the acidifying brine would then need to be 0.40g per 100 ml. Assuming that a canner would aim for an average pH of 4.4 to minimize the risk of individual cans being above 4.59, then from 219–247 mg anhydrous citric acid would be required per 100g fill-in weight of peppercorns. Aside from allowing leeway so that individual cans will not be too high in pH, Johnston (1975) has suggested other considerations which canners should keep in mind.

The peppercorns which had been acidified with known amounts of citric acid agreed in general with our estimates of the amount of citric acid needed to bring the pH below 4.6. Strict comparisons cannot be made because the acidified peppercorns received their preliminary cook in a weak citric acid solution, which is the usual practice in the cannery where they were packed. A composite titration curve for changes in pH above the pH of canned, nonacidified peppercorns and for acidification of peppercorns is given in Figure 1. From the curve and the data given in Table 1, estimates can be made of the amount of citric acid needed to reduce the pH to levels other than 4.4, given above. The

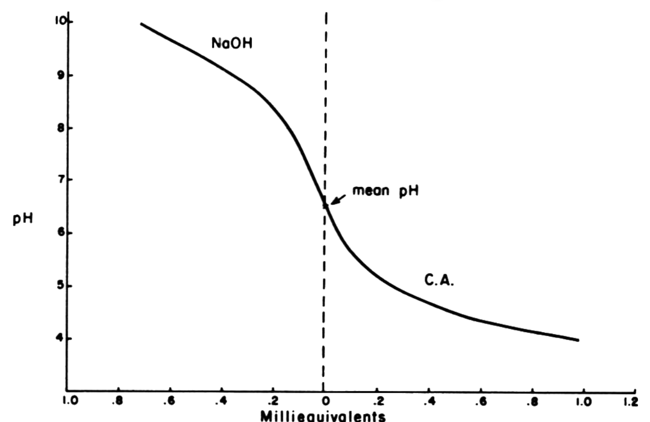

Fig. 1—Composite curve for peppercorn slurry titrated with 0.094N NaOH and 0.063N citric acid solution (curve C.A.).

acidified peppercorns seemed to retain their pungency and pepperiness as compared with fresh peppercorns. Canned products above pH 4.6 were not examined organoleptically.

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MS received 5/26/79; accepted 7/20/79.

The analyses were carried on with State appropriated funds. Appreciation is expressed to Mr. Warren Gaffney Jr. of Malagasy Agencies, Inc., New York, NY, and to Mr. Claude Brunot of Codal Co., Tananarive, Madagascar, for putting up the experimental pack.

A Research Note

BALANCED INCOMPLETE BLOCK DESIGNS WITH A REFERENCE SAMPLE IN EACH BLOCK

E. R. WILLIAMS and P. N. JONES

ABSTRACT

Intrablock analysis of balanced incomplete block designs with a reference sample in each block was discussed by Gacula (JFS 43: 1461). This note illustrates the importance of obtaining treatment estimates with recovery of interblock information, particularly when blocking has proved ineffective and presents several formulas of Gacula in a more natural form.

INTRODUCTION

IN A RECENT PAPER, Gacula (1978) discussed the intrablock analysis of balanced incomplete block designs with a reference sample in each block. The purpose of this note is to illustrate the importance of obtaining treatment estimates with recovery of interblock information, particularly when blocking has proved ineffective. In addition several formulas contained in the paper by Gacula (1978) are presented in a more natural form.

DESIGN & ANALYSIS

WE ASSUME a balanced incomplete block design with t treatments replicated r times in b blocks of size k. This design is then modified by incorporating a single reference sample in each block. Details are given by Gacula (1978). We use the notation of Williams (1977a, b) and assume a model of the form

$$y = X\tau + \epsilon ,$$

where y is an $n \times 1$ vector of mean corrected observations; X is the treatment design matrix, i.e. the $(h,i)^{th}$ element of X is one if treatment i is applied at the hth observation and is zero otherwise; τ is the vector of treatment effects; ϵ is an $n\times 1$ vector of random effects with $E(\epsilon) = 0$ and $E(\epsilon\epsilon') = \sigma^2(I_n + \xi^{-1}(k+1)^{-1} ZZ')$, where Z is the block design matrix and σ^2 and ξ are parameters to be estimated.

For the case of a balanced incomplete block design with a reference sample in every block, it is convenient to write τ' as $[\tau_R \vdots \tau'_I]$ where τ_R is the effect for the reference sample and τ_I is the $t\times 1$ vector of effects for nonreference treatments. Then the weighted least squares estimate $\hat{\tau}$ of the vector of treatment effects satisfies the equation

$$\left[\begin{pmatrix} b & \vdots & 0' \\ \cdots & \vdots & \cdots \\ 0 & \vdots & rI_t \end{pmatrix} - \frac{1}{(k+1)(\xi+1)} \begin{pmatrix} b & \vdots & r1' \\ \cdots & \vdots & \cdots \\ r1 & \vdots & (r-\lambda)I_t + \lambda J_t \end{pmatrix}\right] \begin{bmatrix} \hat{\tau}_R \\ \cdots \\ \hat{\tau}_I \end{bmatrix} = \begin{bmatrix} Q_R \\ \cdots \\ Q_I \end{bmatrix} \quad (1)$$

where $Q = \begin{bmatrix} Q_R \\ \cdots \\ Q_I \end{bmatrix} = \left[X' - [(k+1)(\xi+1)]^{-1} X'ZZ'\right] y$, J_t is a $t\times t$ matrix of ones and $\lambda = r(k-1)/(t-1)$. Note that setting $\xi = 0$ in (1) gives the reduced normal equations for the intrablock estimation of τ. It is straightforward to solve (1) to give

$$\begin{bmatrix} \hat{\tau}_R \\ \cdots \\ \hat{\tau}_I \end{bmatrix} = \begin{bmatrix} b^{-1} & \vdots & 0' \\ \cdots & \vdots & \cdots \\ 0 & \vdots & (rE)^{-1}(I_t - fJ_t) \end{bmatrix} \begin{bmatrix} Q_R \\ \cdots \\ Q_I \end{bmatrix} \quad (2)$$

where $E = [(k+1)\xi + (kt-1)/(t-1)]/[(k+1)(\xi+1)]$ and $f = (1-E)/t$. When $\xi = 0$, E becomes the efficiency factor in the intrablock analysis of the nonreference treatments.

Table 1—Analysis of variance of chopped ham flavor

Source of variation	df	ms	Expectation
Repetitions	1	0.1111	
Treatments (unadjusted)	4	3.8056	
Panelists within repetitions (adjusted)	10	$1.2397 = E_b$	$\sigma^2(1+15\xi/14)$
Error	20	$1.2246 = E_e$	σ^2
Total	35		

From (2),

$$\hat{\tau}_R = b^{-1} Q_R$$

and

$$\hat{\tau}_I = (rE)^{-1}(I_t - fJ_t)Q_I = (rE)^{-1}(Q_I + fQ_R 1_t) \quad . \quad (3)$$

The variance for the comparison of two nonreference treatments is

$$2(rE)^{-1}\sigma^2 \quad , \quad (4)$$

and the variance for the comparison of the reference sample with a nonreference treatment is

$$[(rE)^{-1}(1-f) + b^{-1}]\sigma^2 = (rtE)^{-1}[(t-1) + E(k+1)]\sigma^2 \quad . \quad (5)$$

An estimate for ξ can be obtained by equating lines of the intrablock analysis of variance table to expectation as done by Cochran and Cox (1957). For example if E_e is the residual mean square and E_b is the block mean square adjusted for treatments, then ξ is given by

$$\hat{\xi} = \frac{E_e}{(E_b - E_e)}\left[1 - \frac{t-k}{(b-1)(k+1)}\right] \quad . \quad (6)$$

Formulas (3) through (5) hold when the basic design is repeated say p times provided b and r are interpreted as the total number of blocks and nonreference replications, respectively; however, in (6) the term $(b-1)$ in the denominator must be replaced by $(b-p)$. Putting $\xi = 0$ in (3) through (5) gives the intrablock estimates and variances which may be compared with expressions (11) through (15) of Gacula (1978) and the formulas on page 266 of Pearce (1960). Evidently (3) through (5) above agree with Pearce; formula (3) differs slightly from (11) and (13) of Gacula because the latter uses the average of the nonreference treatments as an estimate for μ whereas we correct the data using the

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0022-1147/79/0006-1790$02.25/0
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general mean of all observations. Whilst expression (5) with $\xi = 0$ agrees with formula (15) of Gacula, the variance for the comparison of two nonreference treatments cannot be obtained from formula (14) of Gacula since he does not present the expression for the covariance of the two treatments, namely $(t-1)(k+1)/[(kt-1)rt]$; however, (4) above gives the correct expression.

When blocking is effective, E_e will be small relative to E_b and consequently ξ will be close to zero; then $\hat{\tau}$ will be similar to the vector of intrablock estimates. However, when blocking is ineffective, E_b and E_e will be nearly equal and consequently ξ will be large; then $\hat{\tau}$ will be similar to the vector of unadjusted treatment effects. In the latter situation, use of the intrablock estimate for $\hat{\tau}$ can result in considerable loss of efficiency as can be seen from the example given by Gacula (1978) of a balanced incomplete block design with reference sample for t = 4, k = 2, b = 12, r = 6 and p = 2. The analysis of variance is given in Table 1. Here E_b = 1.2397 and E_e = 1.2246 hence ξ = 75.6927 and E = 0.9971; hence the vectors of unadjusted, intrablock and combined treatment means are, respectively,

$$\begin{bmatrix} 4.5000 \\ 6.1667 \\ 4.5000 \\ 4.8333 \\ 4.1667 \end{bmatrix} \quad \begin{bmatrix} 4.5000 \\ 5.8810 \\ 4.8810 \\ 4.6667 \\ 4.2381 \end{bmatrix} \quad \text{and} \quad \begin{bmatrix} 4.5000 \\ 6.1638 \\ 4.5039 \\ 4.8316 \\ 4.1674 \end{bmatrix}.$$

The intrablock treatment estimates differ considerably from the unadjusted and combined means. In addition variances (4) and (5) reduce to 0.4094 and 0.3066, respectively, compared with 0.5248 and 0.3499 for the intrablock analysis.

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ERRATUM NOTICE

J. Food Sci. 44(3): 918–919 + 921 (1979), Connective tissue profiles of various raw sausage materials by E.L. Wiley, J.O. Reagan, J.A. Carpenter, and D.R. Campion. On page 919, Table 2, the values in the "soluble collagen" column are values for percentage soluble collagen, not mg/g of soluble collagen as published. Please correct accordingly.

COMPUTER MALFUNCTION CAUSES EXTENSIVE ERRATA IN CAPSICUM DATA

B. J. NAGLE, B. VILLALON, and E. E. BURNS

A NUMBER OF ERRORS occurred in the paper Color Evaluation of Selected Capsicums by B.J. Nagle, B. Villalon, and E.E. Burns [J. Food Sci. 44(2): 416–418] as a result of computer failure while performing the calculations. Since all the errors occurred in the Results & Discussion, this section of the paper is being reprinted in its entirety with all necessary corrections included.

These errata clearly demonstrate that computers (calculators) are not infallible, and point up the necessity for making periodic checks with standard calculations with verified results.

RESULTS & DISCUSSION

THE COLOR OF RED PEPPERS is due to carotenoid pigments in the pepper wall. Over 27 carotenoid pigments have been identified in red bell peppers (Curl, 1972). Like tomatoes, red ripe peppers have basically two pigment colors which combine to give a total color: red and yellow. Red pepper color is primarily caused by the xanthophylls capsanthin and capsorubin which make up the largest pigment percentage. The amount of pigment in the tissues depends upon factors such as species, variety, maturity, and growing conditions.

The beta-carotene and xanthophyll levels found in the different cultivars are presented in Table 1. A significant difference in total pigment content was found between all the cultivars (PR > F = 0.0001). Total pigment content ranged from 176.99 mg/100g in cultivar 73 to 830.65 mg/100g in the small ancho cultivar. A rank correlation coefficient of 0.7431 was found between the beta-carotene content and xanthophyll content of the cultivars. The xanthophyll fraction was found to be much larger than the beta-carotene fraction in all cultivars.

Table 1—Means of extractable pigments in raw red pepper walls

Cultivar description	β-Carotene mg/kg	Xanthophyll mg/kg	Total pigment mg/kg	β-carctene/ Xanthophyll
Small Ancho	1010.5	7296.0	8306.5	0.138
Kalocsai-504 Paprika	899.8	7097.4	7997.2	0.126
7311B Chili	1197.7	6138.6	7336.3	0.195
C76C1 Chili	1211.5	5570.6	6781.9	0.217
Hatuani Paprika	565.6	6011.5	6577.1	0.093
C-76S3 Paprika	449.9	5762.7	6212.6	0.078
AC-2243-2 Paprika	519.9	4997.7	5517.6	0.103
Buketn Paprika	845.0	3777.8	4622.8	0.223
Kaloc Paprika	372.0	3557.2	3929.2	0.104
SC-46255 Cayenne	897.6	2991.8	3889.3	0.299
C-76C4 Paprika	840.0	3239.9	4079.9	0.148
C-76S2 Paprika	311.7	3046.3	3358.0	0.102
7311A Chili	201.2	3027.8	3228.9	0.066
75140-1-4-2 Cherry	66.8	2343.0	2409.8	0.028
73 Small Cherry	57.4	1712.5	1770.3	0.033

Table 2—Means of Gardner color values of pureed peppers

Cultivar description	Color "L"	Color "a"	Color "b"	Hue θ
73 Small Cherry	36.30	44.30	24.10	28°32′
SC-46255 Cayenne	35.80	41.50	23.60	29°37′
75140-1-4-2 Cherry	36.40	39.70	23.30	30°24′
C-76C4 Paprika	32.10	41.10	21.10	27°10′
Small Ancho	23.70	41.00	21.50	27°40′
AC-2242-2 Paprika	34.80	43.70	20.80	25°26′
7311A Chili	30.30	41.60	19.80	25°26′
C-76S2 Paprika	31.00	38.00	19.50	27° 9′
Kaloc Paprika	30.20	39.80	19.40	25°59′
Buketn Paprika	30.20	38.30	19.10	26°30′
Hatuani Paprika	29.10	36.30	18.50	27° 2′
C-76C1 Paprika	28.90	40.80	18.20	24°22′
C-76S3 Paprika	27.10	34.70	17.10	26°16′
7311B Chili	33.50	35.10	16.30	24°50′
Kalocsai-504 Paprika	25.30	28.00	14.50	27°22′

The means of the Gardner color meter values for individual cultivars are presented in Tables 2 and 3. Spearman rank correlation coefficients were calculated for Gardner values and pigment values to determine linear relationships. The b values exhibited high correlation coefficients and the L and a values exhibited very low correlation coefficients (Tables 4 and 5).

Simple and multiple correlation coefficients were calculated between total pigment content, hydrocarbon carotenes, oxy-carotene derivatives, and all possible combinations of the three Gardner values L, a, and b to find a regression equation that is suitable for predicting the pigment content in the pepper cultivars. The common logarithms of pigment values were calculated to determine if these had a more linear relationship to Gardner values and they proved to be better correlated than actual pigment values. The simple and multiple correlation coefficients between pigment values and Gardner values for the regression

Table 3—Means of Gardner color values of raw whole red peppers

Cultivar description	Color "L"	Color "a"	Color "b"	Hue θ
C-76S3 Paprika	28.70	18.66	18.75	45° 0′
Small Ancho	29.10	18.36	16.75	42°13′
75140-1-4-2 Cherry	36.23	22.06	12.86	30°45′
73 Small Cherry	29.45	28.00	12.63	24°34′
AC-2243-2 Paprika	28.50	23.45	11.80	26°45′
Kaloc Paprika	29.20	25.83	10.36	21°50′
C-76C4 Paprika	23.40	23.56	10.23	23°22′
7311A Chili	30.73	25.66	9.83	20°49′
Buketn Paprika	29.33	21.50	8.89	22°28′
SC-46255 Cayenne	33.20	17.10	8.75	26°57′
Kalocsai-504 Paprika	29.20	16.90	8.56	26°58′
Hatuani Paprika	30.65	20.45	8.25	21°48′
7311B Chili	28.80	22.30	8.00	19°24′
C-76S2 Paprika	29.66	20.26	7.05	19° 1′
C-76C1 Paprika	29.26	17.13	6.63	21° 1′

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0022-1147/79/0006-1792$02.25/0
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Table 4—Spearman's rank correlation between Gardner color meter values for whole pods and extractable pigment values[a]

Gardner values	β-carotene	Xanthophyll	Total pigment
"L"	0.161	–0.301	–0.225
"a"	0.139	0.339	0.289
"b"	0.828	0.967	0.989

[a] All correlations are significant at the 0.05 level.

Table 5—Spearman's rank correlation between Gardner color meter values for pureed pods and extractable pigment values[a]

Gardner values	β-carotene	Xanthophyll	Total pigment
"L"	0.579	0.593	0.634
"a"	0.492	0.796	0.778
"b"	0.803	0.975	0.985

[a] All correlations are significant at the 0.05 level.

Table 6—Spearman's rank correlation between subjective values and extractable pigment values[a]

Visual analysis	β-carotene	Xanthophyll	Total pigment
Yellow	–0.325	–0.671	–0.610
Red	0.261	0.451	0.384

[a] All correlations are significant at the 0.05 level.

analysis reported in this paper were determined from the log of pigment values.

Higher correlations were achieved between pigment values and Gardner values for purees. Simple correlations of 0.774, 0.955 and 0.975 were obtained for the Gardner b value and beta-carotene, xanthophyll, and total pigments, respectively. The L and a values alone did not exhibit high correlations with pigment values. Multiple correlation coefficients exhibited higher values, as would be expected (Table 7).

Coefficients determined for whole pods and pigment values showed the same trend as for the purees but were lower. This is especially noted for simple correlations calculated for L and a values and pigment values. The b values correlated well with xanthophyll and total pigment ($r = 0.870$ and $r = 0.860$, respectively) (Table 7).

One of the problems encountered in measuring samples of whole fruit or vegetable matter is that often the geometry of the sample hinders uniform sampling. Another problem is that pigment tends to be unevenly distributed throughout any individual sample as Ezell et al. (1959) found in sweet potatoes. Together, these things could account for a great deal of error in sampling whole pods and the lower correlations found between whole pod Gardner values and pigment values.

The beta-carotene/xanthophyll ratios are presented in Table 1. No correspondence was found between the ratios of the unoxygenated and oxygenated pigments and color attributes. Correlation coefficients were very low comparatively. The hue angles (Table 3), calculated in radians, were compared to pigment ratios and total pigments and no significant correlations could be found.

Low r values were found between subjective evaluation and pigment values (Table 8). The scores for evaluation of yellow color and xanthophyll exhibited the higher correlation coefficient ($r = 0.667$).

Kendall's rank correlation procedure was performed on panelists' scores to determine the amount of agreement between panelists' rankings of red and yellow color of the purees. A very high correlation of 0.947 was shown for evaluation of yellow color but a very low correlation of 0.204 was shown for evaluation of red color. One problem often found in subjective evaluation of color of fruit or

Table 7—Pearson's correlation of hue θ with total pigment content[a]

Hue θ	Total pigment
Purees	–0.5789
Whole pods	–0.4380

[a] All correlations are significant at the 0.05 level.

Table 8—Simple and multiple correlation coefficients between Gardner color values and the logarithms of pigment values

Gardner values	β-carotene	P>F	Xanthophyll	P>F	Total pigment	P>F	β-carotene / Xanthophyll	P>F
			Whole pods					
L	0.223	0.42	0.047	0.86	0.023	0.93	0.339	0.21
a	0.231	0.39	0.388	0.15	0.362	0.18	0.090	0.74
b	0.651	0.0008	0.870	0.0001	0.860	0.0001	0.364	0.18
L,a	0.353	0.44	0.388	0.37	0.370	0.41	0.367	0.41
L,b	0.688	0.02	0.871	0.0002	0.861	0.0003	0.497	0.18
a,b	0.682	0.02	0.935	0.0001	0.917	0.0001	0.371	0.41
L,a,b	0.729	0.03	0.935	0.0001	0.920	0.0001	0.513	0.32
			Pureed pods					
L	0.407	0.13	0.539	0.038	0.544	0.036	0.232	0.40
a	0.707	0.003	0.861	0.0001	0.857	0.0001	0.449	0.09
b	0.820	0.0002	0.961	0.0001	0.972	0.0001	0.542	0.03
L,a	0.708	0.015	0.867	0.0002	0.864	0.0001	0.499	0.20
L,b	0.829	0.0009	0.964	0.0001	0.974	0.0001	0.588	0.10
a,b	0.821	0.0012	0.968	0.0001	0.976	0.0001	0.542	0.12
L,a,b	0.831	0.0038	0.971	0.0001	0.976	0.0001	0.558	0.23

In addition, regression equations for predicting pigment content of pepper cultivars were calculated as:

Whole pods: Log total pigment = 3145 + 0.049b
Standard Error of the Residuals = 0.1042

Pureed pods: Log total pigment = 2.270 + 0.070b
Standard error of the residuals = 0.0479

The statistical model was corrected and results of multiple and simple regression analysis showed that the b value is the better Gardner color attribute to use in prediction of pigment content of red peppers.

vegetable purees is that oxidative browning reactions may occur and not necessarily uniformly in all samples. Hoover and Mason (1961) reported that some purees of sweet potato with high beta-carotene content were dark and lacked luster or brightness and were consequently given a lower subjective score. Other samples, lower in beta-carotene, were bright yellow in color and received high scores.

Panel members of this study revealed similar variations in judgment of red pigments. Some judged high pigment content to be a dark sample while others judged it to be a lighter brighter sample. This may account for the low Kendall correlation coefficient for red color evaluation and would also affect r calculated for pigment values and subjective values.

CONCLUSIONS

UNLIKE the relationships found in sweet potatoes (Ezell et al., 1959; Kattan et al., 1957; Ahmed and Scott, 1962) the a value is not the better index to pigment content of red peppers. Total extracted pigments exhibited higher correlations with Gardner values than either beta-carotene or xanthophylls. The Gardner b value proved to be the better single attribute to use in predicting pigment content. Multiple regression analysis of L, a, and b values with total pigment content showed the higher overall correlation coefficients. The more efficient regression equations to predict the pigment content of red peppers are illustrated in Table 8. The subjective analysis of pigment content was less accurate than the Gardner Color Difference Meter.

Appreciation is expressed to Dr. Angela C. Little, Univ. of California, Berkeley, for calling these errata to our attention.

Institute of Food Technologists

The Institute of Food Technologists is a professional society of scientists, engineers, educators, and executives in the field of food technology. Food Technologists are professionals who apply science and engineering to the research, production, processing, packaging, distribution, preparation, evaluation, and utilization of foods. Individuals who are qualified by education, special training or experience are extended an invitation to join in professional association with the select group of the food industry's scientific and technological personnel who are Institute members. Membership is worth many times its modest cost, reflecting positive benefits, stimulation, and opportunities for the individual in his/her business or profession.

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The Institute, as a non-profit, professional, educational society, has several major aims: to stimulate investigations into technological food problems; to present, discuss, and publish the results of such investigations; to raise the educational standards of Food Technologists; and to promote recognition of the scientific approach to food and the basic role of the Food Technologist in industry. All of these activities have the ultimate objective to provide the best possible foods for mankind.

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Call for Nominations for IFT's 1980 Achievement Awards

□ THE IFT ACHIEVEMENT AWARDS honor outstanding individuals and developments in food science and technology and since the presentation of the first Nicholas Appert Award in 1942, the annual awards presentation has been one of the highlights of nearly every one of IFT's Annual Meetings. (See the June and July issues of *Food Technology* for details of this year's winners.)

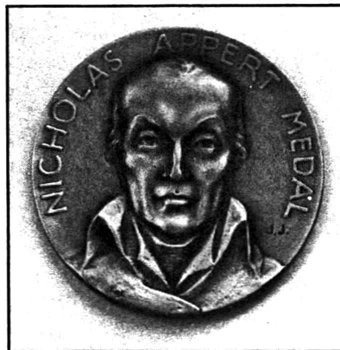
It's time to make the nominations for the 1980 awards, which will be presented at the 1980 Annual Meeting in New Orleans on June 8, 1980. Details are on these two pages. Here are the groundrules:

1. Candidates for any of the awards cannot be considered by the jury unless someone nominates them.
2. Each nomination must be submitted on a prescribed form. Nomination forms for each of the awards are available from: IFT 1980 Achievement Awards, Institute of Food Technologists, 221 N. LaSalle St., Chicago, IL 60601 USA. *When you request an award form, be sure to state the award for which you intend to nominate a candidate.*
3. Except for the International Award, nominees need not necessarily be members of IFT; however only IFT members may make nominations.
4. Members may not nominate themselves for an award. Exception: For the Industrial Achievement Award, a member may nominate his or her own company or organization.
5. Deadlines for submitting nomination forms for each of the awards are given in the following descriptions.

1980 Nicholas Appert Award

Purpose: To honor a person for pre-eminence in and contributions to the field of food technology.

Qualifications: Candidate should fit the purpose of the Award. (Factors of personality or achievement outside the field of food technology cannot be considered.)



Awards and Sponsors: The Awardee receives a special medal and a $1,000 honorarium from the Institute of Food Technologists. Co-sponsored by the Chicago Section, IFT

Deadline for receipt at IFT; **December 15, 1979.**

1980 IFT Food Technology Industrial Achievement Award

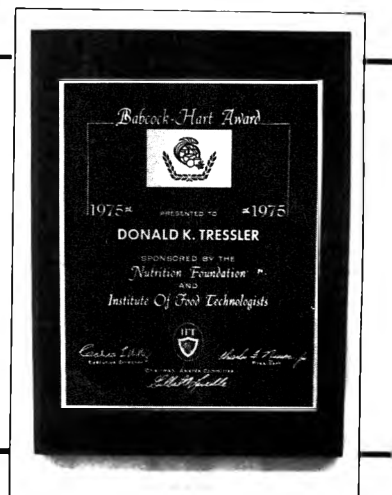
Purpose: To recognize and honor the developers of an outstanding food process and/or product which represents a significant advance in the application of food technology to food production.

Qualifications: The process or product constituting the significant advance must have been in commercial use for no less than six months nor more than four years prior to September 1. Jurors are charged to require high criteria of merit. Therefore, in any year where none of the nominated processes and/or products meet these criteria, an award may not be made.

Award and Sponsor: Bronze plaque(s) will be presented to the winning organization(s); engrossed plaque(s) will go to the individuals responsible for the achievement. As with the other achievement awards, public recognition will be given during the 40th Annual Meeting in New Orleans. An award of the Institute of Food Technologists.

Deadline for receipt at IFT: **December 1, 1979.**

1980 Babcock-Hart Award



Purpose: To honor a person for distinguished contributions to food technology which have resulted in improved public health through some aspect of nutrition or more nutritious food.

Qualifications: Candidate's contributions should fit the purpose of the Award, and should have led to practical application leading to improved nutrition. (Contributions in fundamental studies in nutrition do not qualify for the Award.)

Award and Sponsors: The Nutrition Foundation, Inc., sponsors the Award and provides a $1,000 honorarium. The Awardee also receives an engraved plaque from IFT.

Deadline for receipt at IFT: **January 1, 1980.**

1980 International Award

Purpose: To honor a member of the Institute of Food Technologists who has made outstanding efforts to promote the international exchange of ideas on food technology, or whose work has led to such exchange of ideas or to better international understanding in this field.

Qualifications: Candidate should fit the purpose of the Award.

Award and Sponsors: A suitably inscribed silver salver is furnished by the Australian Institute of Food Science and Technology. A $1,000 honorarium is provided by IFT.

Deadline for receipt at IFT: **December 1, 1979.**

1980 Wm. V. Cruess Award

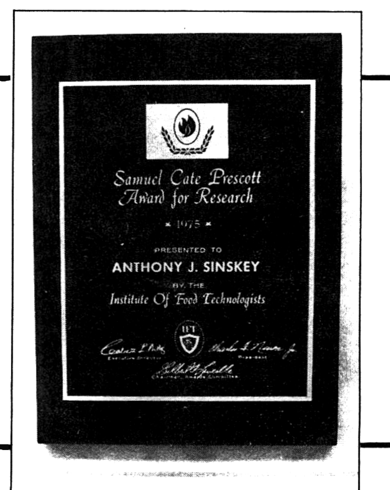
Purpose: To recognize excellence in university teaching in the field of food science and technology.

Qualifications: Candidate should have taught for at least five years in a department offering a bachelor's or higher degree in food science/technology or the equivalent thereof.

Award and Sponsors: The Institute of Food Technologists provides a special medal and a $1,000 honorarium. Co-sponsored by the Northern California Section, IFT.

Deadline for receipt at IFT: **December 15, 1979.**

1980 Samuel Cate Prescott Award



Purpose: To recognize a young research scientist (35 years of age or younger) who has demonstrated outstanding ability in research in some area of food science and technology.

Qualifications: Candidate should fit the purpose of the Award. Special attention will be given to contributions in methodology, competence shown, and effects of the research on food science advances.

Award and Sponsor: The Institute of Food Technologists provides a $1,000 honorarium and an engraved plaque.

Deadline for receipt at IFT: **January 1, 1980.**

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JOURNAL OF FOOD SCIENCE		10/2/79

3. FREQUENCY OF ISSUE	A. NO. OF ISSUES PUBLISHED ANNUALLY	B. ANNUAL SUBSCRIPTION PRICE
BI-MONTHLY	6	$45.00

4. LOCATION OF KNOWN OFFICE OF PUBLICATION *(Street, City, County, State and ZIP Code) (Not printers)*

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GENERAL

THE INSTITUTE of Food Technologists publishes the *Journal of Food Science* bimonthly. Research articles must report original work and have a clear relationship with foods. The journal contains three major sections: "Basic Science," "Applied Science and Engineering," and "Research Notes." Abstracts of all papers are also published in *Food Technology*.

AUTHORSHIP

Membership in the Institute of Food Technologists is not a prerequisite for consideration of research articles for publication.

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Dr. Bernard J. Liska
IFT Scientific Editor
P.O. Box 3067
West Lafayette, IN 47906 U.S.A.

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Acceptance. Each paper is accepted with the understanding that it is the sole document under active consideration for publication covering the work reported. In other words, work reported in a paper submitted

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TYPES OF PAPERS

ARTICLES REPORTING original research, considered for publication in the *Journal of Food Science*, will be classified as either: 1. Basic Science; 2. Applied Science and Engineering; or 3. Research Notes.

Papers offered for publication in an Institute journal are to conform to the style for research articles as set forth in this Style Guide and when applicable, to the Author Guidelines for IFT Papers Reporting Sensory Evaluation Data (available from IFT's Director of Publications).

RESEARCH ARTICLES

A research article should be confined to a coherent well-defined piece of work. Closely related work should be put in one paper and should not be broken down into several papers, as sequential numbering of papers in a series is no longer permitted. Although no limits on length are imposed, authors are expected to report their findings as CONCISELY as possible. The following organization is appropriate.

Title. Informative of the content of the article. Start with a word of value in indexing and general information retrieval.

Author(s). Given name and initials are adequate for proper identification, whether male or female. Omit professional and official titles. Give the complete address for place where work was done and each author's organizational association (company, university, laboratory, etc.).

Add present address of each author, if different, on the title page as a footnote(s).

Abstract. The abstract should state clearly the objective of the study, a concise description of the experiment(s), major observations, results and conclusions. No references should be cited in the abstract. The abstract SHOULD NOT EXCEED 110 WORDS. The abstract will be published in both the *Journal of Food Science* and *Food Technology*.

Introduction. This should be a review of only pertinent previous work and should cite appropriate references. It should also include a statement of the purpose of the investigation.

Experimental. This section is generally headed Experimental but, depending upon its content, may also be headed Materials & Methods, with appropriate subheads indicated for apparatus, reagents, procedures, etc.

Provide sufficient detail of the method and equipment to allow the work to be repeated. Accepted methods should be cited by adequate references. In reporting procedures, the active voice is preferred.

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Appendix. Research papers rarely need an appendix; however, details of calculations, nomenclature, etc., may be placed in an appendix if deemed necessary.

Tables. Numbered with Arabic numerals and each typed on a separate sheet of paper.

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Illustrations. Line drawings, photographs, etc., to be reproduced as figures.

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The principal characteristic of a research note is brevity. It should not exceed four (double-spaced) typewritten pages and two figures (or, two tables).

Research Notes are evaluated for publication by the same high standards as are Research Papers and should be used to report either (a) important preliminary findings in a fast moving research field, or (b) findings of a nature which do not require a full research article. It need not have the more formal organization of a research article, but otherwise should follow the same basic concepts of presentation. An abstract of 110 words or less is required.

Research Notes are given top priority in publication scheduling.

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LANGUAGE

Only papers written in English will be accepted for consideration. Language usage, including spelling, should be in accord with good American practice. Authors whose primary language is not English should have the paper checked for usage prior to submitting it for publication.

Abbreviations, coined expressions, or trade names shall not appear in titles of papers. Use only abbreviations and nomenclature in the text that are accepted by recognized professional organizations, or that are well known through long and common use. At first appearance of coined expressions or trade names in the text, explain the meaning in parentheses.

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IFT does not use a dual system of notation; therefore units of measurement (e.g. temperature) should be consistent. Metric units are preferred whenever practicle.

Type formulas and equations clearly, accurately placing subscripts and superscripts. Distinguish between like figures on the typewriter: e.g., 1 (one) and l (el), zero and cap O.

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PAGINATION

Number all pages consecutively. Do not insert pages not in consecutive numbering; renumber pages, if necessary, to make all consecutive.

Page 1 should contain the following information:
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Name(s) of author(s)
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Running Head–A shortened form of the title, not to exceed 40 letters and spaces
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(Leave one line space here)

The consistency of tomato products can be . . .

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Do not use footnotes in the text for descriptive or explanatory information, source of supply, etc. Include the information at the appropriate place in the text.

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REFERENCES

General. The references cited should be to the original source which should be examined in connection with the citation. If this cannot be done, a secondary source (e.g., abstract, book, etc.) may be used, but it should be cited along with the original reference.

Only references actually cited in the paper are to be included in this section.

Use lower case letters a, b, c, etc., following the year to distinguish among different publications of the same author(s) in any one year.

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When no author (or editor) is indicated, "Anonymous" is cited in the text as well as in the list of References.

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1. With last name of the author as part of the sentence, immediately followed by the year of publication in parentheses:

   Example: Smith et al. (1963) reported growth on vinasse. This was demonstrated by Jones (1966).

2. With last name of author and year of publication in parentheses, usually at the end of a sentence:

   Example: The starch granules are normally elongated in the milk stage (Brown, 1956).

3. To indicate more than two authors, use et al. after the name of the first author:

   Example: This was first observed by Smith et al. (1966) and later confirmed by Smith and Jones (1972).

Complete titles of works should not be cited in the text.

In the list of References. This section is headed References. The entire list MUST be typed double spaced. Each individual citation in the list should begin flush left (with no paragraph indentation). If the citation requires more than one line, indent runover lines about six characters (½ in.).

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EXAMPLES of use **in a Reference list** are given below. The bold-faced parenthetical type of citation above the example is indicated ONLY for information and is **NOT** to be included in the reference list.

(Book–example)

AOAC. 1970. "Official Methods of Analysis," 11th ed. Association of Official Analytical Chemists, Washington, DC.

(Bulletin, circular–example)

Adams, J.Z. and Brakestone, I.J. 1959. Growing apple tissue in a hydrotropic environment. Bull. 1478, Geneva Agric. Exp. Stn., Geneva, NY.

(Chapter of book–example)

Dickerson, R.W. Jr. 1968. Thermal properties of foods. In "The Freezing Preservation of Foods," Vol 2, p. 26. Avi Publishing Co., Westport, CT.

(Journal–example)

Katz, M.H. 1972. Functional properties of carbohydrates. Food Technol. 26(3): 20.

Tsai, R., Cassens, R.G. and Briskey, E.J. 1972. The emulsifying properties of purified muscle proteins. J. Food Sci. 37: 286.

(Non-English reference–example)

Wolfsohn, J.D. 1923. "Apfelgeruch und die Chemische Bestandteile des Apfels." Springer Verlag, Stuttgart, Germany.

(Paper accepted–example)

Delft, J.R. 1972. Apple essence recovery. Food Technol. In press.

(Paper presented–example)

Parrish, F.C. Jr., Young, R.B. and Miner, B.E. 1972. Effect of postmortem conditions on certain properties of bovine muscle. Presented at the 32nd Annual Meeting of the Institute of Food Technologists, Minneapolis, MN, May 22–24.

(Patent–example)

Roth, T.L. 1972. Coating of frozen foods. U.S. Patent 3,607,313.

(Secondary source–example)

Wehrmann, K.H. 1961. Apple flavor. Ph.D. thesis, Michigan State University, East Lansing, MI. Quoted in Wehrmann, K.H. 1966. "Newer Knowledge of Apple Constitution," p. 141. Academic Press, New York.

—or—

Ogandzhanyan, A.M. 1966. Carbohydrate and nitrogenous fractions of corn ears (in Russian). Tr. Arm. Nauch.-Issled. Inst. Zhivotnovod. Vet. 7-8: 283. [In Chem. Abstr. 1968, 68(2): 967.]

(Thesis–example)

Taumer, U. 1965. Identification of volatile constituents of aged Delicious apple juice. Ph.D. thesis, Cornell University, Ithaca, NY.

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BIBLIOGRAPHY

American National Standards Institute, Standards Committee Z39. 1970. International list of periodical title word abbreviations. (Available in N. and S. America from Chemical Abstracts Service, Marketing Dept., University Post Office, Columbus, OH 43210; elsewhere, ICSU-AB Secretariat, 17 rue Mirabeau, 75-Paris 16e, France.)

Committee on Form and Style of the Conference of Biological Editors. 1979. "Style Manual for Biological Journals," 4th ed. American Institute of Biological Sciences, 3900 Wisconsin Ave. NW, Washington, DC 20016.

Editorial Staff, University of Chicago Press. 1969. "A Manual of Style," 12th ed., rev. The University of Chicago Press, Chicago and London.

Spear, M.E. 1952. "Charting Statistics." McGraw-Hill Book Co., Inc., New York.